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Study on the Efficient Method to Produce Cloned Embryos and Species Conservation of Jeju Black Cattle Using Somatic Cell Nuclear Transfer Techniques

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Department of Biotechnology

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Study on the efficient method to produce cloned embryos and species conservation of Jeju Black Cattle using somatic cell transfer technique

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ABSTRACT

Jeju black cattle (JBC), one of five native Korean cattle species characterized by their pitch-black coat color, live in the World Natural Heritage sites in Jeju Special Self-Governing Province. JBC are traditionally regarded as a source of top quality food. However, they are endangered, with approximately 700 animals remaining in Jeju Special Self-Governing Province; therefore, cloning is needed to preserve these endangered animals. The purpose of this study is to improve the production efficiency of SCNT embryos and to develop vitrification technology for providing donor oocytes and SCNT blastocyst to

produce cloned JBC and is to studying ways to contribute to the species conservation of JBC using these technologies.

Experiment 1. It was to compare the effects of two enucleation systems, Hoechst staining and UV irradiation and Oosight imaging, on the *in vitro* production of bovine SCNT embryos. In the Oosight group, the apoptotic index (2.8±0.5 vs. 7.3±1.2) was lower, and the fusion rate (75.6% vs. 62.9%), cleavage rate (78.0% vs. 63.7%), blastocyst rate (40.2% vs. 29.2%), and total cell number (128.3±4.8 vs. 112.2±7.6) were higher than those in the irradiation group (all $p < 0.05$). The overall efficiency after SCNT was twice as high in the Oosight group as that in the irradiation group ($p < 0.05$). The Oosight imaging system may become the preferred choice for enucleation because it is less detrimental to the developmental potential of bovine SCNT embryos.

Experiment 2. The study was to investigate the effect of flavonoid treatment on *in vitro* development of bovine SCNT embryos. To optimize the flavonoid concentration, parthenogenetic day 2 embryos were cultured in 0 (control), 1, 10 and 20 μM flavonoid for 6 days. In the results, *in vitro* development rate was highest in 10 μM flavonoid group (57.1%) among treatment groups, and numbers of total and ICM cells were significantly higher in 10 μM flavonoid group than other groups. The 10 μM treatment group can significantly decrease the apoptotic index and derive high expression of anti-oxidant, anti-apoptotic, cell growth and development marker genes. When the *in vitro* produced day 7 or 8 SCNT blastocysts were transferred into a number of recipients, the 10 μM flavonoid treatment group presented higher pregnancy rate (10.2%, 6/59) than control group (5.9%, 2/34). These results demonstrated that the flavonoid addition in culture medium may have beneficial effects on *in vitro* and *in vivo* developmental capacity of SCNT embryos and pregnancy rate.

Experiment 3. Using a time-lapse monitoring system, we investigated the developmental potential and developmental kinetics of bovine parthenogenetic (PA) and two types of SCNT embryos. Bovine non-transgenic ear cells (bECs) or transgenic cells (bTGCs)

were used as donor cells. The cleavage and blastocyst development rates did not significantly differ among the PA, NT-bEC, and NT-bTGC groups, and first cleavage occurred an average of 19.3 h (n=70), 21.6 h (n=60), and 21.3 h (n=62) after activation, respectively [20.4 h (n=192) for all embryos]. When embryos were classified into early cleaving (20 h) and late cleaving (> 20 h) groups, the blastocyst formation rate was much higher in the early cleaving groups (PA, 46%; NT-bEC, 50%; NT-bTGC, 39%) than in the late cleaving groups (PA, 18%; NT-bEC, 23%; NT- bTGC, 28%), while the percentage of embryos whose development was blocked between the 2- and 8-cell stages was increased in the late cleaving groups. The percentage of embryos classified as early cleaving with a normal morphology was 2-fold higher in the PA group (20.0%, n=14) than in the NT-bTGC group (9.7%, n=6). These results demonstrate that time-lapse monitoring provides novel data regarding individual embryo developmental kinetics and helps to predict developmental potential for improved bovine NT embryo selection based on early cleavage (≤ 20 h) and normal morphology.

Experiment 4. This study sought to optimize the survival and cryopreservation of VT oocytes for SCNT. Co-culture with feeder cells that had been pre-incubated for 15 h significantly improved the survival of VT oocytes and their *in vitro* developmental potential following SCNT in comparison to co-culture with feeder cells that had been pre-incubated for 2, 5, or 24 h ($p < 0.05$). The cloning efficiency of the enucleated-activated-vitrified-thawed (EAVT) group (21.6%) was better than that of the other vitrification groups [enucleated-vitrified-thawed (EVT) group, 13.7%; VT group, 15.0%; $p< 0.05$] and was comparable with that of the non-V group (25.9%). Among the vitrification groups, blastocysts in the EAVT group had the best developmental potential, as judged by their high mRNA expression of developmental potential-related genes (POU5f1, Interferon-tau, and SLC2A5) and their low expression of pro-apoptotic (CASP3) and stress (Hsp70) genes. This

study demonstrates that SCNT using bovine frozen-thawed oocytes can be successfully achieved using optimized vitrification and co-culture techniques.

Experiment 5. For vitrification, JBC-SCNT blastocysts were serially exposed in glycerol (G) and ethylene glycol (EG) mixtures [10% (v/v) G for 5 min., 10% G plus 20% EG (v/v) for 5 min, and 25% G plus 25% EG (v/v) for 30 sec.] which is diluted in 10% FBS added D-PBS. And then SCNT blastocysts were loaded in 0.25 mL mini straw placed in cold nitrogen vapor for 3 min. and then plunged into LN_2. One-step dilution in straw was done in 25 ℃ water for 1 min, by placing vertically in the state of plugged-end up and down for 0.5 min, respectively. When *in vitro* developmental capacity of vitrified SCNT blastocyst was examined at 48 h after one-step dilution, hatched rate (56.4%) was slightly lower than that of control group (62.5%). In field trial, when the vitrified-thawed SCNT blastocysts were transferred into uterus of synchronized 5 recipients, a cloned female JBC was delivered by natural birth on day 299 and healthy at present. This study suggested that our developed vitrification and one-step dilution technique can be applied effectively on field trial for cloned animal production, which is even no longer in existence.

The purpose of this study is to help to the conservation and mass production of JBC through optimization an effective method to produce cloned embryos using somatic cell nuclear transfer techniques.

CHAPTER 1. Background and purpose

1. Somatic cell nuclear transfer

Since the first cloned sheep [1] was born using somatic cell nuclear transfer (SCNT) technology, many mammals have been cloned, including cattle [2], mice [3], and pigs [4]. Cloned animal production technology is expected to be useful for the breeding and research of farm animals, production of transgenic animals for biological medical purposes, and conservation of endangered species. Until recently, many researchers have succeeded in cloning elite bulls [5], cows with good milk production capacity [6] and endangered animals [7]. Furthermore, transgenic animals such as cows that overexpress the casein protein in calves [8] or whose milk is free of prions [9] have also been produced by the SCNT technique. However, despite the many studies that have been done, the efficiency of cloned bovine is low. In particular, the pregnancy rate and overall development rate are significantly lower than those of in vitro fertilization embryos [10, 11]. The reason for the low production efficiency of the cloning site is the stressful, unstable in vitro culture environment due to the length of the mechanical and chemical processes. In addition, cloned calves often exhibit various abnormalities that increase after birth or result in their death [12, 13]. For this reason, the delivery rate of the SCNT embryos is reported to be less than 7% [14]. The application of SCNT depends on improving the production efficiency of healthy cloned calves.

In order to improve the production of cloned bovine the production rate and development status of somatic cloned embryos should be preferentially improved. In this study, Oosight imaging system was used instead of the blind method or Hoechst staining and UV irradiation in order to reduce the physical and chemical damage to cloned embryos in the enucleation process. This reduced the damage to the oocyte and the cell death rate and increased the production rate of the cloned embryo, and the number of inner cell mass (ICM) which improved the development. In addition, the level of gene expression was confirmed, and the expression of the stress-related gene expression and apoptosis-related gene

expression was decreased compared to the control group. In general, the in vitro culture environment has a higher oxygen saturation level than in vivo, which results in the accumulation of reactive oxygen species (ROS) in the cytoplasm. Many studies have decreased oxygen saturation using various anti-oxidant supplements [15, 16]. In this study, we used flavonoids as an additive, to block the oxidative chain reaction by blocking the early development of oxygen radicals, and thereby increase the production rate of SCNT embryos. The flavonoid treated group had a higher pregnancy rate than control group. In fact, cloned calves were produced in 2 out of 6 pregnancies. In addition, in order to increase the pregnancy rate of SCNT blastocysts, it is also important to select and transfer embryos in a good developmental state. These selection parameters are generally based on the morphological characteristics of the embryos and their quality, which is subjectively judged by the researcher. In order to establish more objective selection parameters for excellent embryos, we observed the developmental kinetics and normal morphology of each stage with a time-lapse monitoring system. This technology allows for the safe and continuous monitoring of individual embryos and detailed study of their morphology and developmental kinetics by recording the timing of cleavage at each stage, the blastomere number, compaction status and the extent of cytoplasmic fragmentation. This is confirmed using a time-lapse monitoring system that uses the early cleavage group (≤ 20 h) and normal morphology as parameters.

Cryopreservation could be a useful technique to provide a steady source of oocytes for SCNT. In several studies, SCNT embryos were produced using frozen-thawed embryos [17, 18]. However, when SCNT embryos were produced using frozen-thawed oocytes, both the fusion rate and the embryo development rate were remarkably low. Previous studies have reported that when bovine embryos were cultured using a co-culture system with feeder cells [19], the development rate of embryos increased. This method was used to recover the vitrified oocytes from the pre-incubated 10 μL feeder cell droplet, and the cloning efficiency

of SCNT improved. In addition, when SCNT embryos were produced by injecting somatic cells into enucleated-activated-vitrified-thawed oocytes in advance, they were more efficient than when using vitrified-thawed oocytes without enucleation. This finding has important implications for the establishment of an oocyte bank to study SCNT. There is also the need for a stable SCNT blastocyst frozen-thawed technique that can be used to do the transfer when an excellent recipient cow is prepared. Most of the cloned animals that have been produced are using fresh SCNT blastocysts, and the production of cloned animals by frozen-thawed blastocyst transfer is extremely rare. In this study, we developed a vitrification / one-step dilution and direct transfer method that can be used immediately in the field and confirmed the usefulness of a freezing-thawing method capable of producing cloned bovines.

On the other hand, the potential benefits of using SCNT are not yet known, because it is not clear that somatic cell cloning has adverse effects on animal development and health or that their meat and milk are not harmful to humans. To circumvent these shortcomings, cloned bovine are artificially fertilized to produce offspring so that cloned bovine may be utilized. This not only confirms the reproductive ability of the cloned cattle, but also confirms that they can actually increase populations for species conservation. In this study, we succeeded in producing offspring from two Jeju Black Cattle, the post-death cloned breeding bull (Heuk Oll Dolee) and the breeding cow (Heuk Woo Sunee). Their offspring was born in 2013 (Heuk Woo Dolee) and is still alive as of 2017.

2. Jeju Black Cattle

Jeju Black Cattle (JBC) is one of the five native Korean cattle species and is characterized by their pitch-black coat color. They live in the World Natural Heritage sites in Jeju Special Self-Governing Province. The origin of JBC is a mixed species of Bos primigenius and Bos namadicus which originated on the Korean Peninsula through China and Mongolia, and was established by narrow breeding without other species. A Jeju

University research team analyzed the mtDNA D-loop region of ancient cattle bones from an archaeological site in Jeju and reported that JBC have been bred since ancient times [20]. It is not known exactly when JBC began to be bred, but they have been recognized as a native resource exclusive to Jeju since prehistoric times.

The historical basis for the breeding of JBC is that black cattle were drawn on the tomb murals of Goguryeo, and were recorded to have been offered to the king for the excellent taste of their meat in the Chronicles of King Sejong. Even after that, records of JBC frequently appeared in the Chosun dynasty annals and in various ancient documents. In “Tamna ginyeon”, which was written in 1918 by Kim Seok-ik, there was a record of the establishment of a pasture on Gappa island where 50 animals grazed and were prepared to be offered to the king in the 26th year of the Yeongjo (1750). This showed that the breeding of JBC was strictly nationally managed. JBC breeding was recorded in the “Tamna Sullyeokdo” produced during a tour of the province by Lee Hyung-Sang, who recorded that there were 1,118 breeding animals.

According to the results published in “Chosun Jisan Woo” in 1910, the coat color of Hanwoo, another type of cow, was mainly red, but there were some that were red-brown or black-and-white. On the other hand, in 1920, the Korean Agriculture Newsletter also said that the coat color of Hanwoo was mainly brown, but they could also be reddish brown, yellowish brown, black, dark brown, grayish brown or white. In 1928, the company also reported that the coat color of Hanwoo was 77.8% reddish brown, 10.3% yellowish brown, 8.8% black, 2.6% dark brown, 0.4% brownish black and 0.07% blackish white.

The 10th year of ‘Hannam Livestock’ which was published in 1939, the coat color of Hanwoo was generally reddish brown, but there are also black and tiger spot patterns, and the ratio of color was 77% red-brown, 10% dark brown, 8% black, and 2% others. Hanwoo with varied colors were artificially culled due to the standard coat color of Hanwoo being designated as yellowish brown, based on the “Hanwoo Evaluation Standard” that was been

enacted in 1970. In 1961, 4,924 (10% of the total) cattle in of 23.2% were reported as being black cattle in Jeju. At that time, it was assumed that about 10,000 cattle were bred in Jeju. According to a report in 1971, 1,665 animals out of 6,339 Hanwoo in the Jeju Sangnam region were black, which was 28.8% of the total, but the population of JBC was rapidly declining due to decreased in social interest. In 1981, the JBC breeding project began. The starting point was the purchase of breeding bulls at livestock farms and semen collection and cryopreservation in order to preserve the species at the Nanji Livestock Experimental Station. In the early 1990s, JBC was hybridized with exotic species and almost became endangered before the Livestock Promotion Institute and National Institute of Subtropical Agriculture began to collect genetic resources and bred 23 JBC. In the early days, cows were aged and had difficulty producing calves because of their poor reproductive potential, but since then, the population of JBC has increased to 310 animals in 2005 as a result of steady industrialization and a mass production project. As a result, about 700 animals of JBC original species are breeding in Jeju (2017).

The Jeju Special Self-Governing Province has prohibited the export of JBC resources (including embryos and sperm) outside Jeju in order to create special protection and management. The Korean Animal Improvement Association and the Ministry of Agriculture have established standards similar to those for the Hanwoo and national registration management system by creating separate criteria for the JBC. In 2013, the JBC is designated as National Monument No. 546 and is being protected at the national level.

3. Study purpose

In this study, the effects of two enucleation systems, Hoechst staining and Oosight imaging, were compared using the in vitro production of SCNT embryos and their developmental potential. This study also investigated the effect of flavonoid treatment on the in vitro development of bovine SCNT embryos and investigated their pregnancy and delivery

rate after embryo transfer into the recipient. The developmental potential of bovine activated embryos (PA and NT) classified as early cleaving (≤ 20 h) and/or with a normal morphology was investigated by analyzing the time at which they reached each stage through the use of a time-lapse monitoring system. We then examined the survival optimization for the vitrified oocysts used for SCNT and compared the cloning efficiency of oocytes that were conventionally vitrified, those that were enucleated prior to freezing and those that were enucleated and activated prior to freezing. These were all compared to blastocysts produced from the various groups of oocytes. Finally, this study developed a vitrification and one-step dilution technique for SCNT blastocyst transfer into surrogate cows.

The purpose of this study is to help the conservation and mass production of JBC by optimizing the methods (efficient enucleation of oocytes, improvement of culture environment and development of vitrification and survival techniques) used to produce cloned embryos using SCNT techniques.

CHAPTER 2. Improved cloning efficiency and developmental potential in SCNT with the Oosight imaging system

1. ABSTRACT

In SCNT procedures, exquisite enucleation of the recipient oocyte is critical to cloning efficiency. The purpose of this study was to compare the effects of two enucleation systems, Hoechst staining and UV irradiation and Oosight imaging on the *in vitro* production of bovine SCNT embryos. In the Oosight group, the apoptotic index (2.8±0.5 vs. 7.3±1.2) was lower, and the fusion rate (75.6% vs. 62.9%), cleavage rate (78.0% vs. 63.7%), blastocyst rate (40.2% vs. 29.2%), and total cell number (128.3±4.8 vs. 112.2±7.6) were higher than those in the irradiation group (all $p < 0.05$). The overall efficiency after SCNT was twice as high in the Oosight imaging group as that in the Hoechst staining group ($p < 0.05$). The relative mRNA expression levels of Oct4, Nanog, Interferon-tau, and Dnmt3A were higher and those of Caspase-3 and Hsp70 were lower in the Oosight imaging group compared with the Hoechst staining group ($p < 0.05$). This is the first report to show the positive effect of the Oosight imaging system on molecular gene expression in the SCNT embryo. The Oosight imaging system may become the preferred choice for enucleation because it is less detrimental to the developmental potential of bovine SCNT embryos.

2. INTRODUCITON

Successful application of the SCNT technique to developmental biology has resulted in the production of offspring from various species, including sheep, cattle, mice, and pigs [1–4]. SCNT offers many opportunities in basic and medical research, as well as in endangered species conservation. However, the efficiency of SCNT is still very low. To date, numerous studies have been performed to optimize SCNT procedures, including enucleation, cell injection, and activation [21–23]. Among these, enucleation is recognized as a critically important step. An ideal enucleation technique must completely avoid aneuploidy

abnormalities that could lead to detrimental effects on later development. It must eliminate any genetic contribution from the recipient cytoplasm and must exclude the possibility of parthenogenetic activation [24]. To improve the enucleation procedure and cloning efficiency, several methods have been developed, including blind, Hoechst staining and ultraviolet (UV) irradiation, spindle imaging, and centrifugation [25–28]. The Hoechst staining and UV irradiation approach is used routinely, particularly in domestic species, to ensure error-free enucleation by enabling clear visualization of the chromosomes before removal of the dense cytoplasm from the oocytes prior to nuclear transfer. This procedure involves the removal of a small amount of cytoplasm surrounding the spindle. However, even very brief UV exposure can have negative effects on the membrane integrity, mitochondrial DNA, and further embryo developmental potential [29].

As a more stable enucleation technique, spindle imaging was introduced for the direct enucleation of oocytes using polarized light microscopy [25]. In human IVF programs, spindle imaging (in this study, denoted as the Oosight imaging system) is mainly used to locate the meiotic spindle to avoid disrupting it while injecting oocytes [30]. This noninvasive and reliable technique has been used to improve cloning efficiency in pigs [31]. However, there has been no report of a positive effect of the Oosight imaging system on gene expression in SCNT embryos.

In this study, we examined the effect of two enucleation systems, Hoechst staining and UV irradiation (hereafter, irradiation group) and Oosight imaging (hereafter, Oosight group), on the *in vitro* production of JBC-SCNT embryos and their developmental potential. We analyzed differences in cloning efficiency with different enucleation systems on the basis of apoptosis and specific gene expression by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and semiquantitative real-time PCR.

3. MATERIAL AND METHODS

1) Preparation of donor cell

Donor somatic cells were derived from JBC ear tissue. Sliced ear tissues were incubated in 0.1% collagenase type IV solution at 38℃ for 1.5 h and cultured in donor cell culture medium [Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 1 mM sodium pyruvate, 1% nonessential amino acids, 0.1% β-mercaptoethanol, and 1% penicillinstreptomycin]. The cells were grown and sub-cultured three to five times at intervals of 4–6 days and frozen with $1x10^6$ cells in cryovials (1.5 mL) in a freezing medium [50% donor cell culture medium plus 45% FBS and 5% dimethyl sulfoxide (DMSO)]. For SCNT, frozen-thawed ear cells were washed twice with donor cell culture medium and treated with 3 mg/mL protease for 50sec at room temperature. Treated cells were washed three times and resuspended in a donor cell preparation medium [tissue culture medium 199 (TCM-199) -HEPES (Gibco) supplemented with 0.2 mM sodium pyruvate].

2) Preparation of recipient oocytes

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory within 2 h in 0.9% saline at 35℃. Cumulus oocyte complexes (COCs) were aspirated from visible follicles (2–6mm in size) with an 18-gauge needle attached to a 10 mL disposable syringe. The medium used for COCs collection was HEPES-buffered Tyrode's medium (TL-HEPES). Sets of ten COCs were *in vitro* matured in TCM-199 (Gibco) supplemented with 10% FBS, 0.2 mM sodium pyruvate, 1 μg/mL follicle-stimulating hormone (FolltropinTM, Bioniche Animal Health, Belleville, ON, Canada), 1 μg/mL estradiol-17b, and 1 mM epidermal growth factor (EGF) in mineral oil at 38.8℃ in an incubator (5% CO2, 5% O2, and 90% N2) for 18–20 h.

For enucleation in the irradiation group, denuded oocytes were labeled with 5 μg/mL Hoechst 33342 for 10 min, washed three times, and transferred into enucleation medium (TCM-199-HEPES containing 20% FBS and 7.5 μg/mL cytochalasin B). The metaphase (M) II plate and first polar body (PB1) were visualized by exposure to UV irradiation for 10 sec and removed by squeezing (Figure 1A). In the Oosight group, the MII plate and PB1 were visualized with an inverted microscope (Olympus, Tokyo, Japan) equipped with a Oosight spindle-check system (CRi, Hopkinton, MA, USA) and removed by the same squeezing method (Figure 1A).

3) SCNT

A single treated donor cell was placed in the perivitelline space of the enucleated oocytes in nuclear transfer medium [TCM-199 HEPES containing 0.06% fatty acid-free (FAF) bovine serum albumin (BSA), and 10 μg/mL phytohemagglutinin] through the opening made during enucleation. Oocyte-cell couplets were placed in cell fusion medium (0.3M mannitol, 0.5mM HEPES, 0.05mM CaCl2, and 0.1mM MgSO4) and subjected to an electrical pulse of 1.3 kV/cm for 20 μsec with an Electro Cell Fusion Generator (LF101, NEPAGENE, Chiba, Japan). After fusion, the reconstructed embryos were kept in TCM-199-HEPES supplemented with 20% FBS for 1 h, activated in 10 μM calcium ionophore for 5 min, and exposed to 2 mM 6-dimethylaminopurine for 3 h.

4) Culture of JBC-SCNT embryos

After activation, the reconstructed embryos were cultured in CR1aa medium supplemented with 0.03% FAF-BSA for 2 days. They then were co-cultured on the same JBC ear feeder cell drop in CR1aa medium containing 10% FBS, 1 μM EGF, and 1 μM insulin-like growth factor (IGF) at 38.8℃ in an incubator (5% CO2, 5% O2, and 90% N2) for 6 days.

5) Differential staining

The blastomere, inner cell mass (ICM), and trophectoderm (TE) cell numbers in blastocysts were counted by differential staining. Zona intact blastocysts were incubated in 500 μL of Solution 1 [TLHEPES containing 1% Triton X-100and 100 μg/mL propidium iodide (PI)] for 30sec. Blastocysts were then immediately transferred into 500 μL of Solution 2 (100% ethanol with 25 μg/mL bisbenzimide; Hoechst 33258) and stored at 4°C overnight. Blastocysts were mounted onto a slide glass and observed by fluorescence microscopy equipped with an ultraviolet (UV) filter. The PI and bisbenzimide-labeled TE nuclei appeared pink or red. Bisbenzimide-labeled ICM nuclei appeared blue.

6) TUNEL assay

The numbers of apoptotic cells of day 8 JBC-SCNT blastocysts produced in the two different enucleation systems were determined with the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Briefly, SCNT blastocysts were fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h, permeabilized in 0.3% Triton X-100 for 1 h, and incubated with fluorescein-conjugated deoxyuridine triphosphates (dUTPs) and terminal deoxynucleotidyl transferase enzyme in the dark for 1 h. This process was followed by incubation in 50 μg/mL RNase A for 1 h at 37°C, while the nuclei were simultaneously counterstained with 40 μg/mL PI. Stained blastocysts were loaded onto glass slides and observed by fluorescence microscopy equipped with a UV filter. Red, green, and yellow (merged) indicate chromatin, fragmented DNA, and fragmented DNA of an apoptotic blastomere, respectively. The apoptotic index was determined as the percentage of yellow blastomeres among the total number of red blastomeres.

7) Real-time RT-PCR quantification

Comparative real-time PCR was performed using a Chromo 4 (Bio-Rad) and DyNAmo HS SYBR Green qPCR kit (Finnzymes), according to the manufacturer's instructions, with the primers described in Table 2. Messenger RNA (mRNA) for real-time reverse transcriptase (RT)-PCR was prepared from 15 JBC-SCNT blastocysts from each of the two enucleation systems by using magnetic beads (Dynabeads mRNA purification kit; Dynal, Oslo, Norway). Gene expression was quantified by the 2-DDCt method [32].

8) Experiment design

To examine the effect of the two different enucleation systems on JBC-SCNT embryo production, oocytes enucleated by either Hoechst staining and UV irradiation or by the Oosight imaging system were treated under the same SCNT conditions with the same donor cells. The fusion rate, cleavage rate at day 2, blastocyst rate at day 8, total cell and ICM cell number in blastocysts, and overall efficiency were compared between the two groups. The apoptotic index for *in vitro* produced 8 day old JBC-SCNT blastocysts and the relative mRNA expression levels for seven candidate genes (Oct4, Sox2, Nanog, Interferon-tau, Caspase-3, Dnmt3A, and HSP70) were determined in both groups. Experiments were repeated six times.

9) Statistical analysis

Differences in the fusion rate, developmental rate, total cell and ICM cell number in blastocysts, overall efficiency, apoptotic index, and gene expression levels between the irradiation group and the Oosight group were examined. Differences were evaluated by analyses of variance (ANOVA) with the general linear model (PROC-GLM) in the SAS software program. Differences of $p < 0.05$ were considered significant.

4. RESULTS

Effect of the two different enucleation systems on *in vitro* development of JBC-SCNT embryos

When SCNT was used with two different enucleation systems, as shown in Figure 1A, the enucleation volumes between the two groups were not different. After enucleation by Hoechst staining and UV irradiation or the Oosight imaging system, the cell fusion rates between JBC donor cells and enucleated recipient oocytes were 62.9% (204/324) and 75.6% (242/320), respectively ($p < 0.01$). At day 2 post-activation, the cleavage rates of embryos reconstructed from the fused oocytes were 63.7% (130/204) and 78.0% (189/242) for the irradiation and Oosight group, respectively ($p < 0.05$). When the day 2 JBC-SCNT embryo quality was compared, the good quality four- to eight-cell JBC-SCNT embryo development rates were higher in the Oosight group (71.4%, 135/189) than in the irradiation group (50.8%, 66/ 130), whereas the two- to three-cell (<four cell) JBC-SCNT embryo development rates were higher in the irradiation group (49.2%, 64/130) than in the Oosight group (28.6%, 54/ 189) (each $p < 0.01$) (Table 1 and Figure 1B).

At day 8 post-activation, the blastocyst development rates of JBC-SCNT embryos were higher in the Oosight group (40.2%, 76/189) than in the irradiation group (29.2%, 38/130) ($p < 0.01$). When the expanding numbers of 8 day old JBCSCNT blastocysts were compared, the hatching blastocyst rate was higher in the Oosight group (48.6%, 37/76) than in the irradiation group (39.5%, 15/38), as shown in Figure 1B ($p < 0.05$). The average total cell number in the Oosight group (128.3±4.8) was higher than that in the irradiation group (112.2±7.6) ($p < 0.05$), but the ICM cell numbers were not different between the two groups. Finally, the overall efficiency in producing JBC-SCNT embryos was higher in the Oosight group (23.7%, 76/320) than in the irradiation group (11.7%, 38/324). Therefore, cloning efficiency was significantly different between the two groups ($p < 0.05$).

Effect of two different enucleation systems on cell death in JBC-SCNT embryos

Approximate values for the apoptotic index per blastocyst were determined by fluorescence microscopy with the TUNEL assay (Figure 1C–E). The apoptotic index of the Oosight group (2.8–0.5) was lower than that of the irradiation group (7.3–1.2) ($p < 0.05$).

Relative mRNA expression of candidate genes in JBC-SCNT embryos created by different enucleation systems

The relative mRNA expression levels of genes related to pluripotency (Oct4, Sox, Nanog), maternal recognition of pregnancy (Interferon-tau), apoptosis (Caspase-3), de novo methylation (Dnmt3A), and stress (Hsp70) were analyzed. As shown in Figure 2 and Table 3, the mRNA expression level for the core pluripotency marker gene Oct4 was significantly higher in the Oosight group (1.49-fold) compared with that of the irradiation group. However, the expression levels of Sox2 and Nanog mRNA were not different between the two groups ($p < 0.05$). The relative abundance of Interferon-tau gene expression was significantly higher (1.88-fold) in the Oosight group than in the irradiation group. Conversely, the mRNA expression levels of Caspase-3 and Hsp70 mRNA were lower (0.22- and 0.27-fold, respectively) in the Oosight group than in the irradiation group ($p < 0.05$). The mRNA expression level for the de novo methylation and methylation maintenance marker gene Dnmt3A was slightly higher (1.20fold) in the Oosight group than in the irradiation group.

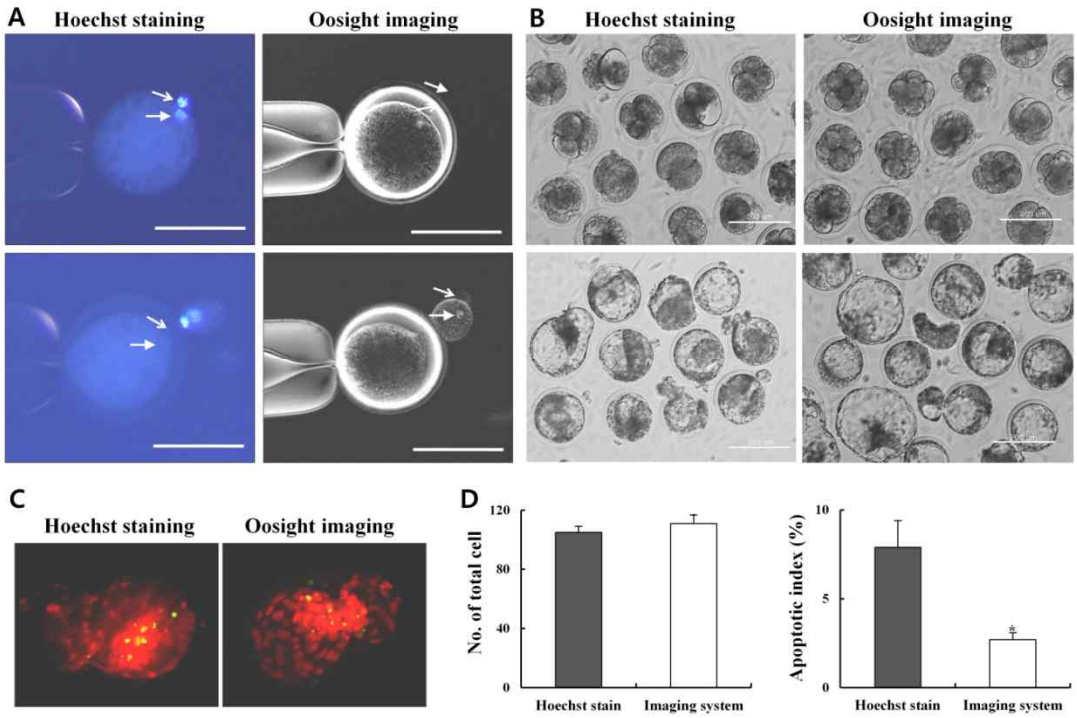


Figure 1. JBC-SCNT embryos produced *in vitro* with different enucleation systems and their TUNEL-stained fluorescence microscopic images. (A) Hoechst staining and UV irradiation and Oosight imaging systems. The arrow and arrowhead in each photograph indicate the location of the first polar body and metaphase II plate, respectively. In both enucleation systems, the karyoplasts were removed by a squeezing method. Bar, 100lm. (B) Day 2 or day 7 JBC-SCNT embryos produced *in vitro* by the two enucleation systems. More good quality four- to eight-cell embryos (arrowheads) and more rapidly developed blastocysts were produced in the Oosight group than in the irradiation group at day 2 and day 7 after SCNT. Bar, 200lm. (C) Representative fluorescence microscopic images of TUNEL-stained 8 day old JBC-SCNT embryos produced with the two enucleation systems. Propidium iodide was used to stain chromatin. Yellow indicates fragmented DNA. Bar, 200lm. The TUNEL-staining results show that the total cell numbers (D) were not different between the Hoechst staining system and the Oosight imaging system, whereas their apoptotic indexes (E) were different ($p<0.05$).

Table 1. *In vitro* Development of JBC-SCNT embryos using two different enucleation systems (r=6)

Enucleation system	No. of enucleated oocytes	No. (%)* of fused oocytes	No. (%)* of embryos developed to							Total no. of cells (ICM)	Overall efficiency (%)
			Day 2			Day 8 blastocysts					
			Total	<4 cell	≥4 cell	Total	Middle	Expanded	≥ Hatching		
Hoechst staining	324	204 (62.9)^{a}	130 (63.7)^{c}	64 (49.2)^{a}	66 (50.8)	38 (29.2)^{a}	7 (18.4)	16 (42.1)	15 (39.5)^{c}	111.2±7.6^{a} (27.4±3.4)	38/324 (11.7)^{c}
Ooisght imaging	320	242 (75.6)^{b}	189 (78.0)^{d}	54 (28.6)	135 (71.4)^{b}	76 (40.2)	10 (13.2)	29 (38.2)	37 (48.6)^{d}	128.3±4.8^{b} (35.3±4.0)	76/320 (23.7)^{d}

*Mean with different superscripts in the same column are significantly different ($^{a,b}p < 0.01$, $^{c,d}p < 0.05$)

Table 2. Oligonucleotide real-Time RT-PCR primer sequences for variable genes

Genes	Primer sequence	Annealing temp (°C)	Product size (bp)
bOct4	5′-CTCTTTGGAAAGGTGTTCAG-3′ 5′-GTCTCTGCCTTGCATATCTC-3′	53	155
bSOx2	5′-GCTGCTCTGGACTGTGCTGA-3′ 5′-ATCCAGTAATCTCCTCCAGC-3′	56	247
bNanog	5′-TGGAACAATCATTTCAACAA-3′ 5′-GCTGGGAATTGAAATACTTG-3′	54	157
bInterferon-tau	5′-ATGGCCGGCGTGCTCTCTCT-3′ 5′-AGGTCCTCCAGCTGCTGTTG-3′	55	356
bCaspase-3	5′-CGATCTGGTACAGACGTG-3′ 5′-GCCATGTCATCCTCA-3′	50	359
bDnmt3A	5′-TGATCTCTCCATCGTCAACCCT-3′ 5′-GAAGAAGGGGCGGTCATCTC-3′	54	221
bHsp70	5′-GACAAGTGCCAGGAGGTGATTT-3′ 5′-CAGTCTGCTGATGATGGGGTTA-3′	51	117
bβ-actin	5′-GTCATCACCATCGGCAATGA-3′ 5′-GGATGTCGACGTCACACTTC-3′	56	111

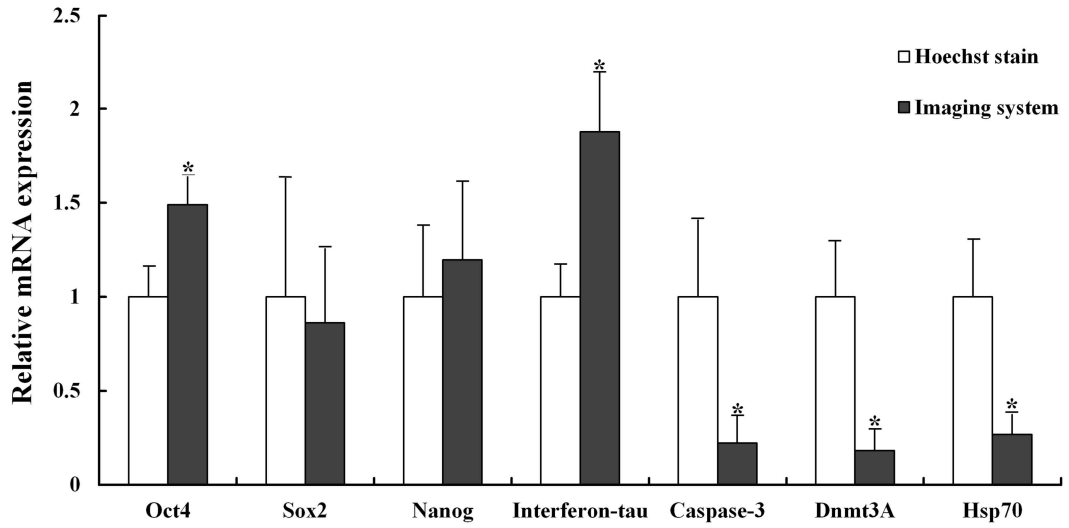


Figure 2. Relative mRNA expression of candidate genes of 8 day old JBC-SCNT embryos produced by different enucleation systems. Expression levels of genes related to pluripotency (Oct4, Sox2, and Nanog), implantation (Interferon-tau), proapoptotic activity (Caspase-3), demethylation (Dnmt3A), and stress (Hsp70) were examined. Bars with different superscripts within a panel differ significantly ($p < 0.05$). Error bars indicate standard deviation.

Table 3. mRNA expression levels of candidate genes of JBC-SCNT embryos in different enucleation system

Gene	Hoechst staining	Oosight imaging	Up-down	Specificity
Oct4	1	1.49	↑	Pluripotency
Sox2	1	0.86	↓	Pluripotency
Nanog	1	1.20	↑	Pluripotency
Interferon-tau	1	1.88	↑	Maternal recognition of pregnancy
Capase-3	1	0.22	↓	Proapoptotic
Dnmt3A	1	1.20	↑	De novo methylation
Hsp70	1	0.27	↓	Stress

5. DISCUSSION AND CONCLUSION

This study demonstrates that the direct enucleation of oocytes using the Oosight imaging system can significantly improve the *in vitro* developmental potential of bovine SCNT embryos compared with the more conservative approach of enucleation using Hoechst staining and UV irradiation. In this study, SCNT embryos were reconstructed using JBC (a species of Korean Native Cattle) ear cells as donor cells. Significant differences between the two enucleation methods were observed in the cell fusion rate, cleavage rate at day 2, blastocyst rate at day 8, and total cell number in the blastocysts. The overall efficiency after SCNT was twice as high in the Oosight group as that in the irradiation group ($p < 0.05$). Comparative TUNEL and semiquantitative RT-PCR assays indicated that the developmental potential of the Oosight group was elevated, with a high expression level of genes related to pluripotency (Oct4 and Nanog), implantation (Interferon-tau), and de novo methylation (Dnmt3A) and a low expression level of pro-apoptotic (Caspase-3) and stress (Hsp70) genes, compared with the irradiation group. This is the first report to determine the positive effect of the Oosight imaging system on molecular gene expression in the SCNT embryo. Enucleation of a recipient oocyte is crucial to cloning efficiency. The use of proper measures in enucleation can avoid problems such as aneuploidy and the related, subsequent detrimental effects on later development genetic interference of the recipient cytoplasm, and possible parthenogenetic activation and embryo development without the participation of a newly introduced nucleus [24]. Enucleation may also affect the ultrastructure of the remaining cytoplast, resulting in the decline or destruction of its cellular compartments [33]. Among the numerous enucleation methods, we selected Hoechst staining and UV irradiation as a conservative (control group) and Oosight imaging as a new (comparison group) technique. The cloning efficiency of Hoechst-stained oocytes is affected negatively by a simultaneous increase in UV irradiation, and some researchers have indicated that oocytes should not be exposed to UV for more than 30 sec [29]. On the other hand, Westhusin et al. [34] found that

exposure to UV irradiation for 10 sec had no effect on embryo viability or the production of live calves. In the present study, we attempted to decrease the UV exposure time of Hoechst-stained oocytes to the briefest time possible (< 10 sec), but differences in cloning efficiency remained between the Hoechst staining and UV irradiation system and the Oosight imaging system. Recently, we produced one cloned female Jeju Black cow through the Oosight imaging system from cells of an elite animal dead before 3 years. The transfer numbers of SCNT embryos were too low to allow valid comparison of the efficiency of cloned animal production between the Hoechst staining and UV irradiation system and the Oosight imaging system. However, TUNEL assay and molecular gene expression analysis results indicated that Oosight imaging may be a more efficient technique than Hoechst staining and UV irradiation.

DNA fragmentation in oocytes associated with apoptotic evidence may indicate poor oocyte quality and lower fertility [35]. Using the TUNEL assay, we confirmed that the DNA fragmentation frequency was significantly lower in the Oosight group than in the irradiation group. In addition, the expression of the pro-apoptotic gene Caspase-3 was significantly lower in the Oosight group compared with their irradiation group. During SCNT procedures, oocytes were exposed to several instances of heat shock stress during the course of the many mechanical and chemical treatment steps involved in these procedures. Heat shock induces apoptosis in preimplantation embryos in a developmentally regulated manner [36]. We observed that heat shock gene (Hsp70) expression was significantly lower in the Oosight group than in the irradiation group. This result is similar to that of the pro-apoptotic Caspase-3 gene expression. These results indicate that the Oosight imaging system is a less stressful method for the production of SCNT embryos than the Hoechst staining and UV irradiation system.

Important genes, such as those related to pluripotency (Oct4, Nanog), de novo methylation (Dnmt3A), and implantation (Interferon-tau), which affect the *in vitro* and *in*

vivo developmental potential of SCNT embryos, were relatively highly expressed in the Oosight group compared with the irradiation group. The octamer-binding transcription factor Oct4 is a master key regulator that is expressed at the beginning of mammalian embryogenesis. Observed variations in the level and pattern of Oct4 expression might be responsible for at least some of the problems related to cloning [37]. The level of Oct4 expression might regulate cell lineage commitment in that a critical level of expression is required to maintain pluripotency [38]. Nanog is also a crucial pluripotency factor, expressed in ICM cells with Oct4. The Nanog expression level is directly related to epiblast formation at later stages [39]. The present study found that the two pluripotency factors, Oct4 and Nanog, were highly expressed in SCNT embryos of the Oosight group compared with those of the irradiation group.

DNA methylation in embryos changes in a very organized manner to set up imprinting patterns that are vitally important for numerous biological events. Dnmt3A is thought to be essential for the establishment of de novo methylation patterns during gametogenesis and mammalian development [40, 41]. Abnormalities observed in cloned animals suggest that imprinted genes and problems related to unsuccessful epigenetic reprogramming might be serious contributors to the failed development of cloned embryos [42]. The Dnmt3A expression level was higher in the Oosight group compared with the irradiation group, although this difference was not significant. This result suggests that the Oosight imaging method may be more helpful in furthering the developmental potential of SCNT embryos than the Hoechst staining and UV irradiation method.

Interferon-tau is exclusively secreted by the trophectodermal cells of blastocysts. It primarily functions in the maternal recognition of pregnancy in cattle [43]. Higher mRNA expression levels of Interferon-tau indicate good quality embryos. In this study, there was a significant difference in the Interferon-tau mRNA expression level between the two groups,

suggesting that SCNT embryos of the Oosight group had better developmental potential than those of the irradiation group.

Taken together, the results of this study demonstrate that direct enucleation using the Oosight imaging system has positive effects on the production of SCNT embryos compared with Hoechst staining and UV irradiation. Using real-time imaging with the easy-to-use software of the Oosight imaging system, the MII plate and PB1 of recipient oocytes were easily removed without the damaging membrane or cytoplasmic integrity [44]. In conclusion, Oosight imaging is an efficient and reliable enucleation technique that could improve the cloning efficiency and developmental potential of SCNT embryos by being less damaging to unknown cellular mechanisms.

CHAPTER 3. The *in vitro* development of SCNT embryos treated with flavonoids and the production of cloned Jeju Black Cattle

1. ABSTRACT

This study was to investigate the effect of flavonoid treatment on *in vitro* development of bovine SCNT embryos, and their pregnancy and delivery rate after embryo transfer into recipient. In experiment 1, to optimize the flavonoid concentration, parthenogenetic day 2 (≥ 2 cell) embryos were cultured in 0 (control), 1, 10 and 20 μm flavonoid for 6 days. In the results, *in vitro* development rate was highest in 10 μm flavonoid group (57.1%) among treatment groups (control, 49.5%; 1 μm, 54.2%; 20 μm, 37.5%), and numbers of total and ICM cells were significantly ($p < 0.05$) higher in 10 μm flavonoid group than other groups. We found that 10 μm flavonoid treatment can significantly ($p < 0.05$) decrease the apoptotic index and derive high expression of anti-oxidant, anti-apoptotic, cell growth and development marker genes such as MnSOD, Survivin, Bax-inhibitor, Glut5, In-tau, compared to control group. In experiment 2, to produce the cloned JBC, beef quality index grad 1 bull somatic cells were transferred into enucleated bovine MII oocytes and reconstructed embryos were cultured in 10 μm flavonoid added medium. When the *in vitro* produced day 7 or 8 SCNT blastocysts were transferred into a number of recipients, 10 μm flavonoid treatment group presented higher pregnancy rate (10.2%, 6/59) than control group (5.9%, 2/34). Total three cloned Jeju Black calves were born. Also, two cloned calves in 10 μm flavonoid group were born and both were all healthy at present, while the one cloned calf born in control group was dead one month after birth. In addition, when the result of short tandem repeat marker analysis of each cloned calf was investigated, microsatellite loci of 11 numbers matched genotype between donor cell and cloned calf tissue. These results demonstrated that the flavonoid addition in culture medium may have beneficial effects on *in vitro* and *in vivo* developmental capacity of SCNT embryos and pregnancy rate.

2. INTRODUCTION

SCNT has been successful in many species of mammals. In 1997, Wilmut et al. produced a cloned sheep using SCNT for the first time. Since then, clones of various species have been reported, including a mouse [2], goat [45], pig [4], and cat [46]. The production of cloned cattle was reported by Kato et al. [47] and Cibelli et al. [48] in 1998, and Im et al. [49] have reported on the production of cloned Hanwoo Korean native cattle. However, the efficiency of SCNT is lower than IVF, and the delivery rate has been reported as less than 7% [50, 51]. The reason for the low production efficiency of the cloned cattle may be found in the mechanical and chemical process stresses due to the long-term, unstable in vitro culture environment, resulting in implantation failures after the embryos are transferred and a low delivery rate caused by incomplete implantation. Therefore, SCNT embryos require better in vitro culture conditions than IVF embryos because the production process of SCNT embryos is more likely to induce unstable proliferation than IVF embryos.

In typical in vitro culture conditions, oxygen saturation is high, causing ROS to accumulate in the cytoplasm of the embryo at greater rates than in vivo conditions, inhibiting development. The ROS react with macromolecules such as proteins, lipids and DNA in the cytoplasm, and result in enzyme activity inhibition, mitochondrial dysfunction, and DNA fragmentation [52, 53]. In vitro oocytes or embryos have no way to prevent high oxygen concentration, and therefore, studies have been done to improve the developmental potential culture with anti-oxidant supplements. For example, cystein or β-mercaptoethanol is known to enhance glutathione synthesis and improve the developmental potential of bovine embryos by controlling the reducing environment, and vitamin E is known to inhibit cell membrane injury caused by ROS [54–56]. As a result, it is very important to supplement the in vitro culture with anti-oxidant. Therefore, the development of new anti-oxidant supplements for culturing in vitro oocytes and embryos is in progress. Flavonoids, which are newly developed anti-oxidant supplements, consist of a shared structure of monomers, such as

flavonol, flavone, flavanol and flavanone, and some flavonoids are known to have potent anti-tumor and anti-oxidant functions that regulate the apoptotic cell cycle and cell differentiation. In addition, the direct function of flavonoids is to prevent the early occurrence of oxygen radicals and thereby block the oxidative chain reaction, and it is also known to form a salt with a transition metal ion to inhibit oxidation (Figure 3) [57].

Although studies on similar components, such as green tea polyphenol, have been reported in the in vitro culture of bovine embryos [58], there has been no report on the direct effect of synthetic flavonoids. This study was carried out with SCNT using the somatic cells of the superior breeding bull for the species conservation of the endangered JBC, and investigated the effect of flavonoid treatment on the in vitro development of bovine SCNT embryos by analyzing their pregnancy and delivery rate after embryo transfer into the recipient.

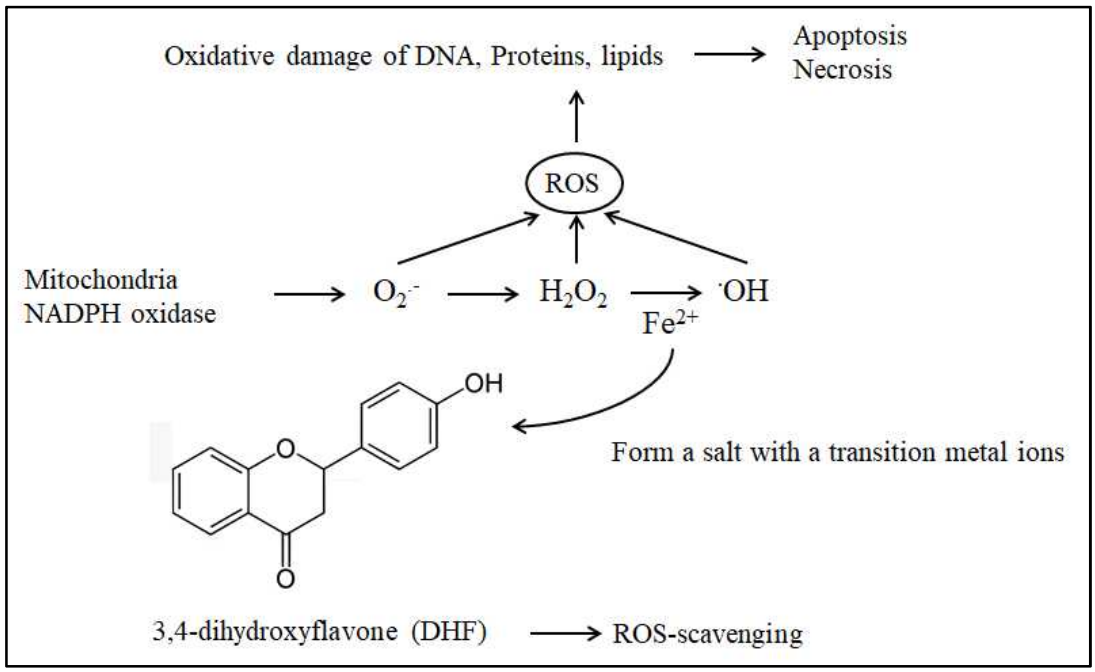


Figure 3. The structures and functions of flavonoid. Flavonoids may inhibit the generation of primary oxygen radicals and subsequent oxidation chains, since they are effective chelators of transition metal ions. The number of hydroxyl substitutions in a flavonoid is thought to be a critical factor in its ROS-scavenging ability [59].

3. MATERIAL AND METHODS

1) Oocyte preparation and *in vitro* maturation

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory within 2 h in 0.9% saline at 35°C. Cumulus oocyte complexes (COCs) were aspirated from visible follicles (2–6 mm in size) with an 18 gauge needle attached to a 10 mL disposable syringe. The medium used for COCS collection was HEPES-buffered Tyrode's medium (TL-HEPES). Sets of 30–40 COCs were in vitro matured in each well of 4-well using 500 uL tissue culture medium 199 (TCM-199, Gibco) supplemented with 10% fetal bovine serum (FBS), 0.2 mM sodium pyruvate, 1 μg/mL follicle-stimulating hormone (FolltropinTM, Bioniche Animal Health, Belleville, ON, Canada), 1 μg/mL estradiol-17β, and 1 mM EGF in mineral oil at 38.8 °C in an incubator (5% CO_2, 5% O_2, and 90% N_2) for 18–21 h.

2) Parthenogenetic activation and *in vitro* culture

The matured oocytes were activated with 10 μM calcium ionophore in Charles Rosenkrans 1 medium with amino acids (CR1aa) containing 3 mg/mL bovine serum albumin (BSA) for 5 min and immediately placed in 2 mM 6-dumethylaminopurine (DMAP) for 3 h. After activation, the reconstructed embryos were cultured in CR1aa medium supplemented with 0.03% FAF-BSA for 4 days. At day 4, they were cultured in CR1aa medium containing 10% FBS at 38.8°C in an incubator (5% CO_2, 5% O_2, and 90% N_2) for 4 days. At this time, embryo development was examined by control group and flavonoid treatment groups with 1, 10 and 20 μm, and flavonoid was added to the culture medium of every 2 days.

3) Differential staining

The blastomere, inner cell mass (ICM), and trophectoderm (TE) cell numbers in blastocysts were counted by differential staining. Zonaintact blastocysts were incubated in

500 μL of Solution 1 [TLHEPES containing 1% Triton X-100and 100lg/mL propidium iodide (PI)] for 30 sec. Blastocysts were then immediately transferred into 500 μL of Solution2 (100% ethanol with 25 μg/mL bisbenzimide; Hoechst 33258) and stored at 4°C overnight. Blastocysts were mounted onto a slide glass and observed by fluorescence microscopy equipped with an ultraviolet (UV) filter. The PI and bisbenzimide-labeled TE nuclei appeared pink or red. Bisbenzimide-labeled ICM nuclei appeared blue.

4) TUNEL assay

The numbers of apoptotic cells of day 8 JBC-SCNT blastocysts were determined with the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Briefly, SCNT blastocysts were fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h, permeabilized in 0.3% Triton X-100 for 1 h, and incubated with fluorescein-conjugated deoxyuridine triphosphates (dUTPs) and terminal deoxynucleotidyl transferase enzyme in the dark for 1 h. This process was followed by incubation in 50 μg/mL RNase A for 1 h at 37°C, while the nuclei were simultaneously counterstained with 40 μg/mL PI. Stained blastocysts were loaded onto glass slides and observed by fluorescence microscopy equipped with a UV filter. Red, green, and yellow (merged) indicate chromatin, fragmented DNA, and fragmented DNA of an apoptotic blastomere, respectively. The apoptotic index was determined as the percentage of yellow blastomeres among the total number of red blastomeres.

5) Real-time RT-PCR quantification

Comparative real-time reverse transcription (RT)-PCR was performed using a Chromo 4 detector (Bio-Rad) and a DyNAmo HS SYBR Green qPCR Kit (FINNZYMES), according to the manufacturer's instructions. The primers are described in Table 5. Magnetic beads (Dynabeads mRNA Purification Kit; Dynal, Oslo, Norway) were used to prepare

mRNA from 15 blastocysts per group. Gene expression was quantified by the 2-DDCt method.

6) SCNT and *in vitro* culture

For SCNT, thawed ear cells were plated in a culture dish with donor cell culture medium and cultured for 2–3 day before preparation as donor cells. On the day of the experiment, donor cells were recovered using TrypLE solution. Donor cells were then washed three times and re-suspended in donor cell preparation medium [TCM199-HEPES (Gibco) supplemented with 0.2 mM sodium pyruvate]. The donor cell was collected and stored frozen from the ear cells of JBC (BK01-10, BK94-13) in Jeju Special Self-Governing Livestock Promotion Agency in 2008. For enucleation, denuded oocytes using 0.1% hyaluronidase were labeled with 5 μg/mL Hoechst 33342 for 10 min, washed three times, and transferred into enucleation medium (TCM-199-HEPES containing 20% FBS and 7.5 μg/mL cytochalasin B). The metaphase (M) II plate and first polar body (PB1) were visualized by exposure to UV irradiation for 10 sec and removed.

A single treated donor cell was placed in the perivitelline space of the enucleated oocyte in nuclear transfer medium [TCM199-HEPES containing 0.6% fatty acid-free (FAF) BSA and 10 μg/mL phytohaemagglutinin] through the opening made during enucleation. Oocyte donor cell couplets were placed in cell fusion medium (0.3 M mannitol, 0.5 mM HEPES, 0.05 mM $CaCl_2$ and 0.1 mM $MgSO_4$) and subjected to an electrical pulse of 1.3 kV/cm for 20 μs (delivered via an Electro Cell Fusion Generator, Model LF101; NEPA#GENE, Chiba, Japan). After fusion, the reconstructed embryos were maintained in TCM199-HEPES supplemented with 20% FBS for 1 h, activated in 10 μM calcium ionophore for 5 min and then exposed to 2 mM 6-dimethylaminopurine for 3 h. After activation, the reconstructed embryos were cultured in CR1aa medium supplemented with 0.3% FAF-BSA for 2 days. They were then co-cultured for 6 days on the same JBC ear

feeder cells in CR1aa medium containing 10% FBS at 38.8°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. From day 2, they were cultured in separating control and flavonoid treatment group.

7) SCNT embryo transfer and production of cloned calf

The recipient was inserted CIDR-PLUS for 7 days in vagina, and then removed it and administered PGF_2 α 25mg on the day, in this way, estrus synchronization was induced. There were selected the recipient that shown the normal estrus and have a good state of corpus-luteum through rectal palpation, and then flavonoid treated or non-treated SCNT blastocysts (each 1–2) were transferred uterine corpus with luteum. At this time, SCNT blastocysts were transported from Seoul to Jeju by airplane, and it took about 4 h to get them out of the incubator and transferred into surrogate recipients. Pregnancy of the recipient was confirmed by rectal palpation at 2 months after the embryo transfer. 10–20 days before the delivery, we observed the sign of delivery of the recipients, and allowed normal delivery at the appropriate time.

8) DNA analysis

DNA from the cloned JBC and donor JBC were analyzed using 11 International Society of Animal Genetics (ISAG) standard microsatellite markers (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225 and BM1824). In brief, a QIAamp DNA Micro Kit (QIAGEN) was used to isolate genomic DNA from umbilical cord tissue obtained during delivery. Bovine autosomal microsatellite markers were then amplified by polymerase chain reaction (PCR) with fluorescent-labeled primers. PCR product polymorphisms were analyzed by electrophoresis using an Applied Biosystems 3130XL Genetic Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and GeneMapper ID software (version 3.2, Applied Biosystems, Life Technologies).

9) Statistical analysis

In vitro developmental rate of parthenogenetic and SCNT embryos, total cell and ICM cell number in blastocysts, apoptotic index, and gene expression levels between control group and the flavonoid treatment group were examined. Differences were evaluated by analyses of variance (ANOVA) with the general linear model (PROC-GLM) in the SAS software program. Differences of $p < 0.05$ were considered significant.

4. RESULTS

Effect of flavonoid treated development of in vitro parthenogenetic embryos

Table 4 shows that the in vitro parthenogenetic embryos were treated in a culture medium with various concentrations of flavonoids. When the culture medium was treated with 0, 1, 10, and 20 μm flavonoids, at day 8, blastocyst development rates were 49.5%, 54.2%, 57.1% and 37.5%, respectively, and there was no significant difference in the development rate of the 10 μm treatment group compared to the control group. On the other hand, the 10 μm treatment group and 1 μm treatment group showed similar development rates, while the 20 μm treatment group had a significantly lower development rate. In addition, there was no significant difference in the total cell number and ICM number in the control, 1 and 20 μm treatment groups according to differential staining. The total cell and ICM cell number in the 10 μm treatment groups was significantly higher than those of the control and other treatment groups (Table 4 and Figure 4). Therefore, 10 μm was selected as the optimal concentration of flavonoid treatment based on the results of in vitro development and the cell count.

Effect of flavonoid treatment on apoptosis of bovine parthenogenetic embryos

Figure 5 shows the results of TUNEL analysis on each group using blastocysts to investigateion the effects of the in vitro culture on apoptosis with or without 10 μm

flavonoids. There were no differences in the total cell number between the control and the flavonoid treatment group; however, the apoptotic index was significantly lower the in 10 μm flavonoid treatment group (4±2.5) than in the control group (12±3.2).

Effects of flavonoid treatment on gene expression in bovine parthenogenetic embryos

The effects of flavonoid treatment on the gene expression of in vitro parthenogenetic embryos were investigated using real-time PCR analysis. The mRNA expression level for the oxidant marker gene SOX was lower for the 10 μm flavonoid treatment group than in the control group, and the mRNA expression level for the anti-oxidant marker gene MnSOD was higher in the 10 μm flavonoid treatment. This result shows that the flavonoids have an anti-oxidant effect. The mRNA expression level for the apoptotic marker genes Caspase-3 and Bax were also lower in the 10 μm flavonoid treatment group, and the anti-apoptotic marker genes Survivin and Bax-inhibitor were significantly increased compared to the control group. It may be said that 10 μm flavonoids had the effect of decreasing apoptosis. We confirmed that the mRNA expression level of Glut5 (metabolism) and Interferon-tau (implantation) were significantly higher in the 10 μm flavonoid treatment group than in the control group (Figure 6).

Effect of flavonoid treated development of in vitro SCNT embryos

After SCNT, the cleaved embryos were treated with 10 μm flavonoids and the in vitro development and total cell number were analyzed using differential staining. As shown in Table 6, the blastocyst development rate was higher in the flavonoid treatment group (30.7%) than in the control group (26.0%) at day 8, and the total number of cells (164.3±13.5) and ICM cell number (50.0±5.8) were significantly higher than the control group (135.6±6.3, 35.6±6.9).

The pregnancy results of SCNT embryo in vitro culture with flavonoid treatment

SCNT embryos were divided and cultured into two groups according to whether they were flavonoid treated or non-treated, and the blastocysts were then transferred into recipients at day 8. As shown in Table 7, in the control group, 2 out of 34 (5.9%) of the recipients became pregnant, whereas in the flavonoid treatment group, 6 out of 59 (10.2%) became pregnant. At 10 months after transference, 1 out of 2 in the control group was delivered, but died about 30 days after birth, while in the flavonoid treatment group, 4 out of 6 pregnancies were miscarried at 6 months, and 2 were born without issue (birth weight of 28kg, bK01-10, and birth weight of 27kg, BK94-13; Figure 7).

Confirming the genetic parentage of the cloned calves

The result of short tandem repeat (STR) marker analysis of the ear cells of the two cloned calves born in the flavonoid treatment group and the muscle cells of the JBC somatic cell and the surrogate mother cell were investigated. The microsatellite loci of 11 numbers matched the genotype between the donor cell and the cloned calf's tissue rather than the surrogate mother's, and the gender was male. Figure 8 shows the analysis of the genetic parentage testing of [BK01-10; 3. 11, 2009].

Table 4. Effect of flavonoid treatment on *in vitro* development of bovine parthenogenetic embryos

Flavonoid treatment (μM)	No. (%) * of embryos developed to				No. of total cells (ICM)*
	Day 2 ≥ 2–4 cell	Day 4 ≥ 4–8 cell	Day 6 ≥ morula	Day 6 ≥ blastocysts	
0	107	98 (91.5)	89 (83.1)	53 (49.5)^{ab}	112.3±13.7^{a} (29.3±2.5) ^{a}
1	103	91 (88.3)	75 (72.8)	56 (54.2)^{a}	117.0±14.2^{a} (34.3±7.6)^{a}
10	112	105 (93.7)	98 (87.5)	64 (57.1)^{a}	139.2±9.0)^{b} (47.8±7.8)^{b}
20	112	102 (91.0)	96 (85.7)	42 (37.5)^{b}	133.2±10.5)^{ab} (26.8±11.0)^{a}

* ($p < 0.05$)$^{a-b}$

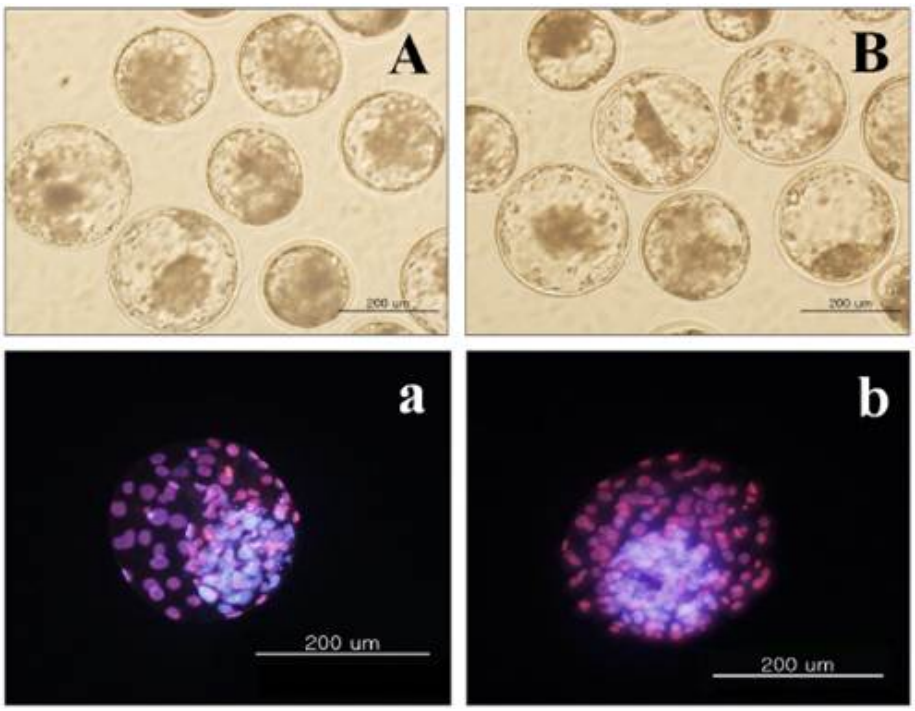


Figure 4. *In vitro* development of bovine parthenogenetic day 8 blastocysts and differential stained inner cell mass and trophectoderm cells. A-a; control, B-b; 10 μM flavonoid treatment group.

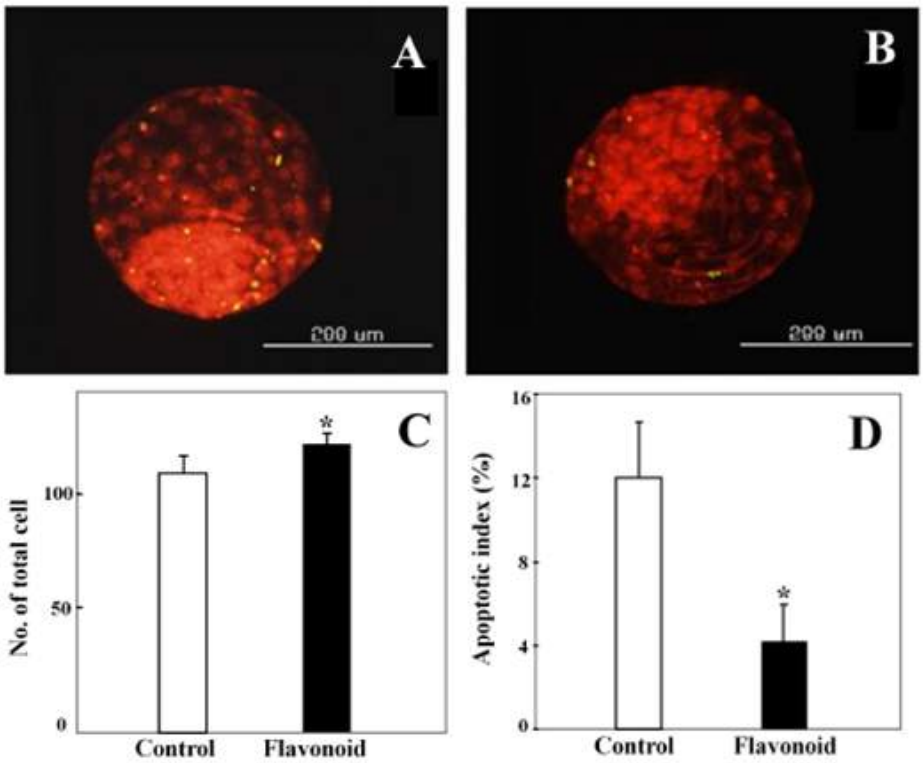


Figure 5. Flurescence microscopy images of nuclei of bovine parthenogenetic day 8 blastocysts by TUNEL asining. (A) control gorup; (B) 10 μM flavonoid treatment group; Green: fragmented DNA, red: chromain. The total coll number of cells (C) and apoptotic index (D) of bovine day 8 blastocysts cells. Star indicated statistically significant differences between two groups ($p < 0.05$).

Table 5. Primer sequences and cycling conditions used in real-time PCR

Genes	Primer sequence	Annealing temp.	Product size (bp)
bH2A	5′-GTCTTGGAGTACCTGACCGC -3′ 5′-ACAACGAGGGCTTCTTCTGA-3′	56C	201
bSox2	5′-GCTGCTCTGGACTGTGCTGA-3′ 5′-ATCCAGTAATCTCCTCCAGC-3′	56C	247
bMnSOD	5′-AGCACGAGCAGGAGACTGGT-3′ 5′-GTCCAGAAGATGCTGTGA-3′	56C	287
bCaspase-3	5′-CGATCTGGTACAGACGTG-3′ 5′-GCCATGTCATCCTCA-3′	50C	359
bSurvivin	5′-CCTGGCAGCTCTACCTCAAG-3′ 5′-TAAGTAGGCCAACACGAAAG-3′	56C	233
bBax	5′-GCTCTGAGCAGATCAAG-3′ 5′-AGCCGCTCTCGAAGGAAGTC-3′	56C	400
bBax inhibitor	5′-GCTCTGGACTTGTGCATT-3′ 5′-GCCAAGATCATCATGAGC-3′	56C	374
bGlut-5	5′-TTGGAGAGCCAGTGAACAGT-3′ 5′-TGCTGATAACTGTCTGCGCT-3′	60C	292
bInterferon-tau	5′-ATGGCCTTCGTGCTCTCTCT-3′ 5′-AGGTCCTCCAGCTGCTGTTG-3′	55C	356

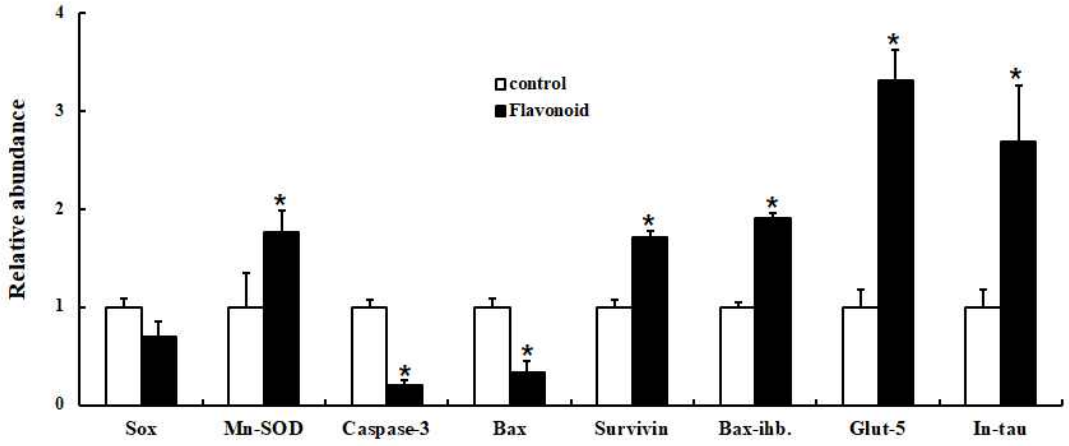


Figure 6. Relative abundance of oxidant, apoptotic and growth mRNA expression in control or 10 μM flavonoid treated bovine parthenogenetic day 8 blastocysts. Significant differences are indicated by * ($p < 0.05$). Values are mean±SEM of three separate experiments.

Table 6. Effect of flavonoid treatment on *in vitro* development of JBC-SCNT embryos

Flavonoid treatment (μM)	No. (%) * of embryos developed to				No. of total cells (ICM)*
	Day 2 ≥ 2–4 cell	Day 4 ≥ 4–8 cell	Day 6 ≥ morula	Day 6 ≥ blastocysts	
0	119	82 (68.9)	69 (57.9)	31 (26.0)	135.6±6.3[a] (29.3±2.5)[a]
10	103	91 (88.3)	75 (72.8)	56 (54.2)[a]	164.3±13.5)[b] (50.0±5.8)[b]

*($p < 0.05$)

Table 7. JBC-SCNT embryo transfer and their pregnancy

Flavonoid treatment (μM)	No. of transfer surrogate mother	No. of pregnancy	No. of birth calf	No. of living calf
0	34	2 (5.9%)	1	-
10	59	6 (10.2%)	2	2

*($p < 0.05$)[a-b]

Figure 7. Breeding bull provided somatic cell (left, BK01-10) and five month old SCNT transfer JBC mal calf “Heuk Young Dolee” (Birth weight; 28kg, Mar. 11, 2009).

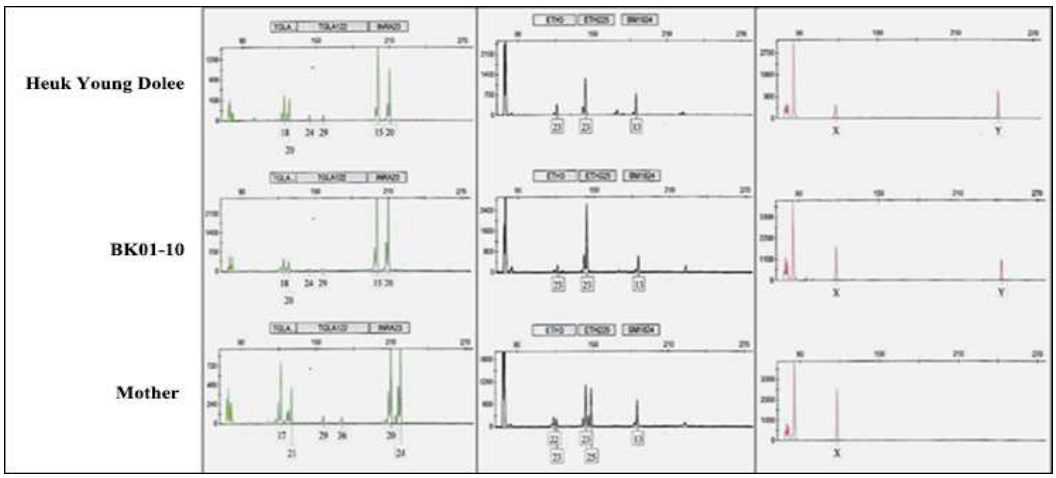


Figure 8. STR (short tandem repeat) profile investigated full DNA fingerprint of cloned calf (Heuk Young Dolee, BK01-10), somatic cell and surrogate mother using STR marker.

5. DISCUSSION AND CONCLUSION

When a JBC-SCNT embryo was cultured in vitro with a synthetic flavonoid as an anti-oxidant, there was an improved in vitro development rate, total cell number and ICM cell number of blastocysts, pregnancy rate, and production rate after embryos were transferred. It was shown that flavonoids were useful as culture supplements for SCNT embryos. In vitro parthenogenetic embryos were cultured with different amounts of flavonoid supplement, in order to determine the proper concentration and treatment effect of flavonoid prior to application to the SCNT embryos. When the 1, 10 and 20 μM flavonoid concentrations were compared with the control group, it was confirmed that the developmental rate and total cell number increased to a 10 μM concentration, and an increase in the ICM cell number was confirmed. There was no difference in the effect of the concentration below 10, but a concentration of 20 showed a significantly lower developmental rate. Anti-oxidant activity was observed at low concentrations, however, similar to in other studies, high concentrations above the appropriate level created oxidation [59].

This study confirmed by TUNEL assay that the apoptotic index of the in vitro parthenogenetic embryos cultured with flavonoids was significantly decreased. As previously mentioned, flavonoids are anti-oxidants that reduce oxygen radicals and nitrogen free radicals and inhibit the chain oxidation reaction by preventing the primary steps that produce oxygen radicals. They are known to have anti-oxidant functions that inhibit tumorigenesis, death, differentiation and cell cycle regulation [57]. This function of flavonoid supplements has been confirmed in gene expression studies. The expression of MnSOD, an endogenous anti-oxidant gene, was significantly higher in the flavonoid treatment group, and the expression of anti-apoptotic genes such as Survivin and Bax-inhibitor 1 was found to be significantly increased when compared to the control group using real-time PCR. The flavonoids used in this study suppressed the death factor and also

significantly increased the expression of Glut5, a growth-related metabolic gene that has anti-oxidative activity and anti-apoptotic effects, and In-tau, an implantation gene. These effects are considered to enable the development of SCNT embryos by promoting healthy embryo development overall.

SCNT is a technique for the mass production of genetically identical cloned animals, in which new embryos are generated by transplanting already-differentiated somatic cells into enucleated, unfertilized embryos to reprogram their nucleus. So far, many researchers have been conducting various studies on SCNT and the development of cloned embryos [60, 61]. In this study, the ear cells of two JBC breeding bulls that were held at the Jeju Special Self-Governing Livestock Promotion Agency were harvested in 2007, cultured and vitrified. Using healthy somatic cells corresponding to 5–15 passages nuclear transfer was performed. In addition, the blastocysts, which were cultured, treated and non-treated with flavonoids for 7–8 days, were delivered by plane from Seoul to Jeju, and approximately four hours elapsed between the incubator and the embryo transfer into the surrogate cow. At this time, SCNT blastocysts were transported in a thermos to maintain the proper temperature, and saved in straws filled with the culture medium in both the flavonoid and no flavonoid conditions. These embryo transfer systems have already been established in this research institute for the production and transplant of elite IVF embryos, resulting in a 44% (18/44) delivery rate. When the in vitro blastocyst is transferred from Seoul to Jeju within 4 hours and the flavonoid is included in the culture medium during transportation, the blastocyst's health is maintained and the pregnancy rate is not decreased [62]. In addition, this study found that flavonoid treatment significantly increased the anti-oxidative and anti-apoptotic effects of the SCNT embryos, increased metabolism-related gene expression and increased the expression of the implantation gene. Anti-apoptotic effects help cell division and the development of embryos. As a result, the total cell number as well as the ICM cell number of SCNT blastocysts with the flavonoid treatment was significantly higher than for the control. In

addition to this, the SCNT embryos cultured in the medium treated with flavonoids were born a without morphological abnormalities or abnormal organs in the body 10 months after their transplantation and grew well, without pathological disorders.

In general, cloned bovines are known to have large offspring syndrome. LEE et al. (2004) [63] reported that the cloned calves were 11– 17% heavier for in vitro fertilization and artificial insemination conditions at 150 days of gestation. On the other hand, Garry et al. (1996) [64] has reported that large offspring syndrome and malformed calves are born even when using IVF embryo blastomeres instead of somatic cells. This is not a problem for SCNT embryos. This suggests that there are various causes for occurrences during in vitro culture or replication. It is known that the production of cloned bovines from SCNT embryos is less than 7% [50], and 80% of the cows that become pregnant have a miscarriage around 90 days after transplantation due to failure of early implantation. In this study, the pregnancy rates were 5.9% (2/34) and 10.2% (6/59) in the control and flavonoid treatment groups, respectively. In the control group, 1 out of 2 died within 30 days of birth, and in the flavonoid treatment group, 2 out of 6 (33.3%) cloned calves were born healthy at 282 days and 292 days of gestation, respectively.

Of these two calves, the first cloned JBC breeding bull ("Heuk Young Dolee", Figure 7) was born on March 11, 2009, and replicated a superior amount of the quality meat traits from the best breeding bull (BK01-10). The second cloned JBC was born on September 9, 2009, and replicated excellent meat quality, a high degree of intramuscular fatness and became the first class incidence rate 95% or more breeding bull (BK94-13). Of note is the fact that the second cloned JBC used cryopreserved somatic cells, confirming that it is possible to restore this excellent species if somatic cells are secured.

One pregnant cow in the control group and four pregnant cows in the treatment group suffered miscarriages due to differences in health and specimen management status of the grazing surrogate cow or due to the abnormal intrauterine implantation of the fetus. The

SCNT process may be weakened by a large number of mechanical processes and chemical exposures during long periods of time, and in order to improve the birth rate of offspring using SCNT blastocysts, flavonoid treatment is required to serve as an anti-oxidant capable of rapidly recovering functions after nuclear transfer. However, various other solutions, such as the selection of healthy surrogate cows and the management of specimens after implantation, may be even more critical.

CHAPTER 4. Investigation of the developmental potential and developmental kinetics of using a time-lapse monitoring system

1. ABSTRACT

A time-lapse monitoring system has predictive value for selecting good quality embryos with the highest implantation potential. Using this new tool, we investigated the developmental potential and developmental kinetics of bovine parthenogenetic (PA) and two types of somatic cell nuclear transfer (NT) embryos. Bovine non-transgenic ear cells (bECs) or transgenic cells (bTGCs) were used as donor cells. The cleavage and blastocyst development rates did not significantly differ among the PA, NT-bEC, and NT-bTGC groups, and first cleavage occurred an average of 19.3 h (n=70), 21.6 h (n=60), and 21.3 h (n=62) after activation, respectively (20.4 h [n=192] for all embryos). When embryos were classified into early cleaving (≤ 20 h) and late cleaving (> 20 h) groups, the blastocyst formation rate was much higher in the early cleaving groups (PA, 46%; NT-bEC, 50%; NT-bTGC, 39%) than in the late cleaving groups (PA, 18%; NT-bEC, 23%; NT-bTGC, 28%), while the percentage of embryos whose development was blocked between the two- and eight-cell stages was increased in the late cleaving groups. The percentage of embryos classified as early cleaving with a normal morphology was twofold higher in the PA group (20.0%, n=14) than in the NT-bTGC group (9.7%, n=6). The timing of each developmental stage varied widely; the timing of first cleavage varied from 7.6 h in the PA group to 34.5 h in the NT-bEC group and the timing of expanded/hatching blastocyst appearance varied from 141.6 h in the PA group to 196.3 h in the NT-bTGC group, differences of 26.9 and 54.7 h, respectively (PA>NT-bEC>NT-bTGC). These results demonstrate that time-lapse monitoring provides novel data regarding individual embryo developmental kinetics and helps to predict developmental potential for improved bovine NT embryo selection based on early cleavage (≤ 20 h) and normal morphology.

2. INTRODUCTION

Successful application of the somatic cell nuclear transfer (SCNT) technique offers many opportunities in basic and medical research, and in endangered species conservation [66–68]. Although many studies have attempted to improve the efficiency of SCNT, it remains low [69, 70] To overcome this, detailed information about developmental kinetics that can be used to predict the developmental potential of individual SCNT embryos may be needed [71–73]. In general, morphological features of developing embryos are mainly used to evaluate their quality, and this depends on the subjective judgment of researchers [74].

Recently introduced innovative technologies, such as a time-lapse monitoring system, can be new alternatives to predict the developmental capacity of SCNT embryos, which are more complicated to produce than *in vitro* fertilized embryos [71]. A time-lapse monitoring system, which was originally developed by Payne et al. [75], is a noninvasive technique to select high-quality human embryos produced *in vitro* by precisely assessing embryo morphology and developmental kinetics, with the ultimate aim of increasing the pregnancy rate and reducing the number of multiple pregnancies. This technology enables safe and continuous monitoring of individual embryos and detailed study of embryo morphology and developmental kinetics by recording the timing of cleavage at each stage, the blastomere number, embryo compaction, and the extent of cytoplasmic fragmentation [76, 77]. This technique has been used in human IVF clinics, which recognize its value [78], while there are a few studies in mouse [79], hamster [80], and cow [81].

Among developmental kinetics, timing of the first cleavage is a valuable parameter of embryo quality [82, 83]. Developmental speed is directly related to developmental potential and may be an important parameter in selecting embryos for maintaining a pregnancy after transfer [84]. The time-lapse system illustrated that continuous monitoring of early embryo morphology may provide useful data for predicting subsequent developmental competence [78, 85]. In addition, analysis of developmental kinetics revealed significant differences

between *in vivo* and *in vitro* derived embryos [86, 87] and even among culture environments [88, 89]. However, there is no study comparing the developmental kinetics of bovine parthenogenetic (PA) and nuclear transfer (NT) embryos using non-transgenic or transgenic donor cells.

The objective of this study was to investigate the developmental potential of bovine activated embryos (PA and NT) classified as early cleaving (≤ 20 h) and/or with a normal morphology by analyzing the timings at which they reached each stage using a time-lapse monitoring system. These data will be helpful for setting criteria to select high quality SCNT embryos and investigating their developmental speed, and they also provide the average timings at which embryos reach each developmental stage and variation in these timings.

3. MATERIALS AND METHODS

1) Oocyte preparation and *in vitro* maturation

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory within 2 h in 0.9% saline at 35℃. Cumulus-oocyte complexes (COCs) were aspirated from visible follicles (2–6mm in size) with an 18 gauge needle attached to a 10 mL disposable syringe. The medium used for COC collection was HEPES-buffered Tyrode's medium. Sets of 30-40 COCs were matured in each well of a four-well plate using 500 μL of tissue culture medium 199 (TCM-199; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 0.2 mM sodium pyruvate, 1 μg/mL follicle-stimulating hormone (Folltropin; Bioniche Animal Health, Belleville, Canada), 1 μg/mL estradiol-17b, and 1mM epidermal growth factor (EGF) in mineral oil at 38.8C in an incubator (5% CO2, 5% O2, and 90% N2) for 18–21 h.

2) PA activation

The matured oocytes were activated with 10 μM calcium ionophore in Charles Rosenkrans 1 medium with amino acids (CR1aa) containing 3 mg/mL bovine serum albumin (BSA) for 5 min and immediately placed in 2 mM 6-dimethylaminopurine for 3 h.

3) Preparation of donor cells

Non-transgenic cells (bovine non-transgenic ear cells [bECs]) were derived from the ear tissue of Hanwoo cattle (Korean native cattle). The cell line preparation was described by Kim et al. [70]. Briefly, minced ear tissue was bAU5 incubated in 0.1% collagenase type I solution at 38 ℃ for 2.0 h and then cultured in donor cell culture medium (Dulbecco's modified Eagle's medium containing 10% FBS, 1mM sodium pyruvate, 1% nonessential amino acids, 0.1% β-mercaptoethanol, and 1% penicillin-streptomycin). After three to five subcultures, cells ($1x10^6$) were frozen in cryovials (1.5 mL; NUNC, Roskilde, Denmark) in donor cell freezing medium (50% donor cell culture medium containing 45% FBS and 5% dimethyl sulfoxide). For SCNT, frozen-thawed bECs were washed twice with donor cell culture medium and then treated with 3 mg/mL protease for 50 sec at room temperature. Treated cells were washed three times and re-suspended using donor cell preparation medium (TCM-199-HEPES supplemented with 0.2mM sodium pyruvate). Bovine transgenic cells (bTGCs) were prepared from human fibroblast growth factor 2 (FGF2) gene-knock-in ear fibroblasts of 1-month-old dairy cattle. The cell line preparation was described by Jeong et al. [90]. Briefly, to construct the knock-in vector, F2A (XhoI-KpnI) and human FGF2 complementary DNA (cDNA) (KpnI-SmaI) fragments were first ligated into the XhoI-SmaI site of the pBKS(+) plasmid. The 5¢-arm fragment (NotI-SalI) was inserted into the NotI-XhoI site of the pBKS-F2A-hFGF2 plasmid. Then, the 5¢-arm-F2A-hFGF2 fragment from the pBKS-5¢-arm-F2A-hFGF2 plasmid was inserted into the NotI-SmaI site of the pMCDT-A (A+T/pau) plasmid (Gibco BRL). The BGH polyA (SmaI-EcoRI) and PGK-neo (EcoRIBamHI) fragments were ligated into the SmaI-BamHI site of the pBKS(+) plasmid.

Next, BGH polyA-PGK-neo (SmaIBamHI) from the pBKS-BGHp-PGKneo plasmid and the 3¢-arm (BamHI-SalI) fragment were ligated into the pBSK(+) plasmid. Finally, to generate the knock-in vector, the BGHp- PGKneo-bbC3¢-arm fragment was inserted into the SmaIXhoI site of the pMCDT-5¢-arm-F2A-hFGF2 plasmid. Bovine ear fibroblasts were electroporated using the linearized knock-in vector on the BTX Electro Cell manipulator (ECM 2001; BTX, Holliston, MA), cultured in a 24-well plate (4000 cells/well) for 48 h, and then selected using 500 μg/mL G418 for 10–12 days. After selection, analyzed transgene-positive colonies were cultured in scale-up conditions, and then cells were frozen using donor cell freezing medium. For SCNT, frozen-thawed bTGCs were treated as described above.

4) SCNT

SCNT of bovine oocytes was carried out as described previously [91]. For enucleation, cumulus cells were completely removed from the oocyte by vortexing for 3 min in the presence of 0.05% hyaluronidase. Oocytes with an extruded first polar body (PB1) were selected, and denuded oocytes were transferred to enucleation medium (TCM-199-HEPES containing 20% FBS and 7.5 μg/mL cytochalasin B). Thereafter, the metaphase II plate and PB1 were visualized using an inverted microscope (Olympus, Tokyo, Japan) equipped with the Oosight imaging system (CRi, Hopkinton, MA) and removed by the squeezing method as reported previously [70].

A single treated donor cell (bEC or bTGC) was placed in the perivitelline space of the enucleated oocytes in NT medium (TCM-199-HEPES containing 0.06% fatty acidfree- BSA and 10 μg/mL phytohemagglutinin) through the opening made during enucleation. Oocyte cell couplets were placed in cell fusion medium (0.3M mannitol, 0.5mM HEPES, 0.05mM $CaCl_2$, and 0.1mM $MgSO_4$) and subjected to an electrical pulse of 1.3 kV/cm for 20 μs with an Electro Cell Fusion Generator (LF101; NEPAGENE, Chiba, Japan). After fusion, the

reconstructed embryos were kept in TCM-199 HEPES supplemented with 20% FBS for 1 h, and then activated as described above.

5) Embryo culture in a Primo Vision dish

The micro-wells form a matrix of four rows with four wells per row. The culture dish was loaded with early embryo culture medium (CR1aa containing 3 mg/mL BSA) on the day before starting time-lapse imaging to allow the media to equilibrate before adding the embryos. The dish was filled with 40–60 μL of culture medium. After activation or reconstruction, PA or NT (bEC or bTGC) embryos were cultured in early embryo culture medium at 38.5 ℃ in 5% O2, 5% CO2, and air with maximum humidity for 2 days, and then in late embryo culture medium (CR1aa supplemented with 10% FBS, 1mM EGF, and 1 μM flavonoid) for 5 days.

6) Time-lapse cinematography

Developmental kinetics was analyzed using Primo Vision as described previously [92]. *In vitro* development was monitored using a real-time cultured cell monitoring system with Imaging Capture. During the 168 h culture period, 2016 images of embryos were taken at 5 min intervals. Image stacks were analyzed using Primo Vision Analyzer Software. The timing of the first appearance of embryos at the following stages was recorded for zygotes in focus with identifiable blastomeres: 2 cell, 4–8 cell, 16 cell, 32 cell, morula, early blastocyst, and expanded blastocyst (PA group) or hatching blastocyst (NTbEC and NT-bTGC groups).

7) Experiment design

In vitro produced bovine PA and NT embryos (using non-transgenic or transgenic donor cells) were cultured in a 16-microwell Primo Vison dish and imaged using the time-lapse monitoring system. Six replicates were performed. From the time-lapse monitoring

data various parameters were analyzed. First, the average timings at which *in vitro* produced embryos in the three treatment groups (PA, NTbEC, and NT-bTGC) reached each developmental stage were examined. Second, according to the average timing of first cleavage for total embryos (20 h), embryos in the three treatment groups (PA, NT-bEC, and NT-bTGC) were categorized into two groups, early or late cleaving, and their developmental potential was analyzed. Finally, for each of the three treatment groups, the developmental potential and average timing (along with the range) at which embryos classified as early cleaving and/or with a normal morphology reached each developmental stage were examined

8) Statistical analysis

Differences were evaluated by an analysis of variance using the general linear model (PROC-GLM) in the SAS software program. $p < 0.05$ was considered significant.

4. RESULTS

***In vitro* development of bovine PA, NT-bEC, and NT-bTGC embryos assessed using a time-lapse monitoring system**

The *in vitro* development rates of bovine PA, NT-bEC, and NT-bTGC embryos were examined using a time-lapse monitoring system. The cleavage and blastocyst formation rates did not differ among the PA group (n=70, 72.9% and 37.1%, respectively), NT-bEC group (n=60, 62.5% and 33.3%, respectively), and NT-bTGC group (n=62, 64.6% and 29.0%, respectively) (PA>NT-bEC>NT-bTGC) (Table 8).

Average timings at which bovine PA, NT-bEC, and NT-bTGC embryos reached each developmental stage

From the time-lapse data of bovine PA, NT-bEC, and NT-bTGC embryos, the average timings at which embryos reached each developmental stage (2 cell, 4–8 cell, 16 cell, 32 cell,

morula, early blastocyst, and expanded or hatching blastocyst) were analyzed. First cleavage occurred an average of 19.3, 21.6, and 21.3 h after activation in the PA (n=70), NT-bEC (n=60), and NT-bTGC (n=62) groups, respectively, and there was a difference of about 2 h between the PA group and the NT groups (PA>NTbTGC‡NT-bEC) (Table 9). The average timing of first cleavage for all three groups was 20.4 h post-activation.

PA, NT-bEC, and NT-bTGC embryos reached the four to eight cell stage an average of 35.6, 36.1 and 41.4 h after activation, respectively, meaning it occurred 5–6 h later in the NT-bTGC group than in the other groups. There was a larger difference in the timings when embryos reached the 16 cell stage (PA, 61.0 h; NT-bEC, 59.4 h, and NT-bTGC, 69.7 h after activation). Embryos reached the 32 cell and morula stages faster in the PA group (80.1 and 104.9 h after activation, respectively) than in the NT-bEC group (89.0 and 109.6 h after activation, respectively) and the NT-bTGC group (91.9 and 109.4 h after activation, respectively). The average timings at which embryos reached the early blastocyst and expanded or hatching blastocyst stages were similar in the PA group (141.3 and 161.8 h after activation, respectively) and the NT-bEC group (141.9 and 162.4 h after activation, respectively), which were faster than the timings in the NT-bTGC group (148.4 and 169.0 h after activation, respectively). Total embryos reached the 4–8 cell, 16 cell, 32 cell, morula, early blastocyst, and expanded or hatching blastocyst stage an average of 37.6, 63.4, 87.0, 108.0, 143.9, and 164.4 h after activation, respectively.

When the mean timings at which embryos reached each developmental stage were plotted, the slope was less steep for the PA group than for the NT-bEC and NT-bTGC groups (Figure 9). The slope was steepest for the NT-bTGC group.

***In vitro* developmental potential of bovine PA, NT-bEC, and NT-bTGC embryos classified into early and late cleaving groups**

In vitro developmental potential of bovine PA, NT-bEC, and NT-bTGC embryos classified into early and late cleaving groups On the basis of the average timing of first cleavage for total embryos (20 h), PA, NT-bEC, and NT-bTGC embryos were classified into the early and late cleaving groups (≤ 20 h and > 20 h, respectively). The *in vitro* development rates of embryos in the early and late cleaving groups were analyzed in bF2 Figure 10. For PA embryos, the cleavage rate was higher in the early cleaving group of PA group than in the late cleaving group, in contrast with NTbEC and NT-bTGC embryos. However, the blastocyst development rate was higher in all the early cleaving groups than in the late cleaving groups (Figure 10A–C). The developmental potential of embryos in each of the groups was also investigated. The blastocyst formation rate was much higher in the early cleaving groups (PA, 46%; NT-bEC, 50%; NT-bTGC, 39%) than in the late cleaving groups (PA, 18%; NT-bEC, 23%; NT-bTGC, 28%), while the percentage of embryos blocked between the two and eight cell stages was higher in the late cleaving groups (PA, 55%; NT-bEC, 47%; NT-bTGC, 55%) than in the early cleaving groups (PA, 38%; NT-bEC, 42%; NT-bTGC, 38%) (Figure 10A'–C').

***In vitro* developmental potential and development timings of bovine PA, NT-bEC, and NT-bTGC embryos classified as early cleaving and/or with a normal morphology**

Embryos in the three treatment groups (PA, NT-bEC, and NT-bTGC) were examined for early cleaving (first cleavage ≤ 20 h), a normal morphology (no fragmentation or abnormal cytoplasmic division at each developmental stage), or both, and were classified as E, N, and E+N, respectively (Table 10). The percentage of embryos classified as E was significantly higher in the PA group (68.6%, n=48) than in the two NT groups (NT-bEC, 40.0%, n=24; NT-bTGC, 41.9%, n=26) ($p < 0.05$). The percentage of embryos classified as N was similar among the treatment groups (PA, 22.9%, n=16; NT-bEC, 26.7%, n=16; NT-bTGC, 19.4%, n=12). The percentage of embryos classified as E+N was twofold higher in

the PA group (20.0%, n=14) than in the NT-bTGC group (9.7%, n=6). *In vitro* development timings were also examined (Table 11). The average timings of first cleavage of embryos classified as E, N, and E+N in each of the treatment groups (PA, 16.4, 15.3, and 14.6 h, respectively; NT-bEC, 16.8, 19.9, and 17.4 h, respectively; NT-bTGC, 16.9, 19.8, and 16.3 h, respectively) were all earlier than that of total early cleaving embryos (20 h). For PA embryos, the E+N group showed the fastest *in vitro* development (4–8 cell, 30.5 h; 16 cell, 55.0 h; 32 cell, 80.2 h; morula, 105.6 h; early blastocyst, 127.6 h; expanded blastocyst, 157.9 h), which were the best data among all the treatment groups. For NT-bEC embryos, the E group showed the fastest *in vitro* development (4–8 cell, 31.0 h; 16 cell, 53.6 h; 32 cell, 85.3 h; morula, 107.3 h; early blastocyst, 138.7 h; hatching blastocyst, 159.8 h). For NT-bTGC embryos, the E+N group showed the fastest *in vitro* development (4–8 cell, 33.7 h; 16 cell, 67.8 h; 32 cell, 88.4 h; morula, 108.4 h; early blastocyst, 144.0 h; hatching blastocyst, 166.2 h). However, the timings at which embryos reached various developmental stages varied widely; the timing of first cleavage varied from 7.6 h in the PA group to 34.5 h in the NT-bEC group. For total embryos (n=192), the timing of first cleavage varied by 26.9 h. This variation was very high during embryo development (4–8 cell, 24.2–61.0 h, a difference of 36.8 h; 16 cell, 38.1–88.5 h, a difference of 50.4 h; 32 cell, 54.6–107.5 h, a difference of 52.9 h; morula, 85.6–125.5 h, a difference of 39.9 h; early blastocyst, 103.4–194.2 h, a difference of 90.8 h; expanded or hatching blastocyst, 141.6–196.3 h, a difference of 54.7 h). However, there was a clear difference in the developmental timings of representative embryos classified as E+N among the three treatment groups (PA>NT-bEC>NTbTGC) (Figure 11).

Table 8. *In vitro* development of bovine parthenogenetic and SCNT embryos with non-transgenic or transgenic donor cells using a time-lapse monitoring system (r=6)

Treatment group	No. of examined oocytes	No. (%) of cleaved embryos ant day 2	No. (%) of developed blastocysts at day 7
PA	96	70 (72.9)	26 (37.1)
NT-bEC	96	60 (62.5)	20 (33.3)
NT-bTGC	96	62 (64.6)	18 (29.0)

Table 9. Average developmental timings of bovine parthenogenetic and SCNT embryos with non-transgenic or transgenic donor cells using a time-lapse monitoring system (r=6)

		Average timings at which embryos reached the specified developmental stage after activation[a] (in hours)						
Treatment group	No. of examined embryos	2 cell	4–8 cell	16 cell	32 cell	Morula	Early blastocyst	Expanded or hatcing blastocyst
PA	70	19.3	35.6	61.0	80.1	104.9	141.3	161.8
NT-bEC	60	21.6	36.1	59.4	89.0	109.6	141.9	162.4
NT-bTGC	62	21.3	41.4	69.7	91.9	109.4	148.4	169.0
Total[b]	192	20.4	37.6	63.4	87.0	108.0	143.9	164.4

[a]Average data included all embryos at each developmental stage.

[b]Average timings of all three groups (PA, NT-bEC and NT-bTGC)

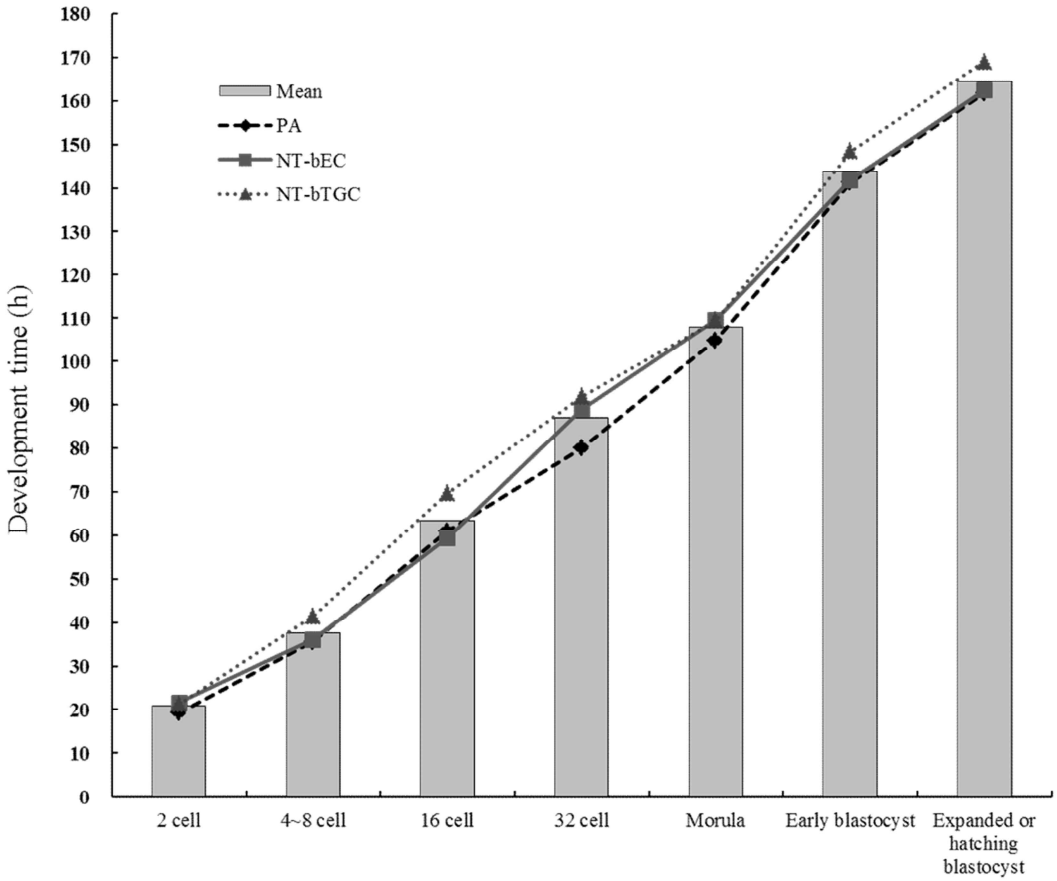


Figure 9. Time taken for bovine PA and somatic cell NT embryos generated using non-transgenic (bEC) or transgenic (bTGC) donor cells to reach the specified stage. The average data included all embryos at each developmental stage. bEC, bovine non-transgenic ear cell; bTGC, bovine transgenic cell; NT, nuclear transfer; PA, parthenogenetic.

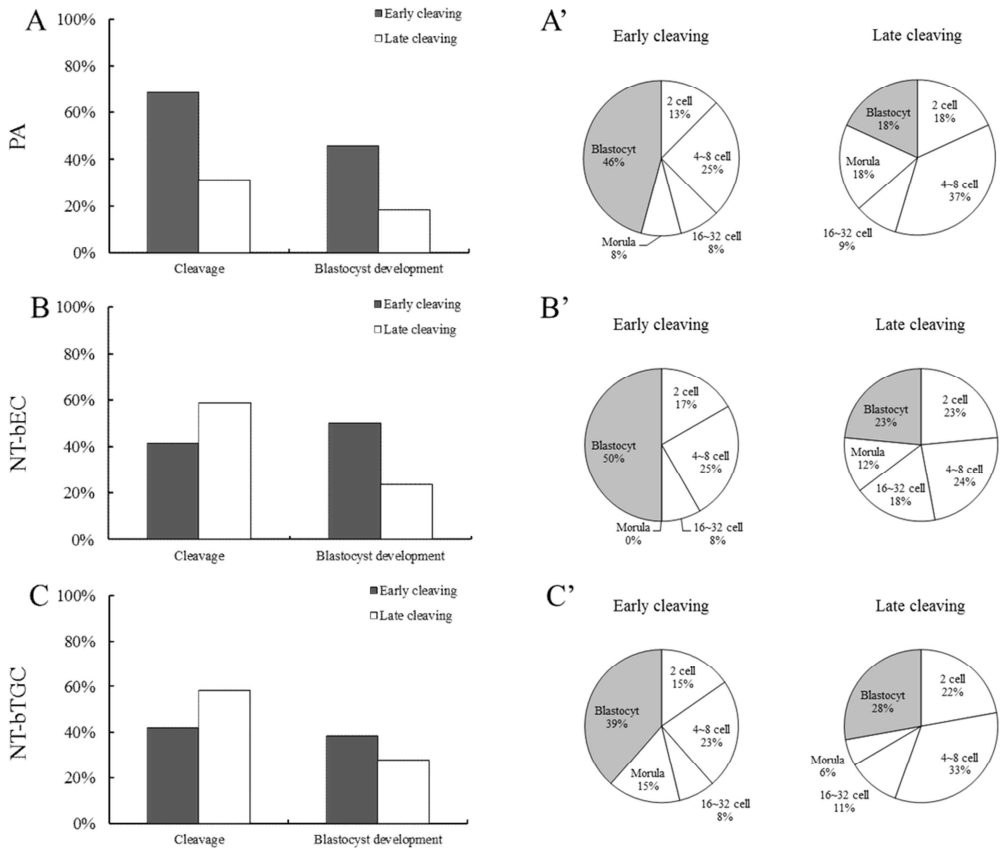


Figure 10. *In vitro* development rates of embryos classified as early and late cleaving in the PA group (A–A', PA) and the NT groups using non-transgenic cells (B–B', NT-bEC) and transgenic cells (C–C', NT-bTGC). On the basis of the average cleavage time (20 h), embryos in each treatment group were classified into the early and late cleaving groups, and their blastocyst development rates (A–C) and final developmental stages (A'–C') were examined. bEC, bovine non-transgenic ear cell

Table 10. *In vitro* development rates of bovine parthenogenetic and SCNT embryos classified as early cleaving and/or with a normal morphology (r=6)

		No. (%) of classified embryos[a]		
Treatment group	No. of examined oocytes	Early cleaving (E)[b]	Normal morphology (N)	Early cleaving and normal morphology (E+N)
PA	70	48 (68.6)[c]	16 (22.9)	14 (20.0)
NT-bEC	60	24 (40.0)[d]	16 (26.7)	10 (16.7)
NT-bTGC	62	26 (41.9)[d]	12 (19.4)	6 (9.7)

[a]Early cleaving: cleavage occurred ≤20 hours after activation

Normal morphology: embryos did not exhibit fragmentation or abnormal cytoplasmic division at each developmental stage

[b]$p < 0.05$

Table 11. *In vitro* development timings of bovine parthenogenetic and SCNT embryos classified as early cleaving and/or with a normal morphology (r=6)

			Timings as which embryos reached the specified developmental stage after activation (in hours)						
Treatment group	Classification type[a]	No. of classified embryos	2 cell	4–8 cell	16 cell	32 cell	Morula	Early blastocyst	Expanded or hatching blastocyst
PA	Total	70	19.3 (7.6–30.1)	35.6 (25.2–45.3)	61.0 (38.1–82.4)	80.1 (54.6–92.4)	104.9 (85.6–118.4)	141.3 (103.4–184.4)	161.8 (141.6–179.2)
	E	48	16.4 (7.6–20.3)	32.4 (24.2–45.1)	59.3 (38.1–79.1)	79.5 (54.6–92.4)	103.9 (85.6–118.4)	140.2 (103.4–184.4)	160.7 (141.6–179.2)
	N	16	15.3 (7.6–20.3)	30.5 (24.2–39.3)	55.9 (38.1–69.1)	78.7 (54.6–92,4)	103.4 (85.6–118.4)	128.6 (103.4–143.5)	158.1 (141.6–175.3)
	E+N	14	14.6 (7.6–20.2)	30.5 (25.2–31.4)	55.0 (38.1–69.4)	80.2 (54.6–92.4)	105.6 (85.6–118.4)	127.6 (103.4–151.2)	157.9 (141.6–175.3)
NT-bEC	Total	60	21.6 (10.6–34.5)	36.1 (24.4–48.3)	59.4 (47.3–88.5)	89.0 (71.1–107.5)	109.6 (91.1–125.5)	141.9 (133.6–165.1)	162.4 (152.1–185.4)
	E	24	16.8 (10.6–20.2)	31.0 (24.4–48.3)	53.6 (46.1–88.5)	85.3 (75.5–107.5)	107.3 (91.1–125.5)	138.7 (132.2–146.4)	159.8 (152.5–171.1)
	N	16	19.9 (15.5–28.2)	35.0 (24.4–48.3)	55.9 (46.1–59.2)	86.5 (75.5–107.5)	110.5 (91.1–125.5)	140.2 (133.6–147.60	160.2 (152.4–171.1)
	E+N	10	17.4 (15.5–18.5)	32.3 (24.4–37.1)	55.1 (46.1–71.6)	85.3 (71.1–107.5)	110.1 (91.1–125.5)	140.1 (135.3–146.4)	160.4 (152.3–171.1)
NT-bTGC	Total	62	21.3 (14.3–29.4)	41.4 (26.5–61.0)	69.7 (53.4–87.2)	91.9 (81.4–107.3)	109.4 (100.5–120.4)	148.4 (128.5–194.2)	169.0 (158.4–196.3)
	E	26	16.9 (14.3–19.6)	39.5 (26.5–61.0)	62.5 (54.3–79.1)	85.6 (79.6–96.3)	108.7 (100.6–117.4)	158.3 (131.0–194.2)	172.9 (153.3–196.3)
	N	12	19.8 (14.3–27.1)	36.4 (32.4–47.0)	71.0 (54.3–87.2)	91.9 (76.6–107.3)	109.9 (100.6–120.4)	143.6 (128.5–170.2)	172.6 (149.5–196.3)
	E+N	6	16.3 (14.3–19.6)	33.7 (33.2–35.2)	67.8 (54.3–79.1)	88.4 (79.6–96.3)	108.4 (100.6–117.4)	144.0 (131.0–170.2)	166.2 (153.3–188.1)

[a]Early cleaving: cleavage occurred ≤ 20 h after activation Normal morphology: embryos did not exhibit fragmentation or abnormal cytoplasmic division at each developmental stage

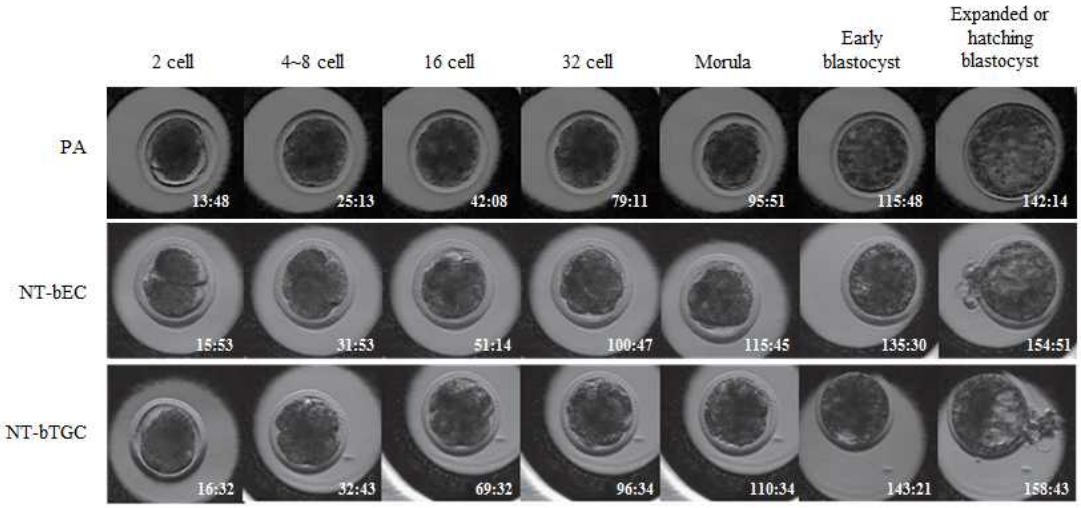


Figure 11. Images from time-lapse monitoring of *in vitro* developed bovine PA and somatic cell NT embryos generated using non-transgenic (NT-bEC) or transgenic (NT-bTGC) donor cells that were classified as early cleaving and with a normal morphology from the two cell to expanded or hatching blastocyst stages. Investigated time is approaching development stage of each embryo.

5. DISCUSSION AND CONCLUSION

This study examined the *in vitro* developmental potential and developmental kinetics of bovine PA and two types of SCNT (NT-bEC and NT-bTGC) embryos using a time-lapse monitoring system. The results demonstrated that early cleavage (≤ 20 h) and a normal morphology may be important criteria to select good quality bovine NT embryos and to predict an enhanced implantation potential. However, there was a 2 h difference in average timing of the first cleavage among PA group (19.3 h) and NT groups (NTbEC, 21.6 h; NT-bTGC, 21.3 h), and there was a difference of ~7 h in the fastest first cleavage among the PA, NT-bEC, and NT-bTGC groups (7.6, 10.6 and 14.3 h, respectively) (PA>NT-bEC>NT-bTGC). Early blastocyst development was faster in the PA group (103.4 h) than in the two NT groups (NT-bEC, 132.2 h; NT-bTGC, 128.5 h).

In this study, PA or reconstructed NT oocytes were cultured individually in a well of a Primo Vision dish and were recorded from day 0 to 7 post-activation. The cleavage and blastocyst formation rates did not significantly differ among the three treatment groups (PA, NT-bEC, and NT-bTGC), although both rates were higher in the PA group than in the two NT groups. However, the NT-bEC group had a slightly better developmental potential than the NT-bTGC group. In general, NT with transfected cells results in a low efficiency and a high rate of developmental abnormalities compared with NT using non-transgenic cells; this may be due to the extended donor cell culture according to transfection and selection procedures [93]. There was little difference in developmental potential and developmental kinetics between the NT-bEC and NT-bTGC groups. Early cleavage is one of the most promising parameters for the selection of viable embryos [94]. Early cleaving embryos are more competent to become blastocysts and have enhanced implantation rates compared with late cleaving embryos [95, 96]. Development occurred faster in the PA group than in the two NT groups, and the average timing of first cleavage was about 2 h earlier in the PA group (19.3 h) than in the NT-bEC (21.6 h) and NT-bTGC (21.3 h) groups. The average timing of

first cleavage for all examined embryos (n=192) was 20 h. Similarly, it was reported that most NT oocytes underwent cleavage between 18 and 24 h after activation, and the cleavage rate was highest at 20 h after activation [97]. According to another report, in bovine IVF, the best quality embryos were cleaved at 30 h post-insemination, the developmental potential decreased over time, and very late (> 42 h post-insemination) cleaving embryos did not usually reach the blastocyst stage [98, 99].

When embryos in the three treatment groups were classified as early cleaving (≤ 20 h) and late cleaving (> 20 h), the blastocyst development rate was significantly (PA and NT-bEC groups) or clearly (NT-bTGC group) higher in the early cleaving groups than in the late cleaving groups, while the cleavage rate was higher in the early cleaving group than in the late cleaving group only for PA embryos. By contrast, for NT-bEC and NT-bTGC embryos, the cleavage rate was higher in the late cleaving groups than in the early cleaving groups, which may have resulted in delayed development. Recent data demonstrated that early cleaving embryos are more likely to develop to the blastocyst stage [100]. Our results showed that the probability of reaching the blastocyst stage was significantly higher in the early cleaving groups than in the late cleaving groups for all three treatment groups (PA, NTbEC, and NT-bTGC).

On the other hand, late cleaving embryos might have a higher incidence of chromosomal abnormalities, altered gene expression, and increased DNA double-strand breaks than early cleaving embryos [101, 102]. The percentage of embryos whose development was blocked was clearly lower in the early cleaving groups than in the late cleaving groups (Figure 10). In time-lapse monitoring, analysis of early cleavage and normal embryo morphology may be a key to predict better implantation. Morphology is classified as normal if the embryo does not exhibit fragmentation or abnormal cytoplasmic division at each developmental stage. The percentage of embryos classified as N did not differ among the three treatment groups (PA, 22.9%; NT-bEC, 26.7%; NTbTGC, 19.4%), while the

percentage of embryos classified as E differed between the PA group and the two NT groups (PA, 68.6%; NT-bEC, 40.0%; NT-bTGC, 41.9%). However, the percentage of embryos classified as E+N was highest in the PA group and lowest in the NT-bTGC group. Under the criteria of the first cleaving time 20 h, normal morphology from E group indicated the higher developmental potential (PA, 14/48=29.2%; NT-bEC, 10/24=41.7%; NTbTGC, 6/26=23.1%) than later normal morphology group (PA, 2/22=9.1%; NT-bEC, 6/36=16.7%; NT-bTGC, 6/36=16.7%). According to our record images, in 32 cell stage embryos were the most easily going abnormal irrespective of PA or NT groups (PA, 43.8%; NTbEC, 18.1%; NT-bTGC, 41.7%) and then the lesser in 16 cell stage (PA, 26.3%; NT-bEC, 6.7%; NT-bTGC, 21.4%) (data was not shown). This result demonstrated that the developmental potential of an individual embryo can be predicted by assessing early cleavage and embryo morphology using the time-lapse system. Using this tool, the timing of embryo development is automatically recorded and can be systematically analyzed and more results were filed up, and this helps to select viable bovine SCNT embryos. In the analysis of embryos classified as E, N, and E+N at each developmental stage, there was large variation and some discrepancies among the groups. Nevertheless, this study is very important because it compares the timings of development for embryos classified as E+N among the three treatment groups. This study may be the first to present detailed timings of development, from first cleavage to the blastocyst stage, of PA, NT-bEC, and NT-bTGC embryos.

This study demonstrated that time-lapse monitoring provides novel data regarding embryo developmental kinetics and helps to predict embryo developmental potential. Early cleavage (≤ 20 h) and a normal morphology may be good parameters to select bovine NT embryo. However, the culture environment varies among laboratories; therefore, further *in vitro* and *in vivo* studies using a time-lapse monitoring system should be performed to confirm the embryo implantation potential.

CHAPTER 5. Effective oocyte vitrification and survival techniques for bovine somatic cell nuclear transfer

1. ABSTRACT

Bovine somatic cell nuclear transfer (SCNT) using vitrified–thawed (VT) oocytes has been studied; however, the cloning efficiency of these oocytes is not comparable with that of non-vitrified (non-V) fresh oocytes. This study sought to optimize the survival and cryopreservation of VT oocytes for SCNT. Co-culture with feeder cells that had been pre-incubated for 15 h significantly improved the survival of VT oocytes and their *in vitro* developmental potential following SCNT in comparison to co-culture with feeder cells that had been pre-incubated for 2, 5, or 24 h ($p < 0.05$). Spindle assessment via the Oosight Microscopy Imaging System and microtubule staining revealed that vitrified metaphase II oocytes (VT group) were not suitable for SCNT. However, enucleating and/or activating oocytes prior to freezing enhanced their developmental potential and suitability for SCNT. The cloning efficiency of the enucleated-activated-vitrified-thawed (EAVT) group (21.6%) was better than that of the other vitrification groups [enucleated-vitrified-thawed (EVT) group, 13.7%; VT group, 15.0%; $p < 0.05$] and was comparable with that of the non-V group (25.9%). The reactive oxygen species level was significantly lower in the EAVT group than in the other vitrification groups ($p < 0.05$). mRNA levels of maternal genes (ZAR1, BMP15, and NLRP5) and a stress gene (HSF1) were lower in the vitrification groups than in the non-V group ($p < 0.05$), whereas the level of phospho-p44/42 mitogen-activated protein kinase did not differ among the groups. Among the vitrification groups, blastocysts in the EAVT group had the best developmental potential, as judged by their high mRNA expression of developmental potential related genes (POU5f1, Interferon-tau, and SLC2A5) and their low expression of pro-apoptotic (CASP3) and stress (Hsp70) genes. This study demonstrates that SCNT using bovine frozen-thawed oocytes can be successfully achieved using optimized vitrification and co-culture techniques.

2. INTRODUCTION

Successful cryopreservation of bovine oocytes would greatly facilitate their use for research and commercial applications. Cryopreservation of oocytes is critical to preserve female genetic resources. In particular, the development of advanced technologies, such as transgenesis and cloning by somatic cell nuclear transfer (SCNT), are reliant upon the quantity and quality of oocytes [67, 103]. Cryopreservation could be a useful technique to provide a steady source of oocytes for SCNT. In general, oocytes are more susceptible to cooling damage than zygotes because metaphase spindle microtubule integrity is disrupted during cooling and high concentrations of cryoprotectants (CPAs) damage oocytes [104].

Vitrification (glass-like solidification) reduces oocyte damage during cryopreservation by increasing the cooling and warming rates, thereby avoiding ice crystal formation [105]. The oocytes of many mammalian species have been successfully vitrified, including those of cattle [106], mice [107], pigs [108], and humans [109]. Many simple and efficient vitrification methods for bovine oocyte cryopreservation have been reported. These use various containers (e.g., electron microscopy grid, open pulled straw, Cryoloop, and solid surface) and a small volume (1–2 μL) of freezing solution, which has a high cooling capacity [110–112]. Nevertheless, it remains difficult to cryopreserve bovine oocytes, and the developmental potential and cloning efficiency of vitrified-thawed (VT) oocytes following SCNT are poor [113–115].

To enhance the cloning efficiency of SCNT using cryopreserved bovine oocytes, it might be necessary to minimize freezing, mechanical, and chemical damage such that all frozen-thawed oocytes survive. Co-culture with somatic cells can be used to overcome the developmental block of frozen-thawed oocytes [116, 117]. In previous studies [67, 70], we performed SCNT with ear cells and co-cultured the result and embryos with the same cells. Optimized feeder cell conditions may be key to improving the survival and developmental potential of oocytes after thawing. Another concern is that microtubule depolymerization

induced by CPA treatment and cryopreservation can cause meiotic spindle disassembly and chromosome misalignment [118]; therefore, the results of SCNT studies using VT metaphase II (MII) oocytes might be highly variable and not comparable with those of SCNT studies using fresh oocytes [114, 119]. In our previous study [120], we successfully vitrified bovine oocytes using the minimum volume cooling (MVC) method and confirmed that these frozen-thawed oocytes can develop to full-term following IVF. However, the use of the MVC method for SCNT was not optimized [121].

The current study sought to optimize conditions for the survival and cryopreservation of VT oocytes used for SCNT. Changes in the reactive oxygen species (ROS) level, expression of maternal-related genes [zygote arrest 1 (ZAR1), bone morphogenenic protein 15 (BMP15), growth differentiation factor 9 (GDF9), NLR family, pyrin domain containing 5 (NLRP5), heat shock transcription factor 1 (HSF1), and superoxide dismutase 1 (SOD1)], and mitogen-activated protein kinase (MAPK) activity may be useful for analyzing the viability of frozen-thawed oocytes used for SCNT and the freezing-related decline in the survival of these oocytes.

In the current study, we show that SCNT can be performed using VT oocytes, as well as non-vitrified (non-V) fresh oocytes. We examined the following: (1) Optimization of the survival of VT oocytes used for SCNT; (2) how the cloning efficiency is affected by changes to spindle integrity following freezing and thawing; and (3) comparison of the cloning efficiency among oocytes that are conventionally vitrified, those that are enucleated prior to freezing, and those that are enucleated and activated prior to freezing. We also compared these various VT oocytes and fresh oocytes in terms of the level of ROS, microfilament localization, mRNA expression of maternal candidate genes (ZAR1, BMP15, GDF9, NLRP5, HSF1, and SOD1), and the level of phosphorylated MAPK. The mRNA levels of developmental potential-related genes [POU class 5 homeobox 1 (POU5f1), Interferon-tau, solute carrier family2 (facilitated glucose/fructose transporter) member5 (SLC2A5), caspase

3 (CASP3), heat shock protein 70 (Hsp70), and DNA (cytosine-5)-methyltransferase 3-alpha (Dnmt3A)] were compared among blastocysts produced from the various groups of oocytes.

3. MATERIALS AND METHODS

1) Oocyte preparation and *in vitro* maturation

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory within 2 h in 0.9% saline solution at 35 °C. Cumulus-oocyte complexes (COCs) were aspirated from visible follicles (2-6mm) with an 18-gauge needle attached to a 10 mL disposable syringe. The medium used for COC collection was HEPES-buffered Tyrode's medium (TLHEPES). Sets of ten COCs were matured *in vitro* in tissue culture medium-199 (TCM-199; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 0.2 mM sodium pyruvate, 1 μg/mL follicle-stimulating hormone (FolltropinTM, Bioniche Animal Health, Belleville, ON, Canada), 1 μg/mL estradiol-17b, and 1mM epidermal growth factor (EGF) in mineral oil at 38.8 °C in an incubator (5% CO_2, 5% O_2, and 90% N_2) for 19–21 h.

2) Vitrification and thawing

The basic medium used for pre-treatment, vitrification, and dilution was Dulbecco's phosphate-buffered saline (D-PBS, Gibco) containing 10% FBS. The pretreatment solution also contained 10% ethylene glycol (EG10). The vitrification solution (VS) contained 30% ethylene glycol and 0.5M sucrose (EG30). For serial dilution after thawing, D-PBS containing 1.0, 0.5, 0.25, or 0.125 M sucrose and 10% FBS was used. Oocytes were freeze-thawed according to the MVC vitrification procedures reported previously [114]. After incubation for 20 h in IVM)medium, cumulus cells were partially (MII oocytes) or completely (enucleated oocytes) removed by treatment with 0.1% hyaluronidaseand mechanical pipetting. Oocytes were washed with TL-HEPES, incubated in a droplet of

previous cultured IVM medium for 1 h to recover, and then frozen with or without prior enucleation and/or activation. Freezing procedures were performed at room temperature. MII oocytes or enucleated oocytes were washed three times in TL-HEPES and then equilibrated in D-PBS for 5 min. For vitrification, oocytes were pretreated with EG10 for 5 min, exposed to EG30 for 30 sec, and then loaded individually onto the inner wall of a modified French ministraw (total length, 2.5-3.0cm) coated with a minimum volume of VS. The straw was plunged directly into liquid N_2, and four to five straws were placed into a pre-chilled cryovial, which was stored in a freezing cane and placed in a liquid nitrogen tank. For thawing, CPAs were removed via a five step procedure using thawing solutions warmed to 37 °C. Straws stored in liquid nitrogen were moved rapidly to D-PBS containing 1.0 M sucrose. Thereafter, oocytes were sequentially transferred to D-PBS containing 0.5, 0.25, and 0.12 5M sucrose, and then into D-PBS lacking sucrose. Oocytes were incubated in each solution for 1 min. Finally, oocytes were cultured with feeder cells (pre-incubated for 2, 5, 15, or 24 h) in TCM-199 medium for 2 h.

3) Preparation of donor cells and feeder cells

Donor somatic cells were derived from the ear tissue of Hanwoo Cattle (Korean Native Cattle). Minced ear tissue was incubated in 0.1% collagenase type IV solution at 38 °C for 1.5 h and then cultured in donor cell culture medium [Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1mM sodium pyruvate, 1% nonessential amino acids, 0.1% b-mercaptoethanol, and 1% penicillin-streptomycin]. The cells were grown and sub-cultured three to five times, with an interval of 4–6 days. Thereafter, cells ($1x10^6$) were frozen in cryovials (1.5 mL) in freezing medium (50% donor cell culture medium containing 45% FBS and 5% dimethyl sulfoxide). For SCNT, frozen-thawed ear cells were washed twice with donor cell culture medium and treated with 3 mg/ mL protease for 50 sec at room temperature. Treated cells were washed three times and re-suspended in donor cell

preparation medium (TCM-199 HEPES supplemented with 0.2 mM sodium pyruvate). A droplet of feeder cells was prepared using cultured bovine ear cells, the same cells as were used for SCNT, to create homogeneous culture conditions for oocytes and embryos. The cells were detached using TrypLE reagent (Gibco), added to PBS, centrifuged at 2000xg for 1 min, resuspended in DMEM containing 10% FBS, and seeded into a 10 μL droplet. The droplet was covered with mineral oil and incubated at 38.8 °C in 5% O_2, 5% CO_2, and 90% nitrogen for 1 or 2 days prior to co-culture with frozen-thawed oocytes.

4) Preparation of recipient oocytes

For enucleation, cumulus cells were completely removed from the oocyte by vortexing for 3 min in the presence of 0.05% hyaluronidase. Oocytes with an extruded first polar body (PB1) were selected, and denuded oocytes were transferred to enucleation medium (TCM-199 HEPES containing 20% FBS and 7.5 μg/mL cytochalasin B). Thereafter, the MII plate and PB1 were visualized using an inverted microscope (Olympus, Tokyo, Japan) equipped with the Oosight Microscopy Imaging System (CRi, Hopkinton, MA, USA) and removed by the squeezing method, as reported previously [69].

5) SCNT

A single treated donor cell was placed in the perivitelline space of an enucleated oocyte in nuclear transfer medium [TCM-199 HEPES containing 0.06% fatty acid-free bovine serum albumin (BSA) and 10 μg/mL phytohemagglutinin] through the opening made during enucleation. Thereafter, oocytes were placed in cell fusion medium (0.3 M mannitol, 0.5 mM HEPES, 0.05 mM $CaCl_2$, and 0.1 mM $MgSO_4$) and subjected to an electrical pulse of 1.3kV/cm for 20 μsec using an Electro Cell Fusion Generator (LF101, NEPAGENE, Chiba, Japan). After fusion, the reconstructed embryos were kept in TCM-199 HEPES supplemented with 20% FBS for 1 h, activated for 5 min in CR1aa medium supplemented

with 1.5 mg/mL BSA and 10 μM calcium ionophore, and exposed to 2mM 6-dimethylaminopurine for 3 h. The reconstructed embryos were cultured in CR1aa medium supplemented with 10% FBS, 1 μM EGF, 1 μM insulin-like growth factor, and 10 μM flavonoid at 38.8 °C in 5% O_2, 5% CO_2, and 90% nitrogen with maximum humidity for 8 days.

6) Immunofluorescence microscopy

Microtubules and DNA were detected by indirect immunocytochemical techniques, as described by Kim et al [119]. Briefly, oocytes were permeabilized in modified Buffer M (25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid, 1 mM β-mercaptoethanol, 50 mM imidazole, pH 6.7, 3% Triton X-100, and 25 mM phenylmethylsulfonyl fluoride; Simerly and Schatten) [122] for 20 min, fixed in methanol at -20 °C for 10 min, and stored in PBS containing 0.02% sodium azide for 7 days at 4 °C. Microtubule localization was determined using an anti-a-tubulin antibody (T-5168). Fixed oocytes were incubated for 90 min at 39 °C with this antibody diluted 1:100 in PBS. After several washes with PBS containing 0.5% Triton X-100 and 0.5% BSA, oocytes were incubated in blocking solution (0.1 M glycine, 1% goat serum, 0.01% Triton X-100, 1% powdered milk, 0.5% BSA, and 0.02% sodium azide) at 38 °C for 1 h. Thereafter, oocytes were incubated with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody. DNA was detected fluorescently by incubation with 50 μg/mL propidium iodide for 1 h. Following extensive washing, samples were mounted onto glass slides. Fluorescence images of individual samples were digitized using a laser scanning confocal microscope (Leica Laser Technik GmbH) and MetaMorph software (v. 6.1, Universal Imaging Corporation, Westchester, PA, USA). To detect the distribution of microfilaments, oocytes were fixed with 4% paraformaldehyde prepared in PBS for 1 h and then transferred to membrane permeabilization solution (0.5% Triton X-100) for 30 min. After 1 h in blocking buffer (1%

BSA prepared in PBS), oocytes were incubated with phalloidin-tetramethylrhodamine (TRITC) (1 μg/mL) for 2 h. After three washes (5min each) with 1% BSA prepared in PBS, samples were co-stained with Hoechst 33342 (1 μg/mL prepared in PBS) for 30 min. Oocytes were mounted onto glass slides and examined using a confocal laser scanning microscope (Leica Laser Technik GmbH). At least 20 oocytes were examined per group.

7) Measurement of intracellular ROS

Intracellular ROS were measured in oocytes by a 2,7dichlorofluorescein assay, as described previously [122]. Briefly, oocytes were incubated with 100 μM 2,7-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR, USA) for 20 min at 38 °C, washed three times in TL-HEPES to remove excess dye, and immediately analyzed by epifluorescence microscopy (Olympus, Tokyo, Japan) using excitation and emission wavelengths of 450-490nm and 515–565nm, respectively. Grayscale images were acquired using a digital camera (Nikon, Tokyo, Japan) attached to the microscope, and mean gray values were measured using ImageJ (NIH, Bethesda, MD, USA). Background fluorescence values were subtracted from the final values prior to statistical analysis. Experiments were repeated three times, with 10–20 oocytes per experiment.

8) Real-time revers transcription PCR

Comparative real-time reverse transcription (RT)-PCR was performed using a Chromo 4 detector (Bio-Rad) and a DyNAmo HS SYBR Green qPCR Kit (FINNZYMES), according to the manufacturer's instructions. The primers are described in Table 14. Magnetic beads (Dynabeads mRNA Purification Kit; Dynal, Oslo, Norway) were used to prepare mRNA from 20 oocytes per group. Gene expression was quantified by the 2-DDCt method [32].

9) Western blot analysis

Oocytes (30 per sample) were added to 20 μL of 1xsodium dodecyl sulfate (SDS) sample buffer [62.5 mM TrisHCl (pH 6.8 at 25℃), 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 50 mM dithiothreitol (DTT), and 0.01% (wt/vol) Bromophenol Blue or Phenol Red] and heated for 5 min at 95 °C. Proteins were resolved on a 5-12% Tris-SDS-polyacrylamide gel electrophoresis (PAGE) gel for 1.5 h at 80–100V. Thereafter, proteins were transferred to a nitrocellulose membrane (Amersham, Hybond-ECL, Buckinghamshire, UK) at 300 mA for 2 h in transfer buffer [25 mM Tris (pH 8.5) containing 200 mM glycine and 20% (vol/vol) methanol]. After blocking for 1 h with 5% skimmed milk prepared in PBS, the membrane was incubated for at least 2 h with anti-phospho-p44/42 MAPK and anti-MAPK antibodies (Cell Signaling Technology, Danvers, MA, USA) diluted 1:500 in blocking solution [1·Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% (wt/vol) nonfat dry milk], washed three times in TBST [20 mM Tris-HCl (pH 7.5) containing 250 mM NaCl and 0.1% (vol/vol) Tween-20], and incubated for 1 h with anti-rabbit immunoglobulin G horseradish peroxidase (IgG-HRP; Cell Signaling Technology, Danvers, MA, USA) diluted 1:2000 in blocking solution. After three washes with TBST, antibody binding was visualized with a chemiluminescence luminol reagent (Invitrogen, Carlsbad, CA, USA).

10) Experiment design

Oocytes were divided into the following groups enucleated-vitrified-thawed (EVT), enucleated-activated-vitrified-thawed (EAVT), VT, and non-V. To determine the condition in which feeder cells best support the survival of VT bovine oocytes used for SCNT, feeder cells were pre-incubated for various amounts of time (2, 5, 15, and 24 h). The suitability of VT MII oocytes for SCNT was determined by assessing the spindle using the Oosight Microscopy Imaging System (live imaging) and immunofluorescence labeling of microtubules (fixed samples). The developmental potentials of oocytes in the EVT and

EAVT groups were compared with those of oocytes in the non-V and VT groups. Furthermore, the levels of ROS, microfilament localization, and mRNA expression of maternal candidate genes (ZAR1, BMP15, GDF9, NLRP5, HSF1, andSOD1) and the level of phosphorylated MAPK in oocytes were compared among the groups. In addition, the mRNA levels of developmental potential-related genes (POU5f1, Interferon-tau, SLC2A5, CASP3, Hsp70, and Dnmt3A) in *in vitro* produced 8 day old SCNT blastocysts were compared among the various groups. Experiments were repeated three to six times.

11) Statistical analysis

Differences were evaluated by an analysis of variance using the general linear model (PROC-GLM) in the SAS software program. $p < 0.05$ was considered significant.

4. RESULTS

Effect of the pre-incubation time of feeder cells on the survival of VT bovine MII oocytes used for SCNT

To determine the feeder cell conditions that best support the survival of bovine frozen-thawed MII oocytes used for SCNT, 10 μL droplets of feeder cells were pre-incubated for 2, 5, 15 and 24 h, and were then co-cultured with the oocytes (Table 12). The survival rates of oocytes co-cultured with feeder cells that had been pre-incubated for 5 h or longer [5 h, 88.2% (82/93); 1 5h, 90.2% (83/92); 24 h, 86.7% (78/90)] were significantly higher than those of oocytes co-cultured with feeder cells that had been pre-incubated for 2 h (73.9%, 65/88; $p<0.05$). Fewer than half (44.9–50.0%) of the surviving VT oocytes could be used for SCNT. The percentage of oocytes that underwent fusion following SCNT was significantly higher among oocytes co-cultured with feeder cells that had been pre-incubated for 5 or 15 h [5 h, 80.5% (33/41); 15 h, 85.0% (34/40)] than among oocytes co-cultured with feeder cells that had been pre-incubated for 2 h (66.7%, 20/30) ($p < 0.05$). However, the percentage of

fused oocytes that underwent cleavage was significantly higher among oocytes co-cultured with feeder cells that had been pre-incubated for 15 h or longer [15 h, 52.9% (18/34); 24 h, 44.0% (11/25)] than among oocytes co-cultured with feeder cells that had been pre-incubated for 5 h or less [2 h, 35.0% (7/20); 5 h, 36.4% (12/33)]. In comparison, the cleavage rate was significantly higher in the non-V group ($p < 0.05$). The percentage of blastocysts was significantly higher among oocytes co-cultured with feeder cells that had been pre-incubated for 15 h or longer [15 h, 27.8% (5/18); 24 h, 18.2% (2/11)] than among oocytes co-cultured with feeder cells that had been pre-incubated for 5 h or less [2 h, 0% (0/7); 5 h, 0% (0/7)]. Pre-incubation of feeder cells for 15 h resulted in the highest percentage of fused VT oocytes developing to the blastocyst stage (14.7%, 5/34); for comparison, the corresponding percentage in the non-V group was 23.6% (13/55; $p < 0.05$).

Assessment of VT MII oocytes in terms of their survival following thawing, spindle visualization using the Oosight microscopy imaging system, and microtubule immunostaining

VT MII oocytes were assessed in terms of survival following thawing, visualization of the spindle using the Oosight Microscopy Imaging System (live imaging), and immunofluorescence labeling of microtubules (fixed samples) (Figure 12). After thawing, 91.4% of MII oocytes survived. Spindle poles and polar bodies were visualized using the Oosight Microscopy Imaging System (Figure 12B). From this, 48.8% (42/86), 34.9% (30/86) and 16.3% (14/86) of oocytes were judged to have spindle poles and polar bodies that were clear, unclear, and could not be observed (negative), respectively. Oocytes with spindle poles and polar bodies that could be visualized (clearand unclear) were subjected to microtubule immunostaining and confocal microscopy (Figure 12C). In normal spindles, chromosomes were clustered in a discrete bundle at the metaphase plate were scattered along microtubules. The percentage of oocytes with normal microtubule staining was significantly higher among

oocytes whose spindle poles and polar bodies were clearly visualized using the Oosight Microscopy Imaging System (64.3%, 27/42) than among those whose spindle poles and polar bodies were unclear using this system (43.3%, 13/30; $p < 0.05$).

Effect of enucleation and activation prior to freezing on the cloning efficiency of bovine oocytes

Oocytes were enucleated (EVT group, Figure 13E-G) or were enucleated and activated (EAVT group, Figure 13I-K) prior to freezing. The developmental potentials of the resulting embryos (Figure 13H, L), following thawing and SCNT, were compared with those of the non-V (Figure 13M, N) and VT (Figure 13A–D) groups. As shown in Table 13, the percentage of thawed oocytes that survived was similar among the VT (93.4%, 170/182), EVT (86.7%, 156/180) and EAVT (88.9%, 160/180) groups, although the percentage of oocytes that were suitable for SCNT significantly differed [VT, 52.9% (90/170); EVT, 78.2% (122/156); EAVT, 81.3% (130/160); $p < 0.05$]. The percentage of vitrified oocytes that underwent fusion following SCNT [VT, 88.9% (80/90); EVT, 83.6% (102/122); EAVT, 89.2% (116/ 130)] was similar to the corresponding percentage in the non-V group [90.6% (116/128)].

At day 2 post-activation, the percentage of embryos that underwent cleavage in the EAVT group [70.7% (82/116)] was significantly higher than that in the other vitrification groups [VT, 52.5% (42/80); EVT, 57.8% (59/102); $p < 0.05$], but was not significantly different to that in the non-V group [72.4% (84/116)]. At day 8 post-activation, the percentage of embryos that had reached the blastocyst stage was higher in the EAVT group [30.5% (25/82)] than in the VT [28.6% (12/42)] and EVT [23.7% (14/59)] groups; the corresponding percentage in the non-V group was 35.7% (30/84). Finally, the overall efficiency with which SCNT embryos were produced in the EAVT group [21.6% (25/ 116)] was higher than that in the other vitrification groups [VT, 15.0% (12/80); EVT, 13.7% (14/102); $p < 0.05$] and did not differ from that in the non-V group [25.9% (30/ 116)].

Therefore, among the vitrification groups, the cloning efficiency was highest in the EAVT group, with two-fold more *in vitro* produced blastocysts (25) in this group than in the VT group (12).

Comparison of the levels of ROS and phosphorylated MAPK, microfilament organization, and mRNA expression among oocytes in the various groups

Oocytes in the VT, EVT and EAVT groups were compared with those in the non-V group in terms of the level of ROS, microfilament localization, mRNA expression of maternal candidate genes (ZAR1, BMP15, GDF9, NLRP5, HSF1, and SOD1), and the level of phosphorylated MAPK (Figure 14). The level of ROS in the EAVT group was significantly lower (Figure 14A, d) than that in the VT (Figure 14A, b) and EVT (Figure 14A, c) groups, but was similar to that in the non-V group (Figure 14A, a) (Figure 14B, $p < 0.05$). Microfilament localization did not differ among the various treatment groups, apart from the absence of spindle and chromatin labeling in the EVT (Figure 14A, g) and EAVT (Figure 14A, h) groups. mRNA levels of maternal genes (ZAR1, BMP15 and NLRP5) and the stress gene HSF1 were lower in the VT, EVT, and EAVT groups than in the non-V group ($p < 0.05$). mRNA expression of the GDF9 (maternal) gene was significantly lower in the EVT group than in the non-V group, whereas mRNA expression of the SOD1 (anti-oxidant) gene was higher in the vitrification groups than in the non-V group (Figure 14C). The level of phosphorylated MAPK did not vary among the groups (Figure 14D).

Comparison of blastocyst mRNA expression of developmental potential-related genes among the groups

The mRNA levels of developmental potential-related genes (POU5f1, Interferon-tau, SLC2A5, CASP3, Hsp70, and Dnmt3A) in *in vitro* produced 8 day old SCNT blastocysts were compared among the groups. The mRNA levels of POU5f1 (pluripotency) and Interferon-tau (implantation) in these blastocysts were significantly lower in the EVT, VT,

and EAVT groups (EVT<VT<EAVT) than in the non-V group ($p < 0.05$; Figure 15). The mRNA level of SLC2A5 (metabolism) did not differ between the non-V and EAVT groups, but was significantly lower in the VT and EVT groups ($p < 0.05$). The mRNA levels of CASP3 (pro-apoptotic) and Hsp70 (stress) were significantly lower in the EVT and EAVT groups than in the non-V and VT groups ($p < 0.05$), whereas the mRNA level of Dnmt3A (methylation) was significantly lower in the EVT group than in the non-V group.

Table 12. Effect of co-culture with feeder cells pre-incubated for various amounts of time on the survival of vitrified-thawed bovine MII oocytes and their subsequent *in vitro* developmental potential following SCNT

Treatment group*	No. (%)** of oocytes/embryos						No. (%) of cloning efficiency
	Thawed	Survived	SCNT	Fused	Cleaved (day 2)	Blastocyst (day 8)	
Non-V			65	55 (84.6)^{b}	40 (72.7)^{c}	13 (32.5)	13/55 (23.6)
VT-I (2h)	88	65 (73.9)^{a}	30 (46.2)	20 (66.7)^{a}	7 (35.0)^{a}	-	-
VT-II (5h)	93	82 (88.2)^{b}	41 (50.0)	33 (80.5)^{b}	12 (36.4)^{a}	-	-
VT-III (15h)	92	83 (90.2)^{b}	40 (48.2)	34 (85.0)^{b}	18 (52.9)^{b}	5 (27.8)	5/34 (14.7)
VI-IV (24h)	90	78 (86.7)^{b}	35 (44.9)	25 (71.4)^{ab}	11 (44.0)^{ab}	2 (18.2)	2/25 (8.0)

*Non-V, non-vitrified MII oocyte group. To support the survival of vitrified-thawed (VT) MII oocystes, feeder cells used for co-culture were pre-incubated for various amount of time (2, 5, 15 and 24 h). Oocytes were co-cultured with feeder cell for 2 h.

**$^{a-c}p < 0.05$.

A

Assessment of vitrified-thawed MII oocytes			
1st step	2nd step		3rd step
No.(%) of oocytes that survived	No.(%) of oocytes viewed spindle by the Oosight imaging system		No.(%)* of oocytes with normal microtubule staining
86/94 (91.4)	clear	42 (48.8)	27 (64.3)[a]
	unclear	30 (34.9)	13 (43.3)[b]
	negative	14 (16.3)	-

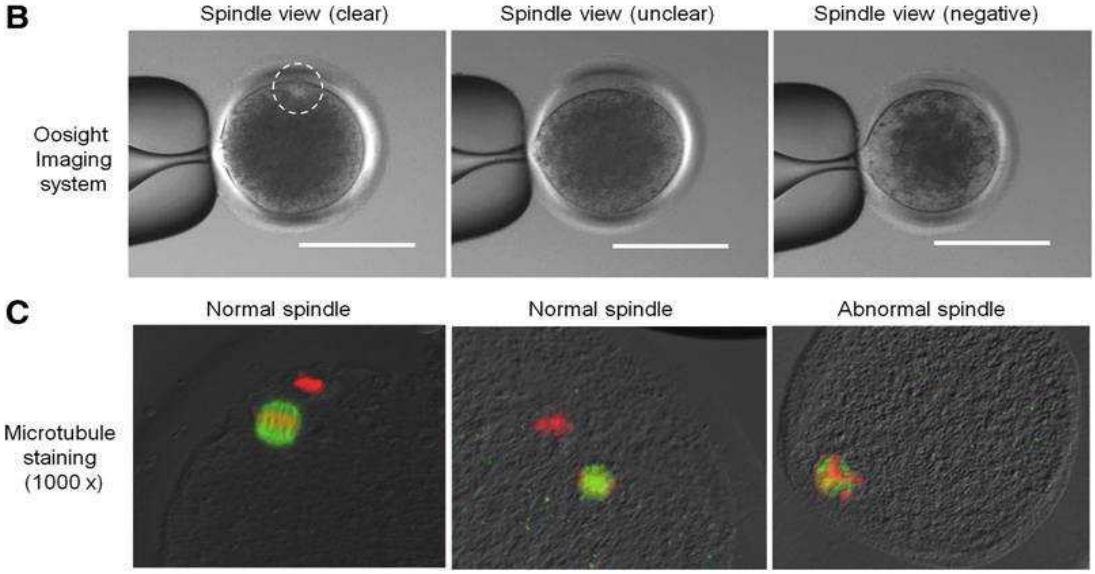


Figure 12. Vitrified-thawed bovine MII oocytes were assessed in terms of their survival following thawing, visualization of their spindle using the Oosight Microscopy Imaging System, and immunofluorescence labeling of microtubules. (A) The percentages of oocytes that survived, had spindle poles and polar bodies that were detectable by the Oosight Microscopy Imaging System, and had normal spindles by microtubule immunofluorescence labeling were calculated. (*) $p<0.05$ compared with the clear group. (B) Spindle poles and polar bodies were classified as clear, unclear, and not detectable (negative). Bar, 100 μm. (C) Confocal microscopy images of immunostained microtubules. Spindles were judged to be normal when chromosomes were clustered in a discrete bundle at the metaphase plate or were scattered along microtubule. Red, chromatin; green, microtubules.

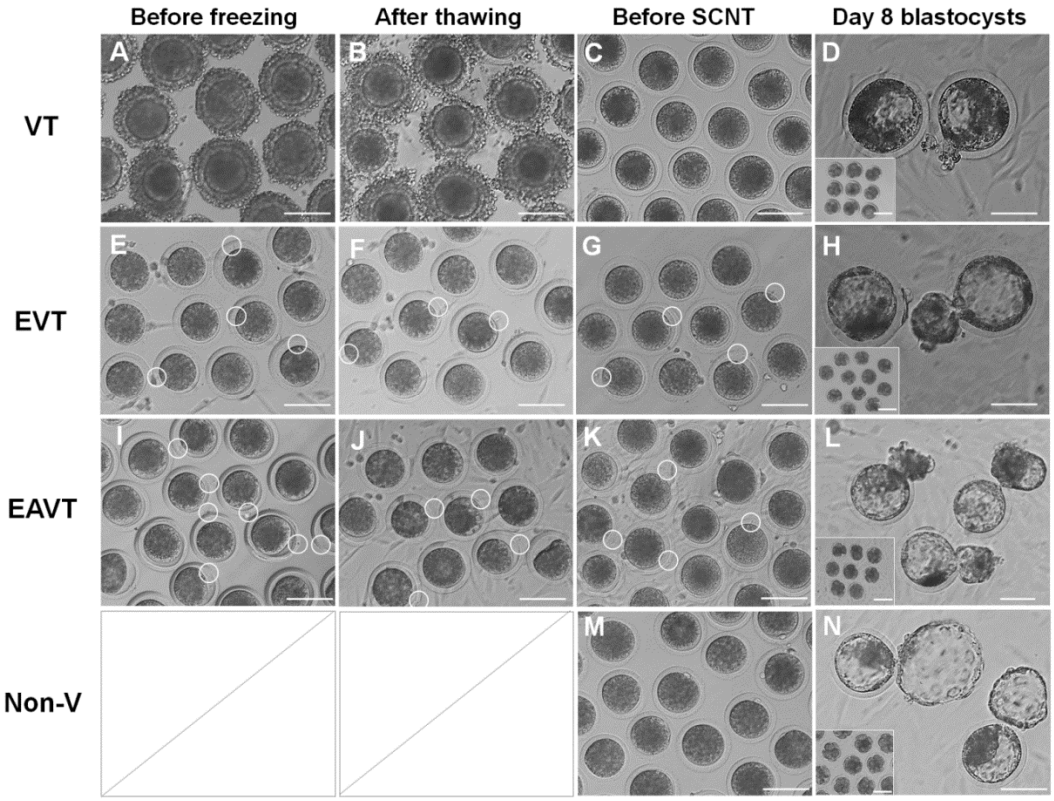


Figure 13. Morphology of oocytes in the various groups, namely, vitrified-thawed (VT; A and B), enucleated-vitrified-thawed (EVT; E and F), and enucleated-activated-vitrified-thawed (EAVT; I and J), and the corresponding 8-day-old SCNT blastocysts (D, H, and L, respectively) were compared with those in the non-vitrified control group (non-V, M and N). The insets show 2-day-old embryos after SCNT. (Open circles) Holes generated by enucleation. In the EVT and EAVT groups, somatic cell nuclei can be directly transferred into enucleated oocytes after thawing (G and K, respectively). In the VT and non-V groups, MII oocytes need to be enucleated prior to SCNT (C and M, respectively). Bar, A–C, E–G, I–K, and M, 150·; D, H, L, and N, 100·

Table 13. Effect of enucleation and enucleation/activation prior to freezing on the *in vitro* developmental potential of vitrified-thawed bovine oocytes following SCNT (r=3)

Treatment group*	No. (%)** of oocytes/embryos						No. (%) of cloning efficiency
	Thawed	Survived	SCNT	Fused	Cleaved (day 2)	Blastocyst (day 8)	
Non-V			128	116 (90.6)	84 (72.4)^{b}	30 (35.7)	30/116 (25.9)^{b}
VT	182	170 (93.4)	90 (52.9)^{a}	80 (88.9)	42 (52.5)^{a}	12 (28.6)	12/80 (15.0)^{a}
EVT	180	156 (86.7)	122 (78.2)^{b}	102 (83.6)	59 (57.8)^{a}	14 (23.7)	14/102 (13.7)^{a}
EAVT	180	160 (88.9)	130 (81.3)^{b}	116 (89.2)	82 (70.7)^{b}	25 (30.5)	25/116 (21.6)^{ab}

*Non-V, nonvitrified MII oocyte group; VT, vitrified-thawed MII oocyte group; EVT, enucleated-vitrified-thawed oocyte group; EAVT, enucleated-activated-vitrified-thawed oocyte group. Oocytes were co-cultured for 2 h with feeder cells that had been preincubated for 15 h.

** $^{a, b}p < 0.05$

Table 14. Sequences of primers used for real-time reverse transcription PCR

Genes	Primer sequence	Annealing temp (℃).	Product size (bp)
bZAR1	5′-GTACAGGGCACTAACAAGGT-3′ 5′-CTGAAAGTGCTATCACAGGA-3′	54	218
bBMP15	5′-GTACAGGGCACTAACAAGGT-3′ 5′-CTGAAAGTGCTATCACAGGA-3′	54	214
bGDF-9	5′-TAGTCAACTGAGGTGGGACA-3′ 5′-AGGAGTCAAGTTTCTCATGG-3′	54	152
bNLRP5	5′-TGTTCTCCTACGTCTTCCTT-3′ 5′-AGCTCTTCGAGTCCATCTAC-3′	54	166
bHSF1	5′-GAGCGAGGACATAAAGATTC-3′ 5′-GAGATGAGGAACTGGATGAG-3′	54	207
bSOD1	5′-TTGGAGAGCCAGTGAACAGT-3′ 5′-TGCTGATAACTGTCTGCGCT-3′	54	203
bPOU5f1	5′-CTCTTTGGAAAGGTGTTCAG-3′ 5′-GTCTCTGCCTTGCATATCTC-3′	54	155
bInterferon-tau	5′-ATGGCCTTCGTGCTCTCTCT-3′ 5′-AGGTCCTCCAGCTGCTGTTG-3′	55	356
bSLC2A5	5′-TTGGAGAGCCAGTGAACAGT-3′ 5′-TGCTGATAACTGTCTGCGCT-3′	60	292
bCAP3	5′-CGATCTGGTACAGACGTG-3′ 5′-GCCATGTCATCCTCA-3′	50	359
bHsp70	5′-GACAAGTGCCAGGAGGTGATTT-3′ 5′-CAGTCTGCTGATGATGGGGTTA-3′	51	117
bDnmt3A	5′-TGATCTCTCCATCGTCAACCCT -3′ 5′-GAAGAAGGGGCGGTCATCTC-3′	54	221
bGAPDH	5′-GAGGGACTTATGACCACTGT-3′ 5′-GTCAGATCCACAACAGACAC-3′	54	224

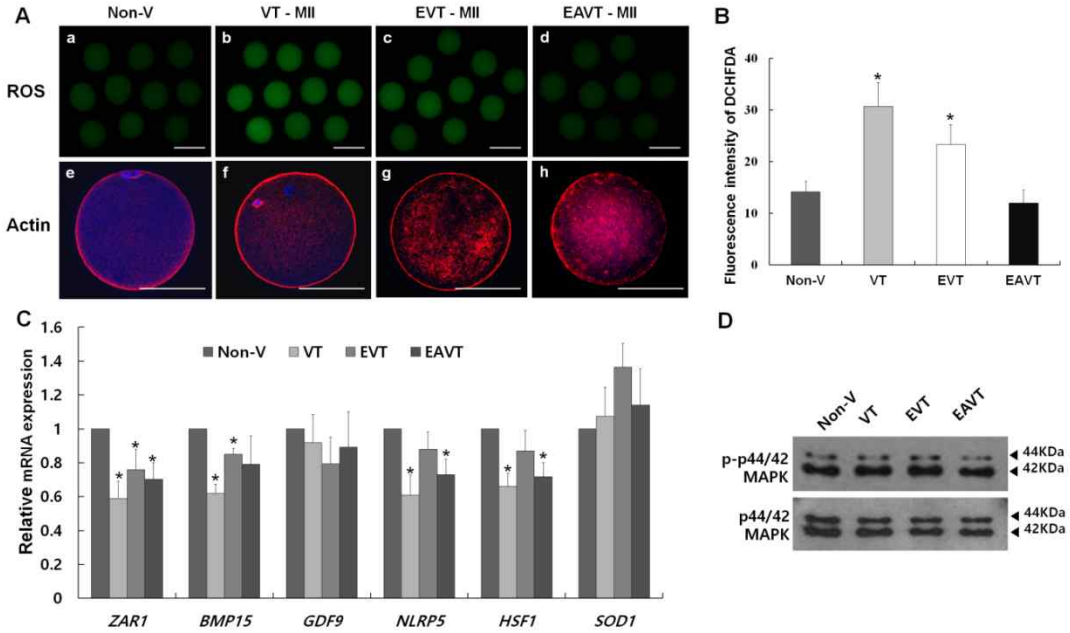


Figure 14. Levels of ROS, mRNA expression, and phosphorylated MAPK in oocytes in the various groups, namely, vitrified-thawed (VT), enucleated-vitrified-thawed (EVT), and enucleated-activated-vitrified-thawed (EAVT), were compared with those in the nonvitrified (non-V) group. (A) Fluorescence images of ROS (A, a–d) and confocal microscopy images of microfilament localization (A, e–h). ROS activity was detected with dichlorodihydrofluorescein diacetate (DCHFDA) (green). Bar, 100 lm. (B) Fluorescence intensity of DCHFDA. (C) Relative mRNA expression of maternal candidate genes (ZAR1, BMP15, GDF9, NLRP5, HSF1, and SOD1). (D) Level of phosphorylated MAPK. (*) $p < 0.05$ compared with the non-V group.

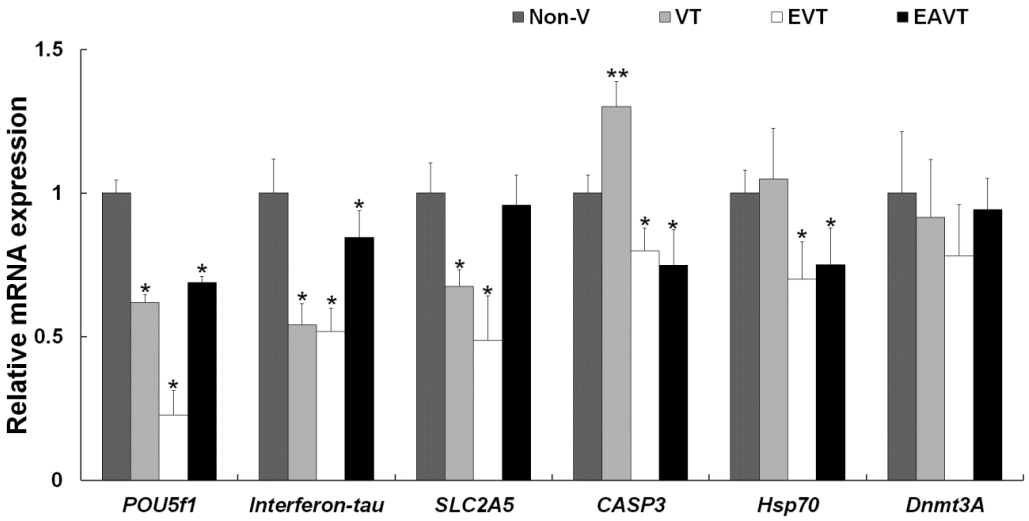


Figure 15. mRNA expression of developmental potential-related genes (POU5F1, Interferon-tau, SLC2A5, CASP3, HSP70, and Dnmt3A) in blastocysts produced via somatic cell nuclear transfer (SCNT) in the non-vitrified (non-V), vitrified-thawed(VT), enucleated-vitrified-thawed (EVT), and enucleated-activated-vitrified-thawed (EAVT) groups. GAPDH was used as an internal standard. (*) $p < 0.05$ compared with the non-V group.

5. DISCUSSION AND CONCLUSION

This study demonstrates that SCNT using bovine VT oocytes can be successfully performed by co-culturing oocytes with feeder cells that have been pre-incubated for 15 h and by enucleating and activating oocytes prior to freezing. To improve the survival of frozen-thawed oocytes, we performed co-culture with feeder cells prior to SCNT. We tested the effect of pre-incubating feeder cells for various amounts of time (2, 5, 15 and 24 h) on the survival of frozen-thawed oocytes and their developmental potential after SCNT. There were differences among the treatment groups ($p < 0.05$). Pre-incubation of feeder cells for 15 h prior to co-culture improved the survival of frozen-thawed oocytes, but the development potential of these oocytes was not comparable to that of non-V oocytes. Therefore, we enucleated and/or activated oocytes prior to freezing. Following SCNT, the developmental potential of oocytes in the EAVT group was the highest among the vitrification groups and was comparable to that of non-V fresh oocytes. To identify the reason underlying the differences among the VT, EVT, and EAVT groups, we examined ROS production, MAPK activity, and maternal related gene expression in oocytes and development-related gene expression in blastocysts. Cryopreservation of oocytes is critical for preserving female genetic resources.

Cryopreservation could be a useful technique to provide a steady source of oocytes for SCNT. However, oocytes are more susceptible to cooling damage than embryos. Their large cell size and low permeability to water and CPAs underlie why oocytes are highly sensitive to cryopreservation [124]. In addition, depolymerization of microtubules induced by CPA treatment and cryopreservation causes meiotic spindle disassembly and chromosome misalignment [118]. Vitrification is a simple, rapid, cost-effective, and reliable method. Many vitrification methods for bovine oocyte cryopreservation have been reported [110–112]. In a previous study [121], we tested the suitability of the MVC method for SCNT, and showed that by day 8 after SCNT, 8–15% of VT oocytes had developed to the morula

stage.

In the present study, we pre-incubated droplets (10 μL) of feeder cells for various amounts of time and determined how well co-culture with these cells supported the survival of VT oocytes. Co-culture with feeder cells that have been pre-incubated for 15 h best supported the survival of VT oocytes. Co-culture systems mimic the *in vivo* environment [125]. Co-culture with somatic cells can overcome the *in vitro* developmental block of cultured embryos. Co-cultured cells may secrete nutrients, cytotropic factors, or cell-specific glycoproteins [126].

Although blastocysts were produced from VT oocytes following SCNT, freezing of cumulus-enclosed MII oocytes (VT group) was not effective for subsequent SCNT. In the VT group, half of the surviving oocytes were suitable for SCNT; however, enucleation was difficult because the spindle was unclear or undetectable via the Oosight Microscopy Imaging System. In a previous study [70], we reported that the Oosight Microscopy Imaging System can be used to observe the meiotic spindles of VT oocytes. Enucleation is critical for SCNT; it must eliminate all genetic material from the recipient cytoplasm without causing parthenogenetic activation [24].

Following co-culture with feeder cells that had been pre-incubated for 15 h, we compared the SCNT cloning efficiency of various groups of frozen-thawed bovine oocytes, namely, the EVT, EAVT and VT groups. Significantly more oocytes were suitable for SCNT in the EVT and EAVT groups than in the VT group ($p < 0.05$). Following SCNT, the percentage of oocytes that underwent cleavage in the EAVT group (70.7%) was comparable to that in the non-V group (72.4%) and was significantly higher than that in the VT and EVT groups ($p < 0.05$). In previous studies, following SCNT using VT oocytes, the cleavage rate of reconstructed embryos was reported to be about 50% [113, 114] and 60–70% and the developmental potential of MII oocytes and enucleated oocytes did not differ [119]

In addition, these studies reported varying percentages of SCNT reconstructed

embryos that developed to the blastocyst stage of 7–10% (Booth et al [113], used blastomeres; Muenthaisong et al [119], used ear fibroblasts; Yang et al [115], used fetal fibroblasts) and 16% (Hou et al [114], used cumulus cells or ear fibroblasts). In the current study, we used ear fibroblasts as donor cells. The overall cloning efficiency in the EAVT group (22%) was higher than that in the VT (15%) and EVT (14%) groups and was comparable with that in the non-V group. This indicates that SCNT can be performed using enucleated VT oocytes, as well as with non-V fresh oocytes. On the basis of the aforementioned data, we examined the levels of ROS and phosphorylated MAPK as well as gene expression in the various groups.

The ROS level in the EAVT group was similar to that in the non-V group and was significantly lower than that in the VT and EVT groups ($p < 0.05$). Oxidative stress is mediated by ROS, which are by products of normal cellular metabolism in mitochondria and serve as key signaling molecules in various physiological and pathological processes [127]. An increase in ROS production may lead to a decrease in the intracellular adenosine triphosphate (ATP) concentration and the glutathione/glutathione disulfide ratio, as well as a concomitant increase in the cytosolic concentration of calcium ions, which are all detrimental to oocyte health. We expected freezing to affect ROS production in oocytes; however, the level of ROS in the EAVT group was lower than that in the VT and EVT groups, and was similar to that in the non-V group. This result suggests that oocytes were exposed to different levels of oxidative stress among the groups, and that, among the vitrification groups, oocytes in the EAVT group are most suitable for SCNT.

Microfilament localization and the level of phosphorylated MAPK did not vary among the groups and did not change upon freezing. Microfilaments, or actin filaments, are the thinnest filaments of the cytoskeleton, a highly versatile structure that functions in cytokinesis, cell shape changes, cell movement, and cell division [128]. In the present study, we found no differences in actin filament localization among the groups, except for the

absence of chromatin and spindle staining in the EVT and EAVT groups. Protein levels of MAPK and phospho-44/42 MAPK did not vary among the treatment groups. MAPK is a signaling molecule that associates with centrosome components and is related with microtubule stabilization and thereby with the control of spindle assembly and microtubule configuration [129].

This is the first report to analyze mRNA expression of maternal and development-specific genes in frozen-thawed SCNT cytoplasts and the resulting SCNT embryos. The mRNA levels of several maternal genes (ZAR1, BMP15, and NLRP5) and the stress gene HSF1 were lower in the VT, EVT, and EAVT groups than in the non-V group ($p < 0.05$). The mRNA levels of the GDF9 (maternal) and SOD1 (antioxidant) genes did not vary between the non-V group and the treatment groups, with the exception that the level of GDF9 mRNA was lower in the EVT group than in the non-V group (Figure 14C). Accumulated maternal mRNA in the oocyte is crucial for successful embryo development prior to activation of the embryonic genome [130].

Among the various genes expressed in germ cells, ZAR1, BMP15, GDF9, and NLRP5 transcripts are detected at much higher levels in oocytes than in gonads and are thought to be oocyte-specific markers [131]. ZAR1 was the first oocyte-specific maternal effect gene to be identified and is critical for early-phase embryo development; knockout of this gene renders embryos incapable of developing beyond the first cleavage [132]. Similarly, NLRP5 is a maternal effect gene required for early development prior to zygotic genome activation [133]. The NLRP5 transcript is detected during oocyte growth from primary follicles and accumulates during oocyte development, and its level decreases after fertilization [134]. BMP15 and GDF9, germ cell-specific members of the transforming growth factor-b superfamily, directly affect oocyte growth and function [135, 136]. The present study indicates that oocyte cryopreservation causes the loss of some cytoplasmic mRNA.

Transcription of heat shock genes is rapidly induced after temperature stress. Heat shock gene expression is crucial for the survival of cells exposed to extracellular stress stimuli and also for normal cellular physiology. HSF1-null oocytes have a normal morphology [137]. SOD1 binds to copper and zinc ions and is one of three superoxide dismutases responsible for destroying free superoxide radicals. The encoded isozyme is a soluble, cytoplasmic/mitochondrial intermembrane space protein that forms a homodimer to convert naturally occurring, but harmful, superoxide radicals into molecular oxygen and hydrogen peroxide [138]. In the current study, the mRNA levels of HSF1 and SOD1 were lower and higher, respectively, in the vitrification groups than in the non-V group. This showed that VT oocytes survived well when cultured with feeder cells that had pre-incubated for a long time.

The mRNA expression of developmental potential-related genes (POU5f1, Interferon-tau, and SLC2A5) in SCNT blastocysts was significantly lower in the EVT, EAVT, and VT groups than in the non-V group; the exception was SLC2A5 expression in the EAVT group, which was comparable to that in the non-V group. The higher mRNA expression of POU5f1, Interferon-tau, and SLC2A5 in the EAVT group than in the VT and EVT groups showed that, among the vitrification groups, oocytes in the EAVT group had the highest cloning efficiency. POU5f1 is a master regulator that is expressed at the beginning of mammalian embryogenesis. Variations in the level and pattern of POU5f1 expression might be responsible for at least some of the problems related to cloning [139]. Interferontau is the primary agent responsible for maternal recognition of pregnancy in cattle [43] and is secreted exclusively by trophectodermal cells of blastocysts. This is consistent with the notion that the mRNA expression of this gene is high in good quality embryos. The Interferon-tau mRNA level in the EAVT group was higher than that in the EVT and VT groups, although it was still lower than that in the non-V group. SLC2A5 (GLUT5) is a fructose transporter that is expressed in 8-/16-cell stage embryos [140] and in skeletal muscle, testis, kidney, fat

tissue, and brain. The mRNA level of SLC2A5 in the EAVT group was significantly higher than that in the VT and EVT groups, but was not different from that in the non-V group.

The mRNA expression of the pro-apoptotic gene CASP3 was significantly lower in the EVT and EAVT groups than in the non-V group. Oocytes are exposed to several instances of heat shock stress during the many mechanical and chemical treatment steps involved in SCNT. Heat shock induces apoptosis in preimplantation embryos in a developmentally regulated manner [36]. Hsp70 expression was significantly lower in the EVT and EAVT groups than in the non-V and VT groups, similar to CASP3 expression. These results indicate that enucleated oocytes are safer for the production of SCNT embryos than MII oocytes. Changes in DNA methylation in embryos occur in an extremely organized manner to set up imprinting patterns that are vital for several biological events. Dnmt3A is thought to be responsible for de novo methylation because its activity appears to be targeted to certain domains of the genome [41]. The Dnmt3A mRNA level was significantly lower in the EVT group than in the non-V group.

This study demonstrates that optimized feeder cell co-culture along with oocyte enucleation and activation prior to freezing can be a novel technique for successful SCNT, resulting in enhanced oocyte survival, low ROS production, and a high cloning efficiency. This finding has important implications for nuclear transfer research and the establishment of oocyte banks for SCNT.

CHAPTER 6. The production of cloned Jeju Black Cattle from SCNT embryos using vitrification, one-step dilution and the direct transfer technique

1. ABSTRACT

One-step dilution and direct transfer would be a practical technique for the field application of frozen embryo. This study was to examine whether JBC can be successfully cloned from vitrified and one-step diluted somatic cell nuclear transfer (SCNT) blastocyst after direct transfer. For vitrification, JBC-SCNT blastocysts were serially exposed in glycerol (G) and ethylene glycol (EG) mixtures [10% (v/v) G for 5 min, 10% G plus 20% EG (v/v) for 5 min, and 25 % G plus 25% EG (v/v) for 30 sec.] which is diluted in 10% FBS added D-PBS. And then SCNT blastocysts were loaded in 0.2 mL mini straw, placed in clod nitrogen vapor for 3 min, and then plugged end up and down for 0.5 min, respectively. When *in vitro* developmental capacity of vitrified SCNT blastocyst was examined at 48 h after one-step dilution, hatched rate (56.4%) was slightly lower than that of control group (62.5%). In cloned female JBS was delivered by natural birth on day 299 and healthy at present. In addition, when the short tandem repeat marker analysis of the cloned JBS was evaluated, microsatellite loci of 11 numbers was perfectly matched genotype with donor cell (BK94-14). This study suggested that our developed vitrification and one-step dilution technique can be applied effectively on field trial for cloned animal production, which is even no longer in existence.

2. INTRODUCTION

Cloned mammalian production by SCNT technology has been reported in various species, such mice [4], goats [45], pigs [3], rabbits [140] and cats [46], since it was first reported by Wilmut et al. in the UK in 1997 [1]. In the case of cloned bovine production, Kato et al. [47] and Cibelli et al. [2] have conducted studies, and in Korea, Im et al. [49] and Kim et al. [141] have reported on the production of cloned Hanwoo. However, the efficiency

of cloned embryos produced by SCNT is much lower than that of artificial insemination or IVF embryos, and it is reported that the delivery rate is less than 7% [10, 14]. The in vitro development rate of SCNT blastocysts has been reported to be 30–50%, which is similar to the blastocyst formation rate of IVF embryos. However, the reason for the low production efficiency of cloned embryos is that they are subjected to mechanical and chemical processes for a protracted time in the unstable in vitro culture environment, which increases the rate of failed embryo implantation and incomplete implantation.

On the other hand, in order to increase the productivity of cloned embryos with superior genetic resources or of endangered animals, cryopreserving produced SCNT blastocysts should be established beforehand. In general, cloned embryos are most likely to be transferred immediately without being frozen because of the risk of quality deterioration due to a number of environmental factors. In order to increase the pregnancy rate and birthrate of cloned embryos, it is necessary to develop a stable cryopreservation technology that can be transferred at any time in a timely manner, together with the selection of a recipient with a superior uterine status. Many studies have reported on the production of cloned bovines using fresh SCNT embryos. However, the production of cloned bovines through the transference of frozen SCNT embryos is rare [142, 143], and none use a simple method that can be directly applied in the field.

This study was carried out to investigate the effect of embryo transfer using SCNT blastocysts produced by JBC that were vitrified and stored in a laboratory, and after a certain period of time, were thawed in a one-step dilution method so that the SCNT embryos could be transferred to a recipient’s uterus immediately. In this way, we investigated the possibility of JBC cloned production and the usefulness of vitrification and one-step dilution techniques using SCNT frozen blastocysts.

3. MATERIALS AND METHODS

1) Oocyte preparation and *in vitro* maturation

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory within 2 h in 0.9% saline at 35 °C. Cumulus oocyte complexes (COCs) were aspirated from visible follicles (2–6 mm in size) with an 18 gauge needle attached to a 10 mL disposable syringe. The medium used for COCS collection was HEPES-buffered Tyrode's medium (TL-HEPES). Sets of 30-40 COCs were *in vitro* matured in each well of 4-well using 500 μL tissue culture medium 199 (TCM-199, Gibco) supplemented with 10% fetal bovine serum (FBS), 0.2 mM sodium pyruvate, 1 μg/mL follicle-stimulating hormone (FolltropinTM, Bioniche Animal Health, Belleville, ON, Canada), 1 μg/mL estradiol-17, and 50 μg/mL gentamycin (Sigma) in mineral oil at 38.8 °C in an incubator (5% CO_2, 5% O_2, and 90% N_2) for 18–21 h.

2) SCNT and *in vitro* culture

In vitro matured bovine oocytes were removed cumulus cells from the TL-HEPES medium supplemented with 0.1% hyaluronidase, and only oocytes were used the released the first polar body. The oocytes were transferred to TCM-HEPES culture medium drop added with 7.5 μg cytochalasin B, and nucleus position was confirmed using Ooight imaging system, and then enucleated. The donor cells were collected from the JBC dam's ear cells in Jeju Special Self-Governing Province, Livestock Promotion Agency, and frozen and stored in 2007, cultured in 4–8 passaged were used. Donor cells were treated with Tryple solution, separated into single cells, treated with 3% preteinase solution for 50 sec, and then injected into the space between the zona pellucida and the cytoplasm of the enucleated oocyte with 10 μm cells. The injected oocytes were fused 1 pulse at direct current 22 volts in a 0.3M mannitol solution using a LF101 Electro Cell Fusion Generator (NEPA GENE, Shioyaki, Japan) and the cell fusion was observed 30 min later. The fused oocytes were exposed to 5

μm Ca-ionophore for 5 min and cultured in 20 mM DMAP for 3 h. The activated oocytes were cultured in CR1aa culture medium containing 3 mg/mL FAF-BSA for 48 h, and cell divided embryos were cultured to induce embryonic development in CR1 aa 10 μL feeder cell droplet with 10% FBS, 1 μg/mL Epidermal growth factor (EGF), 1 μg/mL Insulin-like growth factor (IGF) and 10 μM flavonoid.

3) Vitrification of SCNT blastocyst

10% FBS was added to Dulbecco's phosphate buffered saline (D-PBS) as a base solution, and the vitrification solution was mixed with glycerol (G) and ethylene glycol (EG). SCNT blastocysts were treated in D-PBS (10% FBS) with 10% G (v/v) for 5 min and then for 5 min in D-PBS with 10% G and 20% EG (v/v) to induced dehydration equilibrium. And then they were exposed to D-PBS mixed with 25% G and 25% EG for 20–30 sec using a pipette, loaded on a 0.25 mL french mini straw and thermally sealed. The straw was exposed to the LN_2 vapor for 3 min, frozen and immediately immersed in LN_2 at -196 ℃. The constituent layers in the straw for the one-step dilution were prepared laboratory-developed [145] and were prepared as follows. 7.5 cm (0.5M sucrose) - 0.5 cm (air layer) - 1.0 cm (G24EG24 vitrification solution, loaded SCNT blastocysts layer) - 0.5 cm (air layer) - 1.5 cm (0.5 M sucrose) layers were prepared the sucrose and vitrification solutions at a ratio of 9: 1 to dilute. Two SCNT blastocysts were loaded each straws.

4) One-step dilution

The frozen straws were placed in 25 ℃ water, and then inverted for 30 sec and left in a vertical state for 30 sec to completely mix the contents in the straw. The control group for the *in vitro* development investigation after one-step dilution was treated step by step with the pre-treatment solution and One-step dilution solution, followed by final washing with the culture solution, it was investigated the toxicity.

5) Estrus synchronization of recipient, one-stage dilution SCNT blastocyst transfer and production of cloned calf

The recipient was inserted CIDR-PLUS for 7 days in vagina, and then removed it and administered PGF_2 α 25 mg on the day, in this way, estrus synchronization was induced. There were selected the recipient that shown the normal estrus and have a good state of corpus-luteum through rectal palpation, and then one-step diluted SCNT blastocyst were transferred uterine corpus with luteum, and two professional technicians who have 700 times of embryo transfer experiences performed SCNT blastocyst transplants. Pregnancy of the recipient was confirmed by rectal palpation at 45 day after the vitrified-thawed embryo transfer. 10–20 days before the delivery, we observed the sign of delivery of the recipients, and allowed normal delivery at the appropriate time.

6) DNA analysis

There were performed DNA analysis using somatic cell of donor breeding bull (BK94-14) and ear cell of cloned calf cells, in order that confirmed to cloning of calf born from vitrified-thawed SCNT blastocyst. DNA from each test sample was extracted as a genetic material, and specific genes were amplified at the same time to determine whether donor somatic cells (BK94-14) and the cloned calf were matched. There were analyzed using 11 International Society of Animal Genetics (ISAG) standard microsatellite markers (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225 and BM1824).

7) Statistical analysis

The survival rate of SCNT blastocyst after vitrification and one-step dilution was analyzed by SAS program, and the significant difference of each other groups was tested using Duncan's multiple range tests.

4. RESULTS

In vitro development of vitrified JBC blastocyst after one-step dilution

Table 15 and Figure 16 show the results of the *in vitro* development rate after one-step dilution of the vitrified SCNT blastocyst. When the cloned blastocysts of the control (exposed with vitrification pretreatment solution and one-step dilution solution without LN_2) and treatment groups (vitrified and one-step dilution) were cultured *in vitro* for 48 h, the survival rate after one-step dilution was 84.6% in the treated group, and the development rate of the hatched blastocysts (Figure 16B) among survival blastocysts (Figure 16A) was 56.4%, slightly lower than that of the control group (62.5%), but there was no significant difference. This experiment was investigated 5 times.

Pregnancy rate of direct transfer after vitrification and one-step dilution of SCNT blastocyst

Vitrified and one-step diluted SCNT blastocyst were direct transferred to five uterus of induced estrus synchronized recipient, as a result, a normal pregnancy was identified at 45 days after transplantation in one recipient (Table 16). SCNT cloned breeding calf was successfully normal delivered at 299 days, two weeks after the normal pregnancy period, and the birth weight was 36 kg. It was named “Heuk Woo Sunee”.

Confirming the genetic parentage testing of cloned calves

When the result of short tandem repeat marker analysis of cloned calf ear cells (using vitrified blastocysts, one-step dilution and direct transfer technique) and donor JBC was investigated, all 11 microsatellite loci matched genotype between cloned calf and donor JBC somatic cell, and gender was also matched as the same female (Figure 18).

Table 15. *In vitro* survival of vitrified and one-step diluted bovine SCNT blastocyst

Treatment	No. of SCNT blastocyst	No. (%) of recovery	No. (%) of survived after 48 h	
			≥Hatching blastocyst	Hatched blastocyst
Control*	40	40 (100.0)	40 (100.0)	25 (62.5)
Vitrification & one-step dilution	39	39 (100.0)	33 (84.6)	22 (56.4)

*Fresh SCNT blastocysts were treated sequentially in solution and then in one-step dilution solution except LN_2 exposure, respectively.

Table 16. Production of cloned JBC from vitrified-thawed SCNT embryo

No. of* transferred embryo	No. of recipient	No. of pregnancy	No. of** birth calf	No. of living calf
10	5	1	1	1

*Vitrified SCNT embryos were one-step diluted in 25 ℃ water for 1 min and then directly transferred into surrogate mother in field.

** A healthy and apparently normal cloned Jeju Black calf was born on day 299.

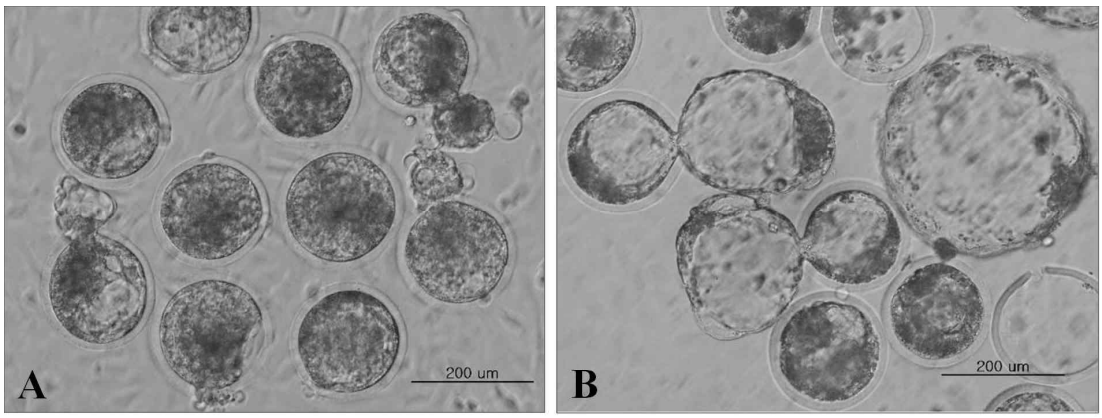


Figure 16. *In vitro* survival morphology of vitrified JBC-SCNT blastocysts after one-step dilution. After co-culture with monolayered ear cell for 24 h (A) and 48 h (B), survived embryos were normally developed into hatching and hatched stages, respectively.

Figure 17. Five month old somatic cell nuclear transfer JBC female calf “Heuk Woo Sunee” (Birth weight: 36kg, Oct. 31. 2009).

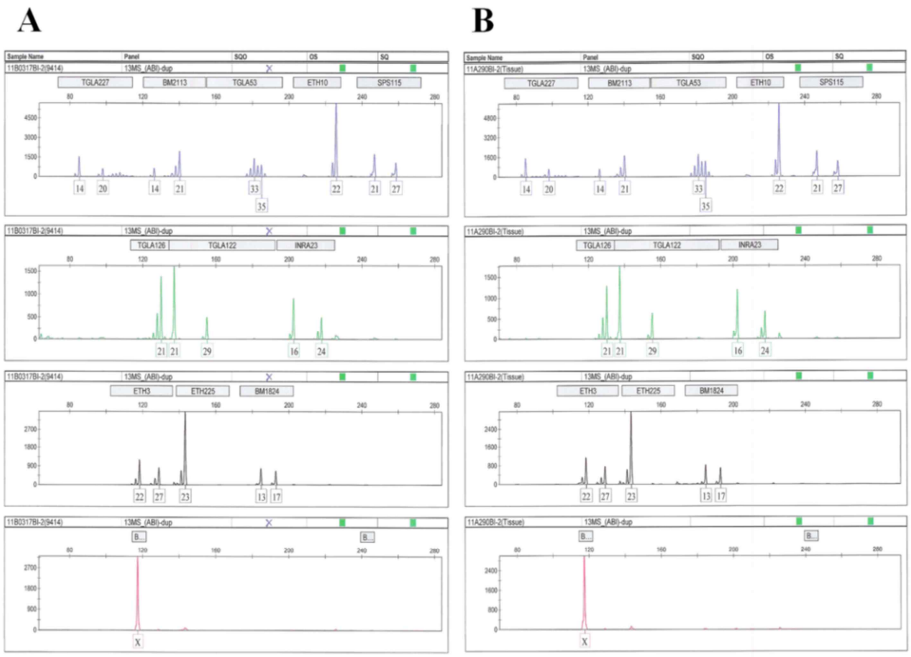


Figure 18. STR profile investigated full DNA fingerprint of elite female JBC somatic cell (BK94-14, A) and it’s cloned female calf (“Heuk Woo Sunee”, B) using 11 STR markers and amelogenin.

5. DISCUSSION AND CONCLUSION

In this study, in order to produce cloned JBCs of an excellent lineage, SCNT blastocysts were vitrified and stored, and then one-step dilution method was used to thaw them when the recipient was been ready. This was done through the typical field frozen-thawed method of direct transfer into the recipient's uterine, which is used to produce cloned calves.

Vitrification is a method of freezing the embryo within a few minutes [104] while preventing ice formation, which is the main cause of cell injury during freezing, by using a high concentration of a cryoprotective agent. It does not require expensive equipment and has high efficiency, and therefore, it is a practical freezing method that has been applied to human embryos and experimental animals alike. The frozen embryo is generally rapid thawed in 36 °C water, and it is common to carry out several stages of washing to remove the cryoprotective agent. In human in vitro fertilization procedures as well as those for experimental animals, the thawing of frozen embryo is carried out in the laboratory before embryo transfer. As such, it is not necessary to study the method of field-based thawing. One-step dilution is a very simple method developed for field applications in industrialized livestock production, and it is a useful method that may be easily done without requiring professional skills [145, 146].

There have been reports of in vivo and in vitro fertilized embryos [147, 148]; however, it is rarely reported that an offspring was created using SCNT embryos that underwent the simple vitrifying and thawing method. Tecirlioglu et al. [142] used the open pulled straw (OPS) method to vitrify the SCNT embryos, and thawing was performed in a two-step transfer from the OPS straw to the diluting straw. One cloned calf was obtained from 14 animals. Gong et al. [143] used frozen SCNT embryos in a two-step method that exposed them to an EFS40 solution, which was poured into the culture dish at the time of thawing, washed with two-steps, and then mounted on a transplanting straw. In this way,

SCNT embryos were transferred to produce one cloned calf among 9 animals. However, both of the above-mentioned methods are difficult to use directly in the field. Therefore, this study is the first example of the production of a cloned calf through the field application of the freezing method. This study establishes the usage of transfer straws as the vitrification method. Since it takes approximately a single minute, this is a short, useful transfer method that requires only one-step dilution and can be used anywhere on the field without environmental restrictions.

The usefulness and safety of the direct transfer technique after the vitrification / one-step dilution method that was developed in this study was already achieved by the 40% (4/10) delivery rate reached in an attempt to produce a Hanwoo elite calf in some farms in Jeju during a previous study that used in vitro fertilization embryos [149]. Based on this stable freezing-thawing technique, SCNT embryos were produced using the ear somatic cells of JBC from the Jeju Special Self-Governing Livestock Promotion Agency, which had been frozen in 2007, and two SCNT blastocysts were placed in each straw. Some were thawed using the ultra-simple thawing method and then transferred to recipients in order to succeed in ‘restoring the most outstanding non-existent pedigree, the JBC breeding cow species.’

When genetic analysis was performed using the 11 STR markers for the donor somatic cell of the breeding cow and the ear cells of the cloned calf, all 11 microsatellite loci between the cloned calf and the donor somatic cells of the JBC breeding cow were completely matched. The gender was also confirmed to be the same, female, and the calf produced in this study was proven to be a cloned calf derived from BK94-14. Therefore, the vitrification / one-step dilution method developed in this study is considered to be a very useful technology for establishing an industrial base by increasing the usefulness of SCNT embryos. Currently, transfer studies of frozen cloned embryos are underway in the production of cloned bovines for the restoration of superior bloodlines.

In our previous study [141], we succeeded in producing a cloned breeding bull (Heuk Young Dolee) with the most meat traits in existing black cattle (BK01-10), and have also reported the production of a second cloned breeding bull (Heuk Oll Dolee) using the cryopreserved somatic cells of a good meat quality breeding bull (BK94-13), which was born and slaughtered at the same time as the black cow used in this study. The third cloned cow lso grew without any abnormalities in health since the pregnancy was confirmed. Unlike the two cloned bulls that were born by cesarean section, the JBC cloned cow that was produced was born in a natural birth after 299 days of pregnancy and weighed 36 kg (Figure 17). Based on these results, our researchers are now equipped with all the technologies and environments necessary for cloned bovine production, such as SCNT, culture environment, the frozen and thawed method, direct transfer, and pregnancy maintenance and management. A valuable research base has been established for industrial development and mass proliferation of the endangered JBC according to successful production of cloned breeding bulls (existing and non-existing) and a cloned breeding cow resulting from the incorporation of vitrification / one-step dilution and direct transfer technology.

Therefore, the vitrification / one-step dilution and direct transfer technology developed in this study can be effectively used for the application of SCNT blastocysts as well as in vivo / in vitro fertilization of embryos, and it is expected that it will be useful for the conservation of excellent species and the restoration of endangered species disappear due to unpredicted natural environmental diseases, such as foot-and-mouth disease, in addition to the establishment of the development of mass production technology for JBC.

CHAPTER 7. OVERALL DISCUSSION AND CONCULUTION

This study was carried out to contribute to the conservation of an endangered species, JBC, by increasing the production efficiency of SCNT embryos and making it possible to produce cloned animals efficiently. SCNT is done through various mechanical and chemical steps that are very damaging to the oocyte in the production process. Therefore, the development rate is lower than for in vitro fertilization embryos, and the pregnancy rate and delivery rate are low when the produced SCNT embryo is transferred. Despite these difficulties, there have been many studies that have successfully produced cloned animals that belong to endangered species [1–3].

To solve these problems, researchers have been conducting various studies. First, a study was conducted to reduce the physical and chemical damage caused by the enucleation process of the oocyte. Especially in bovine species, lipid droplets spreading in the ooplasm hamper the identification and enucleation of metaphase II (MII) chromosomes, and the success rate of cloning remains low as a result. In this study, the efficiency of the two methods (Hoechst staining and Oosight imaging system) at determining the nucleus location was compared, and enucleation was done through the squeezing method. The cloning efficiency of Hoechst-stained oocytes is affected negatively by a simultaneous increase in UV irradiation, and some researchers have indicated that oocytes should not be exposed to UV for more than 30 sec [150]. Hoechst strongly binds to the minor groove of DNA, particularly to the AT-rich regions [151]. Mitochondrial DNA (mtDNA) possesses many AT-rich sites [152]. Recently, it has been demonstrated that Hoechst 33342 can strongly bind to mtDNA and induce an increase in ROS in human malignant glioma cells that leads to cell death [153]. If a similar mechanism occurs in the mitochondria of the oocyte after exposure to Hoechst and UV irradiation, embryonic development might be impaired as a consequence of ROS accumulation inside of the cell [154]. The results of this study also showed that the Hoechst staining group has low overall cloning efficiency. The fusion rate, cleavage rate and

blastocyst development rate were significantly higher in the Oosight imaging group, ; however, the apoptotic index was lower. The gene expression level of Oct4 (maternal recognition of pregnancy-related genes) was higher in the Oosight group than in the Hoechst staining group, and the expression level of Hsp70 (a stress related gene), and Caspase-3 (a pro-apoptotic related gene) was lower than in Hoechst staining. Therefore, the enucleation method of the Oosight imaging system was less harmful to the development of SCNT embryos.

Secondly, to improve the development efficiency of the cloned embryos, an antioxidant supplement iwas added to the culture medium. Unlike in vivo culture, it is generally known that oxygen saturation is accumulated during in vitro culture, and ROS is one of the causes of the inhibition of development. There are many reports that antioxidants are added to the culture to lower the oxygen saturation degree. Flavonoids are a class of plant secondary metabolites and are most commonly known for their antioxidant activity in vitro [155]. In this study, the production of cloned embryos and their efficiency were confirmed by adding flavonoids, and it was confirmed that the apoptotic index was low in the 10 μm flavonoid-treated group. In addition, the expression of Mn-SOD, Survivin and Bax inhibitor were significantly increased, and the expression of Bax and Caspase-3 were decreased. After the transfer of the SCNT embryos into the recipient cow, the rate of pregnancy and delivery rate of flavonoid treated group were high. This indicates that flavonoids can be used as antioxidant additives in the production of in vitro SCNT embryos.

Third, this study was investigated using a time-lapse monitoring system to evaluate the embryos and provide standard data to select healthy embryos and improve embryo implantation potential. The time-lapse monitoring system is an exciting, novel technology with great potential for enhancing embryo selection in the embryology laboratory. This non-invasive objective assessment of embryos has provided a new tool for predicting embryo development and implantation potential. Time-lapse monitoring detects several

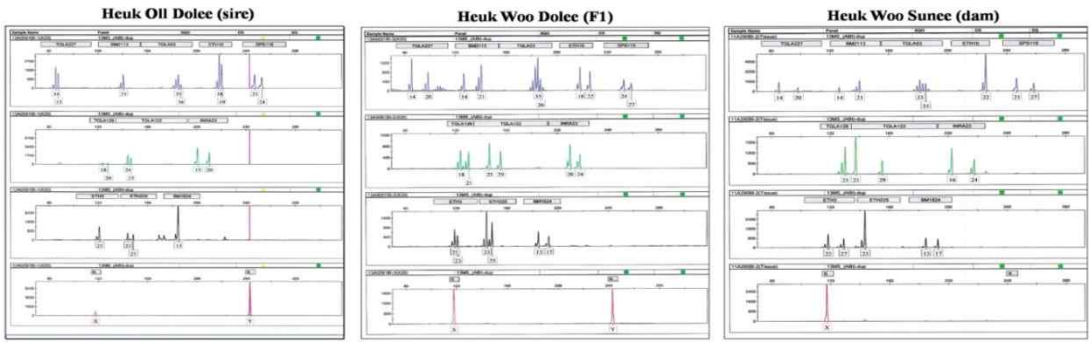
morphological phenomena that are often missed with static observations using conventional incubators, such as irregular divisions, blastocyst collapse and re-expansion, timing of blastocoel appearance, and the timing of the formation and internalization of fragments [156]. In particular, in this study, the average developmental rate was confirmed at each stage of the parthenogenetic embryos using ear cells and transgenic cells, and correlations between normal embryonic development and first cleavage time were investigated. As a result, we could observe that the early cleaving group developed into a normal form at each stage, and it was confirmed that the probability of stopping development was also low. This was similar to that of the PA, NT-bEC and NT-bTGC group; however, the NT-bTGC group showed fewer numbers of embryos that underwent early cleaving and normal morphology than in other groups. In this study, early cleavage and normal morphology were considered to be good parameters for the selection of healthy embryos.

Fourth, for the stable supply of donor oocytes for the production of SCNT embryos and the smooth transfer of the produced SCNT embryos, simple and efficient cryopreservation and survival technology must developed such that they can be applied in the field. Cryopreservation is a process by which biological cells or tissues are preserved at subzero temperatures, resulting in a radical decrease in the rate of the metabolic processes and the ability to store samples for extended periods. However, as expected, freezing cells causes damage. The two major causes of cellular damage are the physical damage caused by the formation of ice crystals and chemical damage [157]. Therefore, this study attempted to simplify the vitrification and thawing step and require as little time as possible to reduce the damage. In particular, pro-incubated feeder cells were used for recovery after oocyte thawing, and the improvement of the oocyte survival rate was confirmed. In the case of SCNT embryo production, it is necessary to undergo the enucleation process of the oocyte. Since the enucleation of the oocyte after thawing is not easy, SCNT embryos were produced through enucleation and activation before freezing, and somatic cells were injected after thawing.

Moreover, cloning efficiency was significantly higher after using this method group than in the other groups. Next, the method used in this study to transfer the SCNT embryos is a simple method in which a transfer straw is used for vitrification and one-step dilution is carried out in one minute. This may be directly applied in the field since it takes only a minute to directly transfer the embryo after thawing, and this study succeeded in producing cloned JBCs as a result.

In addition, my laboratory confirmed that the post-death cloned JBC breeding bull and cow have a reproductive capacity [67]. The post-death cloned JBC cow (Heuk Woo Sunee) and the post-death cloned JBC bull (Heuk Oll Dolee) artificially conceived and succeeded in giving birth to Heuk Woo Dolee, who was healthy and developed normally. We confirmed the biological relationshipbetween the cloned bull (Heuk Oll Dolee), the cloned cow (Heuk Woo Sunee), and their F1 offspring (Heuk Woo Dolee) (Figure 19). This is the first report to prove the reproductive capacity of a post-death cloned bovine.

The conclusion of this study is as follows. 1) Oosight imaging is an efficient and reliable enucleation technique that could improve cloning efficiency and the developmental potential of SCNT embryos by being less damaging to unknown cellular mechanisms. 2) In order to improve the birth rate of offspring of SCNT blastocysts, a flavonoid treatment is necessary to act as an antioxidant capable of rapidly recovering cell functions after nuclear transfer. 3) Early cleavage (≤ 20 h) and a normal morphology may be good parameters to select bovine SCNT embryos. 4) An optimized feeder cell co-culture along with oocyte enucleation and activation prior to freezing is a novel technique for successful SCNT that results in enhanced oocyte survival, low ROS production, and high cloning efficiency. This finding has important implications for nuclear transfer research and the establishment of oocyte banks for SCNT. 5) The vitrification / one-step dilution and direct transfer technology can be effectively used for the application of SCNT blastocysts as well as the in vivo / in vitro fertilization of embryos. Heuk Young Dolee, Heuk Oll Dolee and Heuk Woo Sunee,



Name	Heuk Oll Dolee (sire)		Heuk Woo Dolee (F1)		Heuk Woo Sunee (dam)	
Gender	XY		XY		XX	
Locus	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
TGLA227	14	15	14	20	14	20
BM2113	21	21	14	21	14	21
TGLA53	35	36	35	36	33	35
ETH10	18	19	18	22	22	22
SPS115	21	24	24	27	21	27
TGLA126	18	20	18	21	21	21
TGLA122	24	25	25	29	21	29
INRA23	15	20	20	24	16	24
ETH3	23	23	22	23	22	27
ETH225	23	25	23	25	23	23
BM1824	13	13	13	17	13	17

*The shared alleles between Heuk Oll Dolee and F1 (Heuk Woo Dolee) are represented in blue.
*The shared alleles between Heuk Woo Sunee and F1 (Heuk Woo Dolee) are represented in green.

Figure 19. Cloned sire (Heuk Oll Dolee) and cloned dam (Heuk Woo Sunee) and their F1 offspring (Heuk Woo Dolee). STR analysis revealed that Heuk Oll Dolee and Heuk Woo Sunee were the biological sire and dam of Heuk Woo Dolee, as they shared all alleles at 11 out of 11 loci.

which are clones of good breeding cattle, have been produced using these techniques, and succeeded in producing Heuk Woo Dolee by confirming the reproductive capacity of the post-death cloned JBC.

This study conducted analysis to determine the most effective methods (efficient enucleation of oocytes, improvement of culture environment and development of vitrification and survival techniques) for producing cloned embryos using SCNT techniques. These techniques are intended to contribute to the conservation of JBC and to help establish the basis for their mass production and industrialization.

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체세포 복제 기술을 이용한 복제 수정란의 효율적인 생산 방법 및 제주 흑우 종 보존에 관한 연구

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ABSTRACT

제주흑우는 제주특별자치도에서 서식하는 검은색 모색을 가진 한국 전통 소의 5품종의 하나이다. 제주흑우는 전통적으로 고품질 육류로 여겨져 왔으나 현재 제주특별자치도 내에 원종이 대략 700여 마리 정도 밖에 서식하지 않으며, 멸종위기 동물로 분류되고 있다. 이러한 멸종 위기의 동물의 종 보존에 복제기술이 필요한 기술로 이용 될 수 있다. 본 연구의 목적은 체세포 복제 수정란의 생산 효율을 높이고 복제 시 이용될 난자 및 체세포 복제 배아의 냉해동 기술을 개발하여 복제 소 생산율을 높혀 제주흑우 종 보존에 기여하고자 함이다.

실험1: Hoechst staining 과 Oosight imaging 두 가지 탈핵 방법을 이용하여 체세포 복제 수정란을 생산하였을 때 그 효과를 비교해보았다. Oosight imaging 그룹의 경우 세포사멸율이 낮았고 (2.8-0.5 vs. 7.3-1.2), 체세포 합성율 (75.6% vs 62.9%), 난할율 (78.0% vs 63.7%), 배아 발달율 (40.2% vs 29.2%) 및 총 세포 수 (128.3-4.8 vs 112.2-7.6) 모두 Hoechst staining 그룹에 비해 높게 나타났다 (모두 $p < 0.05$). 최종적인 복제수정란의 생산 효율 또한 Oosight imaging 그룹이 두 배 이상 높게 나타났다 ($p < 0.05$). 이는 Oosight imaging 시스템을 탈핵 방법으로 선택했을 때 Hoechst staining 시스템보다 복제 수정란 발달능에 덜 유해함을 보여준다.

실험2: 본 실험은 플라보노이드 처리를 하였을 때 체외 복제 수정란 발달에 미치는 영향을 조사하였다. 플라보노이드의 적정 농도를 확인하기 위해 단위 생식 수정란의 발달 2일 째 0 (대조군), 1, 10 그리고 20 μM 농도로 처리하여 6일간 배양하였다. 결과적으로 체외 수정란 발달율은 세 그룹 중 10 μM 농도 (57.1%) 에서 가장 높았고 총 세포수가 ICM 세포 수 또한 유의하게 10 μM 농도에서 높게 나타났다. 10 μM 처리군의 경우 세포사멸율이 유의하게 다른 그룹에 비해 낮았으며 항산화 관련 유전자와 항세포사멸 유전자, 세포 성장과 발달 유전자의 발현이 높게 나타났다. 최종적으로 배양 7일 또는 8일째 체세포 복제 배아를 대리모에 이식하였을 때 10 μM 처리군의 수태율 (10.2%, 6/59)이 대조군 (5.9%, 2/34)에 비해서 높았다. 이러 결과는 배양액에 플라보노이드를 첨가하여 배양하는 것이 체외 및 체내 복제 수정란의 발달율과 체세포 복제 수정란의 수태율에 긍정적인 영향을 준다는 것을 알 수 있었다.

실험3: 실시간 모니터링 시스템을 사용하여 단위발생 수정란 (PA)과 두 가지 타입의 체세포 복제 수정란의 발달능 및 발달 속도를 조사하였다. 체세포는 귀 세포 (bECs) 또는 형질전환 체세포 (bTGCs) 두 종류를 사용하였다. 난할율과 배아 발달

율은 세 그룹 (PA, NT-bEC, NT-bTGC)에서 유의한 차이가 나지 않았으나 활성화 이후 첫 번째 난할이 일어나는데 까지 걸리는 시간은 19.3 시간 (n=70), 21.6 시간 (n=60)와 21.3 시간 (n=62)이 걸렸다 (전체 적인 평균은 20.4 시간 (n=192)이었다). 20 시간을 기준으로 early cleaving (20 시간)과 late cleaving (> 20 시간)으로 나누어 배아 형성율을 비교해 본 결과 early cleaving 그룹이 (PA, 46%; NT-bEC, 50%; NT-bTGC, 39%) late cleaving 그룹 (PA, 18%; NT-bEC, 23%, NT-bTGC, 28%) 보다 유의하게 높게 나타났다. 반면에 2 세포기와 8세포기에 수정란의 발달이 멈추는 비율은 late cleaving 그룹이 증가됨을 보였다. 또한 early cleaving과 형태적으로 정상적인 발달을 보인 보인 수정란의 수가 PA 그룹 (20.0%, n=14)이 NT-bTGC 그룹 (9.7%, n=6) 보다 2배 정도 높았다. 이러한 결과는 실시간 모니터링 시스템으로 얻어진 각 개체별 수정란의 발달 속도와 형태학적의 자료를 기반으로, 체세포 복제 수정란의 early cleaving (< 20 시간)과 형태적으로 정상적인 발달 상태를 기준으로 발달능을 예측하는데 도움을 주어 건강한 복제 수정란을 선택할 수 있으리라고 사료된다.

실험4: 본 연구는 체세포 복제를 위해 제공된 난자의 냉동 보존과 해동 후 생존율의 최적화하는 실험을 실시 하였다. 지지세포와 함께 15시간 동안 선 배양했을 때 냉해동 난자의 생존율이 2, 5, 또는 24 시간 배양한 그룹보다 유의하게 높았다 ($p < 0.05$). 복제 효율은 EAVT (탈핵 후 활성화 시켜 냉해동) 그룹 (21.6%)이 non-V (냉동하지 않은) 그룹 (25.9%)보다는 낮았으나, EVT (탈핵 후 냉해동) 그룹 (13.7%), VT (탈핵하지 않고 냉해동) 그룹 (15.0%) 보다 높게 나타났다. 유전자 발현 수준에서 확인한 결과, 냉해동 그룹 중에서 EAVT 그룹의 배아가 발달능 관련 유전자 (POU5f1, Interferon-tau, SLC2A5)의 발현 수준이 높았고, 세포사멸 관련 유전자 (CASP3)와 스트레스 관련 유전자 (Hsp70)의 발현 수준은 낮게 나타났다. 이러한 결과는 냉해동 난자의 최적화된 선배양 및 급속냉동 기술을 이용하여 체세포

복제시 유용하게 냉해동 난자를 제공할 수 있을 것으로 사료된다.

실험5: 초자화동결을 위해 제주흑우 체세포 복제 배아를 D-PBS 용액에 10% FBS가 포함된 글리세롤 (G) 과 에딜글라이콜 (EG) 혼합물로 [10% (v/v) G 5분, 10% G와 20% EG (v/v) 5분, 25% G와 25% EG (v/v) 30초] 처리하여 0.25 mL 미니 스트로에 로딩하여 3분간 액체질소 냉기에 노출한 후 일정시간 액체질소에 보관하였다. 해동 시에는 25 ℃의 물에서 수직상태로 30초 놓아두고 다시 뒤집어서 수직상태로 30초간 방치하여 총 1분간 스트로 내의 내용물이 완전히 섞이도록하는 1-단계 융해법을 사용하였다. 초자화동결된 체세포 복제 배아의 발달능을 실험하기 위해 1-단계 융해를 실시한 48시간 후 부화된 배아율을 확인한 결과 1-단계 융해 방법을 사용하여 동결융해된 처리군 (56.4%)이 대조군 (62.5%)에 비해 미세하게 낮게 나타났으나 유의한 차이는 없었다. 동결융해한 체세포 복제 배아를 5마리의 대리모 소에 이식하여 한 마리 대리모가 수태하였고 임신 299일만에 암소가 건강하게 태어났다. 본 연구에서 제안한 초자와동결법과 1-단계 융해법 기술이 현장에서 복제 동물 생산을 위해 유용하게 적용될 수 있을 것이다.

본 연구는 복제 수정란 생산을 효율적으로 할 수 있는 여러 방법 (난자의 효율적인 탈핵 방법, 복제 수정란 배양환경 개선 그리고 난자와 배아의 초자화동결 및 동결 후 생존율 향상 기술 개발)을 조사 하였고 복제 소를 생산하는데 생공하였고 사후 복제된 제주흑우 씨수소 씨암소의 생식능력도 확인하였다. 이러한 기술은 멸종위기의 제주흑우의 종 보존에 가능하게 하며 나아가 제주흑우 대량증식 및 산업화 기반을 마련하는데 도움을 줄 수 있을 것으로 사료된다.

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