Master's Thesis

Production of Cloned Jeju Black Cattle (Korean Cattle) Using Oosight Imaging System

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Oosight Imaging System을 이용한 제주흑우 (한우) 복제소 생산

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(Directed by Professor Se Pill Park)

ABSTRACT

Somatic cell nuclear transfer (SCNT) is an efficient technique that has been successfully applied to developmental biology, and resulted in the production of offspring from various species including sheep, cattle, mice, and pigs. It offers many opportunities in basic and medical research as well as endangered species conservation. However, the efficiency of SCNT is still very low. To date, numerous studies have been performed to optimize the SCNT procedures such as enucleation, cell injection and activation.Enucleation of a recipient oocyte is critically important to cloning efficiency.

This study compared the effect of two enucleation systems, ‘Hoechst staining and UV irradiation’ and ‘Oosight imaging’, on *in vitro* production of bovine

SCNT embryos and their developmental potential.Enucleation of recipient oocytes by either Hoechst staining and UV irradiation or Oosight imaging prior to transfer of Jeju Black Cattle donor cells.

In result the SCNT group using Oosight imaging, the fusion rate (75.6% vs. 62.9%, $P<.01$), cleavage rate (78.0% vs. 63.7%, $P<.05$), blastocyst rate (40.2% vs. 29.2%, $P<.01$) and total cell number (128.3±4.8 vs. 112.2±7.6, $P<.05$) were significantly higher than the SCNT group using Hoechst staining and UV irradiation. Also, the apoptotic index was significantly lower in the Oosight imaging group (2.8±0.5 vs. 7.3±1.2, $P<.05$) compared with the Hoechst staining and UV irradiation group. In addition, in the Oosight imaging group, the relative expression levels of Oct4, Nanog, Interferon tau, and Dnmt3A mRNA were higher, and of Caspase-3 and Hsp70 mRNA were lower, compared with the Hoechst staining and UV irradiation group.

The Oosight imaging system improves the cloning efficiency and *in vitro* develop- mental potental of bovine SCNT embryos and this technique will be useful for the cloned offspring production.

INTRODUCTION

SCNT is an efficient technique that has been successfully applied to developmental biology, and resulted in the production of offspring from various species including sheep, cattle, mice, and pigs (1-4). It offers many opportunities in basic and medical research as well as endangered species conservation. However, the efficiency of SCNT is still very low. To date, numerous studies have been performed to optimize the SCNT procedures such as enucleation, cell injection and activation (5-7). Among them, the enucleation is recognized as a critically important step, the fine enucleation technique absolutely needs to avoid aneuploidy abnormalities with detrimental effects on later development to eliminate any genetic contribution of the recipient cytoplasm and to exclude the possibility of parthenogenetic activation (8). To improve the enucleation procedure and cloning efficiency, several methods were developed such as 'Blind', 'Hoechst stain and UV irradiation', 'Spindle imaging' and 'Centrifugation' (9-12).

To ensure error-free enucleation 'Hoechst stain and UV irradiation' has been used, particularly in domestic species, easy to visualize the chromosomes before removal of dense cytoplasm in the oocytes prior to NT. Using this procedure, a little cytoplasm removal surrounding the spindle is possible, conversely even very short UV exposure may affect negatively on the membrane integrity, mitochondrial DNA and further embryo developmental potential (13). As a more stable and proper enucleation, 'Spindle imaging' was introduced for the direct enucleation of ocytes using a polarized light microscopy (11). In human IVF program, Spindle imaging (in this study I mentioned as Oosight imaging system) was used to locate the meiotic

spindle to avoid disrupt it while injecting oocytes (14). Also, the noninvasive and reliable technique was used to improve cloning efficiency in pig (15). However, there was no report that the positive effect of Oosight imaging system on the gene expression of SCNT embryos.

In this study, I examined the effect of two enucleation systems, 'Hoechst staining and UV irradiation' and 'Oosight imaging', on *in vitro* production of Jeju Black Cattle (JBC, Korean Native Cattle) -SCNT embryos and their developmental potential. In addition, I analyzed the significant differences in cloning efficiency according to use different enucleation system in the base of apoptosis and specific gene expression by TUNEL assay and semi-quantitative RT-PCR, respectively.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals were purchased from Sigma Chemical Company (St Louis, MO,USA).

1. Preparation of donor cells

Donor somatic cells were derived from Jeju Black Cattle (JBC) ear tissue. Briefly, after removing fur, a small ear tissue was washed three times with PBS, incised with a sterile scissors to exposure basal epidermis and cut into <1.0 mm size. The sliced ear tissues were incubated in 0.1% collagenase type IV solution at 38℃ for 1.5-2.0 hours and then washed twice in donor cell culture medium [Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 1 mM sodium pyruvate, 1% non-essential amino acid, 0.1% β-mercaptoethanol (Gibco) and 1% penicillin-streptomycin (Gibco)]. Ten or eleven pieces of tissues were placed on 60 mm culture dish containing 2- ml of donor cell culture medium in a 5% CO_2 incubator at 38.8℃. The cells were grown and subcultured 3-5 times at intervals of 4-6 days in a 35 mm culture dish. The confluent ear fibroblasts were dissociated with TrypLE solution (Gibco) and freezed with 1 x 10^6 cells in 1.5-ml cryovial in a freezing medium (50% donor cell culture medium plus 45% FBS and 5% DMSO).

For SCNT, the frozen JBC ear cells were thawed and expanded for one or two subcultures. The expanded cells were dissociated using TrypLE solution, washed twice with donor cell culture medium and then treated with 3 mg/ml protease for 50

seconds at room temperature. The treated cells were washed 3 times using donor cell culture medium, and re-suspended in a donor cell preparation medium [TCM199-HEPES (Gibco) supplemented with 0.2 mM sodium pyruvate] and then JBC ear individual cells were provided for SCNT.

2. Preparation of recipient oocytes

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory within 2 hours in 0.9% saline (NaCl 9 g/L, penicillin–streptomycin 0.1 g/L) at 35℃. Cumulus oocyte complexes (COCs) were aspirated from 2 to 6 mm sized visible follicles using 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). Only oocytes with multilayered compact cumulus and evenly granulated cytoplasm were selected for maturation. Sets of 10 COCs were *in vitro* matured (IVM) in TCM199 (Gibco), supplemented with 10% FBS, 0.2 mM sodium pyruvate, 1 ug/ml follicle-stimulating hormone (Folltropin^{TM}, Bioniche Animal Health, Belleville, On, Canada), 1 ug/ml estradiol-17β and 1 mM epidermal growth factor(EGF) and 25 ug/ml gentamycin sulfate under mineral oil at 38.8℃ in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 18-20 hours.

After IVM, cumulus cells were completely removed from the oocyte by vortexing for 4 minutes in the presence of 0.1% hyaluronidase. For enucleation, denuded oocytes which the first polar body (PB1) appeared normal were selected and incubated in 20% FBS supplemented TCM199-HEPES medium for 1 hour. In Hoechst staining and UV irradiation system, denuded oocytes were labeled with 5

ug/mL Hoechst 33342 for 10 minutes at room temperature, washed three times and transferred into the enucleation medium drop (TCM199-HEPES containing 20% FBS and 7.5 ug/ml cytochalasin B). And then the metaphase II plate and PB1 were visualized by exposure to UV (excitation, 330-385 nm; emission, ≥ 420 nm) irradiation for 10 seconds and removed by squeezing method. A portion of the zona pellucida near the PB1 was cut prior to enucleation, and a small volume of cytoplasm surrounding the PB1 was squeezed out through the slit made with a cutting needle (Fig. 1). In Oosight imaging system, denuded oocytes were transferred into the enucleation medium drop overlaid with mineral oil. The metaphase plate and PB1 were visualized using an inverted microscope (Olympus, Tokyo, Japan) equipped with Oosight spindle-check system (CRi, Hopkinton, MA, USA) and removed by the same squeezing method (Fig. 1). Enucleated oocytes were incubated at 38.8 ℃ in a humidified atmosphere of 5% CO_2 in air for 30 minutes.

3. Somatic cell nuclear transfer (SCNT)

Treated single donor cell was placed in the perivitelline space of the enucleated oocytes in nuclear transfer medium [TCM199-HEPES containing 0.06% fatty acid-free (FAF)-BSA and 10 ug/ml PHA] through the opening made during enucleation. Oocyte-cell couplets were placed in cell fusion medium (0.3M mannitol, 0.5mM HEPES, 0.05% FAF-BSA, 0.05mM $CaCl_2$ and 0.1mM $MgSO_4$) and applied an electrical pulse of 20 volts for 20 micro-seconds to fusion. After fusion, the reconstructed embryos were kept in 20% FBS added TCM199-HEPES for 1 hour, activated in 10 uM calcium ionophore for 5 minutes and then exposed in 2 mM 6-

Dimethylaminopurine for 3 hours.

4. Culture of JBC-SCNT embryos

After activation, the reconstructed embryos were cultured in 0.03% FAF-BSA added CR1aa medium for 2 days, and then they were co-cultured on the same JBC ear feeder cell drop in a CR1aa medium containing 10% FBS, 1 uM EGF, 1 uM IGF and 10 uM flavonoid at 38.8 ℃ in 5% O_2, 5% CO_2, and 90% N_2 incubator for 6 days.

5. Blastocyst differential staining

The blastomere, inner cell mass (ICM), and trophectoderm (TE) cell numbers in blastocysts were counted by differential staining according to Thouas et al (17). Zona-intact blastocysts were incubated in 500- μL of Solution 1 (TL-HEPES–buffered medium with 1% Triton X-100 and 100 μg/ml propidium iodide, PI) for up to 30 seconds, or until the TE cells were noticeably changed color to red and shrank slightly. Blastocysts were then immediately transferred into 500-μL of Solution 2 (fixative solution of 100% ethanol with 25 μg/mL bisbenzimide; Hoechst 33258) and stored at 4°C overnight. Fixed and stained blastocysts were transferred from Solution 2 directly into glycerol, taking care to avoid a carry-over of excessive amounts of Solution 2. Blastocysts were mounted onto a slide glass in a drop of glycerol, gently flattened with a cover slip, and visualized for cell counting. Labeled nuclei were observed by fluorescence microscopy equipped with an 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 two different enucleation systems were determined using In Site Cell Death Detection Kit (Roche, Mannheim, Germany). Briefly, SCNT blastocysts were fixed in 3.7% paraformaldehyde in PBS for 1hour at room temperature, washed in PBS/PVP and permeabilized by incubation in 0.3% Triton X-100 for 1hour at room temperature. They were incubated with fluorescein conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme in the dark for 1hour at 37℃. And then they were incubated in 50 μg/ml RNase A for 1 hour at 37℃ and the nuclei of them were counterstained with 40 μg/ml PI at the same time. Stained blastocysts were loaded onto slide glass, covered with coverslip, and then observed by fluorescence microscopy equipped with 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

Messenger RNA for real-time RT-PCR was prepared from JBC-SCNT blastocysts produced in two different enucleation systems using magnetic beads

(Dynabeads mRNA purification kit; Dynal, Oslo, Norway) following the manufacturer's instructions. Briefly, 15 oocytes or embryos were suspended in 100-μL of lysis/binding buffer [100 mM Tris-HCl (pH 7.5), 500 mM LiCl, 10 mM EDTA (pH 8.0), 1% lithium dodecylsulphate (LiDS), and 5 mM DTT] and vortexed at room temperature for 5 minutes. A 50- μL aliquot of an oligo (dT) 25 magnetic-bead suspension was added to the samples and incubated at room temperature for 5 minutes. The hybridized mRNA and oligo (dT) beads were washed twice with washing buffer A [10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA, and 1% LiDS] and once with washing buffer B [10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, and 1 mM EDTA]. The mRNA samples were eluted from beads in 15-μL of double-distilled DEPC-treated water.

After mRNA extraction, cDNA was synthesized using an oligo (dT) 12–18 primer and Superscript reverse transcriptase (Invitrogen, Calsbad, Calif). Real-time RT-PCR (Bio-Rad, Chromo4) was performed using the primer sets shown in Supplementary Table 1. In all experiments, histone β-actin mRNA served as an internal standard. The threshold cycle (Ct) value represents the cycle number at which the sample fluorescence rose statistically above background noise. To monitor the reactions, the protocol provided with the DyNAmo SYBR green qPCR kit was used. This kit contains a modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM $MgCl_2$, and a dNTP mix that includes dUTP (Finnzyme Oy, Espoo, Finland). The PCR protocol used a denaturation step at 95°C for 15 minutes, followed by amplification and quantification cycles that were repeated 40 times at 94°C for 30 seconds, at 50 or 56°C for 1 minute, and at 72°C for 1 minute using a single fluorescence measurement, and a melting curve program of 65–95°C with a

heating rate of 0.2°C/second and continuous fluorescence measurement. Samples were cooled to 12°C. SYBR Green fluorescence was measured after the extension step during the PCR reactions. PCR products were analyzed by generating a sequence-specific melting curve to distinguish non-specific from specific PCR products. The crossing point (CP), defined as the point at which fluorescence rises appreciably above background noise, was determined for each transcript. Gene expression was quantified by the 2-ddCt method (17).

8. Embryo transfer, pregnancy diagnosis and calving

Freezing and thawing of JNU-SCNT embryos method is One-Step Dilution and Direct Transfer Technique (18). Pregnancies were confirmed at first when recipient cows did not return to the subsequent estrus cycle, and 10 days before the expected beginning of the calving every 2 hr. All newborns had nasal passages cleared, were helped to breath, had navels dipped into iodine solution and were encouraged to suckle within 30min of birth.

9. Experimental design

To examine the effect of two different enucleation systems on JBC-SCNT embryo production, enucleated oocytes by either Hoechst staining and UV irradiation system or Oosight imaging system were treated in the same SCNT condition using the same donor cells. The comparison items were fusion rate, cleavage rate at day 2, blastocyst rate at day 8, total cell and ICM cell number of blastocysts, and overall

efficiency. Also, in different enucleation system, *in vitro* produced day 8 JBC-SCNT blastocysts were examined their apoptotic index, and their relative mRNA expression levels of seven candidate genes (Oct4, Sox2, Nanog, Interferon-tau, Caspase-3, Dnmt3A and HSP70). Experiments were replicated six times.

10. Statistical analysis

Differences in the fusion rate, developmental rate, total cell and ICM cell number, and overall efficiency, apoptotic index and the relative abundance of gene expression between Hoechst staining and UV irradiation group and Oosight imaging group were evaluated by analyses of variance (ANOVA) using the general linear model (PROC-GLM) in the SAS software program. Differences of $P<.05$ were considered significant.

RESULTS

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

When the effects of the two different enucleation systems (Hoechst staining and UV irradiation or Oosight imaging) on *in vitro* development rate of JBC-SCNT embryos and their embryo quality were examined, as shown in Table 1, there were significant differences in fusion rate ($P<.01$), cleavage rate at day 2 ($P<.05$), blastocysts rate at day 8 ($P<.01$) and total cell number of day 8 blastocysts ($P<.01$) between the two groups, respectively, while the enucleation volumes between two enucleation groups were not different as shown in Fig. 1.

After enucleation using either Hoechst staining and UV irradiation or Oosight imaging system, the cell fusion rates between JBC donor cells and enucleated recipient oocytes were 62.9% (204/324) or 75.6% (242/320), respectively, which was significantly different ($P<.01$). At day 2 post activation, the cleavage rates of reconstructed embryos against fused oocytes were 63.7% (130/204) or 78.0% (189/242), respectively, it was also significantly different ($P<.05$). When the day 2 JBC-SCNT embryo quality was compared, good-quality 4- to 8-cell (≥ 4-cell) JBC-SCNT embryo development rates were significantly high in Oosight imaging group (71.4%, 135/189) than in Hoechst staining and UV irradiation group (50.8%, 66/130) as shown in Table 1 and Fig. 2, while 2- to 3-cell (< 4-cell) JBC-SCNT embryo development rates were significantly high in Hoechst staining and UV irradiation group (49.2%, 64/130) than in Oosight imaging group (28.6%, 54/189) ($P<.01$).

At day 8 post activation, blastocyst development rates of JBC-SCNT embryos were also significantly high in Oosight imaging group (40.2%, 76/189) than in Hoechst staining and UV irradiation group (29.2%, 38/130) (P<.01). When the expanding levels of day 8 JBC-SCNT blastocysts produced in different enucleation groups were compared, there was significant difference in hatching blastocyst rate [Oosight imaging group, 48.6% (37/76); Hoechst staining and UV irradiation group, 39.5% (15/38)] excluding of middle or expanded blastocyst rates between two groups as shown in Fig. 2, respectively (P<.05). In addition, total cell numbers and ICM cell numbers of day 8 JBC-SCNT embryos in each treatment group were examined, total cell number of Oosight imaging group (128.3±4.8) was significantly higher than that of Hoechst staining and UV irradiation group (112.2±7.6) (P<.05), whereas the ICM cell numbers were not different between two groups. Finally, overall efficiency according to treatment of different enucleation system for production of JBC-SCNT embryos were presented double score, as 23.7% (76/320) in Oosight imaging group and 11.7% (38/324) in Hoechst staining and UV irradiation group, and therefore cloning efficiency was significant difference between two groups (P<.05).

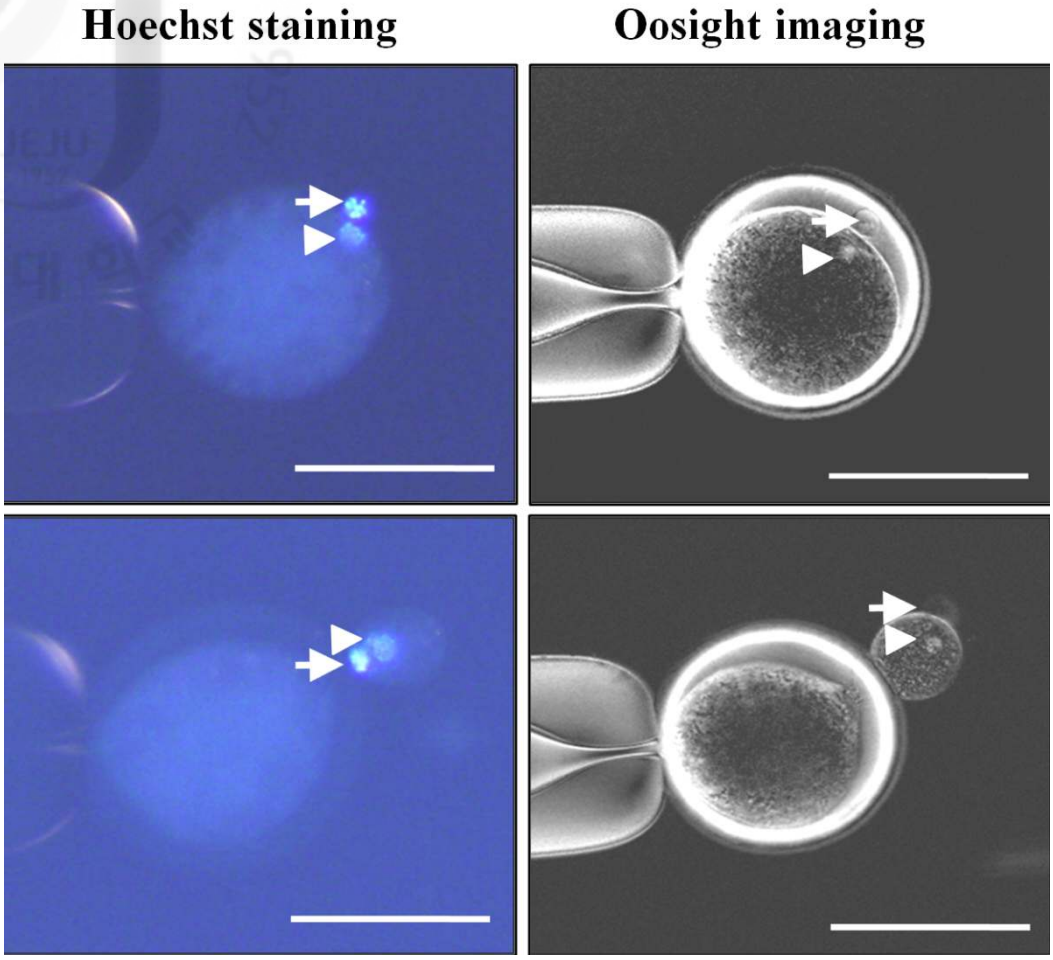

Fig. 1. In Hoechst staining or Oosight imaging system, an arrow and an arrowhead in each picture indicate the location of first polar body and metaphase II plate, respectively. In different enucleation system, those karyoplasts were perfectly removed by squeezing method. Bar = 100 um.

Table 1. ***In vitro*** **development of JBC-SCNT embryos using two different enucleation systems (6 replicates)**

Enucleation system	No. of enucleated oocytes	No. (%)* of fused oocytes	No. (%)* of embryos developed to							No.* of total cell (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)[a]	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]
Oosight Imaging	320	242 (75.6)[b]	189 (78.0)[d]	54 (28.6)[b]	135 (71.4)[b]	76 (40.2)[b]	10 (13.2)	29 (38.2)	37 (48.6)[d]	128.3±4.8[b] (35.3±4.0)	76/320 (23.7)[d]

* Means with different superscripts in the same column are significantly different ([a-b] $P<.01$; [c-d] $P<.05$).

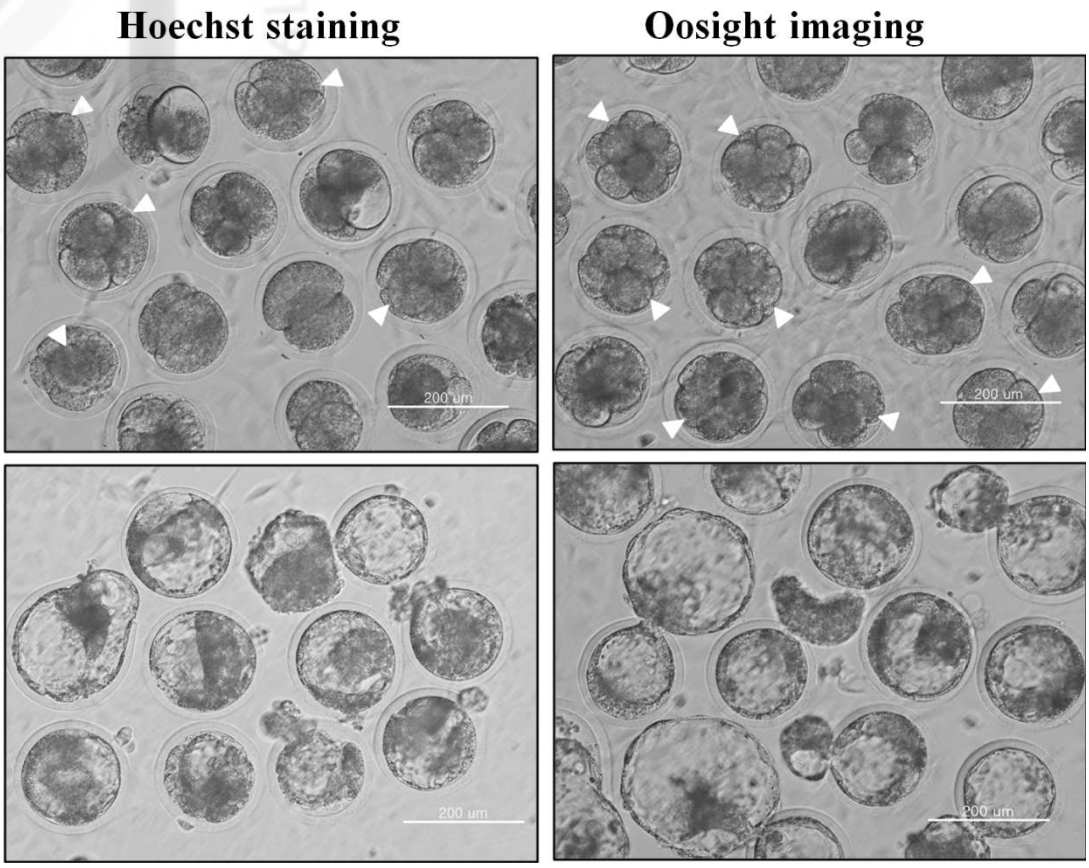

Fig. 2. *In vitro* produced day 2 or day 7 JBC-SCNT embryos in different enucleation systems. More number of good quality 4-8 cell embryos (arrowheads) and faster developed blastocysts were produced in Oosight imaging system than in Hoechst staining system at day 2 and day 7 after SCNT, respectively. Bar = 200 um.

2. Effect of two different enucleation systems on the cell death of JBC-SCNT embryos

Approximate values for the apoptotic index per blastocyst were determined using fluorescence microscopy with the TUNEL assay (Fig. 3). The apoptotic index of the Oosight imaging group (2.8±0.5) was significantly lower than that of Hoechst staining and UV irradiation group (7.3±1.2) ($P<.05$).

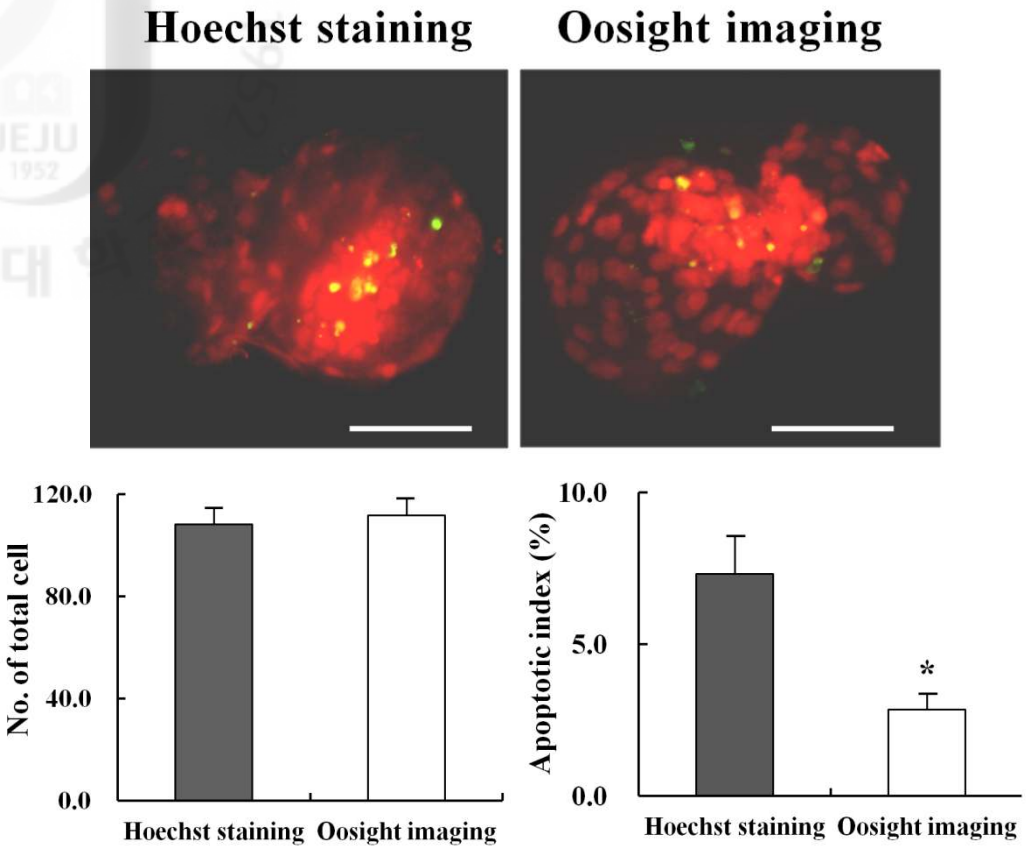

Fig. 3. Representative fluorescence microscopic images of TUNEL stained day 8 JBC-SCNT embryos in different enucleation systems. PI used to stain chromatin and yellow dot presented fragmented DNA. Bar = 200 um. In the result of TUNEL staining, total cell numbers were not different between Hoechst staining system and Oosight imaging system, while their apoptotic indexes were different ($P<.05$).

3. Relative mRNA expression of candidate genes of JBC-SCNT embryos in different enucleation systems

When the relative mRNA expression levels of pluripotency (Oct4, Sox, Nanog), maternal recognition of pregnancy (Interferon-Tau), apoptosis (Caspase-3), the de novo methylation (Dnmt3A) and stress marker gene (Hsp70) were analyzed, as shown in Fig. 4 and Table 2, the core puripotency marker gene Oct4 mRNA expression level of Oosight imaging group was significantly high (x 1.49 fold) compared to that of Hoechst staining and UV irradiation group, while the expression levels of Sox2 and Nanog mRNA were not different between two groups ($P<.05$). Also, the relative abundance of Interferon-tau gene expression was significantly high (x 1.88 fold) in Oosight imaging group than in Hoechst staining and UV irradiation group, and conversely the expression levels of Caspase-3 (x 0.22 fold) and Hsp70 (x 0.27 fold) mRNA were presented significantly low in Oosight imaging group than in Hoechst staining and UV irradiation group ($P<.05$). In addition, Dnmt3A mRNA expression level was slightly high (x 1.20 fold) in Oosight imaging group compared to Hoechst staining and UV irradiation group.

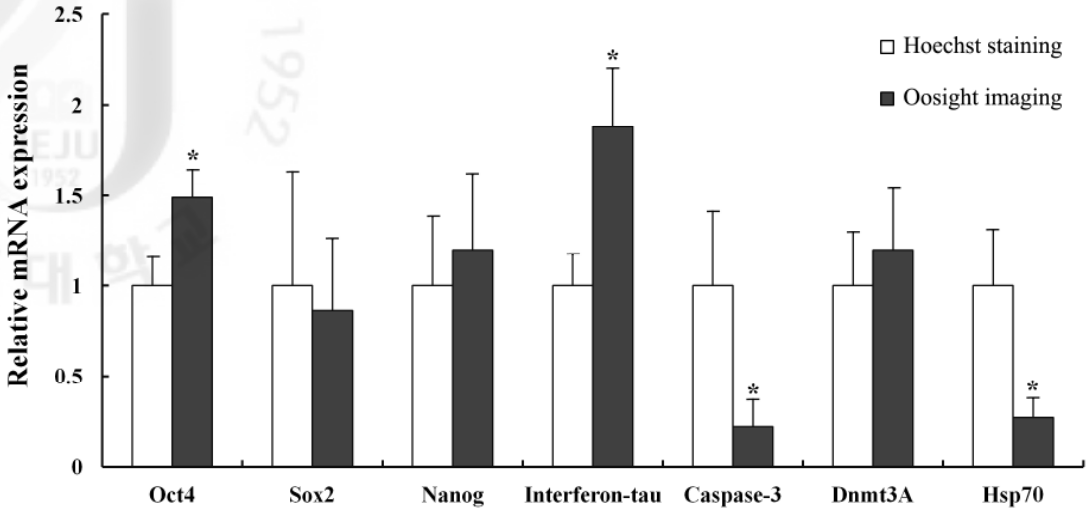

Fig. 4. Relative mRNA expression of candidate genes of day 8 JBC- SCNT embryos produced in different enucleation systems, which was examined in pluripotency (Oct4, Sox2 and Nanog), implantation (Interferon-tau), pro-apoptotic (Caspase-3), demethylation (Dnmt3A) and Stress (Hsp70). Bars with different superscripts within a panel differ significantly ($P<.05$). The standard deviation is indicated by error bars.

Table 2. Relative mRNA expression of candidate genes of JBC-SCNT embryos in different enucleation systems

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
Caspase-3	1	0.22	↓	Pro-apoptotic
Dnmt3A	1	1.20	↑	De novo methylation
Hsp70	1	0.27	↓	Stress

Table 3. Oligonuceotide real-time PCR primer sequences for variable genes

Genes	Primer sequence	Annealing Temp.	Product size	Gene Bank Accession No.
bOct4	5'-CTCTTTGGAAAGGTGTTCAG-3' 5'-GTCTCTGCCTTGCATATCTC-3'	53C	155 bp	NM-174580
bSox2	5'-GCTGCTCTGGACTGTGCTGA-3' 5'-ATCCAGTAATCTCCTCCAGC-3'	56C	247 bp	NM-001105463
bNanog	5'-TGGAACAATCATTTCAACAA-3' 5'-GCTGGGAATTGAAATACTTG-3'	54C	157 bp	NM-001025344
bInterferon-tau	5'-ATGGCCGGCGTGCTCTCTCT-3' 5'-AGGTCCTCCAGCTGCTGTTG-3'	55C	356 bp	NM-174106
bCaspase-3	5'-CGATCTGGTACAGACGTG-3' 5'-GCCATGTCATCCTCA-3'	50C	359 bp	NM-001077840
bDnmt3A	5'-TGATCTCTCCATCGTCAACCCT-3' 5'-GAAGAAGGGGCGGTCATCTC-3'	54C	221 bp	XM-02691463
bHsp70	5'-GACAAGTGCCAGGAGGTGATTT-3' 5'-CAGTCTGCTGATGATGGGGTTA-3'	51C	117 bp	NM-174550
bβ-actin	5'-GTCATCACCATCGGCAATGA-3' 5'-GGATGTCGACGTCACACTTC-3'	56C	111bp	NM-173979

4. Production of cloned JBC "Heuk Woo Sunee"

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 months 14 and healthy at present (Fig. 5). In addition, when the short tandem repeat marker analysis of the cloned JBC was evaluated, microsatellite loci of 11 numbers was perfectly matched genotype with donor cell (BK94-14) (Fig. 6).

Fig. 5. One hundred and one day-old aged cloned female Jeju Black Cattle "Heuk Woo Sunee" by SCNT technique using Oosight imaging system (Birth date; Oct. 31, 2010).

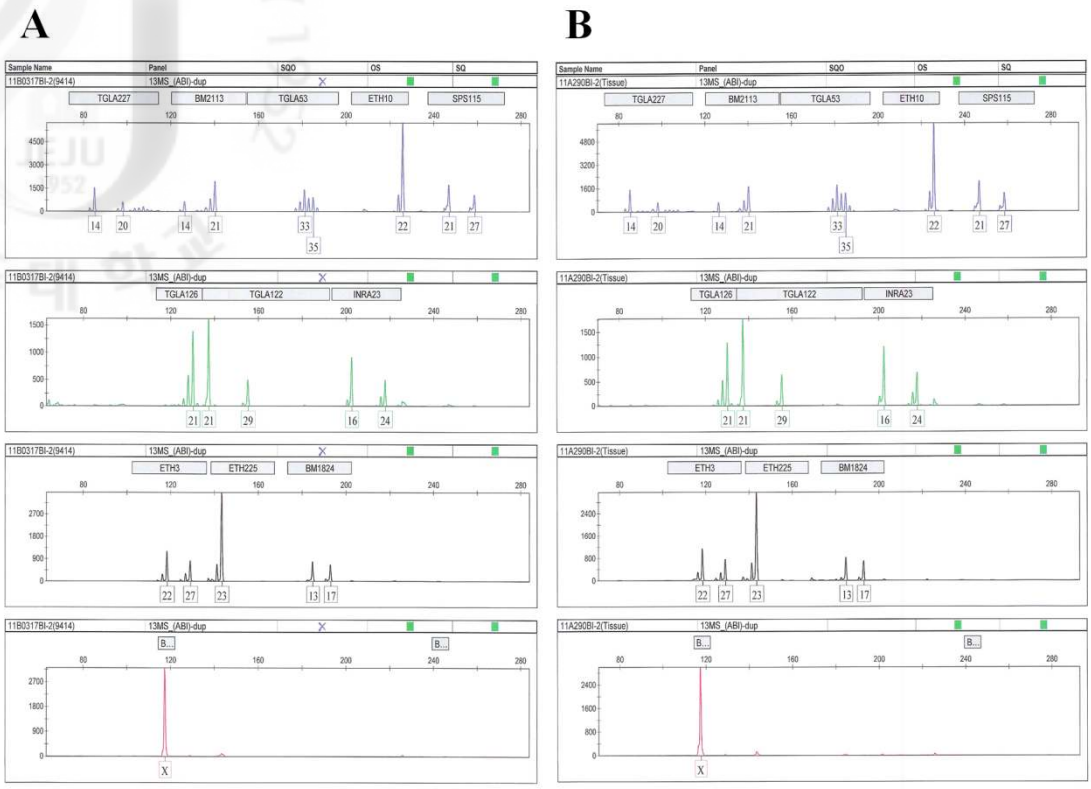

Fig. 6. STR (short tandem repeat) profile investigated full DNA fingerprint of elite female Jeju Black Cattle somatic cell (BK94-14, A) and it's cloned female calf ("Heuk Woo Sunee", B) using 11 STR markers and amelogenin.

DISCUSSION

This study demonstrates that direct enucleation of oocytes using Oosight imaging system improve significantly the *in vitro* developmental potential of bovine SCNT embryos compared to conservative enucleation using Hoechst staining and UV irradiation system. In this study, NT embryos were reconstructed using Jeju Black Cattle (a species of Korean Native Cattle) ear cell as the donor cells, and the differences were consecutively presented in the cell fusion rate ($P<.01$), cleavage rate at day 2 ($P<.05$), blastocyst rate at day 8 ($P<.01$) and total cell number of the blastocyst ($P<.01$). Thus, the overall efficiencies after SCNT were resulted in double high in the Oosight imaging group compared to the Hoechst staining and UV irradiation group ($P<.05$). Also, comparative assay of TUNEL and semi-quantitative RT-PCR presented that the developmental potential of Oosight imaging group is elevated by the high expression of pluripotency (Oct4 and Nanog), implantation (Interferon-tau) and de novo methylation (Dnmt3A) genes, and the low expression of pro-apoptotic (Caspase-3) and stress (Hsp70) genes, compared to that of Hoechst staining and UV irradiation group. This is the first report to determine the positive effect of Oosight imaging system on the molecular gene expression of SCNT embryo.

Enucleation of a recipient ooycte is crucially important to cloning efficiency. Proper measures used in enucleation avoid such problems as aneuploidy with subsequent detrimental effects on later development, genetic interference of the recipient cytoplasm, and possible parthenognentic activation and embryo development without the participation of a newly introduced nucleus (8). Enucleation may also affect the ultrastructure of the remaining cytoplast, thus resulting in a

decline or destruction of its cellular compartments (19).

Among the numerous enucleation methods, I selected the two, Hoechst staining and UV irradiation as a conservative (control group) and Oosight imaging as a new (comparison group) technique. There was reported that the cloning efficiency of Hoechst stained oocytes was affected negatively by the increase of UV irradiation time of and thus they should not be exposed to UV for more than 30 seconds (13). On the other hand, the exposure to UV irradiation for 10 seconds has no affect on embryo viability and production of live calves (20). In the present study, I attempted at the best to decrease the UV exposure time of Hoechst stained oocytes into the least (<10 seconds), but there was difference in cloning efficiency between Hoechst staining and UV irradiation system and Oosight imaging system. In recent, I produced one female cloned Jeju Black Cattle by Oosight imaging system, endangered ranking number 1 animal dead before 3 years. Although the transfer numbers of SCNT embryo were a few and the efficiency of cloned animal production between Hoechst staining and UV irradiation system and Oosight imaging system couldn't compare simultaneously, through the TUNEL assay and molecular gene expression analysis, I knew that Oosight imaging may be more efficient technique than Hoechst staining and UV irradiation.

DNA fragmentation in oocytes associated with apoptotic evidence might be one of the reasons for poor oocyte quality and lower fertility (21). By TUNEL assay, I confirmed that DNA fragmentation frequency is significantly lower in Oosight imaging group than in Hoechst staining and UV irradiation group. In addition, pro-apoptotic genes Caspase-3 expression is significantly low in Oosight imaging group compared to Hoechst staining and UV irradiation group. Also, during SCNT

procedures, oocytes were exposed to a numerous heat shock stress environment according to many mechanical and chemical treatment steps. Heat shock induced apoptosis in preimplantation embryos is a developmentally regulated manner and embryo acquires one or more thermoprotective responses as embryonic development proceeds (22). In this study, heat shock gene (Hsp70) expression is significantly low in Oosight imaging group than in Hoechst staining and UV irradiation group, this result is parallel with the level of pro-apoptotic Caspase-3 gene expression. These results represented that the Oosight imaging system is more safe for production of SCNT embryos than Hoechst staining and UV irradiation system.

On the whole, important genes such as pluripotency (Oct4, Nanog), de no methylation (Dnmt3A) and implantation (Interferon-tau), affect to the *in vitro* and *in vivo* developmental potential of SCNT embryos, were relatively high expressed in Oosight imaging group compared to Hoechst staining and UV irradiation group. The octamer binding transcription factor (Oct)-4 is a master key regulator at the beginning of mammalian embryogenesis, variations observed in the level and pattern of Oct-4 expression might be responsible for at least, some of the problems related to cloning (23). The level of Oct-4 expression might regulate cell lineage commitment in that a critical level of expression is required to maintain pluripotency (24). Nanog, is also a crucial pluripotency factor, expressed in ICM cells with Oct4, and the expression level is directly related on epiblast formation at later stage (25). This study presented the two pluripotency factor (Oct4 and Nanog) expression is high in SCNT embryos of Oosight imaging group compared to those of Hoechst staining and UV irradiation group.

DNA methylation in embryos changes in a very organized manner to set up

imprinting patterns which are vitally important for a number of biological events. Dnmt3A is thought to be responsible for de novo methylation since their activity appears to be targeted to certain domains of the genome (26). Abnormalities observed in cloned animals suggest imprinted genes and problems related to failed epigenetic reprogramming might be a serious contributor to puzzle of failed development of cloned embryos (27). Although there was no significant difference, the Dnmt3A expression level was high in Oosight imaging group compared to Hoechst staining and UV irradiation group. It means the Oosight imaging technique may be more helpful for the epigenetic reprogramming of SCNT embryos to Hoechst staining and UV irradiation technique.

Interferon-tau is exclusively secreted by the trophectodermal cells of blastocysts and primarily functions as a factor responsible for maternal recognition of pregnancy in cattle (28). Higher expression level of Interferon-tau mRNA is in good quality embryos. In this study, there was significant difference in the Interferon-tau mRNA expression level between two groups and this result demonstrates SCNT embryos of Oosight imaging group have the better developmental potential than those of Hoechst staining and UV irradiation group.

Therefore, this study demonstrated that the noninvasive direct enucleation using Oosight imaging system has positive effects on the production of SCNT embryos compared to the Hoechst staining and UV irradiation. Using real-time imaging with easy-to-use software of the Oosight imaging system, MII plate and PB1 of recipient oocytes were easily removed without damaging of membrane or cytoplasm integrity (29). In conclusion, the Oosight imaging is a safe, reliable and efficient enucleation technique; it could improve the cloning efficiency and

developmental potential of SCNT embryos and their related gene expression.

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Oosight Imaging System을 이용한 제주흑우 (한우) 복제 소 생산

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국문초록

체세포 핵 이식은 발생학에 유용한 기술로서 현재 양, 소, 쥐, 돼지를 포함한 다양한 종의 복제 동물 생산에 이용되었다. 또한 체세포 핵 이식 기술은 기초 및 의료 연구 분야 그리고 멸종동물 보존에 기여해 왔다. 그러나 체세포 핵 이식 효율은 매우 낮아, 지금까지 체세포 핵 이식 과정에 있어서의 탈핵, 체세포 이식, 난 활성화 같은 기술의 최적화를 위한 다양한 연구들이 수행되어 왔다. 그 중에서도 공여난자의 탈핵 과정은 복제효율에 매우 큰 영향을 미치는 것으로 알려져 있다.

본 실험은 'Hoechst staining and UV irradiation' 과 'Oosight imaging' 을 이용하는 두 가지 탈핵 방법이 체세포 복제란 생산과 발달능

에 미치는 영향을 비교해 보고자 실시하였다. 제주 흑우 체세포를 이식하기 전 위에서 언급한 두 가지 방법으로 공여란의 탈핵을 실시하였다.

그 결과, Oosight imaging을 이용한 체세포 핵 이식 그룹의 융합율 (75.6% vs. 62.9%, $P<.01$), 난할율 (78.0% vs. 63.7%, $P<.05$), 배반포기배아 생산율 (40.2% vs. 29.2%, $P<.01$) 과 총 세포 수 (128.3±4.8 vs. 112.2±7.6, $P<.05$) 가 Hoechst staining을 이용한 체세포 핵 이식 그룹의 결과에 비해 유의하게 높은 것을 확인하였다. 또한 배반포기배의 세포 사멸율도 Oosight imaging을 이용한 체세포 핵 이식 그룹 (2.8±0.5 vs. 7.3±1.2, $P<.05$) 에서 Hoechst staining을 이용한 체세포 이식 그룹에 비해 유의하게 낮은 것을 확인하였다. 이와 더불어 체외 발생된 복제수정란의 유전자 발현에 미치는 영향을 조사하였던 바, Oosight imaging을 이용한 체세포 핵 이식 그룹의 배반포기 배아에서 Oct4, Nanog, Interferon-tau와 Dnmt3A 유전자의 발현이 Hoechst staining을 이용한 체세포 핵 이식 그룹에 비해 높은 반면, Caspase-3 와 Hsp70 유전자 발현은 현저하게 낮은 것을 확인하였다.

따라서, Oosight imaging 방법은 체세포 핵 이식의 복제 효율과 복제수정란의 체외 발달능을 향상시켜 복제 소 생산에 유용하게 이용될 것으로 사료된다.

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