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Production of Bovine Transgenic Somatic Cell Nuclear Transfer Embryos by Demecolcine-assisted Enucleation

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Demecolcine-assisted Enucleation에 의한 소 형질 전환 핵이식란 생산

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SUMMARY

본 연구는 소 형질 전환 체세포 핵이식에서 용이한 탈핵을 위해 demecolcine을 이용할 시 탈핵율과 핵이식란의 발육능을 높이기 위한 최적의 조건을 알아보고자 실시되었다. 도축장 유래 미성숙 난자를 18시간 체외성숙 후 제1극체가 확인된 성숙 난자를 0.1, 0.2, 0.4 및 0.8 ug/ml의 demecolcine이 첨가된 배지에서 1시간 더 처리한 다음 세포막이 돌출되어 있는 난자를 체세포 핵이식에 공여하여 각 군간 배반포로의 발육능을 비교하였다. Demecolcine 처리 후 핵이 포함된 세포막의 protrusion rates를 각 군간 비교한 결과 0.2, 0.4 및 0.8 ug/ml 군에서 0.1 ug/ml 군보다 유의적으로 높았으며(82.8, 86.2, 90.4 vs. 70.1%), conventional blind 방법과 비교한 결과 demecolcine를 이용한 군에서 유의적으로 높은 탈핵율을 보였다(75.3 vs. 96.2%; $p<0.05$). 체세포 핵이식란의 발육능 비교에서는 0.1 및 0.2ug/ml 군에서 대조군과 함께 유의적으로 높은 분할율 및 배반포로의 발육능을 보였다($p<0.05$). 결론적으로 소 형질전환 체세포 핵이식을 위한 탈핵시 높은 탈핵율과 배반포로의 발육율을 얻을 수 있는 demecolcine의 적정농도는 0.2ug/ml이라고 사료된다.

(Key words: bovine, demecolcine, enucleation, somatic cell nuclear transfer, transgenic)

INTRODUCTION

Enucleation of a recipient oocyte is an essential process in somatic cell nuclear transfer (SCNT) and important to SCNT efficiency (Oback and Wells 2003; Cho *et al.*, 2002). In the SCNT process, enucleated oocytes are sufficiently competent to facilitate genomic reprogramming and to support embryonic development to term (Tian *et al.*, 2003; Heyman *et al.*, 2002). For increasing SCNT efficiency in enucleation process, chromosome must be completely removed with little loss of cytoplasm to avoid genetic interference of remained oocyte nucleus and parthenogenetic activation without the newly introduced nucleus. Additionally, it is important to decrease the decline or destruction of the cellular

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compartment of recipient oocytes in the enucleation process. Even if a donor cell has the potential to support development of SCNT embryos, meiotic division can be arrested if metabolism is limited due to destruction of cytoplasm ultrastructure of recipient oocytes (Simerly *et al.*, 2003).

Many of enucleation methods were conducted to prepare the recipient oocytes in SCNT including with blind enucleation (Cheong *et al.*, 1992; McGrath and Solter 1983), staining of chromosomes and UV light (Smith *et al.*, 1990), squeezing methods (Cho *et al.*, 2002), telophase enucleation (Liu *et al.*, 2000; Bordignon and Smith 1998), sucrose pretreatment (Wang *et al.*, 2001), bisection of oocytes (Vajta *et al.*, 2003; Booth *et al.*, 2001; Vajta *et al.*, 2001), centrifugation (Tatham *et al.*, 1996; Tatham *et al.*, 1995) and chemically assisted enucleation (Ibanez *et al.*, 2003; Kawakami *et al.*, 2003; Elsheikh *et al.*, 1998). Out of these methods, chemically assisted method has been conducted in mouse, bovine and porcine SCNT because this method could increase the enucleation rates without UV irradiation and it's relatively simplicity compared with other methods (Tani *et al.*, 2006; Russell *et al.*, 2005; Vajta *et al.*, 2005; Gasparrini *et al.*, 2003; Ibanez *et al.*, 2003; Yin *et al.*, 2002). However, developmental potential of SCNT embryos using chemically assisted enucleation is controversial and not yet fully demonstrated in bovine transgenic SCNT.

The present study investigated the usefulness of demecolcine in the chemically assisted removal of nucleus in bovine transgenic SCNT and its optimal concentrations. We also compared the developmental competence of SCNT embryos between blind enucleation and chemical enucleation group.

MATERIALS AND METHODS

1. Collection of Somatic Cells and Primary Cell Culture

Ear tissues were obtained from an adult Holstein cow, washed in D-PBS, and minced with a surgical blade. The minced ear tissues were dissociated in DMEM (Life Technologies, Rockville, MD) supplemented with 0.1% (w/v) trypsin and 1 mM EDTA (Life Technologies) for 1 to 2 hrs. Trypsinized cells were washed once by centrifugation at 300 × g for 10 minutes and subsequently seeded into 100 mm plastic culture dishes. Seeded cells were subsequently cultured for 6 to 8 days in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS Life Technologies), 1 mM sodium pyruvate (Sigma, St. Louis, MO), 1% (v/v) non-essential amino acids (Life Technologies) and 10 μg/mL penicillin-streptomycin solution (Sigma) at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cell or explants, attached cells were further cultured until confluent, and then subcultured at intervals of 5 to 7 days.

2. Generation of Transfected Cells

Transgenic ear fibroblast cells were generated using a lipid-mediated gene transfer system. An expression plasmid for human α_1-antitrypsin (αAT) was constructed by inserting a bovine beta-casein promoter (accession number: M55158.1), a green fluorescent protein (GFP) marker gene, and human AT target gene (accession number: X01683) into a pcDNA3 plasmid (Life Technologies) (Fig. 1). For transfection, frozen cells scheduled to be used as donor cells were thawed, cultured until 50% confluency in a 35 mm culture dish and transfected with the plasmid using FuGene6® (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's suggested procedure. The transfected cells were cultured for another 4 days in order to induce chromosomal integration of transgene. Before injection of the donor cells, transfected cells were collected by trypsinization and after centrifugation the pellets were resuspended in PBS supplemented with 0.5% FBS. GFP-expressing cells were selected under ultraviolet (UV) light using

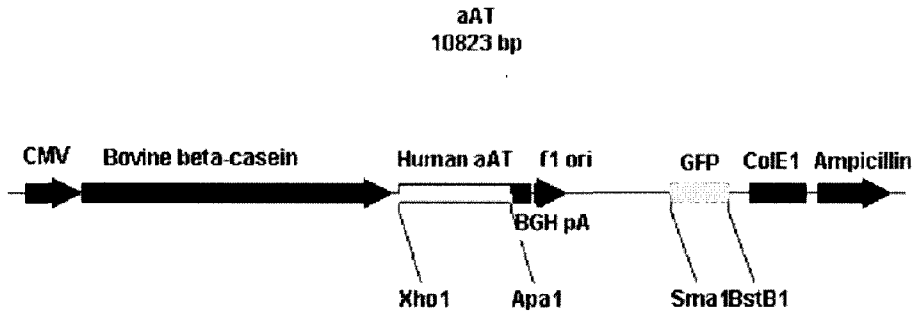

Fig. 1. Diagrammatic representation of the features of the constructed vector containing bovine -casein promoter, human alpha1-antitrypsin gene, ampicillin resistant gene, and green fluorescent gene (GFP) as a marker gene.

a standard fluorescein isothiocyanate filter set (FITC; excitation wavelength: 450~490 nm; B-mode filter, Leica, Germany) and used for nuclear transfer.

3. *In Vitro* Maturation

Ovaries were collected at a local slaughterhouse and transported to the laboratory in 0.9% (v/v) NaCl solution at 30 to 35℃. Follicular fluid and cumulus-oocyte complexes (COCs) from follicles 2 to 8 mm in diameter were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe. COCs with evenly granulated cytoplasm and enclosed by compact cumulus cells of more than three layers were selected, washed three times in HEPES-buffered tissue culture medium-199 (TCM-199; Life Technologies), and cultured for *in vitro* maturation (IVM) in bicarbonate-buffered TCM-199 supplemented with 10% (v/v) FBS, 0.005 IU/ml FSH (Antrin, Teikoku, Tokyo, Japan), 1 mg/ml estradiol (Sigma) at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air for 18 hrs.

4. Chemically Assisted Enucleation

After maturation, COCs were transferred to HEPES-buffered TCM-199 medium (working medium in this experiment) containing 0.1% (w/v) hyaluronidase for 1 min and the cumulus cells were subsequently removed by gentle pipetting. Matured oocytes with the first polar body were selected and then cultured in TCM-199 supplemented with 0.1, 0.2, 0.4, and 0.8 ug/ml of demecolcine for 1 h. Treated oocytes with a protruding membrane were moved to working medium supplemented with 5 μg/ml cytochalasin B (Sigma) and demecolcine at each concentration. And then the protrusion containing maternal chromosome and the first polar body was removed with a beveled pipette (Fig. 2). For the control group, blind enucleation was conducted in untreated oocytes with the first polar body. To determine whether enucleation was successful in the 2 groups, the enucleated oocytes were stained with 5 μg/ml bis-

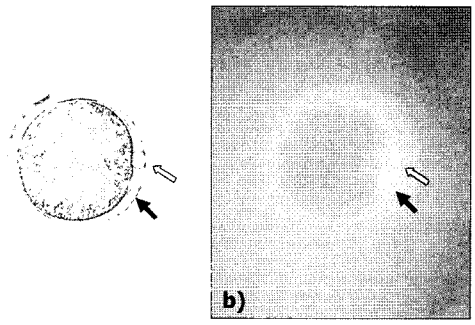

Fig. 2. An oocyte with a membrane protrusion following 1 h of demecolcine treatment. Closed arrow shows the membrane protrusion with maternal chromosome, and open arrow indicates the first polar body. The condensed chromosome mass can be seen in the protrusion under fluorescent field (b) after Hoechst33342 stain.

benzimide (Hoechst 33342, Sigma) for 5 min and observed under UV light (Cho *et al.*, 2004; Cho *et al.*, 2002).

5. Embryo Reconstruction and Culture

GFP-expressing donor cells identified under an FITC filter sets were aspirated into an injection pipette and introduced into the perivitelline space of an enucleated oocyte. Couplets were placed into a 0.3 M mannitol solution containing 0.5 mM HEPES and 0.1 mM $MgCl_2$ for 4 min and transferred to a chamber containing two electrodes overlaid with the mannitol medium. Embryos were fused with double DC pulses of 1.75 kV/cm for 15 μs using a BTX Electro-cell Manipulator 2001 (BTX, Inc., San Diego, CA) and transferred to mSOF medium (Takahashi and First 1992). At 4 hrs after fusion, fused oocytes were chemically activated by treatment with 5 M ionomycin for 4 min, followed by 1.9 mM 6-dimethylaminopurine (DMAP, Sigma) treatment for 4 hrs. All treated embryos were washed in working medium and placed into 25 μl microdrops (5~10 oocytes per drop) of mSOF supplemented with 0.8% (w/v) BSA and cultured at 39℃, 5% CO_2 atmosphere. The reconstructed embryos were cultured for 7 days after fusion, and cleavage and blastocyst formation were monitored under a stereomicroscope and GFP expression were confirmed under FITC filter set (Fig. 3).

6. Experimental Design and Statistical Analysis

In the first experiment, optimal demecolcine concentration was investigated for increasing oocytes with protrusion of maternal chromosome in the 4 different concentrations (0.1, 0.2, 0.4 and 0.8 ug/ml). In the second experiment, comparison of enucleation rates between blind-enucleation and chemical-assisted group was conducted. In the third experiment, developmental potential of bovine transgenic SCNT embryos using chemically treated enucleation was compared with untreated control embryos. All values in each parameter were analyzed using a general linear model (PROC-GLM) in a SAS 8.12 program. Statistical significance was determined when the P value was less than 0.05.

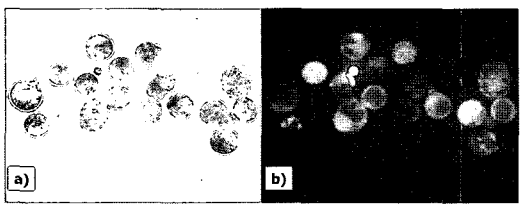

Fig. 3. Bovine transgenic blastocysts with expression of green fluorescent protein produced by demecolcine-assisted enucleation. Under normal light microscope (a) and B-mode filter (b).

RESULTS

1. Concentration of Demecolcine

The proportion of oocytes with a membrane protrusion significantly increased when matured oocytes were treated with 0.2, 0.4 and 0.8 ug/ml demecolcine compared with 0.1 ug/ml (Table 1). The maternal chromosome mass was observed within the membrane protrusion (Fig. 2), and it was easily removed with a small volume of oocyte cytoplasm.

2. Enucleation Rates

In the experiment 2, the enucleation rate using

Table 1. Protrusion of membrane with maternal chromosome after demecolcine treatment in the bovine oocytes

Concentration of demecolcine	No. of oocytes	
	Treated	Protruded (%)
0.1 ug/ml	134	94 (70.1)[a]
0.2 ug/ml	145	120 (82.8)[b]
0.4 ug/ml	123	106 (86.2)[b]
0.8 ug/ml	114	103 (90.4)[b]

[a,b] Within a parameter, values with different superscripts differed significantly, $p<0.05$.

the demecolcine-assisted method was significantly higher than that by the conventional blind method (Table 2).

3. Developmental Rates of Transgenic SCNT Embryos

In the experiment 3, the proportions of transgenic SCNT embryos that fused were not significantly different between control and groups treated with demecolcine at the concentration of 0.1, 0.2, 0.4 and 0.8 ug/ml (Table 3). However, in the proportions of cleavage and developed to blastocyst, there were significant differences between groups (Table 3). When oocytes treated with demecolcine at concentration of 0, 0.1 and 0.2 ug/ml, proportions of SCNT embryos that developed to 2-cell and blastocyst were significantly higher than that of oocytes were treated at 0.4 and 0.8 ug/ml.

Table 2. Comparison of enucleation rates between blind method and demecolcine-assisted method

Enucleation methods	No. of oocytes	
	Enucleated	Confirmed enucleation (%)
Blind	89	67 (75.3)[a]
Demecolcine-assisted	106	102 (96.2)[b]

[a,b] Within a parameter, values with different superscripts differed significantly, $p<0.05$.

DISCUSSION

Demecolcine-assisted enucleation methods were reported in the several studies including with mice, porcine and bovine SCNT (Russell *et al.*, 2005; Gasparrini *et al.*, 2003; Ibanez *et al.*, 2003; Yin *et al.*, 2002). In this study, the high enucleation efficiency was also achieved by demecolcine treatment. Enucleation rates were increased in the demecolcine-assisted enucleation compared with control group in the all 4 groups (0.1, 0.2, 0.4 and 0.8 ug/ml). However, development rates to blastocyst stage were significantly lower than that of control group.

In the experiment 1, we investigated the protrusion rates of membrane containing maternal chromosome after treatment of demecolcine with 4 different concentrations. As results of this experiment, there were no significant differences in the protrusion rates of membrane regardless of concentration except 0.1ug/ml group. In the previous studies chemically-induced enucleation in bovine SCNT, demecolcine has been used at the concentration of 0.4 ug/ml or 0.5 ug/ml (Tani *et al.*, 2006; Russell *et al.*, 2005; Vajta *et al.*, 2005). However, in the present study,

Table 3. *In vitro* development of bovine transgenic SCNT embryos reconstructed with chemical-treated oocytes according to concentration of demecolcine

Concentration of demecolcine	No. of embryos			
	Injected	Fused (%)*	Cleaved (%)†	Develop to bl.#
Control (0 ug/ml)	143	88 (61.5)	54 (61.4)[ab]	33 (37.5)[a]
0.1 ug/ml	102	62 (60.8)	40 (64.5)[ab]	19 (30.6)[a]
0.2 ug/ml	107	68 (63.6)	48 (70.6)[a]	17 (25.0)[a]
0.4 ug/ml	91	64 (70.3)	28 (43.8)[c]	6 (9.4)[b]
0.8 ug/ml	98	65 (66.3)	31 (47.7)[bc]	7 (10.8)[b]

* Percentage of the number of embryos injected, † Percentage of the number of embryos fused.
\# Blastocyst, [a~c] Within a parameter, values with different superscripts differed significantly, $p<0.05$.

efficient protrusion rates were could be achieved using the 0.2 ug/ml of demecolcine without significant difference with 0.4 and 0.8 ug/ml group. Therefore, it was suggested that there was no need to use demecolcine higher than 0.2 ug/ml.

In the experiment 2, significant higher enucleation rates were obtained in the demecolcine-induced enucleation than blind-enucleation. In most domestic species, some points as an indicator are required for enucleation because large amount of cytoplasmic lipid droplets hamper visualization of nucleus under a common light microscope. Using the first polar body as an indicator is one possibility to remove chromatin; however, it has been revealed that up to 30% of zona-included oocytes are improperly enucleated using blind enucleation method (Dominko *et al.,* 1999; Mitalipov *et al.,* 1999; Nour and Takahashi 1999; Prather *et al.,* 1989). Although enucleation with detection of nucleus with Hoechst 33342 staining and UV light was reported in many studies, it has been shown to cause a significant decrease in development of the NT embryos to the blastocyst stage mainly due to induction of alterations of the membrane and intracellular components of the bovine oocytes and of chromatic defects (Velilla *et al.,* 2002; Dominko *et al.,* 1999; Smith 1993). Additionally, the mitochondrial DNA remains in the enucleated cytoplast and may be damaged during UV irradiation, altering the metabolism of the reconstructed embryo. In this present study, treatment of M Ⅱ-stage oocytes with demecolcine produced a membrane protrusion that contained chromosome mass, as in the previous studies (Tani *et al.,* 2006; Vajta *et al.,* 2005; Kawakami *et al.,* 2003; Yin *et al.,* 2002). Therefore, significantly higher enucleation rates were achieved easily by simple process without detrimental effects of chemical staining and UV light or removing large volume of oocyte cytoplasm near the first polar body in the blind enucleation.

For using the demecolcine in the enucleation process, no detrimental effects must be demonstrated in the SCNT. However, in the experiment 3, developmental competences of bovine SCNT embryos were gradually decreased after demecolcine treatment. Demecolcine is a specific microtubule inhibitor that binds to tubulin dimers and prevents microtubule polymerization, thus resulting in the loss of the dynamic spindle microtubules. Russell *et al.* (2005) reported that high levels of heteroploidy observed in the bovine NT embryos after demecolcine-induced enucleation. Low developmental competence in this study might be related to this high levels of heteroploidy and several karyokinetic and cytokinetic alterations induced by demecolcine treatment. In the previous studies, demecolcine treatment of M Ⅱ oocytes increased maturation promoting factor (MPF) activity which maed oocytes remain arrested in the M phase despite the occurrence of a normal pattern of calcium oscillations (Tani *et al., 2006*; Ibanez *et al., 2003*; Moses *et al., 1995*; Kubiak *et al.,* 1993). Although delayed activation improved the development rates of SCNT embryos in mouse and cattle (Wells *et al.,* 1999; Wakayama *et al.,* 1998), abnormal MPF concentrations by demecolcine treatment might affect the development competences. In the experiment 3, although significantly lower development rates were shown in the high concentration groups (0.4 and 0.8 ug/ml), higher development competences were achieved in the low concentration groups (0.1 and 0.2 ug/ml) similar to control group. However, we could not achieve relatively high rates of membrane protrusion containing chromatin after treatment of 0.1 ug/ml demecolcine in the experiment 1. Therefore, it was suggested that 0.2 ug/ml demecolcine-induced enucleation is most appropriate concentration with respect to high rates of membrane protrusion containing chromatin and development to blastocyst in the bovine SCNT.

In conclusion, an efficient and reliable chemically enucleation method for bovine oocytes has been suggested in this study by increasing the enucleation

rates and development competence in the 0.2 ug/ml demecolcine groups. Further studies to investigate the effect of demecolcine on developmental ability of oocytes are also required to increase the efficiency of bovine SCNT.

CONCLUSION

The present study was conducted to investigate the usefulness of demecolcine in the chemically assisted enucleation in bovine transgenic SCNT and its optimal concentrations. Additionally, developmental competence of SCNT embryos was compared between blind enucleation and chemical enucleation group.

1. The proportion of oocytes with a membrane protrusion significantly increased when matured oocytes were treated with 0.2, 0.4 and 0.8 ug/ml demecolcine compared with 0.1 ug/ml.
2. The enucleation rates of oocytes using the demecolcine-assisted method were significantly higher than that by the conventional blind method.
3. The fusion rates were not significantly different between groups regardless of demecolcine concentration.
4. SCNT embryos produced by 0.1 and 0.2 ug/ml demicolcine-induced enucleation showed significantly higher developmental rate to blastocyst than that of 0.4 or 0.8 ug/ml.

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