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## INFLUENCE OF NOCODAZOLE ON THE DEVELOPMENT OF DONOR BLASTOMERES FROM 16-CELL STAGE BOVINE EMBRYOS IN NUCLEAR TRANSFER

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### ABSTRACT

The aim of the present study was to establish a reliable procedure with nocodazole treatment for the synchronous cleavage of blastomeres of bovine embryos used as nuclear donors for nuclear transfer. Sixteen-cell stage embryos derived from *in vitro*-maturation, fertilization and culture were used. In three initial experiments, embryos were incubated in mTCM-199+FCS with various concentrations (0–20  $\mu$ M) of nocodazole under 5 % CO<sub>2</sub> in air. The concentrations required to arrest the blastomeres in the mitotic phase were examined. The effects of 10  $\mu$ M nocodazole were also examined by observation of the division rate of blastomeres after the removal of nocodazole.

Ninety percent (90 %) of the blastomeres were arrested in the mitotic phase when embryos were exposed to 10 and 20  $\mu$ M nocodazole. Exposure to 10  $\mu$ M nocodazole had the highest blastomere-cleavage rate (47 %). When the exposure period to 10  $\mu$ M nocodazole was prolonged to 36 hr, the division rate of the blastomeres decreased.

Furthermore, the effects of 2 culture conditions (mTCM-199 under 5 % CO<sub>2</sub> in air vs modified synthetic oviduct fluid medium under 5 % CO<sub>2</sub>, 5 % O<sub>2</sub> and 90 % N<sub>2</sub>) were compared on the division rate of blastomeres of embryos exposed to 10  $\mu$ M nocodazole for 12 hr. When the embryos were exposed to nocodazole in mSOF, the division rate of blastomeres was improved to about 60 %.

The blastomeres produced by this treatment condition were used as nuclear donors and the developmental potential of the reconstituted embryos was investigated. The developmental rate to the blastocyst stage was 30.1 % (58 / 193). Five embryos were transferred to 5 recipient cows and 2 of the 5 recipients (40 %) became pregnant. Subsequently, one normal calf was born.

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#### INTRODUCTION

The ability to produce a large number of identical bovine offspring is of great importance for the evaluation, selection and multiplication of genotypes with superior economic value. Since the first calf derived from nuclear transfer was reported<sup>27)</sup>, there have been many successful transfers of cloned bovine embryos<sup>5,7,11,17,18,28,30,38,42,43,46,47)</sup>, but the developmental rate to the blastocyst stage of the reconstituted embryos produced by nuclear transfer is low.

The factors which affect the development of reconstituted embryos are regarded to be the cell cycle stage of recipient cytoplasm<sup>19,32,33,47)</sup>, nuclear donors<sup>32,33)</sup> and the culture conditions<sup>47)</sup>. Moreover, it has been confirmed that the cell cycle stage of donor blastomeres is the most important factor in the development of the reconstituted embryos<sup>8-10)</sup>. To improve the development rate of the reconstituted embryos, donor blastomeres need to be synchronized with the cell cycle stage. Cytoskeletal inhibitors that have the potential to arrest the cell cycle at specific stages need to be used for synchronization. Microfilament-inhibitors (cytochalasin B & D) and microtubule-inhibitors (colcemid, colchicine, vinblastin & nocodazole) have been used as cytoskeletal inhibitors to control the cell cycle in early-stage mouse embryos<sup>15,16,20,21,26,31)</sup>. Snow<sup>34)</sup> showed that cytochalasin B induces polyploidy in mouse embryos. Siracusa et al.<sup>31)</sup> found the effect of colcemid to be far more reversible than that of colchicine or vinblastin. Furthermore, the effect of nocodazole as a microtubule-inhibitor for the early-stage mouse embryo was more reversible than that of colcemid. It seems reasonable to conclude that nocodazole can be used to control the cell cycle in early-stage mouse embryos<sup>16)</sup>.

Otaegui et al.<sup>26)</sup> observed that synchronization of the cell cycle of donor blastomeres with nocodazole at the G<sub>1</sub> phase can improve *in vitro* development to the blastocyst stage of reconstituted mouse embryos and that the efficiency of blastomeres of mouse embryos arrested in the mitotic phase depends upon the concentration and the duration of exposure to nocodazole. When treatment with over-concentration and over-exposure to nocodazole is done, the harmful effect of nocodazole may be reflected as the disruption of microtubules other than those required for spindle formation<sup>21,26)</sup>. Consequently, the division rate of blastomeres decreases and the subsequent development of mouse embryos is negatively affected<sup>26)</sup>.

It has been reported that colcemid<sup>35)</sup> and nocodazole<sup>7,12,35,39)</sup> are useful to synchronize the cleavage of blastomeres for bovine nuclear transfer. However, the harmful effects of nocodazole on bovine embryos were not considered. Thus, there are no reliable methods for synchronizing cell divisions of early embryos without reducing their ability to develop into fetuses in cattle.

The object of this study was to establish a reliable procedure using nocodazole for

the synchronous cleavage of blastomeres of 16-cell stage bovine embryos derived from *in vitro* embryos used as nuclear donors for nuclear transfer. When the embryos were treated with various concentrations of nocodazole, the concentration of nocodazole required to arrest the blastomeres and the division rate of blastomeres after the removal of nocodazole were examined. The effects of the exposure period to nocodazole and culture conditions when the embryos are treated with nocodazole were also examined by observation of the division rate of blastomeres after the removal of nocodazole. Subsequently, the blastomeres treated with nocodazole were used as nuclear donors for nuclear transfer and the developmental capacity of the reconstituted embryos to the blastocyst stage *in vitro* and to full term *in vivo* were confirmed.

#### MATERIALS AND METHODS

##### *In vitro fertilization of ovarian oocytes*

Bovine ovaries were collected from a slaughterhouse and brought to the laboratory within 4 hr after slaughter. Oocytes were collected by aspiration of antral follicles (2–7 mm in diameter) with an 18-gauge needle and a sterile syringe. Cumulus-oocyte complexes (COCs) were washed twice with modified Tyrode's solution (TALP)<sup>4)</sup>. For oocyte maturation, the COCs were cultured in TCM-199 supplemented with 25 mM HEPES (Gibco Laboratories, Grand Island, NY, USA), 10 % fetal calf serum (FCS; Gibco), 0.02U/ml FSH, 1  $\mu$ g/ml estradiol, 0.2 mM sodium pyruvate and 50  $\mu$ g/ml gentamicin sulfate (Sigma Chemical Co., St Louis, MO, USA) for 22 hr at 39°C in an atmosphere of 5 % CO<sub>2</sub> in humidified air.

Frozen-thawed semen from one Holstein bull was used for *in vitro* fertilization. Frozen semen was thawed in a water bath for 1 min at 35°C. The motile sperm were separated using 45 and 90 % Percoll (Pharmacia BioProcess, Uppsala, Sweden) diluted with an isotonic defined medium (BO)<sup>6)</sup> by centrifugation at 700 $\times$ g for 20 min. The sperm were subsequently washed using BO medium without bovine serum albumin (BSA; Sigma) by centrifugation at 500 $\times$ g for 5 min. The sperm were co-incubated with about 15 COCs at the concentration of 5 $\times$ 10<sup>6</sup> cells/ml in a 100  $\mu$ l microdrop of BO medium containing 3 mg/ml BSA and 2.5 mM theophylline (Sigma) covered with paraffin oil for 18 hr at 39°C under 5 % CO<sub>2</sub> in air.

##### *In vitro culture of one-cell embryos*

After insemination, the cumulus cells were removed from oocytes by vortexing in TALP. Cumulus-free oocytes were cultured in 50  $\mu$ l microdrops of modified synthetic oviduct fluid medium (mSOF)<sup>36)</sup> supplemented with 3 mg/ml BSA, 20 amino acids and 10  $\mu$ g/ml insulin (Sigma) for 81 hr at 39°C under 5 % CO<sub>2</sub>, 5 % O<sub>2</sub> and 90 % N<sub>2</sub>. The 16-cell stage embryos at 99 hr post-insemination (hpi) were selected to examine the effect of nocodazole on the blastomeres (Experiments 1 to 4) or to prepare donor nuclei for nuclear transfer (Experiment 5).

##### *Preparation of recipient cytoplasm for nuclear transfer*

The COCs collected from ovaries were cultured in the maturation medium for 20–22 hr. After the removal of the cumulus cells, the oocytes with first polar bodies were selected as recipients.

The oocytes were placed in microdrops of Dulbecco's phosphate-buffered saline (DPBS)<sup>44)</sup> containing 20 % FCS, 5  $\mu\text{g/ml}$  cytochalasin B (Sigma) and 0.3  $\mu\text{g/ml}$  nocodazole (Aldrich Chemical Co., Inc. Milwaukee, USA), and enucleated by removing the first polar body and the adjacent cytoplasm presumably containing the nuclear material<sup>27)</sup>. Enucleation was confirmed by staining the oocytes with 5  $\mu\text{g/ml}$  Hoechst 33342 (Sigma)<sup>43)</sup>. Enucleated oocytes were placed in the maturation medium until 38 hr postmaturation. They were then exposed to 7 % ethanol in TCM-199 supplemented with 24 mM HEPES, 10 % FCS, 0.2 mM sodium pyruvate and 50  $\mu\text{g/ml}$  gentamicin (mTCM-199+FCS) for 7 min for activation<sup>3,24)</sup>. Enucleated oocytes were further cultured in the maturation medium for a total of 42 hr post-maturation<sup>19)</sup>.

#### *Nuclear transfer and membrane fusion*

The zonae pellucidae of 16-cell stage donor embryos were removed by incubation in 0.5 % pronase (Actinase E, Kaken Pharmaceutical Co., Inc., Tokyo, Japan) dissolved in DPBS. Blastomeres of donor embryos were isolated by pipetting the embryos in  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -free DPBS. Nuclear transplantation was carried out as described by McGrath and Solter<sup>22,23)</sup>. A blastomere of the donor embryo was introduced into the perivitelline space of the enucleated oocyte using an injection pipette with inner diameter of 35–40  $\mu\text{m}$  through a slit in the zona.

The blastomere-oocyte complexes were placed in an electrode chamber (BTX, San Diego, CA, USA) filled with 0.3 M mannitol solution containing 0.1 mM  $\text{MgSO}_4$ , 0.05 mM  $\text{CaCl}_2$  and 0.05 mg/ml BSA<sup>8)</sup>. They were aligned by exposure to alternating current (A.C.) pulses of 0.6 MHz, 10 V for 6 sec. Thereafter, 2 direct current (D.C.) pulses of 1.0 KV/cm for 70  $\mu\text{sec}$  (each pulse 1 sec apart) were applied for membrane fusion of the blastomere and enucleated oocyte<sup>9)</sup> by an Electro Cell Fusion instrument (LF100, Life Tec., Tokyo, Japan).

#### *In vitro culture of nuclear transfer embryos*

About 10 blastomere-oocyte complexes were cultured in a 50  $\mu\text{l}$  drop of mSOF supplemented with 3 mg/ml BSA, 20 amino acids and 10  $\mu\text{g/ml}$  insulin for 6 days (147 hr) at 39°C under 5 %  $\text{CO}_2$  and 90 %  $\text{N}_2$ . The fusion rates of the blastomeres and oocytes, and cleavage rates of the reconstituted embryos were investigated at 2 and 48 hr post-fusion, respectively. The nuclear transfer embryos were examined for development to the blastocyst stage under a stereomicroscope. The embryos that developed to blastocysts were assigned for cell counting<sup>36)</sup> or transfer to recipient cows.

#### *Experiment 1*

The effects of various concentrations (0, 1, 3.3, 5, 10 & 20  $\mu\text{M}$ ) of nocodazole (Aldrich Chem.) on the mitosis of the blastomeres were investigated. The 16-cell

stage embryos at 99 hpi were incubated in mTCM-199+FCS and each concentration of nocodazole for 12 hr at 39°C in an atmosphere of 5 % CO<sub>2</sub> in air. After nocodazole treatment, the zonae pellucidae were removed by incubation with 0.5 % pronase in DPBS. Blastomeres were then isolated by pipetting the embryos in Ca<sup>2+</sup> and Mg<sup>2+</sup>-free DPBS. The blastomeres were stained with 5 µg/ml Hoechst 33342 for 10 min and the nuclei were observed under fluorescence microscope. The proportion of blastomeres in the mitotic phase was determined.

#### *Experiment 2*

The effects of various concentrations (0, 1, 3.3, 5, 10 & 20 µM) of nocodazole on the division of the blastomeres after removal from nocodazole were examined. The 16-cell stage embryos at 99 hpi were incubated in mTCM-199+FCS and each concentration of nocodazole for 12 hr at 39°C under 5 % CO<sub>2</sub> in air. Immediately after release from nocodazole, the zonae pellucidae were removed and the blastomeres were isolated as in Experiment 1. Isolated blastomeres were incubated in mTCM-199+FCS in multi-well plates (Sumitomo Bakelite Co. Ltd., Tokyo., Japan) for 5 hr at 39°C in an atmosphere of 5 % CO<sub>2</sub> in air.

#### *Experiment 3*

The effects of various exposure periods to nocodazole on the division of the blastomeres after removal from nocodazole were examined. The 16-cell stage embryos at 99 hpi were incubated in mTCM-199+FCS and 10 µM nocodazole for 12, 24 and 36 hr at 39°C in an atmosphere of 5 % CO<sub>2</sub> in air. After removal of the zonae pellucidae and isolation of the blastomeres, the blastomeres were incubated in mTCM-199+FCS in multi-well plates for 5 hr at 39°C in an atmosphere of 5 % CO<sub>2</sub> in air. The division of blastomeres was then examined.

#### *Experiment 4*

The effects of culture conditions for the nocodazole treatment on the division of the blastomeres after removal from nocodazole were examined. The 16-cell stage embryos at 99 hpi were incubated for 12 hr at 39°C in mTCM-199+FCS under 5 % CO<sub>2</sub> in air or mSOF supplemented with 3 mg/ml BSA and 10 µg/ml insulin under 5 % CO<sub>2</sub>, 5 % O<sub>2</sub> and 90 % N<sub>2</sub>. The embryos were also cultured in the medium with or without 10 µM nocodazole. After removal of the zonae pellucidae and isolation of the blastomeres, the blastomeres were cultured for 24 hr under the same conditions as in the nocodazole treatment except for the absence of nocodazole. The division of blastomeres was examined at 3, 5 and 24 hr of incubation.

#### *Experiment 5*

The developmental capacity of nuclear transfer embryos derived from the nocodazole treatment was examined *in vitro* and *in vivo*. The 16-cell stage embryos at 99 hpi were incubated in mSOF supplemented with 3mg/ml BSA, 10 µg/ml insulin and 10 µM nocodazole for 12 hr at 39°C under 5 % CO<sub>2</sub>, 5 % O<sub>2</sub> and 90 % N<sub>2</sub>. After the removal of the zonae pellucidae and isolation of the blastomeres, the blastomeres were

incubated in mSOF without nocodazole in multi-well plates for 3 hr at 39°C. The blastomeres that divided within 3 hr were used as donors for nuclear transfer. The nuclear transfer embryos were cultured for 6 days (147 hr). The embryos that developed to the blastocyst stage were transferred non-surgically to the uteri of Holstein-Friesian cows that had shown estrous behavior 6–8 days before. Pregnancy was examined by ultrasonography 40 and 60 days after the embryo transfer.

#### Statistical analysis

In Experiments 1 to 4, the results were obtained from 3–18 replicates. The data were analyzed by *the Student's t-test*.

## RESULTS

### Experiment 1

The proportion of blastomeres arrested in the mitotic phase was increased significantly ( $p < 0.01$ ) by the addition of nocodazole to the medium (Table 1). Moreover, the embryos treated with 10 and 20  $\mu\text{M}$  nocodazole showed significantly ( $p < 0.05$ ) higher proportion of blastomeres arrested in the mitotic phase than those in the other groups (1, 3.3 & 5  $\mu\text{M}$ ). The nucleus was not observed in 3.9–7.4 % of the blastomeres.

### Experiment 2

The embryos treated with 10 and 20  $\mu\text{M}$  nocodazole possessed significantly ( $p < 0.05$ ) higher blastomeres division rates compared with the other groups (Table 2).

### Experiment 3

No significant difference was observed in the division rate of blastomeres between the embryos treated with 10  $\mu\text{M}$  nocodazole for 12 and 24 hr (Table 3). However,

Table 1. Influence of concentration of nocodazole on the mitosis of blastomeres of 16-cell stage embryos<sup>a)</sup>

Concentration of nocodazole ( $\mu\text{M}$ )	No. of embryos (blastomeres)	% of blastomeres with different nuclear features		
		Mitotic phase	Interphase	No nucleus
0	4 (100)	14.8 ± 1.4 <sup>b)</sup>	79.8 ± 4.1 <sup>c)</sup>	5.4 ± 4.2
1	3 (67)	66.9 ± 2.6 <sup>c)</sup>	26.8 ± 3.9 <sup>d)</sup>	6.4 ± 1.8
3.3	5 (97)	67.3 ± 3.2 <sup>c)</sup>	25.3 ± 4.0 <sup>d)</sup>	7.4 ± 1.5
5	4 (72)	75.0 ± 3.4 <sup>c)</sup>	17.5 ± 4.7 <sup>f,g)</sup>	7.2 ± 1.8
10	5 (78)	90.2 ± 3.9 <sup>d)</sup>	5.9 ± 4.1 <sup>g)</sup>	3.9 ± 0.2
20	5 (78)	88.6 ± 1.1 <sup>d)</sup>	4.1 ± 2.7 <sup>g)</sup>	7.3 ± 3.8

<sup>a)</sup> Embryos were treated with nocodazole for 12 hr. Values are mean  $\pm$  s. e. m. from 3-5 replicates.

<sup>b,c,d)</sup> Values with different superscripts are significantly different (b-c, b-d :  $P < 0.01$ , c-d :  $P < 0.05$ ).

<sup>e,f,g)</sup> Values with different superscripts are significantly different ( $P < 0.01$ ).

Table 2. Influence of concentration of nocodazole on the division of blastomeres of 16-cell stage embryos after removal from nocodazole<sup>a)</sup>

Concentration of nocodazole ( $\mu$ M)	No. of embryos (blastomeres)	% of blastomeres divided <sup>b)</sup>
0	13 (300)	13.0 $\pm$ 3.1 <sup>c)</sup>
1	14 (327)	21.8 $\pm$ 4.1 <sup>c)</sup>
3.3	12 (243)	16.8 $\pm$ 4.9 <sup>c)</sup>
5	12 (268)	19.5 $\pm$ 4.5 <sup>c)</sup>
10	14 (248)	47.1 $\pm$ 6.3 <sup>d)</sup>
20	12 (208)	38.0 $\pm$ 6.0 <sup>d)</sup>

<sup>a)</sup> Embryos were treated with nocodazole for 12 hr.

<sup>b)</sup> The blastomeres were incubated in mTCM199 + FCS for 5 hr and investigated. Values are mean  $\pm$  s. e. m. from 12-14 replicates.

<sup>c,d)</sup> Values with different superscripts are significantly different ( $P < 0.05$ ).

Table 3. Influence of exposure period to nocodazole on the division of blastomeres of 16-cell stage embryos after removal from 10  $\mu$ M nocodazole

Exposure period (hr)	No. of embryos (blastomeres)	% of blastomeres divided <sup>a)</sup>
12	8 (122)	48.4 $\pm$ 4.9 <sup>b)</sup>
24	18 (282)	52.4 $\pm$ 7.7 <sup>b)</sup>
36	16 (257)	29.4 $\pm$ 5.8 <sup>c)</sup>

<sup>a)</sup> The blastomeres were incubated in mTCM199 + FCS for 5 hr and investigated. Values are mean  $\pm$  s. e. m. from 8-18 replicates.

<sup>b,c)</sup> Values with different superscripts are significantly different ( $P < 0.05$ ).

when the embryos were treated for 36 hr, the proportion of divided blastomeres decreased significantly ( $p < 0.05$ ).

#### Experiment 4

In the embryos treated without nocodazole, there was no difference in division rates of the blastomeres between the 2 different culture conditions at 3, 5 and 24 hr after isolation of the blastomeres (Table 4). The embryos treated with 10  $\mu$ M nocoda-

Table 4. Influence of culture conditions on the division of blastomeres of 16-cell stage embryos at 3, 5 and 24 hr after removal from 10  $\mu$ M nocodazole<sup>a)</sup>

Culture condition <sup>b)</sup>	Nocodazole treatment <sup>c)</sup>	No. of embryos (blastomeres)		% of blastomeres divided		
				3 hr	5 hr	24 hr
mTCM-199	—	12	(203)	15.4 $\pm$ 3.9 <sup>d,A)</sup>	21.7 $\pm$ 4.0 <sup>d,A)</sup>	67.6 $\pm$ 3.8 <sup>B)</sup>
mSOF	—	12	(187)	15.8 $\pm$ 4.8 <sup>d,A)</sup>	22.3 $\pm$ 5.7 <sup>d,A)</sup>	77.4 $\pm$ 3.7 <sup>B)</sup>
mTCM-199	+	12	(191)	33.7 $\pm$ 3.7 <sup>e,A)</sup>	40.1 $\pm$ 4.6 <sup>e,A)</sup>	68.3 $\pm$ 4.0 <sup>B)</sup>
mSOF	+	12	(193)	53.7 $\pm$ 4.6 <sup>f,A)</sup>	61.7 $\pm$ 4.5 <sup>f,A)</sup>	80.0 $\pm$ 4.2 <sup>B)</sup>

a) Values are mean  $\pm$  s. e. m. from 12 replicates.

b) mTCM-199 + FCS under 5 % CO<sub>2</sub> in air. mSOF supplemented with 3 mg / ml BSA and 10  $\mu$ g / ml insulin under 5 % CO<sub>2</sub>, 5 % O<sub>2</sub> and 90 % N<sub>2</sub>.

c) Treatment with (+) or without (—) nocodazole for 12 hr.

d,e,f) Values with different superscripts in the same column are significantly different (P<0.01).

A,B) Values with different superscripts in the same line are significantly different (P<0.01).

zole showed significantly ( $p < 0.01$ ) higher division rates of the blastomeres at 3 and 5 hr than those treated without nocodazole. There was no difference in the division rate at 24 hr among the 4 groups indicated in Table 4. In the treatment with nocodazole, the embryos cultured in mSOF supplemented with 3 mg/ml BSA and 10  $\mu$ g/ml insulin under 5 % CO<sub>2</sub>, 5 % O<sub>2</sub> and 90 % N<sub>2</sub> had significantly ( $p < 0.01$ ) higher division rates of the blastomeres at 3 and 5 hr than those cultured in mTCM-199 + FCS under 5 % CO<sub>2</sub> in air. There was no difference in the division rate of blastomeres between 3 and 5 hr within the 4 groups. The division rate of the blastomeres in the embryos treated in mSOF with nocodazole and cultured for 3 hr was 68 % of those at 24 hr of culture.

#### Experiment 5

When the blastomere-oocyte complexes were cultured, the fusion rate was 89.8 % (193/215) and the cleavage rate of the reconstituted embryos was 94.3 % (182/193). The developmental rate of reconstituted embryos to the blastocyst stage was 30.1 % (58/193). The cell number of blastocysts was  $132.9 \pm 5.1$  ( $n = 53$ , mean  $\pm$  s.e.m.). Five blastocysts were transferred to the uteri of 5 recipient cows. Two recipient cows (40 %) became pregnant at 40 days. One cow delivered a male calf, the other aborted at 60 days after embryo transfer.

#### DISCUSSION

In the present study, the experiments were conducted first to determine the lowest concentration of nocodazole able to arrest blastomeres in the mitotic phase and second to observe the division rate of blastomeres after the removal of nocodazole. As the cell cycle length of 16-cell stage bovine embryo was inferred to last 24 hr<sup>2,3)</sup>

and the embryos have been incubated in a culture medium containing nocodazole for 12–18 hr<sup>7,12,29,35,39)</sup>, the 12 hr duration of exposure to nocodazole was adopted.

Samaké and Smith<sup>29)</sup> observed that cell division of 8-cell stage bovine embryos was not inhibited when they were exposed up to 1  $\mu\text{M}$  nocodazole. In the present study, 16-cell stage embryos treated with 1  $\mu\text{M}$  nocodazole showed a significantly higher rate (67 %) of blastomeres arrested in the mitotic phase than in the control, and our results agree with theirs. When bovine embryos of the morula stage were incubated in B2 medium supplemented with 3.3  $\mu\text{M}$  nocodazole for 15 hr, 90 % of the cells were arrested in the mitotic phase<sup>39)</sup>. In the present study however, 67 % of blastomeres of 16-cell stage embryos were arrested in the mitotic phase after treatment with 3.3  $\mu\text{M}$  nocodazole. When the embryos were exposed to 10 and 20  $\mu\text{M}$  nocodazole for 12 hr, 90 % of the blastomeres were arrested in the mitotic phase. The difference between their results<sup>39)</sup> and our observations may reflect the differences in the media (mTCM-199 vs B2 medium) used or in the stage of development of the embryos at the time of exposure. In fact, 33 % of the blastomeres were arrested in the mitotic phase when blastocyst-stage embryos were exposed to 10  $\mu\text{M}$  nocodazole for 15 hr<sup>12)</sup>. These results indicate that the effect of nocodazole may depend upon the stage of development of the embryo at the time of exposure. In the future, we need to examine the optimum condition of nocodazole treatment for various stages of bovine embryos.

It has been reported that most blastomeres of bovine embryos divide within 1 to 5 hr after the removal of colcemid<sup>35)</sup>. Therefore, the present study examined the division rate of blastomeres within 5 hr after the removal of nocodazole. The embryos exposed to 10 and 20  $\mu\text{M}$  nocodazole had higher division rates of the blastomeres within 5 hr after the removal of nocodazole, suggesting that treatment with these concentrations of nocodazole synchronize the division of blastomeres the most within 5 hr after the removal of nocodazole. Though there was no difference in the population of cells arrested in the mitotic phase and the division rate of cells between the embryos treated with 10 and 20  $\mu\text{M}$  nocodazole, treatment with 10  $\mu\text{M}$  nocodazole tends to result in a higher resumption of cleavage after the removal of nocodazole. As the harmful effect of a high concentration of nocodazole may affect the synchronous cleavage of the embryos, it seems reasonable to suppose that 10  $\mu\text{M}$  is the optimum concentration.

If the exposure period to nocodazole for donor blastomeres is extended, the timing between the preparation of recipient oocytes collected from ovaries under time limit and micromanipulation can be regulated easily. When treatment with nocodazole results in overexposure, it is likely that the harmful effect of nocodazole may reflect on the subsequent development of the embryos. To confirm the maximum permissible exposure to 10  $\mu\text{M}$  nocodazole, the duration of exposure was prolonged to 24 and 36 hr, and the division rate of the blastomeres was examined. Consequently, the

cleavage rate of the blastomeres decreased significantly after the 36 hr exposure. It seems likely that the harmful effects of nocodazole reflect, at least in part, on the resumption rate of the cleavage of blastomeres after its removal. The 24 hr exposure period was regarded to be the maximum permissible exposure to 10  $\mu\text{M}$  nocodazole.

When mouse embryos at the 4-cell stage were treated with 10  $\mu\text{M}$  nocodazole for 9 hr, more than 95 % of the cells were arrested in the mitotic phase and the blastomeres underwent synchronous cleavage which was completed within 90 min after the removal of nocodazole<sup>26)</sup>. In the present study, when 16-cell stage bovine embryos were exposed to 10  $\mu\text{M}$  nocodazole for 12 hr, 90 % of the blastomeres were arrested in the mitotic phase but mitosis was completed in only about 50 % of the blastomeres within 5 hr after the removal of nocodazole. These results indicate that exposure to 10  $\mu\text{M}$  nocodazole for 12 hr may have a harmful effect on the subsequent division of blastomeres of 16-cell stage bovine embryos. Therefore, another experiment was done to expose 16-cell stage bovine embryos to 10  $\mu\text{M}$  nocodazole for 12 hr and to observe the mitosis of embryos for 24 hr after its removal. The results indicate that exposure to 10  $\mu\text{M}$  nocodazole for 12 hr did not have a harmful effect on the subsequent division of the blastomeres because there was no difference in the division rate of blastomeres within 24 hr after the isolation of blastomeres between treatment with and without nocodazole. Moreover, the present study indicates that the difference of medium and oxygen concentration when the embryos are treated with nocodazole may affect the next mitosis of embryos.

Culture with mSOF in 5 %  $\text{O}_2$  was compared with mTCM-199 in air. Consequently, in the embryos treated without nocodazole, there was no difference in the resumption rate of cleavage of blastomeres between those cultured in mTCM-199 and mSOF after the isolation of the blastomeres. However, the embryos treated with nocodazole and cultured in mSOF gave a significantly higher rate of resumption of the cleavage of blastomeres within 3 and 5 hr after the removal of nocodazole. It has been reported that the development of embryos is affected by different culture conditions (with and without co-culture)<sup>13)</sup> and medium components (with and without glucose)<sup>37)</sup>. Likewise, the present study suggests that different media and oxygen concentrations have greater effects on the resumption rate of cleavage of blastomeres after exposure to nocodazole. Oxygen concentration affects the development of embryos in culture and 5–10 % concentration of oxygen gives better developmental rate of embryos<sup>25,40,41)</sup>.

When 16-cell stage bovine embryos were treated with 10  $\mu\text{M}$  nocodazole, the resumption rate of cleavage of blastomeres was improved by the application of the optimum culture conditions in mSOF in 5 %  $\text{O}_2$ . However, the division rate of blastomeres within 24 hr after isolation of the blastomeres was low (67.6–80.3 %). It has been reported that the isolated blastomeres of mouse and sheep embryos have reduced developmental capacity<sup>19,45)</sup>. In the present study, it seems likely that

isolation of blastomeres may have a harmful effect on the blastomeres' subsequent division.

When 16-cell stage bovine embryos were treated with 10  $\mu$ M nocodazole for 12 hr, the blastomere division of the embryos within 5 hr after the removal of nocodazole was 62 % in TCM-199 in 95 % air and 77 % in mSOF after 24 hr. The present study showed that blastomeres of 16-cell stage embryos underwent synchronous cleavage within 5 hr after the removal of nocodazole. This result is in agreement with previous reports that most blastomeres divide within 4 to 5 hr after the removal of nocodazole<sup>12,35,39</sup>). In contrast, the blastomeres of 4-cell mouse embryos undergo synchronous cleavage that is completed in 90 min after the removal of nocodazole<sup>26</sup>). Thus, these results suggest that between mouse and bovine embryos, time differences exist in the synchronous cleavage after the removal of nocodazole.

In the present study, 16-cell stage embryos were treated with 10  $\mu$ M nocodazole for 12 hr in mSOF in 5 % O<sub>2</sub>, which was concluded to be the optimum concentration, exposure period and culture condition. The blastomeres underwent synchronous cleavage within 3 hr after the removal of nocodazole and enucleated oocytes were used as nuclear donors and cytoplasm for nuclear transfer. Consequently, there was no difference in the developmental rate to the blastocyst stage between our result (about 30 %) and the previous studies (24–42 %)<sup>1,18,35</sup>). When the reconstituted embryos were transferred, the pregnancy rate obtained in previous studies was 23–47 %<sup>5,18,46</sup>). Although a small number of embryos were examined, our result is similar with those of previous studies (40 %).

In conclusion, the present study confirmed that it is possible to use nocodazole to initiate synchronous cleavage of blastomeres in 16-cell stage embryos derived from IVF-IVM-IVC for bovine nuclear transfer and that reconstituted embryos are able to develop to the blastocyst stage *in vitro* and to full term *in vivo*. When 16-cell stage bovine embryos are exposed to 10  $\mu$ M nocodazole in mSOF for 12 hr at 39°C in 5 % CO<sub>2</sub>, 5 % O<sub>2</sub> and 90 % N<sub>2</sub>, mitosis can be synchronized in about 60 % of the blastomeres within 3 hr after the removal of nocodazole. Thus, exposure to 10  $\mu$ M nocodazole at 39°C in 5 % CO<sub>2</sub>, 5 % O<sub>2</sub> and 90 % N<sub>2</sub> for 12 hr is recommended to obtain a population of donor blastomeres after the synchronous cleavage of 16-cell stage embryos before bovine nuclear transfer. In the future, studies must be carried out to confirm whether the blastomeres that divided within 3 hr after the removal of nocodazole are at the defined stage of the cell cycle (G<sub>1</sub>, S, G<sub>2</sub> & M).

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