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INFLUENCE OF TIME AFTER THE REMOVAL OF NOCODAZOLE FROM NUCLEAR DONORS ON THE DEVELOPMENT OF RECONSTITUTED EMBRYOS IN BOVINE NUCLEAR TRANSPLANTATION

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ABSTRACT

The present study examined the influence of post-cleavage time of nuclear donors on the development of reconstituted embryos in bovine nuclear transfer. Blastomeres of 16-cell stage embryos derived from *in vitro*-maturation, fertilization and culture were used as nuclear donor source. They were treated with 10 μM nocodazole for 12 hr. Blastomeres that cleaved within 3 hr after the removal of nocodazole were used for the study. Metaphase II (M-II) oocytes were used as recipient cytoplasm. In experiment 1, donor blastomeres at 6, 11 and 15 hr after the removal of nocodazole and donor blastomeres not treated with nocodazole were transferred into ethanol-exposed and enucleated oocytes. The reconstituted embryos produced by donor blastomeres at 6 hr after the removal of nocodazole had a significantly higher developmental rate to the blastocyst stage than those at 15 hr and the untreated groups ($P<0.01$). In experiment 2, blastomeres at 6 hr after the removal of nocodazole used as nuclear donors were transferred into ethanol-exposed and enucleated M-II oocytes. The reconstituted embryos with ethanol-exposed and enucleated oocytes as recipient cytoplasm had a significantly higher rate of initial-cleavage ($P<0.05$) and development to the blastocyst stage ($P<0.01$) than non ethanol-exposed and enucleated M-II oocytes. These results demonstrate that the development of reconstituted embryos was improved when cleaved donor blastomeres after the removal of nocodazole were immediately transferred (at 3–6 hr post-cleavage) into activated enucleated oocytes by exposure to ethanol.

Key Words : nuclear transfer, blastomere, nocodazole, activation, cattle

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INTRODUCTION

Nuclear transfer protocols generally involve enucleation of recipient cytoplasm by micromanipulation and introduction of nuclear donors by fusion. Smith et al.[20] suggested that defined cell cycle-synchronous transplantations between nuclei and cytoplasm were important to the development of reconstituted mouse embryos. The frequency of development to the blastocyst stage of reconstituted embryos is improved when donor blastomeres synchronized to the G1-phase are fransferred to enucleated M-II oocytes in mouse and rabbit nuclear transfer[6,8,17]. The improved development of reconstituted embryos is showed when donor blastomeres at a defined time are transferred to activated enucleated oocytes in bovine nuclear transfer[1,11,21]. However, the defined times reported vary from study to study. It is not clear which stage of the cell cycle of donor blastomeres is optimal for the improvement of the development of reconstituted embryos. Microtubule-inhibitors, nocodazole and colcemid can be used to synchronize the cleavage of donor blastomeres for bovine nuclear transfer[5,21,24,25]. If donor blastomeres at defined post-cleavage times are used for nuclear transfer, it may be possible to determine which stage of the cell cycle of donor blastomeres is optimum for the improvement in the development of reconstituted embryos.

In bovine[1,11,26] and ovine[4] nuclear transfer, it has been reported that reconstituted embryos developmental rates to the blastocyst stage were improved with the use of activated recipient cytoplasm by using ethanol[26] or electro pulse[4,11], or by combining the above methods and cycloheximide treatment[1]. Though ethanol activation has been used for parthenogenesis[15,16,19], its application in nuclear transfer appears in only one report[26]. Therefore, further studies are needed to confirm the effect of ethanol activation for nuclear transfer.

The object of this study was to investigate the influence of post-cleavage time of nuclear donors on the development of reconstituted embryos to the blastocyst stage. Experiments were also carried out to determine whether the exposure of recipient oocytes to ethanol have an influence on the development of the reconstituted embryos.

MATERIALS AND METHODS

Nuclear donor embryos

Bovine ovaries were collected from a slaughterhouse and brought to the laboratory within 4 hr after slaughter. Oocytes were collected from antral follicles (2–7 mm in diameter) by aspirating with an 18-gauge needle and a sterile syringe. Cumulus-oocyte complexes (COC) were washed twice with modified Tyrode's solution (TALP)[3]. For oocyte maturation, the COC were cultured in TCM-199 supplemented with 25 mM N-(2-hydroxyethly) piperazine-N' (2-ethanesulfonic acid) (HEPES, Gibco Laboratories, Grand lsland, NY, USA), 10% fetal calf serum (FCS ; Gibco), 0.02 U/ml

follicule stimulating hormone (Sigma Chemical Co., St. Louis, MO, USA), 1 μg/ml estradiol-17β (Sigma), 0.2 mM sodium pyruvate and 50 μg/ml gentamicin sulfate (Sigma) for 22 hr at 39°C in an atmosphere of 5% CO_2 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 modified Brackett and Oliphant (mBO) medium[23] by centrifugation at 700×g for 20 min. The sperm were subsequently washed, using mBO medium without bovine serum albumin (BSA ; Sigma) by centrifugation at 500×g for 5 min. The sperm were co-incubated with about 15 COC at the concentration of 5×10^6 cells/ml in a 100 μl microdrop of mBO 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_2 in air as described previously[23].

After insemination, the cumulus cells were removed from oocytes by vortex agitation in TALP. Cumulus-free oocytes were cultured in 50 μl microdrops of modified synthetic oviduct fluid (mSOF)[22] supplemented with 3 mg/ml BSA, 20 amino acids and 10 μg/ml insulin (Sigma) for 81 hr at 39°C under 5% CO_2, 5% O_2 and 90% N_2. The 16-cell stage embryos at 99 hr post-insemination (hpi) were selected to prepare donor nuclei for nuclear transfer.

Recipient cytoplasm

COC collected from ovaries were cultured in the same maturation medium. At 20–22 hr post-onset maturation, the cumulus cells were removed and the oocytes with first polar bodies were judged as the metaphase II (M-II) stage were selected as recipients. The oocytes were then placed in microdrops of Dulbecco's phosphate-buffered saline (DPBS)[28] containing 20% FCS, 5 μg/ml cytochalasin B (Sigma) and 0.3 μg/ml nocodazole (Aldrich Chemical Co., Inc., Milwaukee, WI, USA), and enucleated by removing the first polar body and the adjacent cytoplasm presumably containing the nuclear material[18]. Enucleation was confirmed by staining the oocytes with 5 μg/ml Hoechst 33342 (Sigma)[26]. Enucleated oocytes were then placed in the maturation medium. For activation[2,15,16] at 38 hr post-onset maturation, they were exposed to 7% ethanol in TCM-199 supplemented with 25 mM HEPES, 10% FCS, 0.2 mM sodium pyruvate and 50 μg/ml gentamicin for 5 min. Enucleated oocytes were further cultured in the maturation medium for a total of 42 hr post-onset maturation[12].

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 Ca^{2+} and Mg^{2+}-free DPBS. Nuclear transplantation was carried out as described by McGrath and Solter[13,14]. A blastomere of the donor embryo was introduced into the perivitelline space of the enucleated (M-II) oocyte at 42 hr post-onset maturation using

an injection pipette with an inner diameter of 35–40 μm through a slit in the zona pellucida.

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 $MgSO_4$, 0.05 mM $CaCl_2$ and 0.05 mg/ml BSA[6]. They were aligned by exposure to alternating current (AC) pulses of 0.6 MHz, 10 V for 6 sec. Thereafter, 2 direct current (DC) pulses of 1.0 KV/cm for 70 μsec (each pulse 1 sec apart) were applied for membrane fusion of the blastomere and enucleated oocyte[7] by an Electro Cell Fusion instrument (LF 100, Life Tec., Tokyo, Japan).

In vitro culture of nuclear transfer embryos

About 10 blastomere-oocyte complexes were cultured in a 50 μl drop of mSOF supplemented with 3 mg/ml BSA and 10 μg/ml insulin for 6 days (147 hr post-fusion) at 39°C under 5% CO_2, 5% O_2 and 90% N_2.

Experiment 1

The effects of timing after blastomere division on the development of reconstituted embryos were examined when the blastomeres were transferred into ethanol-exposed enucleated oocytes. Resumption of cleavage was induced in donor embryos using the method described by Tanaka et al.[24]. In brief, the 16-cell stage embryos at 99 hpi were incubated in mSOF supplemented with 10 μM nocodazole (Aldrich) for 12 hr at 39°C under 5% CO_2, 5% O_2 and 90% N_2. The zonae pellucidae were then removed by incubation with 0.5% pronase in DPBS. Blastomeres were isolated by pipetting the embryos in Ca^{2+} and Mg^{2+}-free DPBS. Isolated blastomeres were incubated in mSOF without nocodazole in multi-well plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) for 3 hr at 39°C under 5% CO_2, 5% O_2 and 90% N_2. The blastomeres that divided within 3 hr were used as cleaved donor blastomeres and further cultured until use.

Cleaved donor blastomeres were classified into 3 groups at 3 hr after the removal of nocodazole. They were fused with enucleated (M-II) oocytes at 6, 11 and 15 hr after the removal of nocodazole. The presumptive periods of these blastomeres at the fusion were 3–6, 7–11 and 12–15 hr post-cleavage, respectively. The 16-cell stage embryos at 99 hpi were also incubated in mSOF at 39°C under 5% CO_2, 5% O_2 and 90% N_2 for 17 hr. After the removal of the zonae pellucidae and isolation of the blastomeres, these blastomeres were used as control nuclear donors without nocodazole treatment. Blastomeres used as nuclear donors were fused with activated enucleated M-II oocytes at 42 hr post-onset of maturation. The reconstituted embryos were cultured for 6 days (147 hr). 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[22].

Experiment 2

The effect of the activation of recipient cytoplasm was examined when blastomeres that cleaved synchronously were used as nuclear donors for nuclear transfer. The 16-cell embryos were treated with nocodazole as in Experiment 1. Blastomeres at 6 hr after the removal of nocodazole were used as nuclear donors. The cleaved donor blastomeres were fused with either activated or untreated enucleated M-II oocytes at 42 hr post-onset of maturation. The reconstituted embryos were cultured for 6 days (147 hr). The developmental rate of the reconstituted embryos to the blastocyst stage and their cell numbers were examined.

Statistical analysis

In all experiments, the results were obtained from 5 replicates. In Experiment 1, the data were analyzed by One-way ANOVA and Fisher's PLSD. The results of Experiment 2 were analyzed by Student's *t*-test.

RESULTS

Experiment 1

When activated enucleated oocytes were used as recipient cytoplasm for nuclear transfer, there was no difference in the fusion rate of the blastomere-oocyte complexes and the cleavage rate of the reconstituted embryos among untreated donor blastomeres and 3 kinds of cleaved donor blastomeres (Table 1). However, the reconstituted embryos produced by donor blastomeres at 6 hr after the removal of nocodazole had a significantly higher developmental rate to the blastocyst stage than those of 15 hr and the untreated groups ($P<0.01$). There was no difference in the cell numbers of the reconstituted embryos that developed to the blastocyst stage among the 4 groups.

Table 1. Influence of post-cleavage time of nuclear donor on the developmental rate of bovine nuclear transfer embryos[a].

Defined time after division (hr)[c]	No. of oocytes used	% of oocytes fused	% of oocytes cleaved[b]	% of blastocysts[b]	Cell no. in blastocyst
Control[d]	72	90.4±0.9	91.9±3.7	12.5±6.6[e]	119.2±15.8
3− 6	108	93.9±1.1	93.2±2.3	29.7±2.9[f]	110.9± 6.4
7−11	94	89.6±0.6	90.2±2.2	19.0±5.4[e, f]	104.7±11.3
12−15	81	87.2±3.2	92.6±2.8	15.2±2.0[e]	103.7±10.7

[a] Values are mean±s.e.m. from 5 replicates.

[b] Percentage of oocytes cleaved and blastocysts calculated using number fused.

[c] The presumptive post-cleavage time after the removal of nocodazole.

[d] Untreated blastometers at 116 hr post-insemination.

[e, f] Values with different superscripts are significantly different ($P<0.01$).

Table 2. Influence of activation of recipient cytoplasm on the development rate of bovine nuclear transfer embryos[a].

Type of recipient cytoplasm[c]	No. of oocytes used	% of oocytes fused	% of oocytes cleaved[b]	% of blastocysts[b]	Cell no. in blastocyst
Non-activated	126	87.2±1.6	78.4±3.3[d]	13.0±2.2[f]	125.6±12.0
Activated	81	92.0±4.2	96.0±4.2[e]	28.6±4.5[g]	132.9± 5.1

[a] Values are mean±s.e.m. from 5 replicates.
Donor blastojeres for 3-6 hr after the presumptive division were used.
[b] Percentage of oocytes cleaved and blastocysts calculated using number fused.
[c] Oocytes were exposed to 7% ethanol for 5 min for activation.
[d, e, f, g] Values with different superscripts are significantly different (d-e : $P<0.05$, f-g : $P<0.01$).

Experiment 2

When donor blastomeres at 6 hr after the removal of nocodazole were used for nuclear transfer, there was no difference in the fusion rates of the blastomere-oocyte complexes in both recipient cytoplasm groups. However, the reconstituted embryos with activated enucleated oocytes as recipient cytoplasm had a significantly higher rate of cleavage ($P<0.05$) and development to the blastocyst stage ($P<0.01$) than non-activated enucleated M-II oocytes. There was no difference in cell numbers of the reconsituted embryos developing to the blastocyst stage between the 2 groups.

DISCUSSION

The results of Experiment 1 as well as those of Stice et al.[21] showed improved development of reconstituted embryos with the use of cleaved donor blastomeres at a defined time after the removal of nocodazole.

The present study could not identify the mechanism that improves the development of reconstitued embryos by using donor blastomeres at a defined time (6 hr after the removal of nocodazole ; 3–6 hr post-cleavage) as nuclear donors for nuclear transfer, but the subsequent probability was considered. 1) Smith et al.[20] found that reconstituted mouse embryos had a greater developmental rate to the blastocyst stage when nuclei from late 2-cell stage embryos as compared with that of early and mid 2-cell were transferred to enucleated late stage-zygotes. Therefore, it is likely that the development of reconstituted embryos can be improved by using nuclear donors at a defined phase of the cell cycle stage for nuclear transfer. The present study could not identify which defined phase of the cell cycle stage is situated in interphase. Further studies are needed to determine the cell cycle stage of the defined time of donor blastomeres. 2) When the embryos without nocodazole treatment are used as donor blastomeres, not all blastomeres of the donor embryos may have the capacity to

cleave. On the other hand, when blastomeres of nocodazole-treated embryos confirmed of the next mitosis after the removal of nocodazole transferred as donor nuclei, the development of reconstituted embryos is likely improved. The developmental capacity of isolated blastomeres had reflected a damage subsequently[9,29]. If isolated blastomeres are not rapidly transferred, it is possible that the development of reconstituted embryos is decreased.

Oocytes in M-II have high levels of maturation promoting factor (MPF)[2,5]. It seems reasonable to conclude that the reconstituted embryos do not have the ability to maintain correct ploidy in the nuclei and normal development cannot be expected when nuclear donors other than G1-phase nuclei are transferred into enucleated M-II oocytes[5,10]. On the other hand, if M-II oocytes undergo optimum activation, the level of MPF decreases and disappears in the oocytes[2,5]. When nuclear donors at interphase are transferred into oocytes with lower levels of MPF activity, the reconstituted embryos maintain correct ploidy and their normal development is expected[2,5]. Therefore, if M-II oocytes undergo optimum activation, the reconstituted embryos must have a higher developmental rate than untreated M-II oocytes. In Experiment 2, when M-II oocytes were activated with ethanol, the development of the reconstituted embryos was improved. This result as well as those of previous reports has confirmed that the activation of recipient cytoplasm is important in the development of reconstituted embryos. This indicates that M-II oocytes undergo optimum activation with ethanol. However, further studies are needed to determine the level of MPF activity of recipient cytoplasm with ethanol.

In conclusion, M-II occytes and 16-cell stage embryos were used in the present study for nuclear transfer. The development of reconstituted embryos was improved when donor blastomeres were treated with nocodazole and were immediately transferred at 3–6 hr post-cleavage into activated enucleated oocytes by exposure to ethanol.

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