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Cytogenetic Properties of Bovine Reconstituted Embryos by Cell Cycle-Controlled Nuclear Transfer

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소 수정란의 세포주기조절 핵이식에 의한 재구축배의 세포학적 특성

정희태 · 박춘근 · 양부근 · 김정익
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ABSTRACT

This study was conducted to investigate the cytogenetic properties, *in vitro* development, and their relationship in the bovine reconstituted embryos following cell cycle-controlled nuclear transfer. Sixteen-cell stage embryos were treated by nocodazole, and after release from nocodazole treatment, their blastomeres were separated and allowed to subsequent cleavage. Blastomeres within 1.5 h post cleavage(hpc) and at 3hpc were transferred to enucleated oocytes at M Ⅱ-phase or S-phase. Donor nuclei transferred into M Ⅱ-phase recipients underwent various nuclear remodeling, such as extrusion of a polar body(PB)-like structure, premature chromosome condensation(PCC) and chromatin modifications. These nuclear remodeling patterns varied by the time post cleavage of donor blastomeres. Developmental rate to the blastocyst stage differed with time post cleavage of donor blastomeres and existence of a PB-like structure. Whereas donor nuclei transferred into S-phase oocytes did not undergo PCC and other major modifications, and their developmental potentials less depended on the nuclei types. This result confirms that the nuclear remodeling type differs with donor and recipient cell cycle stage, which affect the development of reconstituted bovine embryos.

(Key words: Nuclear transfer, Cell cycle stage, Nuclear remodeling, *In vitro* development, Bovine embryo)

Ⅰ. INTRODUCTION

Nuclear transfer of embryonic cell is affected by the interactions between a donor nucleus and recipient cytoplasm. Especially, cell cycle stages of nucleus and cytoplasm critically affect the development of reconstituted embryos (Cheong et al., 1993), which relate to the various chromosome constitutions following transfer of nuclei in different cell cycle stage(Cheong et al., 1994). A nucleus transferred into an

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enucleated oocyte undergoes various morphological modifications. A characteristic change is a premature chromosome condensation(PCC) shortly after transfer(Collas et al., 1992; Cheong et al., 1993, 1994). The morphology of the PCC depends on the cell cycle stage of the donor nucleus, which affects the development of reconstituted embryos(Cheong et al., 1993). In the mouse, PCC in G1-phase results in normal chromosome constitution and improved development, whereas, PCC in S-phase leads to abnormal spindle structure, chromosome constitution and poor development(Cheong et al., 1993). Thus, it has been recognized that synchronization of the donor nucleus in the G1-phase is an important factor for successful development of reconstituted embryos(Collas et al., 1992; Cheong et al., 1993). This aspect was somewhat confirmed in somatic cell nuclear transfer. Live offsprings were obtained from the nuclear transfer of cultured embryonic cells(Campbell et al., 1996b), fetal fibroblast(Wilmut et al., 1997; Vignon et al., 1999; Zakhartchenko et al., 1999) and adult somatic cells(Wilmut et al., 1997; Kato et al., 1998; Wakayama et al., 1998, 1999; Wells et al., 1998, 1999) in G0 or G1/G0-phases.

With different from the mouse and rabbit embryonic cells, it was suggested that blastomeres of bovine embryos were very difficult to synchronize in G1, because the G1-phase is very short or nonexistent(Barnes and Eyestone, 1990). In bovine embryos, nontreated blastomeres were transferred into enucleated and activated oocytes(Barnes et al., 1993; Aoyagi et al., 1994; Kono et al., 1994). This system may produce the S-phase-synchronized nuclear transfer, because the most of blastomeres(about 80%) from the 25- to 48-cell stage bovine *in vivo* embryos were in S-phase(Barnes et al., 1993).

Cell cycle-controlled nuclear transfer is popular in embryonic and somatic cell nuclear transfer researches. However, nuclear remodeling types following cell cycle-controlled nuclear transfer and relationship between nuclear remodeling and subsequent development of bovine reconstituted embryos was not evaluated. This study was conducted to investigate the cytogenetic properties, development, and their relationship in the bovine reconstituted embryos following cell cycle-controlled nuclear transfer.

Ⅱ. MATERIALS AND METHODS

1. Preparations of Oocytes and Embryos

Bovine follicular oocytes were obtained by aspiration of 2~7 mm follicles. About 10 cumulus-oocytes complexes(COCs) were placed into a 50㎕ droplet of maturation medium previously prepared in a 35mm culture dish overlaid with mineral oil(Sigma, St Louis, MO, USA), and cultured for 20-22 h at 39℃, 5% CO_2 in air. The maturation medium comprised TCM-199 supplemented with 10% fetal bovine serum(FBS; Gibco-BRL, NY, USA), 0.2mM Na-pyruvate, 50㎍/ml gentamycin(Sigma), 0.02U/ml FSH(Sigma) and 1㎍/ml estradiol-17(Sigma). After maturation, cumulus cells of some COCs were removed by vortexing in 200IU/ml hyaluronidase (Sigma), and oocytes extruded first polar body were selected as recipient cells.

For donor embryos, *in vitro* matured COCs were incubated in 50㎕ drops(10 COCs per a drop) of BO medium(Brackett and Oliphant, 1975) containing 5mM Caffeine, 10㎍/ml heparin, 3mg/ml BSA and 2.5×106sperm/ml for 18~20 h at 39℃, 5% CO_2 in air. After insemination, 10 presumptive zygotes with cumulus cells were transferred into 50㎕ droplets of *in vitro* culture medium prepared in a 35mm culture dish overlaid with mineral oil, and cultured for 4~5 days at 39℃, 5% CO_2 in air. The embryo culture medium was TCM-199 supplemented with 0.2

mM Na-pyruvate, 50μg/ml gentamycin and 3mg/ml BSA. Embryos developed to the 16- to 32-cell stage were selected for nuclear donor.

2. Cell Cycle Control of Donor Cells

Embryos developed to the 16-cell stage at Day 4 were cultured with embryo culture medium (above) containing 1.0μg/ml nocodazole(Aldrich Chem. Milw. WI. USA) for 16 h to arrest their cell cycle stages in mitotic(M)-phase. After treatment with nocodazole, embryos were washed in culture medium, and their zona pellucidae were digested for 3 min at 39℃ with 0.5% pronase(Sigma) in PBS containing 3mg/ml BSA. Embryos were washed in PBS containing 3mg/ml BSA, and gentle pipetting completed removal of the zona pellucida. The zona-free embryos were transferred into Mg^{2+} and Ca^{2+}-free PBS and pipetting isolated their blastomeres. Each of blastomeres were incubated with Mg^{2+} and Ca^{2+}-free PBS in each well of the 72-well plate(Nunc, Roskilde, Denmark) at 39℃, and were monitored every 30 min for 3~4 h to assess cleavage. Cleaving blastomeres were separated with two blastomeres by pipetting. Subsequently cleaved blastomeres were used immediately or cultured in embryo culture medium at 39℃ for 2 h before nuclear transfer. Blastomeres from nontreated 32-cell embryos were used as control.

3. Micromanipulation

Micromanipulation procedures were performed in PBS containing 5μg/ml cytochalasin B (Sigma) and 3mg/ml BSA. Oocytes were enucleated by aspirating the first polar body(PB) and the metaphase Ⅱ(MⅡ) plate with a small volume of surrounding cytoplasm. The oocytes were stained with 1μg/ml Hoechst 33342(Sigma) for 15~20 min in TCM-199 containing 3mg/ml BSA and examined under fluorescence to confirm enucleation. About 65% of oocytes were successfully enucleated with this system. The enucleated oocytes were held in embryo culture medium until injection of donor cells. Some enucleated oocytes were activated by incubation in 10μM Ca^{2+}-ionophore(A23187; Sigma) for 5 min, followed by incubation with cycloheximide (CHXM) for 6 h before the nuclear transfer. A single blastomere was injected into the perivitelline space of an enucleated unactivated (MII-phase nuclear transfer) or activated (S-phase nuclear transfer) oocyte through the same slit in the zona.

4. Electrofusion and Activation

Reconstituted embryos were placed in embryo culture medium for 10~20 min prior to fusion. Then they were transferred into a fusion chamber consisting of two wires, 0.5 mm apart, overlaid with 0.3M mannitol containing 0.1mM $MgSO_4$, 0.05mM $CaCl_2$ and 0.05mg/ml BSA. The reconstituted embryos were manually aligned with a pipette, after which two direct current(DC) pulses of 1.25kV/cm for 70μsec were applied to the chamber using a BTX Electrocell Manipulator 200(BTX, San Diego, CA, USA). Fusion was completed within 1.5 h post cleavage(1.5hpc) when received a blastomere immediately after cleavage, and 3 h post cleavage (3.0hpc) when received a blastomere cultured for 2 h after cleavage. After fusion treatment, the reconstituted embryos were placed in embryo culture medium, and checked for fusion. Embryos fused with MⅡ cytoplasm were activated 1 h after fusion by exposing to 10μM A23187 for 5 min at 39℃, followed by incubation in 10μg/ml cycloheximide in embryo culture medium for 6 h at 39℃, 5% CO_2 in air.

5. Culture of Nuclear Transfer Embryos

Fused embryos were checked for a polar

body(PB)-like extrusion after activation(MII-phase nuclear transfer) or culture for 6 h in embryo culture medium(S-phase nuclear transfer). Embryos with or without a PB-like structure were separately transferred into 50μl drops of embryo culture medium and cocultured with cumulus cell clumps prepared previously for 7~9 days at 39℃, 5% CO_2 in air.

6. Fixation of Reconstituted Embryos

Some reconstituted embryos were fixed by whole-mount method at 2~3 h after fusion, and their chromatin structures were examined. Embryos were mounted on a slide, fixed with a mixture of ethanol and acetic acid(3:1) for 48 h. Embryos were then stained with 0.5% aceto-orcein, washed with 25% aceto-glycerol, and examined under a phase contrast microscope(×400).

7. Statistical Analysis

Data were analyzed by chi-square test.

Ⅲ. RESULTS

1. Morphology of Reconstituted Embryos

A PB-like structure was observed in reconstituted embryos with MII-phase oocytes. The proportions of embryos with a PB-like structure were 55.8%(53/95) when reconstituted with a 1.5hpc donor, which lower($P<0.01$) than those when reconstituted with 3.0hpc(76.6%) and control donors(82.7%). Most of reconstituted embryos in S-phase nuclear transfer(97.6~98.8%) did not extrude a PB-like structure(Table 1).

2. Chromatin Modification of Reconstituted Embryos

Whole-mount preparations showed that the most of embryos(94.6~97.4%) reconstituted with MII recipients underwent PCC, and showed one or more chromatin clumps in their cytoplasm(see Cheong et al., 1996). The proportion of embryos had only one chromatin clump was 37.8%(17/45) when reconstituted with a 1.5hpc donor, but significantly lower when reconstituted with 3.0hpc(16.2%) and control donors(13.6%), respectively(Table 2). Contrarily, most of reconstituted embryos in S-phase nuclear transfer(95.0%) did not undergo PCC and had a decondensed nucleus.

3. Development of Reconstituted Embryos

Cleavage rates of reconstituted embryos were 75.0~80.0%, regardless of the cell cycle stage of nuclear and recipient. Developmental rate to the blastocyst stage was 18.0%(9/50) when a 1.5hpc donor was transferred into a MII oocyte, however, only 7.0~7.5% when 3.0hpc and control donors were transferred into MII oocytes.

Table 1. Morphology of reconstituted embryos following cell cycle-controlled nuclear transfer*

Donor	Recipient	No. (%) of oocytes fused/manipulated	No. (%) of embryos	
			without PB	with PB
1.5hpc	MII	95/102(93.1)	42(44.2)[a]	53(55.8)[a]
3.0hpc		77/ 80(96.3)	18(23.4)[b]	59(76.6)[b]
Control		81/ 85(95.3)	14(17.3)[b]	67(82.7)[b]
3.0hpc	S	84/ 90(93.3)	83(98.8)[c]	1(1.2)[c]
Control		85/ 88(96.6)	83(97.6)[c]	2(2.4)[c]

[a,b,c] Values with different superscripts in the same column differ($P<0.01$).

*PB: polar body-like structure, hpc: hour post cleavage, MII: metaphase-II, S: S-phase

On the other hand, 18.2 and 15.6% of reconstituted embryos were developed to the blastocyst stage when 3.0hpc and control donor were transferred into S-phase recipients, respectively (Table 3).

In the M II-phase nuclear transfer, most of blastocysts were obtained from the embryos did not extrude a PB-like structure(Table 4). Re-

Table 2. Chromatin morphologies of reconstituted embryos following electrofusion and activation*

Donor	Recipient	No. (%) of treated embryos	No. (%) of embryos with different types			
			PCC with different number of chromatin clumps			NPCC 1PN
			1	2	3	
1.5hpc	M II	45	17(37.8)[a]	21(46.7)[a]	5(11.1)	2(4.4)
3.0hpc		37	6(16.2)[b]	25(67.6)[ab]	4(10.8)	2(5.4)
Control		38	5(13.6)[b]	26(68.4)[b]	6(15.8)	1(2.6)
3.0hpc	S	40	−	1(2.5)[c]	1(2.5)	38(95.0)
Control		40	−	2(5.0)[c]	−	38(95.0)

[a,b,c] Values with different superscripts in the same column differ($P<0.05$).

*Nuclear transfer embryos were fixed at 2-3 h post fusion.

PCC: premature chromosome condensation, NPCC: non-PCC, PN: pronucleus, hpc: hour post cleavage, M II: metaphase-II, S: S-phase

Table 3. *In vitro* development of reconstituted embryos following cell cycle-controlled nuclear transfer*

Donor	Recipient	No. (%) of embryos cultured	No. (%) of embryos developed to		
			2-Cell	Morula	Blastocyst
1.5hpc	M II	50	38(76.0)	12(24.0)	9(18.0)
3.0hpc		40	30(75.0)	6(15.0)	3(7.5)
Control		43	33(76.7)	7(16.3)	3(7.0)
3.0hpc	S	44	34(77.3)	12(27.3)	8(18.2)
Control		45	36(80.0)	10(22.2)	7(15.6)

*hpc: hour post cleavage, M II: metaphase-II, S: S-phase

Table 4. *In vitro* development of reconstituted embryos derived from M II-phase nuclear transfer*

Donor	Morphology	No. (%) of embryos cultured	No. (%) of embryos developed to		
			2-Cell	Morula	Blastocyst
1.5hpc	NPB	23	20(87.0)	11(47.8)[a]	8(34.8)[a]
	PB	27	18(66.7)	1(3.7)[b]	1(3.7)[b]
3.0hpc	NPB	8	7(87.5)	5(62.5)[a]	3(37.5)[a]
	PB	32	23(71.9)	1(3.1)[b]	0(0.0)[b]
Control	NPB	9	7(77.8)	4(44.4)[a]	2(22.2)[a]
	PB	34	27(79.4)	3(8.8)[b]	1(2.9)[b]

[a,b] Values with different superscripts in the same column differ($P<0.05$).

*hpc: hour post cleavage, PB: polar body-like structure, NPB: without PB

gardless of donor cells, 22.2~37.5% of reconstituted embryos without a PB-like structure were developed to the blastocyst stage, whereas, 0.0~3.7% of embryos with a PB-like structure were developed to blastocysts($P<0.05$).

Ⅳ. DISCUSSION

Cell cycle-controlled nuclear transfer was conducted by synchronization of donor cell cycle stage in G1(Collas et al., 1992; Cheong et al., 1993) or M-phases(Kwon et al., 1996), or of recipient cell cycle stage in S-phase(Barnes et al., 1993). In mouse and rabbit, blastomeres immediately after cleavage(Cheong et al., 1993) or treated with the DNA synthesis inhibitor aphidicolin(Collas et al., 1992) were used for G1-synchronized donor. However, it was suggested that aphidicolin is not effective for the G1 synchronization of blastomere nuclei from bovine embryos(Cheong, unpublished data). In the present study, therefore, blastomeres within 1.5 h post cleavage and at 3 h post cleavage were used as nuclei donor respectively, to examine the possibility of G1-synchronized nuclear transfer and nuclear remodeling patterns.

In bovine nuclear transfer, few report has been made on the nuclear remodeling such as PCC, chromatin behavior, and extrusion of a polar body-like structure following cell cycle-controlled nuclear transfer. It has been suggested that the nuclear remodeling type of bovine reconstituted embryos is different from that of mouse reconstituted embryos(reviewed by Campbell et al., 1996a). In the present study, however, bovine reconstituted embryos showed the similar nuclear remodeling types as shown in the mouse reconstituted embryos. Furthermore, these nuclear remodeling types of bovine reconstituted embryos varied with different cell cycle stages of donor blastomere and recipient cytoplasm, which was also similar to mouse reconstituted embryos(Cheong et al., 1993).

Cell cycle stage synchronization of donor cells can be estimated by existence of a PB-like structure and chromatin structure if the nuclear remodeling types of bovine reconstituted embryos are affected by cell cycle stage of donor and recipient cells. In the present study, 44% of reconstituted embryos received a 1.5hpc donor extruded a PB-like structure, and 38% showed a single chromatin clump. These results suggest that almost 40% of 1.5hpc donors were synchronized in G1. Whereas, about 80% of reconstituted embryos received a 3.0hpc and control donor extruded a PB-like structure and have two or more chromatin structures, suggesting donor cell cycle stage already advanced to subsequent stages.

In mouse nuclear transfer, *in vitro* development as well as nuclear remodeling type of reconstituted embryos were affected by the cell cycle stage of donor cells(Cheong et al., 1993). A similar result was obtained in this study, in which developmental rates to the blastocyst stage varied with different donor cell types when nuclei were transferred into MⅡ oocyte cytoplasts. Difference in developmental rates might be due to the different nuclear remodeling types depending on the time post cleavage of donor blastomeres. Embryos that did not extrude a PB-like structure had high developmental potentials, whereas development of embryos with a PB-like structure was very limited. On the other hand, S-phase-synchronized nuclear transfer leaded to few major nuclear modifications, then development less depended on the nuclear remodeling types. In bovine embryonic cell nuclear transfer, blastomeres were often transferred into S-phase oocyte cytoplasts(Barnes et al., 1993; Aoyagi et al., 1994; Kono et al., 1994), and relatively high blastocyst develop-

ment and offsprings were obtained.

In conclusion, this result confirms that the nuclear remodeling type differs with donor and recipient cell cycle stage, which affects the development of reconstituted embryos.

V. 요 약

본 연구는 핵이식 기술에 의한 재구축 배의 작성과정에서 핵의 세포주기단계에 따른 재구축 배의 염색체의 핵상 변화를 검토하고, 염색체의 핵상 변화와 핵이식란의 체외발육과의 관계를 검토하였다. 공핵란은 nocodazole 처리에 의해 분할구가 분열기에 정지되도록 한 후, 분열개시 1.5시간 이내, 분열 후 3시간째 핵 및 무처리 분할구 핵을 활성화 전(metaphase-II기; MⅡ기), 후(S 기)의 탈핵 미수정란 세포질에 이식하였다.

MⅡ기 수핵란에 핵이식 된 재구축 배의 극체상 방출, 미성숙 염색체응축, 염색질구조 변화 등과 같은 핵형 변화 형태 및 재구축 배의 발육능은 핵의 세포주기 단계에 의해 영향을 받았으며, 극체상 방출 유무에 따라서도 재구축 배의 발육율에 차이가 있었다. S기 수핵란에 핵이식 된 경우에는 이식된 핵의 형태변화가 거의 없었다. 본 연구의 결과는 재구축 배의 핵형 변화가 핵 및 세포질의 세포주기단계에 따라 다양하며, 이것이 핵이식 재구축 배의 발육에 영향을 미칠 수 있음을 확증한다.

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