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Effect of Nuclear Transfer Methods on *In Vitro* Development of Reconstituted Bovine Embryos. I. Effect of Transfer Time of IVF Donor Nuclei and Electric Stimulation on Fusion and *In Vitro* Development

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소에서 핵이식 방법이 재구축배의 체외발달에 미치는 영향. I. 체외수정 공핵배 핵의 이식시기와 전기자극에 따른 융합과 체외발달

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요 약

본 연구는 핵이식의 시기가 융합과 체외발달에 미치는 영향을 조사하고 체외수정 공핵배의 핵과 체외성숙난자간에 전기융합의 최적 통전전압과 통전시간을 결정하기 위하여 시도하였다. 수핵난자는 체외성숙후 25~27시간에 제핵하였고 난자의 추가성숙을 위하여 융합전에 18~20시간 더 배양하였다. 공핵체외수정란은 16~32세포기까지 BOEC와 공배양하였다. 공핵배의 할구 이식시간은 조기핵이식구에서는 제핵후 1~3시간, 지연핵이식구는 제핵후 16~18시간이었다. 융합은 체외성숙후 43~45시간에 행하였다. 융합율은 할구 이식시기에 따라 차이가 없었으나 난할율과 8~16세포기 발달은 지연핵이식구에서 높았다. 융합, 난할 및 상실배-배반포 발달율은 1.0kV/cm보다 0.75kV/cm전압에서 높았으며 0.7kV/cm에서 상실배-배반포율은 17.6%였다. 통전시간에 따라 융합율은 차이가 없었으나 50 μsec와 70 μsec에서 난할율과 상실배-배반포 발달율이 다소 높았다.

본 결과에서 핵이식의 최적시기는 수핵난자의 체외성숙후 42~44시간이었으며 최적 전기융합 조건은 50~70 μsec의 1회 0.75kV/cm 임을 알 수 있었다.

I. INTRODUCTION

Embryo cloning by nuclear transfer (NT) is a commercial and experimental interst in cattle production and breeding. The *in vitro* techniques related to IVM, IVF and *in vitro* culture(IVC) are now provided a great potential for the pro-

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duction of large numbers of embryos in ET industry and NT procedures (Leibfried-Rutledge et al., 1989). To date, the IVM oocytes and IVF embryos have been effectively utilized as the sources of recipient oocytes or donor nuclei for the bovine NT, respectively (Barnes et al., 1993; Yang et al., 1993). It has been also reported that the proportoin of blastocyts obtained from the IVC of bovine NT embryos is almost similar to that obtained through sheep oviducts (Chesne et al., 1993). However, the overall efficiency of NT procedures is still low and the information on factors determining NT efficiency is also restricted (Prather et al., 1989). Among these factors, the problems in relation to the causes of low oocyte activation and enucleation effeciency of the recipient oocytes (Leibfried-Rutledge et al., 1992; Westhusin et al., 1992) and low subsequent development of fused oocytes remains a key of inadequate NT steps (Moraghan et al., 1992). Recently, these steps have been greatly improved by the use of aged oocytes as recipients (Ware et al., 1989; Sims et al., 1991; Barnes et al., 1993; Yang et al., 1993) and by the electrofusion techniques (Willadsen, 1986; Prather et al., 1987; Clement and Brem, 1989). On the other hand, several new approaches have demonstrated that the efficiency of oocyte enucleation and the oocyte activation in NT embryo development in vitro could be simultaneously improved through the delay of electrofusion to 12~20h after enucleation at 20-24h of IVM for oocyte aging (Sims et al., 1991; Yang et al., 1993; Heyman et al., 1994; Chung et al., 1995; Tsunoda et al., 1996). In these studies, the transfer of donor blastomeres into the enucleated oocytes was performed immediately after enucleation or after further aging of the recipient oocytes. In our previous study (Chung et al., 1995), we have observed that blastomere divesion arised somtimes during the additional culture prior to fusion as assumed by Yang et al. (1993) and resulted in low NT efficiency (unpublished data). In addition, the current methods for electrofusion in bovine NT procedures greatly vary according to the investigators, especially 0.7~1.3kV/cm in a DC field strength (Heyman et al., 1994; Chung et al., 1995; Tsunoda et al., 1996) and 20~70 μsec in DC pulse duration (Clement-Sengewald et al., 1992; Yang et al., 1993; Chung et al., 1995). This study was conducted to investigate the effect of transfer time of donor blastomeres on the rates of fusion and subsequent development of reconstituted oocytes and determine the optimal condition of electrofusion using IVM oocytes and IVF donor nuclei.

Ⅱ. MATERIALS AND METHODS

1. Recipient oocytes

Ovaries of the Korean native cattles were obtained at an local abattoir within 30min of slaughter and transferred to the laboratory with in 2h in a thermos of 30~35℃ saline solution. Cumulus-oocyte complexes (COC) were aspirated from 2~6mm follicles in diameter using a 10-ml disposable syringe with a 18-G needle and pooled in a 50-ml conical centrifuge tube. The COC were washed 3 times through 25mM Hepes-buffered TCM199 (Gibco, USA) containing 10%FCS and antibiotics. Only intact oocytes with unexpended dense cumulus layers and normal cytoplasm were chosen for IVM culture. Fifteen to 20 oocytes were cultured in a 500μl drop of TCM199 supplemented with 10%FCS, hormones (5μg/ml FSH, 10IU/ml HCG, 1μg/ml estradiol-17β) and antibiotics under the co-culture with granulosa cells (1×10^6cells/ml) in a humidified incubator with an atmosphere of 5% CO_2 in air. After 23~24h of IVM, all oocytes were stripped of cumulus cells by vortexing for

app. 2min in DPBS containing 0.25% hyaluronidase and by gentle pipetting. The stripped oocytes having a polar body (PB) were used for preparing recipient cytoplasm.

2. IVF donor embryos

Donor embryonic blastomeres were obtained from 16~32-cell stage embryos produced by IVM and IVF oocytes. The IVM was performed by the same method with that for recipient oocytes. For IVF, frozen-thawed sperm from two Korean native bulls were capacitated with caffeine and heparin according to the procedure of Niwa and Oghoda (1988) and 15-20 matured oocytes were transferred into a 200μl sperm microdrop with concetration of $1 \sim 2 \times 10^6$ sperm/ml under paraffin oil. After 18h of incubation with sperm, the IVF oocytes were co-cultured in the development medium containing bovine oviductal epithelial cells (BOEC) prepared according to Eystone and First (1989) for further develepemnt to 8~16-cell stage.

3. Micromanipulation of embryos and oocytes

Micromanipulation for NT was essentially done according to the non-disruptive procedure of McGrath and Solter (1983) using an inverted microscope (Diaphot, Nikon, Japan) equipped with a Norishige micromanipulator system. Donor embryos were treated with 0.5% pronase for zona removal and then placed in DPBS containing 4% BSA and 7.5μg/ml cytochalasin B. The blastomeses of donor embryos were separated by gentle pipetting or aspiration with a micropipette. Recipient oocytes were enucleated at 24~25h after the onset of IVM. The PB and a small amount of cytoplasm adjacent the PB was aspirated into a enucleation micropipette and then checked using Hoechst 33342 whether metaphase chromosome is present in the removed cytoplasm. The enucleated oocytes were incubated for an additional 18-20h prior to fusion to serve as a recipients. The isolated blastomeres from each donor were randomly allotted to the recipient oocytes and was placed in the perivitelline space of the enucleated oocyte with the same pipette through the slit made on the zona during the enucleation. The transfer time of blastomeres was assigned to two treatments ; at 1-3h post-enucleation (early group) and 16~18h post- enucleation (late group), respectively.

4. Electrofusion and *in vitro* culture

At 43~45h of IVM, the reconstituted oocytes were transferred to a fusion chamber in 0.3M mannitol solution containing 0.1mM $MgSO_4$, 0.05mM $CaCl_2$ and 0.05mg/ml BSA and aligned by a short AC (6V, 6sec). Fusion was induced by a single DC pulse of 0.75kV/cm and 1.0kV/cm for 70μsec, respectively or a DC pulse of 0.75kV/cm for 50, 70 and 90μsec, respectively according to the experimental design. Electrostimulation was provided by an electro cell manipulator (BTX 200, USA). After electrofusion, the NT embryos were transferred to TCM199 containing 10%FCS and monitored after 1~2h for the fusion. The fused embryos were co-cultured in a BOEC monolayer established 2~3 days previously by flushing slaughterhouse oviducts to observe the cleavage and development to morula and blastocyt stage.

Ⅲ. RESULTS AND DISCUSSION

1. Effect of transfer time of donor nuclei

As shown in Table 1, the effect of NT time was compared between two groups (early and late NT). The late NT group was designed to remove problem that the inserted blastomeres may be divided during awaiting period before fusion in the early group. The cleavage rate and the proportion of 8- to 16-cell embryos in the

Table 1. Effect of transfer time of donor nuclei on fusion and cleavage of reconstituted oocytes

Time(h)	No.(%) of oocytes fused/pulsed	No.(%) of embryos cleaved to		
		2～4-cell	8～16-cell	Total
Early(24～26)	26/32(81.0)	9	4(15.4)	13(50.0)
Late (42～44)	101/120(84.2)	49	29(28.7)	78(77.2)

Time after onset of IVM.

Electrofusion : a single 0.75kV/cm DC pulse of 70μsec duration at 43-45h post-IVM.

late group greatly increased more than those in the early group. However, the fusion rate did not differ between two groups. In this experiment, such a evidence of the difference in cleavage rates was not detected, but we could observed that the frequency of blastomere division tended to be greatly reduced in the late group (data not shown). Until recently, most of NT experiments has involved the use of aged oocyte as nuclear recipients. To solve the problem that the enucleation elficieny of recipient bovine oocyte decreased with oocyte age, new approach has been demonstrated by Yang et al. (1991, 1993) who performed (a) enucleation and NT immediately after IVM and conducted electrofusiom 6 or 18h later or (b) enucleated after IVM and cutured for an additional 18～20h before NT and fusion. According to the (a) method (Westhusin et al., 1991; Park et al., 1994; Heyman et al., 1994; Chung et al., 1995) and (b) method (Sims et al., 1991; Kim et al., 1993; Yang et al., 1993; Tsunoda et al., 1996), high M+B devolopment rates have been obtained. The results from the late group in this experiment agreed with those of these previous reports. Our results indicate that as suggested by Yang et al. (1993), the methods of late NT may be useful to not only capitalize both the high enucleation and oocyte activation but also easy the NT procedures.

2. Effect of DC voltage and pulse duration

The 2 experiments (Table 2 and 3) were conducted to further improve the results of Table

Table 2. Effect of electric voltage on fusion and *in vitro* development of reconstuted oocytes

DC voltage (kV/cm)	No.(%) of oocytes fused/pulsed	No.(%) of embryos developed to			
		2～4-cell	8～16-cell	M+B	Total
0.75	34/43(79.0)	10	9	6(17.6)	25(74.0)
1.0	15/17(56.0)	4	3	1(0.1)	8(53.0)

Electrofusion : a single DC voltage for 70μsec.

Time of NT and fusion : 42-44h post-IVM and 43-45h post-IVM, respectively.

Table 3. Effect of pulse duration on fusion and *in vitro* developement of reconstituted oocytes

Duration (μ sec)	No.(%) of oocytes fused/pulsed	No.(%) of embryos developed to			
		2～4～cell	8～16～cell	M+B	Total
50	31/35(85.7)	13	5	7(22.6)	25(80.6)
70	29/33(87.7)	10	5	6(20.7)	21(72.4)
90	35/39(89.7)	12	6	5(14.3)	23(65.7)

Transfer time of nuclei and electrofusion condition : see footnote in Table 1, except pulse duration.

1. The NT and fusion was done 42~44h post-IVM and 43~45h post-IVM, respectively. In Table 2, higher fusion, cleavage and M+B development was obtained from 0.75kV/cm DC voltage. The pulse duration greatly affected the cleavage and development of NT embryos (Table 3). M+B development from 90μsec was more lower than from 50 and 70μsec as shown in Table 3. Therefore, the results indicate that a single 0.75kV/cm DC voltage for 50~70μsec leads to more efficiency development of bovine NT embryos. The result that 0.75kV/cm DC produced higher M+B development than 1.0kV/cm DC voltage agreed with the reports of Kim et al.(1995) and Chung et al.(1995) who produced M+B embryos from NT 1-3h post-IVM and fusion 17-18h post-enucleation, respectively. Hewever, it was not similar to the report of Park et al.(1994) who produced 11.4% and 12.6% M+B stage from a single 0.75kV/cm and 1.0kV/cm for 60μsec respectively. In addition, we observed that DC voltage for high M+B development slightly differed between IVF (0.75kV/cm) and *in vivo* produced embryos (1.0kV/cm) as nuclear donor cells, as compared to the result of Chung et al.(1997). In our previous study (Kim et al., 1995), M+B development did not differ between IVF and *in vivo* donor nuclei, but the M+B development greatly differed between two nuclear sources when the NT and fusion was done 18-20h post-IVM (Chung et al., 1997), similar to the other reports (Clement-Sengewld et al., 1992; Yang et al., 1993). The result that pulse duration of 90μsec showed low cleavage and M+B development was the same tendency with the report of Kono et al.(1989), in which activation rate of oocytes tended to be decreased by prolongation of pulse duration.

Ⅳ. SUMMARY

The present study was undertaken to determine the effect of NT time on the rate of fusion and suhseguent development *in vitro* and determine the optimal strength and duration of DC pulse for electrofusion of IVF donor embryo nuclei and IVM recipient oocytes. The recipient oocytes were enucleated 25~25h after IVM and further cultured for 18~20h prior to fusion for oocyte aging. IVF embryos as donor nuclei were co-cultured with BOEC for 16- to 32-cell stage development. The transfer time of donor blastomeres was 1~3h post-enucleation in early NT group and 1~18h post-enucleation in late NT group, respectively and fusion was performed 43~45h post-IVM. The fusion rate did not differ between the early NT and late NT group, but the rate of cleavage and 8- to 16-cell stage embryos in the late NT group was more higher than that in the early NT group. The fusion, cleavage and M+B development was high from 0.75kV/cm DC than from 1.0kV/cm DC voltage, resulting in 17.6% M+B from 0.75kV/cm DC voltage. No difference in fusion rate was among pulse durations, but 50 and 70 μsec pulse duration showed slight high cleavage and M+B development. The results indicate that the best NT time of IVF donor blastomeres into the enucleated oocytes was 42~44 post-IVM and the most suitable condition for electrofusion was a single 0.75kV/cm DC voltage for 50~70μsec.

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