STUDIES ON PRODUCTION AND EFFICIENT UTILIZATION OF LIVESTOCK EMBRYOS BY *IN VITRO* FERTILIZATION AND MICROMANIPULATION
Ⅳ. NUCLEAR TRANSPLANTATION AND ELECTROFUSION FOR CLONING IN BOVINE FOLLICULAR OOCYTES

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Summary

This study was conducted to develop a method for production of nuclear transplant bovine embryos using *in vitro*-matured (IVM) oocytes and to examine the effect of different conditions of electrofusion on fusion rate and developmental capacity of donor nucleus transplanted to enucleated oocytes. Eight- to sixteen-cell embryos derived from oocytes matured and fertilized *in vitro* used as donor blastomeres and IVM oocytes were used as recipient oocytes. Oocytes were enucleated immediately after 23-24 h IVM and then reconstituted with a donor blastomere in two different micromainpulation media. Fusion rate and subsequent development of the reconstituted oocytes was compared under the different electric stimuli and recipient oocyte ages. Success rate of enucleation was significantly higher in TCM-199 medium containing FCS than in DPBS. The high fusion rate (75-94%) and development (6.4-14.8%) to morulae and blastocyst (M + B) were obtained from 0.6-0.75 kV/cm DC voltage, although total cleavage was not different among the electric pulses. Most optimal condition of electric stimulation for fusion and development was 1 DC voltage of 0.75 kV/cm, in which 80.5% of oocytes were fused, 80.0% and 31.7% of which was cleaved and developed to M + B, respectively. No M + B was obtained from 1.2 kV/cm DC voltage regardless of pulse frequency. Recipint oocyte age at electrofusion greatly affected the cleavage and subsequent development to M + B, showing high rate at 40-41 h oocyte maturation. These results suggest that a suitable condition of electrofusion for donor nuclei derived from IVF may be 1-2 DC pulses of 0.7 kV/cm for 70 μsec and that processing of a transplanted nucleus in IVM oocytes may be affected by maturation age of recipient oocytes.

(Key Words : Blastomere, Bovine, Electrofusion, Micromanipulation, Nuclear Transplantation)

Introduction

Bovine embryo cloning by nuclear transplantation has greatly been developed with much interests in its commercial application for the production of a large number of genetically identical offsprings from a superior genotype individual. However, the overall efficiency of nuclear transfer procedures is still quite low and the proportion of nuclear transplant embryos developing to full term is approximiately 1 to 4% (Prather et al., 1987; Bondioli et al., 1990). In addition, information on factors affecting the efficiencies and results of nuclear transfer is still limited and many problems also remain to be solved for the commercialization of embryo cloning technology in cattle. It has been recently shown that the *in vitro* matured (IVM) slaughterhouse oocytes may serve in large amounts as an alternative and a low cost source of recipient cytoplast (Sims et al., 1991; Ushijima et al., 1991) and normal calves have been obtained by the nuclear transfer using these IVM oocytes (Bondioli, 1992; Clement-Sengewald et al., 1992; Barnes et al., 1993). The IVM oocytes for nuclear transfer have, however, obtained a lower freguency of development than with the *in vivo* matured oocytes (Prather et al., 1987). Various important factors related to this problem have been studied by

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several investigators. Key factor of inefficient step in the nuclear transfer procedures is greatly associated with the age, enucleation and activation of recipient oocytes (Sirard et al., 1989; Ware et al., 1989; Sims et al., 1991; Leibfried-Butledge et al., 1992; Moraghan et al., 1992; Yang et al., 1992; Westhusin et al., 1992; Ushijima and Eto, 1993) and electrofusion condition for the manipulated oocytes (Prather et al., 1987).

This study was designed to investigate the effect of micromanipulation media on oocyte reconstitution and the effect of electrical stimulations and recipient oocyte ages on fusion rate and subsequent development of the fused oocytes.

Materials and Methods

Recipient oocytes and donor embryos

Ovaries were transported within 2 h from a slaughterhouse to the laboratory in 0.9% saline solution of 30℃ to 35℃. Cumulus-oocyte complexes from vesicular follicles of 2 to 6 mm in diameter were sapirated using a 10 ml syringe with a 20 gauge needle. The oocytes were selected in accordance with their cumulus appearance and washed 3 times in the Hepes-buffered TCM-199 (Gibco, USA) with 10% fetal calf serum (FCS) and antibiotics. Fifteen and twenty oocytes in a 50 μl drop of 4-well dish (Nunc, Germany) were matured *in vitro* in the TCM-199 containing 10% FCS supplemented with hormones (50 μg/ml follicle-stimulating hormone, 10 IU/ml human chorionic gonadotrophin and 1 μg/ml estrodiol-17β) under the co-culture with granulosa cells of 1×10^6 cells/ml in a 5% CO_2 incubator of 95% air and 39℃. After maturation culture of 23 to 24 h, the matured oocytes having the first polar body for producing recipient oocytes were treated 2-3 min with 0.25% hyaluronidase solution (300 IU/ml, Sigma) and the cumulus cells were removed by gentle pipetting. For producing donor blastomere nuclei, the matured oocytes were fertilized *in vitro* (IVF) with sperm of 1.5×10^6/ml capacitated according to the method of Niwa and Oghoda (1988). The 15 to 20 oocytes were transferred into 200 μl droplet of caffeine-treated sperm in a plastic petri dish (35 × 10 mm, Falcon plastic, USA) and cultured 18 to 20 h for IVF in the TCM-199. The IVF oocytes were washed 3 times and co-cultured with bovine oviduct epithelial cells (Eyestone and First, 1989) for further development to 8- to 16-cell stage in the TCM-199 supplemented with 10% FCS and hormones under the incubation condition as mentioned in the IVM method.

Micromanipulation of donors and recipients

Micromanipulation prodcedures were essentially performed as described by McGarth and Solter (1983) using a Narishige micromanipulator system mounted on an inverted microscope (Diaphot, Nikon, Japan). For isolating single blastomere from IVF 8- to 16-cell embryos, the donor embroys were treated with 0.5% pronase and incubated in Ca^{2+}-Mg^{2+} free phosphate-buffered saline solution (PBS) or TCM-199 for 5 min and blastomeres were separated by aspiration and gentle disaggregation with an injecting pipette. For the enucleation of recipient oocytes, the first polar body and a small amount (about 1/3) of cytoplasm proximal to the first polar body was removed by aspiration with a micropipette and the extracted cytoplasm was confirmed to be successfully removed using DNA specific stain (Hoechst 33342). Immediately following the enucleation, an isolated intact blastomere was introduced into the perivitelline space of the enucleated oocytes through the opening made in zona pellucida during the enucleation. The reconsituted oocytes were incubated in the culture medium for 1h to allow the close contact between the introduced karyoplast and enucleated cytoplasm. All micromanipulation procedures were carried out in the Dulbecco's PBS (DPBS) plus 4% bovine serum albumin (BSA) or the TCM-199 plus 10% FCS supplemented with 7.5 μg/ml cytochalasin B.

Electrofusion and culture of reconstituted oocytes

The reconstitued oocytes were transferred to a fusion chamber consisting of two 0.5 mm round wire stainless steel electrodes in the electrofusion solution of 0.3 M mannitol containing 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 0.05 mg/ml BSA and aligned by the exposure to alternating current (AC) of 6V for 6sec. Membrane fusion was electrically initiated by applying 1 to 3 direct current (DC) pulses of 0.6 to 1.2 kV/cm for 70 μsec, each 1 sec apart, with an electro cell manipulator (BTX 200, USA). To examine the effect of recipient oocyte ages on the efficiency of nuclear transfer, fusion was done 26-27 h, 33-34 h and 41-42 h after the onset of IVM incubation, respectively. The fused oocytes were co-cultured with bovine oviduct epithelial cells for 7 days for the further development to morula and blastocysts in the TCM-199 containing 10% FCS supplemented with hormones under the atmosphere of 5% CO_2 and 95% air at 39℃.

Results and Discussion

Reconstitution of oocytes by nuclear transter

The effect of micromanipulation media on the efficiency of oocyte enucleation and nucleus insertion into

the enucleated oocytes is shown in table 1. The success rate of enucleation was significantly affected by the medium. The TCM-199 containing FCS showed a high successive reconstitution rate. In contrast, the proportion of enucleated oocytes that blastomere was successfully inserted was not different between two medial. The beneficial result in TCM-199 probably seemed to be due to the effect of FCS. In the rabbit, it has been observed that FCS in manipulation medium and incubation of reconstituted oocytes before electrical stimulation affected the activation and development of nuclear transplant oocytes and that mature oocytes were more sensitive to manipulation medium than the fertilized oocytes (Collas and Robl, 1990). In addition, Saito and Niemann (1993) showed that TCM-199 provides more optimized environmental and survival conditions for pig embryo micromanipulative procedures as compared with PBS or Ham's F10. These results indicate that it is necessary to ensure in detail whether the effect of medium prior to electrofusion is one of considerable factors for the improvement of nuclear transfer procedures.

TABLE 1. EFFECT OF MICROMAIPULATION MEDIUM ON NUCLEAR RECONSITITUTION OF OOCYTES

Manipulation medium	No. (%) of oocytes					
	Enucleation			Blastomere insertion		
	No.	Succeed	Ruptured	No.	Succeed	Ruptured
DPBS+BSA	96	60(62.5)	36(29+5)*	102	80(78.4)	22(14+8)
TCM-199+FCS	128	109(85.1)	19(17+2)	116	111(95.7)	5(3+2)

*Front and rear figures in parentheses : No. of oocytes ruptured during(front)and after(rear) manipulation, respectively.

Fusion rate and *in vitro* development

As shown in table 2, high fusion rate and development to morula and blastocysts was obtained when the reconstituted oocytes were exposed to DC voltage of 0.6 and 0.75 kV/cm. Total cleavage rate ranged from 44% to 63% without the difference among the voltages of pulses. The effect of pulse frequency is shown in table 3. One or two pulses with 0.75 kV/cm showed high fusion and development and the cleavage rate was also high under one DC pulse of 0.75 kV/cm. However, no morula and blastocysts were obtained in 1.2 kV/cm DC voltage regardless of the frequency of DC pulse. In table 4, the recipient oocyte age at electro fusion with donor nucleus had no influence on fusion rate, but higher cleavage rate and development to morula and blastocysts was observed at 40-41 h oocyte age than at the other oocyte ages. Based on the results of this study, one or two pulse durations of 70 μsec with 0.75 kV/cm DC pulse strength seemed to be the most suitable condition for the fusion and subsequent development of donor embryo nuclei derived from IVF. Under this fusion condition, the fusion rate, 2-8 cell stage embryos and morula plus blastocysts could be obtained 79%, 30-40% and 25%, respectively. The same tendency with our results was observed by Celement-Sengewald et al. (1992) and Tatham et al. (1995), although the electrical stimuli were exactly not the same conditions. However, our result that DC pulse strength of 1.2 kV/cm was too strong for fusion and subsequent development is greatly different from those of other studies in cattle and other animals. The effective range of pulse strength in these studies was 0.8-1.3 kV/cm (Clement-Sengewald et al., 1992) and 1.0-1.5 kV/cm (Kim et al., 1993) for bovine oocytes and 2.0-2.4 kV/cm (Yang et al., 1992; Lee et al., 1993) and 3.6 kV/cm (Collas and Robl, 1990) for rabbits 1.0-1.5 kV/cm for mouse (Ondera and Tsunoda, 1989; Cheong et al., 1992) and 180 V for pigs and 2.0 kV/cm for sheep (Pugh et al., 1992), respectively. In contrast, the results that there was no difference in pulse frequency is not similar to the result of Collas and Robl (1990), who reported that the fusion and morula production in rabbit was increased by multiple pulses. The result that the oocyte age greatly affected the developmental capactiy of the fused oocytes to M+B stage is similar with those of several studies (Sims et al., 1991; Kato et al., 1993; Kim et al., 1993; Yang et al., 1993). Similar age effect has been observed in rabbit oocytes (Stice and Robl, 1988; Lee et al., 1993). In addition, it has been also shown that the ability of IVM oocytes to be activated increased with the recipient oocyte age (Sirard et al., 1989; Ware et al., 1989; Leibfried-Rutledge et al., 1992; Barnes et al., 1993). Especially, Moraghan et al. (1992) indicates that the activation of IVM bovine oocytes remians a key of insufficient step in the current nuclear transfer procedures. The above studies show that the conditions and results of electrofusion varied considerably according to the species, sources of recipient cytoplasm and donor kasyoplast and

investigators. Therefore, it is still defficult to determine the constant, optimal fusion conditions to provide a sufficiently high developmental capacity of embryonic cells in the reconstituted embryos.

TABLE 2. EFFECT OF PULSE STRENGTH ON FUSION RATE AND DEVELOPMENT OF FUSED OOCYTES

DC voltage (kV/cm)	No. (%) of oocytes fused / manipulated	No. (%) of embryos developed to				
		≤ 4 cells	5-8 cells	9-32 cells	M+B	Total
0.6	27 / 32(84.4)	6	4	3	2 (7.4)	15(55.6)
0.75	27 / 36(75.0)	6	2	4	4(14.8)	16(59.3)
1.0	16 / 30(53.3)	4	2	1	1 (0.6)	8(50.0)
1.2	18 / 37(48.6)	6	2	0	0 (0.0)	8(44.4)

Oocytes were electrofused with 2 pulses of 70 μsec under different DC after AC of 6V for 6 sec at 17-18 h post-manipulation.

TABLE 3. EFFECT OF STRENGTH AND NUMBER OF PULSES ON FUSION FATE AND DEVELOPMENT OF FUSED OOCYTES

DC voltage (kV / cm)	No. of pulses	No. (%) of oocytes fused / manipulated	No. (%) of embryos developed to				
			≤ 4 cells	5-8 cells	9-32 cells	M+B	Total
0.75	1	41 / 50(82.0)	13	6	1	13(31.7)	33(80.5)
	2	27 / 36(75.0)	6	3	4	4(14.8)	17(63.0)
	3	19 / 35(54.2)	7	1	2	2(10.5)	12(63.2)
1.2	1	17 / 34(50.0)	6	1	0	0 (0.0)	7(41.2)
	2	18 / 37(48.6)	6	2	0	0 (0.0)	8(44.4)
	3	2 / 35 (5.7)	0	0	0	0 (0.0)	0 (0.0)

TABLE 4. EFFECT OF FUSION TIME ON FUSION RATE AND DEVELOPMENT OF FUSED OOCYTES

Fusion time* (h)	No. (%) of oocytes fused / manipulated	No. (%) of embryos developed to				
		≤ 4 cells	5-8 cells	9-32 cells	M+B	Total
26-27	16 / 35(74.0)	4	2	3	0 (0.0)	9(34.6)
33-34	35 / 47(74.5)	10	10	3	4(11.4)	27(77.1)
40-41	40 / 49(81.6)	16	6	1	9(22.0)	32(80.0)

*Time after onset of 23- to 24-h maturation (fusion at 2-3 h, 9-10 h and 17-18 h post-manipulation for further cytoplasmic maturation).

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