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Effect of Nuclear Transfer Methods on *In Vitro* Development of Reconstituted Bovine Embryos. Ⅱ. Effect of Electric Voltage and *In Vivo* Produced Donor Embryo Quality on Fusion and *In Vitro* Development

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소에서 핵이식 방법이 재구축배의 체외발달에 미치는 영향 Ⅱ. 통전전압과 체내생산 공핵배의 질에 따른 융합과 체외발달

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요 약

본 연구는 체내생산 공핵배 핵이식의 최적 통전전압을 결정하기 위하여 그리고 공백배의 질이 핵이식 난자의 융합과 체외발달에 미치는 영향을 조사하기 위하여 수행되었다. 수핵난자는 체외성숙후 25~27시간에 제핵하였고 난자의 추가성숙을 위해서 융합전에 18~20시간 더 배양하였다. 상실배시기의 공핵배는 다배란 처녀우에서 채란한 후 질에 따라 양질과 저질로 구분하여 공시하였다. 공핵배의 핵이식은 체외성숙후 42~44시간에 행하였고, 융합은 0.75kV/cm 또는 1.0 kV/cm DC 전압으로 체외성숙 후 43~45시간에 실시하였다. 융합율은 두 전압간에 차이가 없었으나 난할율과 M+B발달율은 1.0 kV/cm DC에서 더 높았으며 0.75kV/cm 와 1.0kV/cm DC의 M+B 발달율은 19.0%와 29.4%였다. 공핵배의 질은 융합율과 난할율에 크게 영향이 없었으나 저질공핵배의 핵이식으로 부터는 상실배의 발달이 없었다.

본 결과에서 체내생산 공핵배 핵이식의 최적전압은 1.0kV/cm DC이었으며 공핵배의 질은 핵이식배 발달에 영향하는 중요 요인 중에 하나이었다.

Ⅰ. INTRODUCTION

Embryo transfer(ET) has become the most powerful technique in cattle breeding. In the near future, If large scale embryo cloning from genetically valuable elite cows and its economic feasibility would reached acceptable levels, more substantial and speedy improvement should be expected in cattle industry. It has been known that the embryos recovered from superovulated cows is a great variable in embryonic cell stage and embryo quality within and among donors (Lindner et al., 1983; Bondioli et

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al., 1990). When ET was done with the retarded 2 days or more, the success rate of ET greatly decreased as compared to the embryos of normal development stage (Elsden et al., 1978) and the embryo quality seemed to be a more accerate predictor of ET pregnancy (Lindner et al., 1983). In addition, Chesne et al. (1993) reported that the considerable variability in embryo development and quality among embryos or donors is still an obstacle to improve the bovine NT procedures and several studies showed that the rate of blastomere recovery from IVF or frozed donor embryos is lower than from the *in vitro* fresh embryos (Westhusin et al., 1991; Yang et al., 1993). The above-mentioned findings suggest that the quality of *in vitro* or *in vivo* donor embryos may also be one of very important factors affecting NT efficiency. Morphological embryo evaluation in ET industry has been widely used to describe embryo quality (Elsden et al., 1978), but is still limited value in determining survival of individual embryos (Shea, 1981). In our previous studies (Chung et al., 1995, 1997; Kim et al., 1995), we have observed that the optimal DC pulse strength for NT slightly differed between the *in vitro* and *in vivo* donor nuclei. In contrast, the results in the developmental capacity to morula and blastocyts among the sources of donor nuclei is not consistent among investigators (Clement-Sengewald et al., 1992; Yang et al., 1993; Heyman et al., 1994). This study was designed to determine the optimal DC voltage for NT from in vivo produced donor nuclei and investigate the effect of donor embryo quality on the fusion and *in vitro* development of NT embryos.

Ⅱ. MATERIALS AND METHODS

1. Recipient oocytes

Ovaries were obtained from the slaughtered Korean native cows at an local abattoir and brought to the laboratory in saline solution at 30~35℃ within 2h. The detailed procedures for oocyte collection and *in vitro* maturation (IVM) were given in our previous paper (Chung et al., 1997). The oocytes of 2~6mm follicles in diameter were collected by aspiration with a 18-G needle and cumulus-oocyte complexes compactly covered by cumulus cells of several layers were chosen for IVM. The selected oocytes were co-cultured with granulosa cells in TCM199 containing 10% FCS, hormones and antibiotics for 23~24h in a humidifed 5%CO_2 incubator at 39℃. After IVM, all oocytes were denuded by vortexing in DPBS containing 0.25%hyaluronidase. The stripped oocytes with a polar body (PB) were only used as recipient oocytes.

2. *In vitro* donor embryos

Fresh donor embryos at morula stage with 16~50 blastomeres were nonsurgically collected from the superovulated Korean native heifers on day 5.0 or 5.5 postestrus and Dulbecco's PBS supplemented with calf serum was utilized as collection medium. For superovulation, an injection of 1.6ml of SUPER-OV solution (12.5 units, AUSA Int'l Inc, Canada) was intramuscularly administrated to the donor twice a day, morning and evening, for 3 days (6 injections). At the time of the 6th injection, $PGF_{2\alpha}$ was given to induce estrus. The heifers were artificially inseminated twice with the frozen-thowed Korean native bull semen 4 to 22h after the onset of estrus. The collected embryos were evaluated whether the embryos has attained an approximate stage of development and whether blastomeres is symmetrically in size or a few extruded and degenerated blastomeres is present. The embryos were morphologically classified into two groups according to the criteria of Lindner et al. (1983) : high (excellent and high, ideal

embryos) and low (fair and poor, not severe problems, but a few extruded and degenerated blastomeres, or retarded embryos).

3. Micromanipulation of embryos and oocytes

Each donor embryo was firmly held by a holding pipette until all blastomeres were repeatedly removed through a pulcture on the zona of donor embryos. The blastomeres were aspirated one at a time with a beveled micropipette having approximately the same diameter as the blastomere. The isolated blastomere was then inserted to the perivitelline space of the enucleated oocytes through the slit made on the zona at oocyte enucleation. The oocyte enucleation and aging of the enucleated oocytes before fusion were performed according to our previous method (Chung et al., 1997). The IVM oocytes were enucleated by removing the PB and adjacent cytoplasm(1/3), washed and cultured for an additional 18~20h in TCM199 plus 10%FCS supplemented with hormones to allow further cytoplasmic maturation. All micromanipulation procedure was done in micromanipulation drops of TCM199 plus 10%FCS and 7.5μg/ml cytochalasin B and using a Narishige micromanipulator mounted on an inverted microscope (Diaphot, Nikon, Japan).

4. Electrofusion and *in vitro* culture

The reconstituted oocytes were plased into fusion medium containing 0.1mM $MgSO_4$, 0.05mM $CaCl_2$ and 0.05mg/ml BSA for equilibration and then transferred into another drop of the same fusion medium on a fusion chamber consisting of two round wire electrodes. The oocyte and blastomere was aligned by an AC (6V, 6 sec) and for membrane fusion, a DC pulse of 0.75 or 1.0kV/cm for 70μsec was used. After electrofusion, the embryos were assessed for fusion and then co-cultured on BOEC monolayer in TCM199 plus 10%FCS and hormones for 6~7days to evaluate the cleavage and morula-bastocyst development at 39℃ in a 5%CO_2 incubator.

Ⅲ. RESULTS AND DISCUSSION

1. Effect of DC voltage

To determine the optimal electrostimulation 3 different voltages of pulses were exposed to the reconstituted oocytes as shown in Table 1. In this experiment, the cleavage response was based on up to 8-cell stage, because the removal of culture block of 8-to 16-cell stage. The 1.0kV/cm DC voltage produced the highest fusion rate followed by 0.7kV/cm and 1.2kV/cm DC. Based on the result of Table 1, the rates of cleavage and development to morula and blastocyst (M+B) between 0.75kV/cm and 1.0kV

Table 1. Effect of electric voltage on fusion and early cleavage of reconstituted oocytes

DC voltage (kV/cm)	No. (%) of oocytes fused/pulsed	No. (%) of embryos cleaved to			
		2-cell	4-cell	8-cell	Total
0.75	15/19(78.9)	1	3	4	8(53.3)
1.0	23/31(74.2)	4	7	5	16(69.6)
1.2	18/30(60.0)	1	3	5	9(50.0)

Pulse duration and time : a single DC voltage of 70μsec duration.

Enucleation time : 25~27h post-IVM.

Fusion time : 42~44h post-IVM.

Donor embryos : 16- to 50-cell stage.

Table 2. Effect of electric voltage on fusion and *in vitro* developement of reconstituted oocytes derived from high donor embryo quality

DC voltage (kV/cm)	No. (%) of oocytes fused/pulsed	No. (%) of embryos developed to 2~4-cell	8~16-cel	M+B	Total
0.75	21/30(70.0)	4	8	4(19.0)	16(76.2)
1.0	51/78(65.4)	9	18	15(29.4)	42(82.4)

Electrofusion conditions : see footnote in Table 1.

Embryo quality : 32- to 50-cell stage showing symmetrical and unform blastomeres.

/cm DC were again assessed with NT embryos derived from good donor embryo quality (Table 2). No significant difference in the rate of fusion and cleavage was between two DC voltages, but M+B development from 1.0kV/cm DC was more higher than 0.75kV/cm DC voltage (19.0% : 29.4%). In addition, from the cleaved embryos, the proportion of M+B in two groups was 25.0% and 35.7%, respectively. These results (Table 1 and 2) indicate that the NT of in vivo donor blastomeres into IVM recipient oocytes appears to be most suitable using a 1.0kV/cm DC pulse voltage. This field strength for in vivo donor nuclei was slightly higher than that for *in vitro* donor nuclei (Kim et al., 1995; Chung et al., 1995, 1997), who suggested that a single 0.75kV/cm DC for *in vitro* donor nuclei was optimal. In contrast, as compared to other previous studies which were investigated with similar conditions to our experiments, the 1.0kV/cm DC voltage is exactly similar to Tanaka et al. (1996), but was lower than those of other reports (Clement-Sengewald et al., 1992; Heyman et al., 1994), in which the voltage and pulse duration used ranged from 0.8~1.3kV/cm, 20 to 50μsec and twice. In our study, the M+B development from 0.75 to 1.0kV/cm nearly agreed with those of the above studies, although the proportion of blastocysts was lower than that of Heyman et al. (1994) and Tanaka et al. (1996).

2. Effect of donor embryo quality

The rates of fusion, cleavage and M+B development in the 3 groups of low embryo quality are presented in Table 3. The donor embryos showing more high and low fusion rate was group C and A, respectively and overall cleavage rate did not differ among the 3 groups. However, none of morula development was produced from all the groups and the high percent embryos (55~65%) in group A and C arrested at 2- to 4-cell stage which may represent nonviable embryos or culture block of 8- to 16-cell. Table 3

Table 3. *In vitro* development of reconstituted oocytes derived from low donor embryo quality

Donor embryos (cell stage)	No. (%) of oocytes fused/pulsed	No. (%) of embryos developed to 2~4-cell	8~16-cell	M+B	Total
A (8~15)	23/44(50.3)	15(65.2)	1	–	16(69.6)
B (16~30)	8/12(66.7)	3(37.5)	3	–	6(75.0)
C (31≤)	29/36(80.6)	16(55.2)	5	–	21(72.4)

Electrofusion : a single DC voltage of 1.0kV/cm for 70μsec.

Embryo quality : A (embryos retarded 1~2days), B and C (irregular embryos with a few extruded and degenerated blastomeres).

shows that donor embryo quality was considerably related to development capacity of NT donor blastomeres. Our these results agreed with that of Barnes et al. (1993) who suggested that low development of NT embryos was not only due to low donor embryo quality but also a source of variation in development capacity. Yang et al. (1993) demostrated that NT embryos showed 8~16cell block and more than half of blocked embryos arrested at 2- to 4-cell stage. In addition, Chesne et al. (1993) suggested that very wide variability in blastocyst development among donor embryos still remains an very great obstacle for the development of NT techniques. Also, Westhusin et al. (1991) indicated that the most difficult problem in NT procedures was to determine which donor embryos were viable or could be used as blastomere donors. Morphological evaluation is used in determing survival of individual embryo in ET industry is also still limited (Shea, 1981). Generally, it has been known that the numbers of blastomere recovered frozen-thawed embryos are considerably less due to low quality or lysis after thawing (Westhusin et al., 1991; Ushijima and Eto, 1992; Yang et al., 1993) and that the pregnancy rate in ET industry is greatly influenced by embryo quality (Elsden et al., 1978; Lindner et al., 1983). The embryos recovered from superovulated cows is a great variable in development stage and quality of embryos (Lindner et al., 1983; Bondioli., 1990).

Ⅳ. SUMMARY

This study was conducted to determine the optimal DC voltage for NT of *in vivo* donor embryo nuclei and investigate the effect of donor embryo guality on fusion and *in vitro* development of NT embryos . Recpient oocytes were enucleated 25~27h after IVM and further cultured for 18~20h prior to fusion for oocyte aging. Donor embryos of molura stage were recovered from superovulated heifers and classified into good and low quality group. Their nuclei were transferred in to the emucleated oocytes 42~44h post-IVM and fused 43~45h post-IVM with a single 0.75kV/cm or 1.0kV/cm DC voltage for 70μsec. The fusion rate of oocytes was not different between two DC voltages. However, the cleavage and M+B developmnent was more high at 1.0kV/cm DC voltage and the proportion of M+B was 19.0% at 0.75kV/cm DC and 29.4% at 1.0kV/cm DC voltage. Donor embryo qualtiy did not greatly affect the fusion and cleavage of NT oocytes, but none of NT embryos derived from low embryo quatity reached the morula stage.

The results indicate that the most suitable DC voltage for electrofusion of *in vivo* donor muclei was a single 1.0kV/cm DC voltage and donor embryo quality was an important factor affecting the development *in vitro* of NT embryos.

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