韓國受精卵移植學會誌(2004) 第 19 卷 第 3 號
Korean J. Emb. Trans. (2004) Vol. 19, No. 3, pp. 191~199

Effect of Media, Synchronization of Fibroblast Cells, Culture Time, O_2 Concentration and Activation on Developmental Rate of Nuclear Transferred Porcine Oocytes

J. H. Quan, M. H. Rhee[1] and S. K. Kim[†]

College of Veterinary Medicine, Chungnam National University, Daejeon 305-764, South Korea

배양액, 섬유아세포, 배양시간, 산소 농도 및 활성화 처리가 돼지 핵이식 배의 체외발달에 미치는 영향

전연화 · 이만휘[1] · 김상근[†]
충남대 수의대

SUMMARY

본 연구는 돼지 태아 섬유아세포유래 공여세포를 미세주입에 의해 주입 후 재 조합한 핵 이식 배에 대한 배양액, 세포주기의 동기화, 배양시간 및 난자의 활성화에 따른 융합율과 체외발생율에 대해 조사하였다. 핵 이식 배를 NCSU-23, TL Hepes 및 TZM-3 배양액으로 1시간 및 8시간 배양하였을 때 배반포로의 분할율은 각각 15.6%, 14.0%, 15.0% 및 13.9%, 10.5%, 13.3%로서 배양액 및 시간에 따른 분할율의 유의적인 차이는 없었다. 공여핵원용 세포를 0, 8, 15시간 배양했을 때 G2/M기로의 체외발달율은 12.0%, 18.0%, 48.0%였다(p<0.01). 공여핵원용 세포를 12~24시간 배양했을 때 G2/M기로의 체외발달율은 유의한 증가를 나타내지 않았다. 공여핵원용 세포를 10% FBS + NCSU-23 배양액으로 1~2, 6~8, 12~14일간 배양 후 핵 이식한 배의 융합율은 각각 60.0%, 73.3%, 62.5%였으며, 분할율은 각각 36.0%, 56.7%, 50.0%였다. 0.5% FBS + NCSU-23, 0.5% + TL-Heaps 및 0.5% + TZM-3 배양액으로 5% O_2 조건 하에서 배양하였을 때 핵 이식배의 ≥2 cell 및 배반포로의 발생율은 각각 12.5±1.6%, 11.1±1.8%, 11.7±1.0%였으며, 10% O_2 조건 하에서 배양하였을 때 핵 이식배의 ≥2 cell 및 배반포로의 발생율은 각각 10.5±1.5%, 9.8±1.4%, 10.0±0.8%였다. 배양액과 O_2 조건에 따른 유의한 발생율에 차이는 인정되지 않았다.

(Key words : nuclear transfer, porcine fetal fibroblast, fusion and cleavage rate)

INTRODUCTION

Among attempts to produce transgenic animals by nuclear transplantation, Briggs and King (1952), Collas and Barnes(1994) and Wakayama et al.(1997) succeeded in amphibians, and Prather et al.(1987) and Tao et al.(1999) were successful in mammals. Wilmut et al.(1997) produced a cloned sheep originating from an adult mammary gland cell. Subsequently, there were reports of live births from embryos cloned using fetal fibroblast cells(Tao et al., 1999), fetal skin and muscle cells (Vignon et al., 1998), and stem cells(First et al., 1994). Numerous experiments have shown that culture medium,

[1] College of Veterinary Medicine, Kyungpook National University, Daegu 305-764, South Korea.
[†] Correspondence : E-mail : kskkim@cnu.ac.kr

culture duration, serum starvation culture procedures, cell size, cell maturational stage, and synchronization of the cell cycle (Prather et al., 1987; Tao et al., 1999; Campbell, 1999; Onishi et al., 2000; Urakawa et al., 2001), can affect *in vitro* development of nuclear transfer embryos. Verma et al.(2000) reported *in vitro* developmental rates of porcine nuclear transfer embryos constructed using porcine fetal fibroblast cultured by cycle of donor cell and serum starvation were 81% and 77%, respectively. Kuhholzer et al.(2001) reported that the fusion rates of donor cells originated from porcine fetus fibroblast cells when fused by electric stimulation after nuclear transfer by cycle of donor cell and serum starvation culture were 58% and 62%. Dinnyes et al. (2001) reported that fusion rates did not differ significantly according to whether serum starvation was applied during the culture of rabbit ear cells. Hill et al. (2000) reported that the fusion rates with electric fusion after nuclear transferred using bovine fetus vs. adult fibroblast cell were 58%, 60% respectively, and there was no significant difference.

Thus, successful production of cloned animals using somatic cells may be affected by cell size, cultural conditions, synchrony of donor and recipient cells, and maturation rate of recipient oocytes. Research to improve fusion rate and *in vitro* development of nuclear transfer embryos will allow more reliable and stable production of cloned animals.

The purpose of this study was to investigate the impact of culture medium, culture duration, and atmospheric condition on the fusion and *in vitro* development rates of nuclear transfer porcine embryos constructed by the microinjection of fetal fibroblast cells into *in vitro* matured oocytes.

MATERIALS AND METHODS

1. Preparation of Donor Cells

To establish donor nuclei cell lines, limb and skin tissues were collected from a 40-day porcine fetus. Sliced tissue was washed in PBS, vortexed for 3 min in D-PBS medium (Gibco, USA) supplemented with 0.25% trypsin/EDTA (Gibco, U.S.A.), and centrifuged with NCSU-23 medium (Sigma, U.S.A.) supplemented with 5% FBS (Gibco, U.S.A.). After removal of the trypsin and EDTA, separated cells in NCSU-23 medium supplemented with 5% FBS were cultured at 38.5℃ in a CO_2 incubator (5% CO_2, or 5% O_2 and 95% air). When a cultured cell successfully formed a monolayer on a dish, serum starvation culture in NCSU-23 medium supplemented with 5% FBS was carried out for 3 days. Induced G_0 or G_1 stage cells cultured for 15~20 days were used as donors of nuclei in subsequent experiments.

2. Preparation of Recipient Oocytes

Immature oocytes collected from porcine ovaries in a slaughterhouse were plunged into NCSU-23 culture medium supplemented with 5% FBS, 10 IU/ml hCG, 10 IU/ml eCG, and 10 ng/ml EGF. Oocytes were covered with mineral oil (Sigma, U.S.A.) and then cultured in a CO_2 incubator. After *in vitro* maturation, enlarged cumulus cells were removed and cultured in D-PBS medium supplemented with 0.2% hyaluronidase (Sigma, U.S.A.). Matured oocytes with normal-appearing cytoplasm and a confirmed 1st polar body were used as recipient oocytes for nuclear transplantation. Matured oocytes were transferred to drops of medium supplemented with 7.5 μg/ml of cytochalacin B (Sigma, U.S.A.). Zona pellucida were incised by micromanipulator, and the cytoplasm with 1st polar body and nucleus were removed. Enucleated oocytes awaiting nuclear transfer were cultured in a CO_2 incubator in medium supplemented with 4 mg/ml BSA and 0.1 mg/ml cystine.

3. Nuclear Transfer and Culture

Cultured donor oocytes were treated with 0.25% trypsin-EDTA, and separated single cells were suspended in TCM-199 drops supplemented with 5% FBS. Recipient oocytes were grasped with a holding pipette of a micromanipulator. Fibroblast cells were pre-synchronized in medium supplemented with 0.2% serum for 48 h or 0.5% and 1% serum for 7 days. Pre-synchronization in early S-phase before incubation in medium containing 0.1 μg/m Hoechst 33342 an increase from 0 and 8 versus 15 h culture an increased percentage of porcine fibroblast cells in G2/M at the end of the synchronization period. Donor oocytes were loaded into a 20~30 μm pipette and injected singly into the zona pellucida and vitelline space of the recipient cells. These cells were then fused using direct current 1.90 kv/cm for 30 μsec, then alternating current for 5 v/mm 5 sec, and with count current 1.50 kv/cm for 30 μsec. Nuclear transferred embryos were treated with 5 μg/ml ionomycion, activated with 1.5 mM 6-DMAP for 4 hr, cultured in NCSU-23 or TL-Heaps medium (both containing 3 mg/ml BSA) in a CO_2 incubator for 8~9 days, stained with 1% aceto-orcein after fixing with methanol: acetic acid (3:1), and microscopically examined ($\times$200) to assess developmental competence and stage using procedures of Lee and Kim(1991).

4. IVF

In vitro fertilization was performed at 38.5℃, 100% humidity in an atmosphere of 5% CO_2 in air. After the maturation period, oocytes were denuded by vortexing 1 min in 2 ml of PBS and washed three times in the same medium and once in fertilization medium before being transferred in groups of 25 oocytes into 4-well plates. Each well contained 250 μl of fertilization medium. A 250 μl aliquot of the sperm suspension was added to each fertilization well to obtain a final concentration of 2×10^5 cells/ml, and the gametes coincubated for 22 h. Parthenogenetically activated oocytes were cultured in NCSU-23 or PZM-3 and TL-Heaps medium + 5% FBS for 6~8 days.

5. Experimental Design

In the first of three experiments using porcine donor and recipient embryos, we evaluated the effect on developmental competence of nuclear transfer embryos of culturing the donor embryos in NCSU-23 or TL Heaps medium for either 1 or 8 days duration. In the second experiment, we evaluated the effect on nuclear-transfer embryo fusion and cleavage rates of culturing donor cells in 5% FBS + NCSU-23 medium for either 1~2, 6~8, or 12~14 days duration. In the third experiment, we evaluated the effect on developmental rates of nuclear transfer embryos of culturing donor cells for 6~8 days in 5% FBS with the addition of either NCSU-23 or TL-Heaps in an atmosphere containing either 5 or 10% O_2. In a fourth experiment, developmental competence of oocytes transferred with nuclei from porcine fibroblasts was compared to that of developmental competence of IVF porcine embryos.

6. Assessment of Developmental Rate

The oocytes was treated with 0.2% hyaluronidase(Sigma, U.S.A.) for 1~5 min. After cumulus cells were removed, oocytes were fixed in acetic acid : ethanol(1:3) solution for 24 h and stained with 1% aceto-orcein or 10 μg/ml bisbenzimide (Hoechst 33342, Sigma, U.S.A.). The development to the G2/M stage was determined by observing stained oocytes and developmental stage of *in vitro* culture.

7. Statistical Analysis

Data were expressed as mean $\pm$ SD. For comparison of means, Duncan's multiple verification was performed using SAS package of General Linear Model (GLM) procedures (SAS Institute,

1996)

RESULTS

1. Nuclear Formation Rate on Different Cultural Media

The nuclear formation and cleavage rates for donor nuclear transfer embryos cultured in NCSU-23 (with Ca) or TL Heaps (without Ca) and PZM-3 (without Ca) medium for 1 or for 8 hr are shown in Table 1. Blastocyst formation rates were not significantly different among treatments(15.6±1.5 %, 13.9±1.0%, 14.0±1.2%, 10.5±0.6%, 15.0± 1.2%, 13.3±1.2%). The nuclear formation and cleavage rates for donor nuclear transfer embryos was similar with both media and culture times.

2. Synchronization of Porcine Fetal Fibroblast Cells

Development to G2/M oocytes on time of synchronization of porcine fetal fibroblast cells with topoosomerase inhibitor are shown in Table 2.

There was no significant difference in the percentage of porcine fibroblast cells in G2/M whether cells were pre-synchronized in medium supplemented with 0.2% serum for 48 h or 0.5% and 1% serum for 7 days. Pre-synchronization in early S-phase before incubation in medium containing 0.1 μg/ml Hoechst 33342 an increase from 0 and 8 versus 15 h culture an increased percentage of porcine fibroblast cells in G2/M at the end of the synchronization period (12.0% and 18.0% versus 48.0%, $P<0.01$). Neither an increase in the concentration of H 33342 (0.2~1.6 μg/ml) nor a longer exposure time (12 h versus 18 h versus 24 h) increased the proportion of porcine G2/M fibroblasts.

Table 2. The percentage of porcine fibroblast cells in G2/M at the end of the synchronization period

Time of synchronization	No. of oocytes examined	No. (%) of G2/M oocytes
0	50	6(12.0)±2.4[b]
8	50	9(18.0)±3.7[b]
15	50	24(48.0)±2.8[a]

[a,b] : Values within column with different superscripts differ ($p<0.01$).

3. Fusion and Cleavage Rates following Different Durations of Culture

The fusion and cleavage rates of nuclear transfer embryos using donor cells cultured in 5% FBS + NCSU-23 medium for 1~2, 6~8, or 12~14 days are shown in Table 3. The percentage of oocytes in which there was successful fusion did not differ significantly with duration of culture, but the percen-

Table 1. Effects of exposure time prior to activation and of medium on developmental rates in nuclear transfer embryos derived from microinjection of fibroblasts

Medium	Duration of culture (h)	No. of oocytes examined	No. (%) of oocytes developed to	
			≥ 2 cell	Blastocyst
NCSU-23(with Ca)	1	90	66(73.3)±2.3	14(15.6)±1.5
	8	72	52(72.2)±2.8	10(13.9)±1.0
TL-Heaps(Ca-free)	1	100	78(78.0)±2.6	14(14.0)±1.2
	8	86	62(72.1)±2.0	9(10.5)±0.6
PZM-3(Ca-free)	1	60	43(71.7)±3.0	9(15.0)±0.9
	8	60	44(73.3)±2.7	8(13.3)±1.0

Table 3. Effect of the number of days of culture on fusion and cleavage rates of nuclear transfer oocytes in porcine fibroblast cells

No. of passages	No. of oocytes microinjected	No. (%) of oocytes fused	No. (%) of oocytes cleaved
1~ 2	50	30(60.0)±1.8	18(36.0)±1.2[a]
6~ 8	60	44(73.3)±1.4	34(56.7)±1.4[b]
12~14	64	40(62.5)±1.0	32(50.0)±1.6[b]

[a,b] : Values within column with different superscripts differ ($p<0.05$).

tage of cells with successful cleavage was higher for cells cultured for 6~8 or for 12~14 days than for cells cultured only 1~2 days ($p<0.05$).

4. Developmental Rate as Affected by Medium and O_2 Concentration

The effects of culture medium (5% FBS + NCSU-23 or 5% FBS + TL-Heaps) and O_2 concentration (5 or 10%) on *in vitro* development of nuclear transfer embryos after 6~8 days of culture are shown in Table 4. Approximately two thirds of all cells reached the two or greater cell stage and approximately one in 10 became blastocysts, but neither media nor oxygen concentration significantly affected the percentages of cells successfully achieving fusion or cleavage.

DISCUSSION

In the present study, our purpose was to investigate the impact of culture medium, culture duration, and atmospheric condition on the fusion and *in vitro* development rates of nuclear transfer porcine embryos constructed by the microinjection of fetal fibroblast cells into *in vitro* matured oocytes. NCSU-23 (with Ca) and TL-Heaps (Ca-free) medium have been used to culture porcine nuclear transfer embryos derived from microinjection of fibroblasts.

The nuclear formation and cleavage rates for donor nuclear transfer embryos cultured in NCSU-23 (with Ca) or TL Heaps (without Ca) medium for 1 or for 8 hr are shown in Table 1. Success rates at both developmental stages were similar for both media and for both culture times as well. None of the differences was statistically significant. Embryos in NCSU-23 medium for 1hr duration had greater developmental ability *in vitro* but embryos in TL-Heaps medium for 1 hr duration had worse developmental ability. Nuclear formation and clea-

Table 4. Effect of media and oxygen concentration on the development of porcine nuclear transfer embryos

Medium	O_2 concentration (%)	No. of oocytes examined	No. (%) of oocytes developed to	
			≥ 2 cell	Blastocyst
NCSU-23	5	88	62(70.1)±2.2	11(12.5)±1.6
	10	76	50(65.8)±2.4	8(10.5)±1.5
TL-Heaps	5	90	62(68.9)±1.8	10(11.1)±1.8
	10	82	54(65.9)±2.0	8(9.8)±1.4
PZM-3	5	60	40(66.7)±2.2	7(11.7)±1.0
	10	60	39(65.0)±2.1	6(10.0)±0.8

vage rates to the 2-cell and blastocyst stage when donor embryos were cultured in NCSU-23 (with Ca) or TL Heaps (Ca-free) medium for 1 or 8 hr duration (Table 1) were higher than corresponding results of Tao et al.(1999), who reported nuclear formation rates ranging from 22.9% to 30.9% for nuclear transfer porcine embryos cultured in NCSU-23 or TL-Heaps for 1 or 8 hr duration. Onishi et al.(2000) reported a 31.2% rate of blastocyst formation for nuclear transfer embryos cultured in NCSU-23 medium, which was significantly higher than that of embryos cultured in BECM-3 (15.2%) or mWM (4.0%) media. NCSU-23 medium includes hypotaurine, while BECM-3 has no hypotaurine. It has been reported that hypotaurine has a beneficial effect for early porcine embryo development *in vitro* (Petters and Well, 1993).

There was no significant difference in the percentage of porcine fibroblast cells in G2/M whether cells were pre-synchronized in medium supplemented with 0.2% serum for 48 h or 0.5% and 1% serum for 7 days. Pre-synchronization in early S-phase before incubation in medium containing 0.1 μg/m Hoechst 33342 an increase from 0 and 8 versus 15 h culture an increased percentage of porcine fibroblast cells in G2/M at the end of the synchronization period (12.0% and 18.0% versus 48.0%, $P<0.01$). Neither an increase in the concentration of H 33342 (0.2~1.6 μg/ml) nor a longer exposure time (12 h, 18 h and 24 h) increased the proportion of porcine G2/M fibroblasts.

The fusion and cleavage rates of nuclear transfer embryos using donor cells cultured in 5% FBS + NCSU-23 medium for 1~2, 6~8, or 12~14 days are shown in Table 2. The percentage of oocytes in which there was successful fusion did not differ significantly with duration of culture, but the percentage of cells with successful cleavage was higher for cells cultured for 6~8 or for 12~14 days than for cells cultured only 1~2 days ($p<0.05$). Cleavage rates were higher, however, for oocytes cultured either 6~8 or 12~14 days (56.7 and 50.0 %) than for oocytes cultured 1~2 days (44.9%). These results are slightly lower than those of Urakawa et al.(2001), who reported fusion rates of nuclear transfer embryos using bovine fetal fibroblast cells for 13 or 20 days of 79.3% and 60.0%, respectively, and cleavage rates of nuclear transfer embryos using bovine fetal fibroblast cells activated with Ca^{2+} ionophore or cycloheximide of 59.8% and 72.6%, respectively. Developmental rates in our experiment were similar or a little lower than those of Hill et al.(2000), who reported fusion rates of nuclear transfer embryos using bovine fetal fibroblast cells cultured for 2 or 18 days of 60% and 55%, respectively, and Cheong et al.(2000), who reported fusion rates of nuclear transfer embryos using bovine fetal fibroblast cells cultured for 4, 5, or 6 days of 69.6%, 73.1%, and 72.6%, respectively.

Nuclear transfer embryos cultured in NCSU-23, TL-Heaps and PZM-3 medium under low oxygen concentrations had a higher developmental rate to the 2 cell and blastocyst stage. The oxygen concentration in the genital tract is lower than that in air (Fischer and Bavister, 1993). Therefore, a low oxygen concentration may be beneficial to early embryo development. When bovine, porcine, and ovine early stage embryos were cultured under a lowe-oxygen concentration, development was reportedly higher than those under high oxygen concentration (Machaty et al., 2000). It was suggested that a low oxygen concentration was beneficial for *in vitro* development of porcine embryos ; it could shift ATP production from oxidative phosphorylation to glycolysis at compaction and blastulation (Krischer et al., 2001), and therefore could improve *in vitro* embryonic development. Embryos cultured under low oxygen concentration (5% CO_2) had higher total cell number and lower apoptotic cell number (Yuan et al., 2003). In this study, the developmental rate to the blastocyst stage was 8~

11% in NCSU-23 and 8 to 10% in TL-Heaps, respectively. Koo et al.(2000) and Betthauser et al.(2000) reported 10 and 7% blastocyst formation rate, respectively. The blastocyst formation rate of the nuclear transfer embryos cultured in NCSU-23 under high oxygen concentration was similar to previous results.

In vitro developmental rates of nuclear transfer embryos using donor cells originating from porcine fibroblast cells cultured in 5% FBS + NCSU-23 medium for 6~8 days were lower than those of Hwang et al.(1999), who reported that cleavage and developmental rates to the blastocyst stage of nuclear transfer embryos from fetal fibroblast cells cultured for 3 or 30 days were 51.8%~70.7% and 13.9%~21.7%, respectively. Our result also was lower than Tao et al.(1999), who reported that developmental rate to morula and blastocysts of nuclear-transferred embryos using porcine fetal fibroblast cells was 44.3%, that parthenogenesis of oocytes was 18.3%, and that 19.3, 10.8% of blastomeres advanced to blastocysts. The proportion of nuclear transfer embryos in our experiment that reached the blastocyst stage also was lower than control embryos produced by IVF.

SUMMARY

This study was conducted to examine *in vitro* development of porcine embryos constructed by the microinjection of cultured fetal fibroblast cells into porcine oocytes matured *in vitro*. Single fetal donor cells were deposited into the perivitelline space of enucleated oocytes, followed by electrical fusion and activation. Activated embryos were cultured in NCSU-23 medium supplemented with 5% FBS, at 38.5 C for 6 to 8 days in 5% CO_2 and air.

In experiment 1, fusion rates of nuclear transfer embryos did not differ for fetal fibroblast cells incubated in 5% FBS + NCSU-23 or 5% FBS + TL Heaps medium, nor did fusion rates of donor cells differ between 1~8 hr incubation durations. Fusion rates for the four treatment subclasses ranged from 72.1% to 78.0%.

In experiment 2, Pre-synchronization in medium containing 0.1 μg/m Hoechst 33342 an increase from 0 and 8 versus 15 h culture an increased percentage of porcine fibroblast cells in G2/M at the end of the synchronization period (12.4%, 17.5 % and 47.6%). Neither an increase in the concentration of H 33342 (0.2~1.6 μg/ml) nor a longer exposure time (12 h, 18 h and 24 h) increased the proportion of porcine G2/M fibroblasts.

In experiment 3, fusion rates did not differ significantly for nuclear transfer embryos constructed using donor cells cultured in 5% FBS + NCSU-23 medium for 1~2, 6~8 or 12~14 days (60.0%, 73.3% and 62.5%), respectively. The cleavage rate for nuclear transplant embryos using fetal fibroblast cells cultured for 1~2 days was 44.0%, significantly less than 56.7% and 50.0%, for 6~8 or 12~14 days duration of culture, respectively.

In experiment 4, the proportions of nuclear transfer embryos that developed to the $\geq$2 cell and to the blastocyst stage were not affected significantly by culture medium (5% FBS + NCSU-23 or 5% FBS + TL-Heaps) or by O_2 concentration of the culture (5% vs 10%). Rates of development to the $\geq$2 cell stage ranged from 65.9% to 70.1%, and development rates to the blastocyst stage ranged from 9.8% to 12.5% for the four treatment subclasses. Developmental rate was highest for embryos cultured in 5% FBS + NCSU-23 under a gas atmosphere of 5% O_2 in air.

REFERENCES

Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Strelchenko N, Voelker G, Watt S, Thomp-

son S and Bishop M. 2000. Nat. Biotechnol. Oct., 18(10):1055-1059.

Briggs R and King TJ. 1952. Transplantation of living cell nuclei from blastula cells into enucleated frog's eggs. Proc. Natl. Acad. Sci. U.S.A., 38: 455-463.

Campbell KH. 1999. Nuclear equivalence, nuclear transfer and the cell cycle. Cloning, 1:3-15.

Cheong HT, Kwon DJ, Park YS, Hwang HS, Park CK, Yang BK and Kim CI. 2000. Developmental potentials of clone embryo derived from bovine fetal fibroblast cells. Korean J. Anim. Reprod., 24(2):49-57.

Collas P and Barnes FL. 1994. Nuclear transplantation by microinjection of inner cell mass and granulosa cell nuclei. Mol. Reprod. Dev., 38: 264-267.

Dinnyes A, Dai Y, Barber M, Liu L, Xu J, Zhou P and Yang X. 2001. Development of cloned embryos from adult rabbit fibroblasts: effect of activation treatment and donor cell preparation. Biol. Reprod., 64:257-263.

First NL, Sims MM, Park SP and Kent-First MJ. 1994. Systems for production of calves from cultured bovine embryonic cells. Reprod. Fertil. Dev., 6:553-562.

Fischer B and Bavister BD. 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J. Reprod. Fertil., 99: 673-679.

Hill JR, Winger QA, Long CR, Looney CR, Thompson JA and Westhusin ME. 2000. Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells. Biol. Reprod., 62:1135-1140.

Hwang WS, Park JI, Cho JK, Kim KY, Shin SJ, Yong HY and Lee BC. 1999. Establishment of bovine fetal fibroblast line for production of cloned calves in Korean native cattle; the effects of culture period and various cell size on the efficiency of nuclear transfer. Korean J. Emb. Trans., 14(2):93-97.

Koo DB, Kang YK, Choi YH, Park JS, Han SK, Park IY, et al. 2000. *In vitro* development of reconstructed porcine oocytes after somatic cell nuclear transfer. Biol. Reprod., 63:986-992.

Krischer RL, Ghandi DK and Lane M. 2000. Developmentally related changes in nutrient uptake and metabolism by *in vitro* produced porcine embryos. Theriogenology, 53:274.

Kuhholzer B, Hawley RJ, Lai L, Kolber-Simonds D and Prather RS. 2001. Clonal lines of transgenic fibroblast cells derived from the same fetus result in different development when used for nuclear transfer in pigs. Biol. Reprod., 64: 1695-1698.

Lee MH and Kim SK. 1991. Studies on the detrimental factors affecting *in vitro* maturation and fertilization of bovine follicular oocytes. Korean J. Vet. Res., 31(2): 179-187.

Machaty Z, Thopmson JG, Abeydeera LA, Day B and Prather RS. 2001. Inhibitors of mitochondrial APT production at the time of compaction improve development of *in vitro* produced porcine embryos. Mol. Reprod. Dev., 58:39-44.

Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H and Perry AC. 2000. Pig cloning by microinjection of fetal fibroblast nuclei. Science, 289:1188-1190.

Petters RM and Well KD. 1993. Culture of pig embryos. J. Reprod. Fertil., 48(Suppl):61-73.

Prather RS, Barnes FL, Sims ML, Robl JM, Eyestone WH and First NL. 1987. Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. Biol. Reprod., 37:859-866.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scoot AR, Ritchie M, Wilmut I, Colman A and Campbell KH. 1997. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science,

278:2130-2133.

Tao T, Machaty Z, Boquest AC, Day BN and Prather RS. 1999. Development of pig embryos reconstructed by microinjection of cultured fetal fibroblast cells into *in vitro* matured oocytes. Anim. Reprod. Sci., 56(2):133-141.

Urakawa M, Uruno K, Ideta A and Aoyaki Y. 2001. Effect on the development of bovine embryos by nuclear transfer using different cultured days of fetal fibroblast. Theriogenology, 55: 294(abstract).

Verma PJ, Du ZT, Crocker L, Faast R, Grupen CG, Mcilfatrick SM, Ashman RJ, Lyons IG and Nottle MB. 2000. *In vitro* development of porcine nuclear transfer embryos constructed using different cultured days of fetal fibroblast. Mol, Reprod, Dev., 57:262-269.

Vignon X, Chesne P, Le Bourhis D, Flechon JE, Heyman Y and Renard JP. 1998. Developmental potential of bovine embryos reconstructed from enucleated matured oocytes fused with cultured somatic cells. CR Acad. Sci., 321(9): 735-745.

Wakayama T, Perry AC, Zuccotti M, Johnson KR and Yanagimachi R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature, 23:394(6691): 369-374.

Wilmut I, Schnieke AE, McWhir J, Kind AJ and Campbell KHS. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature, 27:385:810-813.

Yuan YQ, Van Soom, Coopman FOJ, Mintiens K, Boerjan ML, Van Zeveren A, et al. 2003. Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured *in vitro*. Theriogenology, 59:1585-1596.

(접수일: 2004. 6. 5 / 채택일: 2004. 8. 8)