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Effects of EGF, β-ME, Glucose, O_2 Concentrations and Fibroblasts Subculture on the Development of Porcine NT Embryos

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EGF, β-ME, Glucose, O_2 농도 및 Fibroblast Subculture가 핵이식 배의 체외발생에 미치는 영향에 관한 연구

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SUMMARY

본 연구는 NCSU-23과 PZM-3 배양액에 EGF, β-ME와 glucose의 첨가가 돼지 난자의 체외성숙에 미치는 영향과 배양조건을 다르게 하여 계대배양한 섬유아세포를 이용한 핵이식 배를 다른 배양액과 산소조건에서 배양하였을 때 체외발생율에 미치는 영향을 조사하였다.

핵이식 배를 20ng/ml EGF를 첨가 또는 첨가하지 않은 NCSU-23 및 PZM-3 배양액에서 배양하였을 때 배반포로의 체외 발생율은 각각 12.0±1.3%, 9.6±1.9%, 10.9±2.1%, 9.1±2.3%였다. 핵이식 배를 25 μM β-ME를 첨가 또는 첨가하지 않은 NCSU-23 및 PZM-3 배양액에서 144시간 배양하였을 때 배반포로의 체외 발생율은 각각 9.6±1.7%, 7.3±2.3%, 11.9±1.8%와 7.4±2.1%였다. β-ME를 첨가한 PZM-3 배양액에서 배양하였을 때 배반포로의 체외 발생율은 β-ME를 첨가하지 않은 배양액에서 배양한 배보다 높은 체외발생율을 나타냈다 (p<0.05). 핵이식 배를 1.5mM glucose를 첨가 또는 첨가하지 않은 NCSU-23 및 PZM-3 배양액에서 배양하였을 때 배반포로의 체외 발생율은 각각 9.4±2.2%, 6.8±2.7%, 10.9±2.4%와 8.9±2.6%였다. Glucose를 첨가한 NCSU-23과 PZM-3 배양액에서 배양하였을 때 배반포로의 체외 발생율은 glucose를 첨가하지 않은 배양액에서 배양한 배보다 높은 체외 발생율을 나타냈다. 핵이식 배를 NCSU-23 및 PZM-3 배양액과 5% 및 20% 산소 조건에서 배양하였을 때 배반포로의 체외 발생율은 각각 11.1±1.8%, 9.8±1.4%, 12.5±1.6%와10.9±1.5%였다. NUSU-23과 PZM-3 배양액에서 5% 산소 조건에서 배양하였을 때 20% 산소 조건에서 배양한 난자보다 높은 체외 발생율을 나타났다. 섬유아세포를 NCSU-23 배양액에서 배양하여 공여자세포로 이용하여 10 및 11~15 passage 이내로 계대배양하였을 때의 융합율은 60.0~73.3%, 52.5%였다. 섬유아세포를 PZM-3 배양액에서 배양하여 공여자세포로 이용하여 10 및 11~15passage 이내의 계대배양시의 융합율은 60.4~75.1% 및 58.7%였다.

(Key words : *in vitro* maturation, EGF, β-ME, glucose, NT embryos)

INTRODUCTION

Nuclear transfer (NT) has been considered very

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important because genetically modified pigs might be able to provide organs and tissues for xenotransplantation. However, in the pig, the viability of NT embryos is poor, with an extremely low rate of cloned piglet production. Several types of donor cells have been used including: fetal fibroblasts (Wilmut *et al.*, 1997; Baguisi *et al.*, 1999; Cibelli *et al.*, 1998; Schnieke *et al.*, 1997), mammary-derived cells (Wilmut, *et al.*, 1997), cumulus cells (Wakayama *et al.*, 1998), and oviductal cells (Wakayama *et al.*, 1998; Kato *et al.*, 1998). So far, by using granulosa cells (GCs) cloned calves and piglets have been born (Wells *et al.*, 1999; Polejaeva *et al.*, 2000).

An important factor governing developmental rates of reconstructed embryos is the phase of the cell cycle in which donor nuclei exist prior to nuclear transfer. This is undoubtedly due to the differences in DNA content of donor nuclei, which varies according to the phase of the cell cycle. Serum deprivation is a commonly used method to synchronize cell lines in the G_0 phase of the cell cycle (Hayes *et al.*, 2005). For successful reprogramming of the donor nucleus, it must be in G_0 or G_1 when transferred to metaphase II arrested oocytes with greater amounts of maturation promoting factors. This strict synchrony will allow chromosomes to condense properly and will enhance the correct ploidy in the resulting embryos (Kim *et al.*, 1988). Furthermore, Cibelli *et al.* (1998) suggested to use actively dividing cells in the M phase of the cycle because these cells are supposed to develop in a fashion similar to blastomeres and, therefore, will increase the success rate at embryo transfer.

Oxygen concentration in the embryo culture system has been considered to affect rates of development (Machaty *et al.*, 1998; Olson and Seidel, 2000; Fischer and Bavister, 1993). Oxygen level in the uterine cavity is lower than that in the oviduct (Davis, 1985), and *in vivo* porcine embryos reach the urerine horn before the compact morlula stage (Berthelot and Terqui, 1996). It was suggested that a low oxygen concentration was beneficial for *in vitro* development of porcine embryos (Krischer *et al.*, 2000), which could shift ATP production from oxidative phosphorylation to glycolysis (Johnson and Nasr-Esfahani, 1994), and therefore could improve *in vitro* embryonic development. In addition, it is well known that a higher oxygen concentration than that in genital tract can produce the formation of reactive oxygen species (ROS) during the embryo culture. ROS are known to have deleterious effects on cells, including DNA damage, lipid peroxidation and oxidative modification of proteins (Kues *et al.*, 2000). For successful reprogramming of the donor nucleus, it must be in G_0 or G_1 when transferred to metaphase II arrested oocytes with greater amounts of maturation promoting factors. Successful production of cloned animals using somatic cells may be affected by maturation rate of recipient oocytes, culture media and gas atmospheres.

The purpose of this study was to investigate *in vitro* maturation rate of oocytes cultured in maturation medium with supplementation of EGF, β-ME, glucose, and further development of NT embryos reconstructed with differently subcultured fibroblast cells cultured in different media and gas atmospheres.

MATERIALS AND METHODS

1. Media

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical (St Louis, USA). The basic medium used for oocyte maturation were NCSU-23 and PZM-3 media supplemented with 0.1 mg/ml cysteine, 10% (v/v) porcine follicular fluid (pFF), 10 μg/ml FSH, 10 μg/ml LH, 20ng/ml epidermal growth factor (EGF), 25 μM β-mercaptoethanol (β-ME). The pFF as-

pirated from 3~7 mm follicles was centrifuged at 1,500×g for 30 min (−4℃) to remove blood cells and debris. The supernatant was collected and filtered through 1.2 μm syringe filters. The prepared pFF was transferred to sterile centrifuge and then stored at −20℃ until use.

2. *In Vitro* Maturation of Oocytes

Porcine ovaries were collected at a local slaughterhouse and transported to the laboratory in 0.9% (w/v) NaCl containing 75 μl/ml penicillin G at 30℃. Oocytes were aspirated from medium size follicles (3~6 mm) with an 18 gauge fixed to a 10 ml disposable syringe. The cumulus-oocytes complexes (COCs) that had an evenly distributed cytoplasm and washed three times in oocyte maturation medium containing hormonal supplements. Then each group of 50 COCs was cultured in 500 μl of maturation medium, which had previously been covered with mineral oil and equilibrated in a humidified atmosphere of 5% CO_2 and 95% air at 38.5℃. After culturing for 22 h, COCs were washed three times in the maturation medium without hormonal supplements and transferred into 500 μl drops of the same medium for another 20 h.

3. Preparation of Donor Cell

A Day 35 fetus was obtained from a pregnant gilt. The head of the fetus was removed using iris scissors, and the brain, four limbs, and soft tissues such as liver and intestines were also discarded by scooping out with two watchmaker's forceps. After twice washing with Dulbeccos's Phosphate Buffered Saline (DPBS), the carcass was minced with a surgical blade on a 100 mm culture dish. Cells were dissociated using DMEM supplemented with 0.1% (w/v) trypsin and 1 mM EDTA for 1~2 h at 39℃. And the suspension was centrifuged at 500×g for 5 min and subsequently seeded into culture dishes. The cell pellet was resuspended and cultured for 6~8 days in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin G (75 μg/ml), streptomycin (50 μg/ml), 1 mM sodium pyruvate, 1% (v/v) nonessential amino acids at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cells or explants, attached cells were further cultured until confluent, subcultured (1:4 dilution) at intervals of 4 days by trypsinization for 5 min using 0.1% trypsin and 1 mM EDTA and were stored in freezing medium in liquid nitrogen at −196℃. Frozen media consisted of 80% (v/v) DMEM, 10% (v/v) DMSO and 10% (v/v) FBS. Before nuclear transfer, frozen cells intended for use as donor cells were thawed, cultured in serum starved DMEM supplemented with 0.5% FBS for 3 days until 80% confluency. And Cells for nuclear transfer were retrieved from the monolayer by trypsinization for 30 s.

4. Nuclear Transfer and Culture

After 44 h IVM, a denuded oocyte with the first polar body was held with a holding micropipette and the zona pellucida was partially dissected with a fine glass needle to make a slit near the first polar body. The first polar body and adjacent cytoplasm presumably containing the MⅡ chromosomes were enucleated by a micropipette (30 μm in diameter) in HEPES-buffered NCSU-23 supplemented with 4 mg/ml bovine serum albumin (BSA) and 7.5 μg/mL cytochalasin B (Sigma, USA) at 38 ℃. Round glossy cells were chosen as donor cells and transferred into the perivitelline space of the enucleated recipient oocytes through the hole made at enucleation. After enucleation, oocytes were stained with 5 μg/mL bisbenzimide (Hoechst 33342, Sigma, USA) for 5 min and observed under a with fluorescence microscope. Oocytes still containing DNA materials were excluded from experiments. Couplets were equilibrated with 0.3 M mannitol solution containing 0.5 mM HEPES, 1.0 mM $CaCl_2 \cdot 2H_2O$ and 0.1 mM $MgSO_4$ for 4 min and

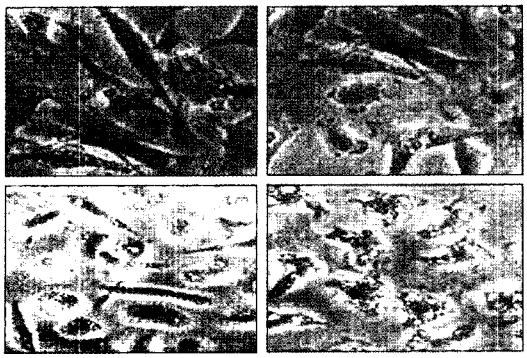

Fig. 1. Fibroblast cells cultured in DMEM supplemented with 0.5% FBS for 7~12 days.

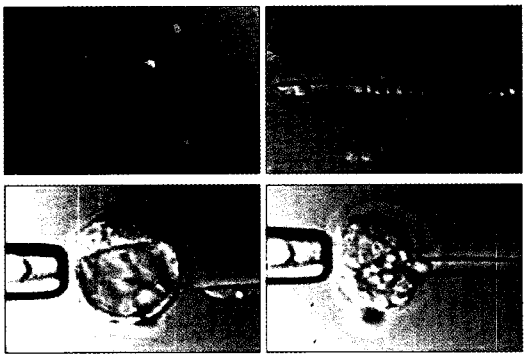

Fig. 2. Nuclear transfer by microinjection.

transferred to a chamber containing two electrodes that were overlaid with fusion and activation solution. And these Couplets were fused and activated simultaneously with a single direct current pulse of 2.1 kV/cm for 30 μs using an Electro-Cell Manipulator (BTX, Inc., San Diego, U.S.A.). This pulse was also utilized to simultaneously induce oocyte activation. After microinjection, reconstructed embryos were placed in Ca free NCSU-23 or PZM-3 supplemented with 4 mg/ml BSA and 0.1 mg/ml cysteine at 39℃ in a humidified atmosphere containing 5% CO_2 in air for 30 min (Machaty *et al.*, 1998). This time period has been inserted to allow donor nuclei remodeling which includes nuclear envelope breakdown and premature chromosome condensation. The reconstructed embryos were cultured for 7 days after activation. Stained with 1% aceto-orcein after fixing with methanol: acetic acid (3:1), and microscopically examined (× 200) to assess developmental competence and stage using procedures of Kim *et al.* (1988).

5. Statistical Analysis

All data were subjected to a Generalized Linear Model procedure (PROC-GLM) of the statistical analysis system (SAS Institute, Gary, NC, USA). Differences among treatment means were determined using Duncan's multiple range test and *t*-test. All the data were expressed as least square (LS) mean±S.D. Differences among treatment effects were considered at $P<0.05$.

RESULTS

1. Effects of EGF and Different Media on the Development of NT Embryos

The developmental rates to 2 cell and blastocyst stage of embryos cultured in NCSU-23 or PZM-3 supplemented with or without 20 ng/ml EGF were shown in Table 1. When the embryos were cultured in NCSU-23 or PZM-3 supplemented with or without 20 ng/ml EGF for 48 h and 144 h, the development rates to 2 cell and blastocyst stage were 56.4±2.7%, 12.0±1.3%, 53.2±1.9%, 9.6±1.9%, 70.5±2.1%, 10.9±2.1%, 62.7±2.4% and 9.1±2.3%, respectively. The developmental rate to the blastocyst stage of NT embryos cultured in both NUSU-23 and PZM-3 supplemented with EGF was higher than cultured in none supplementation of EGF.

2. Effects of β-ME and Different Media on the Development of NT Embryos

The developmental rates to 2 cell and blastocyst stage of embryos cultured in NCSU-23 or PZM-3 supplemented with or without 25 μM β-ME were shown in Table 2. When the embryos were cultured in NCSU-23 or PZM-3 supplemented with or without 25 μM β-ME for 144 h, the development

Table 1. Developmental ability of NT embryos cultured in different maturation media supplemented with or without EGF

IVM condition		No. of oocytes examined	No. of oocytes developed to*	
Media	EGF		≥ 2 cell	Blastocyst
NCSU-23	+	58	33 (56.4±2.7)	7 (12.0±1.3)
NCSU-23	-	52	28 (53.2±1.9)	5 (9.6±1.9)
PZM-3	+	54	38 (70.5±2.1)	6 (10.9±2.1)
PZM-3	-	55	34 (62.7±2.4)	5 (9.1±2.3)

* Data are presented as Mean±S.D.

rates to the blastocyst stage were 9.6±1.7%, 7.3± 2.3%, 11.9±1.8% and 7.4±2.1%, respectively. The developmental rate to the blastocyst stage of NT embryos cultured in PZM-3 supplemented with β-ME was significantly higher cultured in none supplementation of β-ME ($p<0.05$).

3. Effects of Glucose and Different Media on the Development of NT Embryos

The developmental rates to 2 cell and blastocyst stage of embryos cultured in NCSU-23 or PZM-3 supplemented with or without 1.5 mM glucose were shown in Table 3. When the embryos were cultured in NCSU-23 or PZM-3 supplemented with or without 1.5 mM glucose for 144 h, the development rates to the blastocyst stage were 9.4±2.2%, 6.8±2.7%, 10.9±2.4% and 8.9±2.6%, respectively. The developmental rate to the blastocyst stage of NT embryos cultured in NCSU-23 and PZM-3 supplemented with glucose was higher than cultured in none supplementation of glucose.

4. Effects of Media and O_2 Concentration on the Developmental Rate

Effects of media and oxygen concentration on the development of NT embryos were shown in Table 4. When NT embryos were cultured in NUSU-23 and PZM-3 at 5% and 20% O_2 concentration, the developmental rates to blastocyst stage were 11.1±1.8%, 9.8±1.4%, 12.5±1.6% and 10.9± 1.5%, respectively. The developmental rate to the blastocyst stage of NT embryos cultured in both NCSU-

Table 2. Developmental ability of NT embryos cultured in different maturation media supplemented with or without β-ME

Culture condition		No. of oocytes examined	No. of oocytes developed to*	
Media	β-ME		≥ 2 cell	Blastocyst
NCSU-23	+	52	29 (55.1±2.9)	5 (9.6±1.7)
NCSU-23	-	55	30 (54.3±2.7)	4 (7.3±2.3)
PZM-3	+	59	41 (69.6±1.5)	7 (11.9±1.8)[a]
PZM-3	-	54	34 (62.3±2.6)	4 (7.4±2.1)[b]

* Data are presented as Mean±S.D.

[a,b] Values with different superscripts are significantly different ($p<0.05$).

Table 3. Developmental ability of NT embryos cultured in different maturation media supplemented with or without glucose

Culture condition		No. of oocytes examined	No. (%±S.E.M.) of oocytes developed to*	
Media	Glucose		≥ 2 cell	Blastocyst
NCSU-23	+	53	29 (54.7±2.3)	5 (9.4±2.2)
NCSU-23	-	51	27 (52.5±2.5)	3 (6.8±2.7)
PZM-3	+	55	36 (65.5±2.6)	6 (10.9±2.4)
PZM-3	-	56	34 (60.7±2.9)	5 (8.9±2.6)

* : Data are presented as Mean±S.D.

Table 4. Developmental rate of NT embryos cultured in different maturation media and O_2 concentrations

Medium	O_2 (%)	No. of oocytes fused	No. of oocytes developeted to*	
			≥ 2 cell	Blastcyst
NCSU-23	5	90	62 (68.9±1.8)	10 (11.1±1.8)
NCSU-23	20	82	54 (65.9±2.0)	8 (9.8±1.4)
PZM-3	5	88	62 (70.1±2.2)	11 (12.5±1.6)
PZM-3	20	76	50 (65.8±2.4)	9 (10.9±1.5)

* Data are presented as Mean±SD.

[a,b] Values with different superscripts are significantly different ($p<0.05$).

23 and PZM-3 at 5% O_2 concentration was higher than cultured at 20% O_2 concentration.

5. Effects of Fibroblast Subculture on the Development of NT Embryos Cultured in NCSU-23

When fetal fibroblasts were cultured in NCSU-23 and used as donor cells for NT embryos, the fusion and cleavage rates were as shown in Table 5 and 6. The fusion rate of less than 10 passage was higher than those of 11~15 passage (60.0~73.3%

Table 5. Effects of the fibroblasts subculture on the development of NT embryos cultured in NCSU-23

No. of passage	No. of oocytes examined	No. of oocytes fused (%)*	No. of oocytes cleaved (%)*
1~ 5	50	30 (60.0±1.8)	13 (26.0±1.2)[a]
6~10	60	44 (73.3±1.4)	25 (40.8±1.4)[b]
11~15	64	33 (52.5±1.0)	13 (20.1±1.6)[b]

* Data are presented as Mean±S.D.

Table 6. Effects of the fibroblasts subculture on the development of NT embryos cultured in the PZM-3

No. of passage	No. of oocytes examined	No. of oocytes fused (%)	No. of oocytes cleaved (%)
1~ 5	53	32 (60.4±2.1)	17 (32.2±1.8)
6~10	48	36 (75.1±1.8)	21 (42.8±1.5)
11~15	50	29 (58.7±2.3)	14 (27.9±2.4)

* : Data are presented as Mean±S.D.

versus 52.5%). However, more embryos cleaved in NCSU-23 when reconstruction was made with fetal fibroblasts of 6~10 passages than other passage ($p<0.05$). When fetal fibroblasts were cultured in PZM-3 and used as donor cells for NT embryos, fusion and cleavage rates were as shown in Table 9. The fusion rate of less than 10 passage was higher than those of 11~15 passage (60.4~75.1% versus 58.7%). However, more embryos cleaved in PZM-3 when reconstruction was made with fetal fibroblasts of less than 10 passages than those of 11~15 passages (32.2~42.8% versus 27.9%).

DISCUSSION

NT has been considered very important because genetically modified pigs might be able to provide organs and tissues for xenotransplantation. However, in the pig, the viability of NT embryos is poor, with an extremely low rate of cloned piglet production.

In the present study, our purpose was to investigate *in vitro* maturation rate of oocytes cultured in different media with or without supplementation of EGF, β-ME, glucose and further development of NT embryos cultured in different media and gas atmospheres. *In vitro* maturation rate of oocytes cultured in NCSU-23 or PZM-3 supplemented with 20ng/ml EGF, 25 μM β-ME 1.5 mM glucose were examined.

The maturation rate of oocytes cultured in PZM-3 supplemented with EGF, β-ME and glucose were higher compared to cultured in NUSU-23. In previous reports, supplementation of both EGF and β-ME to the maturation media enhanced *in vitro* maturation rate. EGF and β-ME are important for cytoplasmic maturation: the addition of EGF or β-ME to a maturation medium stimulated meiotic maturation (Ding and Foxcroft, 1993; Abeydeera *et al.*, 1998; Kim *et al.*, 2004; Chance *et al.*, 1979). This result was similar with Kim *et al.* (2004) reported that supplementation of β-ME or EGF improved IVM of canine oocytes to MⅡ stage. The effects of these two compounds were suggested to be mediated through the synthesis of GSH which is known to play an important role in protecting the cell or embryos from oxidative damage (Kim *et al.*, 2004). Glucose is an important energy sources for mammalian embryos (Kim *et al.*, 2004), while it is necessary for the last part of *in vitro* culture (Petters *et al.*, 1990).

When the NT embryos were cultured in NCSU-23 and PZM-3 supplemented with or without 20 ng/ml EGF for 48 h and 144 h, the development rates to the 2 cell and blastocyst stage were higher than cultured in none supplementation of EGF. This result was similar with Grupen *et al.* (1997) reported that exposure to EGF during IVM significantly increased blastocyst formation.

In this study, the developmental rate to the blastocyst stage was 10.9~12.5% in PZM-3 and 9.8~11.1% in NCSU-23, respectively. Im *et al.* (2004) reported 17~18% in PZM-3 and 7~12% in NCSU-2, respectively. The blastocyst formation rate of the NT embryos culturd in PZM-3 under low oxygen concentration rate was similar with previous results. NT embryos cultured in PZM-3 under low and high oxygen concentration had a higher blastocyst formation rate. Therefore, PZM-3 can support more development of porcine NT embryos with increased number of nuclei. PZM-3 contains essential and non-essential amino acids, whereas NCSU-23 does not. Amino acids have beneficial effects on preimplantational embryo development in several different species. Non-essential amino acids can provide favorable blastocysts, whereas essential amino acids can increase the total cell number and inner cell mass cell number (Thuan *et al.*, 1996). Therefore, further research on the role of amino acids in the medium is needed.

In the present study, PZM-3 supported higher rates of blastocyst formation after culturing NT

embryos under 5% O_2 versus 20% O_2. It has been suggested that early embryonic development is most dependent on the intracellular ratio of NAD^+ /NADH. Oxygen concentration, as well as the ratio of pyuvate and lactate, may also influence the maintenance of the ratio of NAD^+/NADH (Yoshioka *et al.*, 2002; Machaty *et al.*, 1998; Bavister and Troike, 1979). It can be speculated that the presence of pyruvate and lactate in PZM-3 medium maintained the intracellular ratio of NAD^+/NADH that compensated for the high concentration of oxygen. And oxygen concentration in the embryo culture system has been considered to affect development rate (Machaty *et al.*, 1998; Olson and Seidel, 2000). Oxygen level in the uterine cavity is lower than that in the oviduct (Fischer and Bavister, 1993), and *in vivo* porcine embryos reach the urerine horn before the compact morula stage (Davis, 1985). It was suggested that a low oxygen concentration was beneficial for *in vitro* development of porcine embryos (Berthelot and Terqui, 1996) it could shift ATP production from oxidative phosphorylation to glycolysis (Krischer *et al.*, 2000), and therefore could improve *in vitro* embryonic development.

It appeared that the frequency of donor cell subculture affects developmental competence of reconstructed embryos in the bovine (Hill *et al.*, 2000; Roh *et al.*, 2000). In previous reports, use of fetal fibroblasts subcultured in PZM-3 for 1~10 passage was effective; further increases in preimplantation development could not be achieved by increasing the number of subcultures more than 10 passages. This may indicate that changing the number of subcultures of adult cells used for NT would not enable them to surpass the efficacy of fetal fibroblasts prepared under optimal conditions. This result was similar with Lee *et al.* (2003) reported that when reconstruction was made with fetal fibroblasts of less than 10 passages than those of 11~15 passages (89.2~92.2% vs 73.5%).

In conclusion, the present study indicates that EGF and glucose have beneficial effect on the *in vitro* maturation of oocytes and β-ME have improved developmental ability of NT embryos. Furthermore, the developmental rate was improved when reconstruction was made with less than 10 passages subcultured fibroblast cells cultured in PZM-3 at 5% O_2 gas atmospheres.

CONCLUSION

This study was investigated *in vitro* maturation rate of oocytes cultured in maturation media with or without supplementation of EGF, β-ME, glucose and further development of NT embryos reconstructed with differently subcultured cells cultured in different media and gas atmospheres.

1. When the embryos were cultured in NCSU-23 or PZM-3 supplemented with or without 20 ng/ml EGF for 144 h, the development rates to blastocyst stage were 12.0±1.3%, 9.6±1.9 %, 10.9±2.1% and 9.1±2.3%, respectively.
2. When the embryos were cultured in NCSU-23 or PZM-3 supplemented with or without 25 μM β-ME for 144 h, the development rates to the blastocyst stage were 9.6±1.7%, 7.3± 2.3%, 11.9±1.8% and 7.4±2.1%, respectively. The developmental rate to the blastocyst stage of NT embryos cultured in PZM-3 supplemented with β-ME was significantly higher cultured in none supplementation of β-ME ($p<0.05$).
3. When the embryos were cultured in NCSU-23 or PZM-3 supplemented with or without 1.5 mM glucose for 144 h, the development rates to the blastocyst stage were 9.4±2.2%, 6.8± 2.7%, 10.9±2.4% and 8.9±2.6%, respectively. The developmental rate to the blastocyst stage of NT embryos cultured in NCSU-23 and PZM-3 supplemented with glucose was higher than cultured in none supplementation of glu-

cose.

4. When NT embryos were cultured in NUSU-23 and PZM-3 at 5% and 20% O_2 concentration, the developmental rates to blastocyst stage were 11.1±1.8%, 9.8±1.4%, 12.5±1.6% and 10.9±1.5%, respectively. The developmental rate to the blastocyst stage of NT embryos cultured in both NCSU-23 and PZM-3 at 5% O_2 concentration was higher than cultured at 20% O_2 concentration.
5. When fetal fibroblasts were cultured in NCSU-23 and used as donor cells for NT embryos, the fusion rate of less than 10 passage was higher than those of 11~15 passage (60.0~73.3% versus 52.5%). When fetal fibroblasts were cultured in PZM-3 and used as donor cells for NT embryos, the fusion rate of less than 10 passage was higher than those of 11~15 passage (60.4~75.1% versus 58.7%).

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