韓國受精卵移植學會誌(2005) 第 20 卷 第 2 號
Korean J. Emb. Trans. (2005) Vol. 20, No. 2, pp. 137～145

Effects of EGF, β-ME, Glucose on the *In Vitro* Maturation and Development of Porcine NT Embryos

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EGF, β-ME와 Glucose가 돼지 난자의 체외성숙에 미치는 영향과 핵 이식 배의 발생에 대한 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.

난자를 20ng/ml EGF를 첨가 또는 첨가하지 않은 NCSU-23 및 PZM-3 배양액에서 44시간 배양하였을 때 난자의 체외성숙율은 각각 85.7±3.1%, 75.2±2.8%, 87.1±2.4%와 78.2±2.6%였으며 EGF를 첨가한 배양액에서 배양한 난자의 체외성숙율은 EGF를 첨가하지 않은 배양액에서 배양했을 때의 난자보다 높은 체외성숙율을 나타냈다(p<0.05). 난자를 25μM β-ME를 첨가 또는 첨가하지 않은 NCSU-23 및 PZM-3 배양액에서 44시간 배양하였을 때 난자의 체외성숙율은 각각 79.5±2.6%, 74.7±2.5%, 80.2±2.3%, 78.6±2.7%였고 β-ME를 첨가한 PZM-3 배양액에서 배양한 난자의 체외성숙율이 가장 높게 나타났다. 난자를 1.5mM glucose를 첨가 또는 첨가하지 않은 NCSU-23 및 PZM-3 배양액에서 44시간 배양하였을 때 난자의 체외성숙율은 각각 79.2±2.3%, 75.0±2.6%, 85.5±2.5%와 78.9±2.7%였고, glucose를 첨가한 PZM-3 배양액에서 배양한 난자의 체외성숙율은 glucose를 첨가하지 않은 PZM-3 배양액에서 배양한 난자보다 높은 체외성숙율을 나타냈다(p<0.05). 핵이식 배를 20ng/ml EGF, 25μM β-ME 및 1.5mM glucose를 첨가한 NCSU-23 및 PZM-3 배양액에서 48시간, 144시간 배양하였을 때 2세포기 및 배반포로의 체외발생율은 각각 56.4±2.7%, 54.3±2.9%, 70.5±2.1%,, 69.6±1.5% 및 12.0±1.3%, 9.6±1.7%, 10.9±2.1%, 11.9±1.8%였다.

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

INTRODUCTION

Nuclear transfer (NT) might not only provide useful tools to reproduce genetically superior animals, but also to produce transgenic animals. After the birth of cloned sheep produced by NT from a cultured cell line (Campbell *et al*., 1996) cloned animals have been reported in sheep (Wilmut *et al*., 1997), mice (Wakayama *et al*., 1998), goat (Baguisi *et al*., 1999), and cattle (Cibelli *et al*.,

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1998; Kato *et al.*, 1998; Wells *et al.*, 1999) by NT from a variety of somatic cell types. By using transfected donor cells, transgenic sheep (Schnieke *et al.*, 1997), cattle (Cibelli *et al.*, 1998), and a goat (Baguisi *et al.*, 1999) were also produced.

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. 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, ett 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).

Initially, most cloned animals were produced by using cell fusion as the method of NT. In the pig, NT embryos can develop to the blastocyst stage by microinjection of fetal fibroblasts (Tao *et al.*, 1999), and one cloned piglet was born as a result of microinjection (Onish *et al.*, 2000). One of the advantages of microinjection is that it lessens the volume and cytoplasmic materials (i.e. mitochondria, RNA, cellular proteins, enzymes) of the donor cell that is transferred to the oocyte.

Epidermal growth factor (EGF) or β-mercaptoethanol (β-ME) is 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).

North Carolina State University (NCSU)-23 medium is one of the more successful media for *in vitro* culture of porcine nuclear transfer embryos to the blastocyst stage. Recently, however, it was reported that Porcine Zygote Medium-3 (PZM-3), based on the composition of pig oviductal fluid with supplementary amino acids, supported more development to the blastocyst stage than NCSU-23 (Yoshioka *et al.*, 2002).

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, pyruvate and lactate are important energy supplements for early stages of embryo development *in vitro* (Petters *et al.*, 1990). The NCSU-23 medium has only glucose as an energy substrate. Recently, it has been reported that pyruvate/lactate supplementation instead of glucose in porcine zygote medium (PZM) improved the *in vitro* development of porcine IVF embryos (Yoshioka *et al.*, 2002; Kikuchi *et al.*, 2002).

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). The importance of serum starvation of the donor cells was highlighted in the paper that announced the birth of Dolly. 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.

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 aspirated 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 ℃ (Fig. 1).

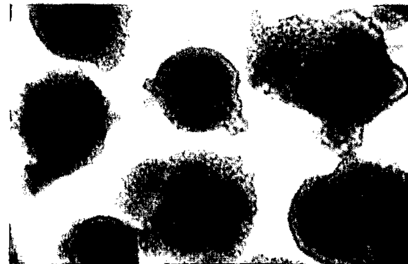

Fig. 1. Oocytes with expanded cumulus cells.

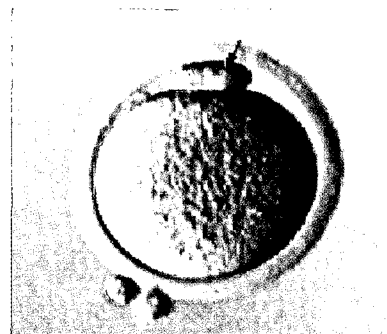

Fig. 2. Oocytes with 1st polar body.

3. Assessment of Meiotic Stage

After maturation for 44 h, oocytes were freed from cumulus cells by gentle pipetting in the same IVM medium containing 0.1% (w/v) hyaluronidase for 1 min. Some of the denuded oocytes were fixed for 5 min in ethanol : acetic acid : chloroform (6:3:1) and 2 h in ethanol: acetic acid (3:1) at room temperature. They were then stained with 10 μg/ml Hoechst 33342 in mounting medium containing PBS and glycerol (1:1). Oocytes were mounted on slide and evaluated under fluorescent microscope to determine the stage of meiosis.

4. Preparation of Donor Cell

After twice washing with Dulbeccos's Phosphate Buffered Saline (DPBS), the carcass was minced with a surgical blade on a 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)

FBS, penicillin G (75 μg/ml), streptomycin (50 μg/ml), 1 mM sodium pyruvate, 1% (v/v) non-essential amino acids at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cells, 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. Before nuclear transfer, 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.

5. Nuclear Transfer and Culture

After 44 h IVM, a denuded oocyte was held with a holding micropipette and the first polar body and adjacent cytoplasm presumably containing the MII chromosomes were enucleated by a micropipette 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. 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 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., U.S.A.). 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. 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.

6. 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 Media and EGF on the *In Vitro* Maturation Rate

This experiment was conducted to investigate the EGF effect on the *in vitro* maturation of oocytes cultured in NCSU-23 or PZM-3. When oocytes were cultured 44 h in different maturation media supplemented with or without 20 ng/ml EGF, the *in vitro* maturation rates were shown in Table 1. The *in vitro* maturation rate of oocytes matured in NCSU-23 and PZM-3 with or without supplementation of EGF were 85.7±3.1%, 75.2±2.8%, 87.1±2.4% and 78.2±2.6%, respectively. The *in vitro* maturation rate of oocytes cultured in medium supplemented with EGF was significantly higher than cultured in none supplementation of EGF ($p<0.05$).

2. Effects of Media and β-ME on the *In Vitro* Maturation Rate

This experiment was conducted to investigate the β-ME effect on the *in vitro* maturation of oocytes cultured in NCSU-23 or PZM-3. When oocytes were cultured 44 h in different maturation

Table 1. *In vitro* maturation of oocytes cultured in different maturation media with or without supplementation of EGF

IVM condition		No. of oocytes examined	No. of oocytes at the stage of			Maturation rate*
Media	EGF		GV	GVBD	MⅡ	
NCSU-23	+	91	5	86	78	85.7±3.1[a]
NCSU-23	–	93	5	88	70	75.2±2.8[b]
PZM-3	+	85	4	81	74	87.1±2.4[a]
PZM-3	–	87	5	82	68	78.2±2.6[b]

* Data are presented as Mean±S.D.
[a,b] Values with different superscripts are significantly different ($p<0.05$).

media supplemented with or without 25 μM β-ME, the *in vitro* maturation rates were shown in Table 2. The *in vitro* maturation rate of oocytes matured in NCSU-23 and PZM-3 with or without supplementation of 25 μM β-ME were 79.5±2.6%, 74.7±2.5%, 80.2±2.3% and 78.6±2.7%, respectively. The *in vitro* maturation rate of oocytes cultured in PZM-3 supplemented with β-ME was higher than other group.

3. Effects of Media and Glucose on the *In Vitro* Maturation Rate

The *in vitro* maturation of oocytes cultured in different maturation media with or without supplementation of 1.5 mM glucose were shown in Table 3. The *in vitro* maturation rate of oocytes cultured for 44 h in NCSU-23 or PZM-3 maturation medium supplemented with or without 1.5 mM glucose were 79.2±2.3%, 75.0±2.6%, 85.5±2.5% and 78.9±2.7%, respectively. The *in vitro* maturation rate of oocytes cultured in PZM-3 supplemented with glucose was significantly higher than cultured in none supplementation of glucose ($p<0.05$).

4. Effects of EGF, 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 20 ng/ml EGF or 25 μM β-ME were shown in Table 4. When the embryos were cultured in NCSU-23 or PZM-3 supplemented with 20 ng/ml EGF or 25 μM β-ME for 48 h and 144

Table 2. *In vitro* maturation of oocytes cultured in different maturation media with or without supplementation of β-ME

IVM condition		No. of oocytes examined	No. of oocytes at the stage of			Maturation rate*
Media	β-ME		GV	GVBD	MⅡ	
NCSU-23	+	83	4	79	66	79.5±2.6
NCSU-23	–	87	4	83	65	74.7±2.5
PZM-3	+	91	5	86	73	80.2±2.3
PZM-3	–	89	4	85	70	78.6±2.7

* Data are presented as Mean±S.D.

Table 3. *In vitro* maturation of oocytes cultured in different maturation media with or without supplementation of glucose

IVM condition		No. of oocytes examined	No. of oocytes at the stage of			Maturation rate*
Media	glucose		GV	GVBD	MⅡ	
NCSU-23	+	53	3	50	42	79.2±2.3
NCSU-23	−	52	2	50	39	75.0±2.6
PZM-3	+	55	3	52	47	85.5±2.5[a]
PZM-3	−	57	3	54	45	78.9±2.7[b]

* Data are presented as Mean±S.D.
[a,b] Values with different superscripts are significantly different ($p<0.05$).

Table 4. 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*	
EGF	Media		≥ 2 cell	Blastocyst
NCSU-23	EGF	58	33 (56.4±2.7)	7 (12.0±1.3)
NCSU-23	β-ME	52	29 (54.3±2.9)	5 (9.6±1.7)
PZM-3	EGF	54	38 (70.5±2.1)	6 (10.9±2.1)
PZM-3	β-ME	59	41 (69.6±1.5)	7 (11.9±1.8)

* Data are presented as Mean±S.D.

h, the development rates to 2 cell and blastocyst stage were 56.4±2.7%, 12.0±1.3%, 54.3±2.9%, 9.6±1.7%, 70.5±2.1%, 10.9±2.1% and 69.6±1.5%, 11.9±1.8%, respectively. The developmental rate to the blastocyst stage of NT embryos cultured in both NUSU-23 supplemented with EGF was higher than control. The developmental rate to the blastocyst stage of NT embryos cultured in both PZM-3 supplemented with β-ME was higher than the control.

DISCUSSION

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 and 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, 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 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.

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. The *in vitro* maturation rate of oocytes matured in NCSU-23 and PZM-3 with or without supplementation of 20 ng/ml EGF were 85.7±3.1%, 75.2±2.8%, 87.1±2.4% and 78.2±2.6%, respectively. The *in vitro* maturation rate of oocytes cultured in medium supplemented with EGF was significantly higher than cultured in none supplementation of EGF ($p<0.05$).
2. The *in vitro* maturation rate of oocytes matured in NCSU-23 and PZM-3 with or without supplementation of 25 μM β-ME were 79.5±2.6%, 74.7±2.5%, 80.2±2.3% and 78.6±2.7%, respectively. The *in vitro* maturation rate of oocytes cultured in PZM-3 supplemented with β-ME was higher than other group.
3. The *in vitro* maturation rate of oocytes cultured for 44 h in NCSU-23 or PZM-3 maturation medium supplemented with or without 1.5 mM glucose were 79.2±2.3%, 75.0±2.6%, 85.5±2.5% and 78.9±2.7%, respectively. The *in vitro* maturation rate of oocytes cultured in PZM-3 supplemented with glucose was significantly higher than cultured in none supplementation of glucose ($p<0.05$).
4. When the embryos were cultured in NCSU-23 or PZM-3 supplemented with 20 ng/ml EGF or 25 μM β-ME for 48 h and 144 h, the development rates to 2 cell and blastocyst stage were 56.4± 2.7%, 12.0±1.3%, 54.3±

2.9%, 9.6±1.7%, 70.5±2.1%, 10.9±2.1% and 69.6±1.5%, 11.9±1.8%, respectively. The developmental rate to the blastocyst stage of NT embryos cultured in NUSU-23 supplemented with EGF was higher than control.

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(접수일: 2005. 6. 3 / 채택일: 2005. 7. 12)