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Study on Effects of Media, EGF, β-ME and Hormones on IVM of Porcine Oocytes

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배양액 종류, EGF, β-ME 및 호르몬이 돼지 난자의 체외 성숙율에 미치는 영향에 관한 연구

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SUMMARY

본 연구는 안정된 돼지 체외 성숙 난자를 얻을 목적으로 배양액의 종류 및 배양액에 EGF, β-ME, 호르몬 첨가가 돼지 난포란의 체외 성숙에 미치는 영향을 조사하였다.

난포란을 TCM-199, NCSU-23 및 PZM-3으로 48시간 배양했을 때 체외 성숙율은 각각 22.1±0.70%, 30.6±0.70% 및 30.4±2.82%였다. TCM-199로 48시간 배양했을 때 체외 성숙율은 NCSU-23 및 PZM-3 보다 약간 낮은 체외 발생율을 나타냈다. 난포란을 25 ng/ml의 EGF를 첨가한 TCM-199, NCSU-23 및 PZM-3로 48시간 배양했을 때 체외 성숙율은 각각 46.3±2.8%, 76.6±3.1% 및 72.2±2.6%로 나타났다. 난포란의 배양 시 배양액에 25 및 50 ng/ml의 EGF를 첨가 후 48시간 배양했을 때 첨가하지 않은 군에 비해 높은 체외 성숙율을 나타냈다($p<0.05$). 난포란을 NCSU-23 및 PZM-3 배양액에 25 μM/ml의 β-ME를 첨가한 후 48시간 배양했을 때 체외 성숙율은 각각 43.9±1.41%, 41.7±1.41%, 44.4±0.70%, 40.6±0.70%로 나타났다. 난포란을 25 μM/ml의 β-ME를 첨가한 NCSU-23로 48시간 배양했을 때 첨가하지 않은 군에 비해 높은 체외 성숙율을 나타냈다($p<0.05$). 난포란의 배양 시 NCSU-23에 PMSG, hCG, PMSG+hCG, hCG+β-estradiol, PMSG+β-estradiol을 첨가 후 배양하였을 때 체외 성숙율은 각각 75.6%, 77.8%, 80.0%, 86.4% 및 84.8%로서 무첨가 군(64.4%)에 비해 높게 나타났다($p<0.05$).

(Key words : porcine oocytes, EGF, β-ME, hormones, IVM rates)

INTRODUCTION

Several media support *in vitro* nuclear maturation of pig oocytes but not cytoplasmic maturation. Problems in cytoplasmic maturation interfere with the formation of the pronuclei after penetration of the sperm, despite normal germinal vesicle (GV) breakdown and extrusion of the first polar body (Abeydeera, 2002). Based on observations of maturation media supplemented with porcine follicular fluid (pFF), Yoshiba *et al.* (1992) concluded that pFF contains substances that improve the expansion of the cells of the cumulus oophorus, nuclear maturation and normal fertilization. Because pFF contains high concentrations of superoxide dismutase, it also plays an important role in the protection of oocytes

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against oxidative stress (Tatemoto *et al.*, 2004).

The main beneficial effect of Epidermal growth factor (EGF) is to stimulate the synthesis of intracellular glutathione (Abeydeera *er al.*, 2000). In addition to the beneficial effect on embryo development, glutathione protects the DNA, acts on the protein synthesis and amino acid transport, and promotes male pronuclear formation (Whitaker and Knight, 2004). 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; Quan *et al.*, 2004; Chance *et al.*, 1979). NCSU-23 medium is one of the more successful media for *in vitro* culture of porcine oocytes to MⅡ stage. Recently, however, it was reported the 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).

In the ovary, mammalian oocytes are arrested in the initial stage of meiotic division (germinal vesicle) until the release of the pre-ovulatory gonadotrophin that stimulates the resumption of meiosis (Richard and Sirard. 1996). During the final stage of maturation, immature oocytes develop from germinal vesicle (prophase I) to metaphase Ⅱ (MⅡ). At this stage, meiosis is again arrested, and this second arrest is normally interrupted by fertilization (Lonergan *et al.*, 2000). When fresh spermatozoa were cultured in various concentrations of β-ME for 2 h, β-ME neutralized the stimulatory effect of caffeine-benzoate on sperm capacitation and the spontaneous acrosome reaction at 50~250 mM/l. When 50 mM/l. and β-ME were added during a transient co-culture of gametes for 10 min, the sperm penetration rate was reduced 9 h after insemination (70.5 ~82.0% vs 90.5~94.0% in the absence of β-ME), but the incidence of monospermic penetration was not affect (Funahashi, 2005). When embryos from electro-activation (parthenotes) or *in vitro* fertilization (IVF-embryos) were cultured in PZM supplemented with 3 mg/ml bovine serum albumin (PZM-3) in 4-well dishes, in medium covered with oil in 4-well dishes or in droplets under oil, 0%, 33% and 20% of the parthenotes, and 11%, 23% and 20% of the IVF-embryos developed to blastocysts (Okada *et al.*, 2005). GV breakdown inhibition is possible through the inhibition of metaphase promoting factor (MPF). This is a protein complex composed of two main subunits, one a cyclin dependent kinase ($p34^{cdc2}$) and the other a cyclin (Alberts *et al.*, 1994). However, an urgent subject need to be increasement of the developmental rate porcine immature oocytes are low than that of other animals.

In order to determine better conditions for *in vitro* maturation of pig oocytes, this study also evaluated the arrest/resumption of oocyte maturation with media, EGF, β-ME and hormones.

MATERIALS AND METHODS

1. Collection 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 with an 18 gauge fixed to a 10 ml disposable syringe. The cumulus-oocytes complexes that had an evenly distributed cytoplasm and washed three times in oocyte maturation medium containing hormonal supplements.

2. *In Vitro* Maturation of Oocytes

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. The basic medium used for oocyte maturation were TCM-199, NCSU-23 and PZM-3 media supplemented with 25, 50 ng/ml epidermal growth factor (EGF), 25, 50 μM/ml β-ME) β-ME, 10 IU/ml hCG, 10 IU/ml PMSG + 10 IU/ml hCG, 10 IU/ml hCG + 1 μg/ml β-estradiol and 10 μg/ml LH.

3. Assessment of Meiotic Stage

Oocytes were fixed in acetic acid : ethanol (1:3) solution for 24 h then stained using with 1% acetoorcein (Sigma, U.S.A.) or 10 μg/ml bisbenzimide (Hoechst 33342, Sigma, U.S.A.) and observed under an fluorescence microscope. The judgement of oocytes maturation *in vitro* was carried out depending on the criteria of maturation by cell and nuclear division, and survival rate or *in vitro* development by investigating embryo development.

4. Statistical Analysis

The results were expressed by treatment as mean ±SD. For comparison of means, Duncan's multiple verification was performed using SAS package of general Linears Model procedures (SAS Institute).

RESULTS AND DISCUSSIONS

1. Effects of Kinds of Media on the IVM Rate

This experiment was conducted to investigate the effects of kinds of media on the IVM rate of porcine oocytes cultured in maturation medium for 0, 24, 48 and 72 hrs, the *in vitro* maturation rate were shown in Table 1.

The IVM rate of oocytes cultured in TCM-199, NCSU-23 and PZM-3 for 48 hrs were 25.4±0.70%, 30.4±0.70% and 30.4±2.82%, respectively. When the oocytes were cultured in TCM-199, NCSU-23 and PZM-3 for 48 hrs, the IVM rate were higher than cultured 24 or 72 hrs. When the oocytes were cultured in NCSU-23 and PZM-3 for 48 hrs., the IVM

Table 1. *In vitro* maturation rate of porcine oocytes cultured in different maturation media

Medium	No. of oocytes examined	Incubation time (hrs)	*In vitro* maturation rate (%) of oocytes		
TCM-199	250	0	18.8±0.70	24.8±0.00	56.4±0.70[a]
		24	22.1±0.70	33.3±0.70	44.6±0.70
		48	25.4±0.70	31.0±0.70	43.5±1.41[b]
		72	23.4±0.00	24.2±0.00	52.4±0.00
NCSU-23	250	0	19.2±1.41	24.4±0.70	56.4±0.70[a]
		24	25.6±0.70	31.1±0.70	43.3±1.44
		48	30.4±0.70	27.3±0.70	42.3±0.70[b]
		72	20.4±0.70	23.4±0.70	56.3±1.41
PZM-3	251	0	17.9±0.70	23.9±1.41	58.2±1.41[a]
		24	27.6±2.12	32.4±0.70	40.0±1.45[b]
		48	30.4±2.82	28.8±1.41	40.8±1.41[b]
		72	19.8±0.70	23.4±1.41	56.9±0.70

[ab] Values with different superscripts within column were significantly different (p<0.05).

rate were higher than cultured in TCM-199 for 48 hrs ($p<0.05$). This result was similar to Yeon *et al.* (2004) reported that the maturation rate to MII stage of porcine oocytes cultured in NCSU-23 and PZM-3 for 48 hrs, the IVM rate were higher than cultured in TCM-199 for 48 hrs.

2. Effects of EGF Concentration on the IVM Rate

This experiment was conducted to investigate the effects of concentration on the IVM rate of oocytes cultured maturation media for 0～72 hrs, the *in vitro* maturation rate were shown in Fig. 1 and 2.

The IVM rate of oocytes cultured in NCSU-23 supplementation with 25 ng/ml EGF for 48 hrs were 21.8±2.8% and 5.24±3.1%, respectively. When the oocytes were cultured in NCSU-23 and PZM-3 supplemented with 25 ng/ml for 48 hrs, the IVM rate were higher than cultured in none supplementation of EGF. The IVM rate of oocytes cultured in medium supplemented with EGF was significantly higher than cultured in non supplementation higher of EGF ($p<0.05$). This result was similar to Yeon *et al.* (2004) and Quan and Kim (2005) reported that the maturation rate of oocytes cultured in TCM-199 supplemented with 25 ng/ml EGF for 48 hrs higher than cultured in non supplementation higher of EGF.

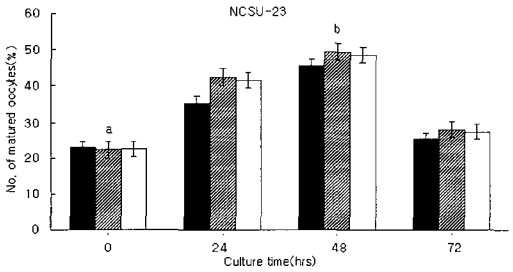

Fig. 1. The IVM rate of oocytes cultured in NCSU- 23 supplemented with 25 or 50 ng/ml EGF for 0~72 hrs(■ Control, ▨ 25 ng/ml, □ 50 ng/ml).

* ab Different letters (between ■ and ▨ group) indicate significant differences ($p<0.05$).

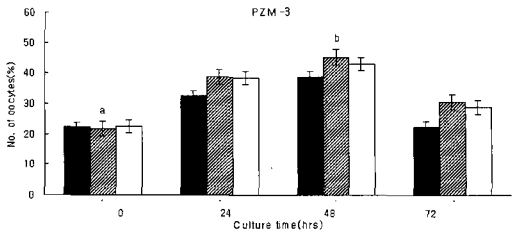

Fig. 2. The IVM rate of oocytes cultured in PZM-3 supplemented with 25 or 50 ng/ml EGF for 0~72 hrs(■ Control, ▨ 25 ng/ml, □ 50 ng/ml).

* ab Different letters (between ■ and ▨ group) indicate significant differences ($p<0.05$).

3. Effects of β-ME Concentration on the IVM Rate

The IVM rate of oocytes cultured in different maturation media with or without 25, 50 μM/ml β-ME for 0～72 hrs were shown in Fig. 3.

The IVM rate of oocytes were cultured in NCSU-23 and PZM-3 supplementation with 25, 50 μM/ml β-ME for 24 or 48 hrs were 35.5±1.41%, 32.2±0.70% and 43.9±1.41%, 41.7±1.41%, 44.4±0.70%, 40.6±0.70%, respectively. When the oocytes were cultured in NCSU-23 and PZM-3 supplemented with 25 μM/ml β-ME for 48 hrs, the IVM rate were higher than cultured in non supplementation of β-ME ($p<0.05$). This result was similar to Quan and Kim (2005) reported that the maturation rate of oocytes cultured in NCSU-23 and PZM-3 supplemented with 25 ng/ml β-ME for 48 hrs higher than culturedin non supplementation of β-ME.

4. Effects of Hormone Supplementation on the IVM Rate

The IVM rate of oocytes cultured in NCSU-23 with supplementation of hormones were shown in Table 2.

The IVM rate of oocytes cultured in NCSU-23 with supplementation of PMSG, hCG, PMSG+hCG, hCG+β-estradiol, PMSG+β-estradiol for 48 hrs were 75.6%, 77.8%, 80.0%, 86.4% and 84.8%, respectively, and the IVM rate of oocytes cultured in NCSU-23 supplemented with hormones for 48 hrs

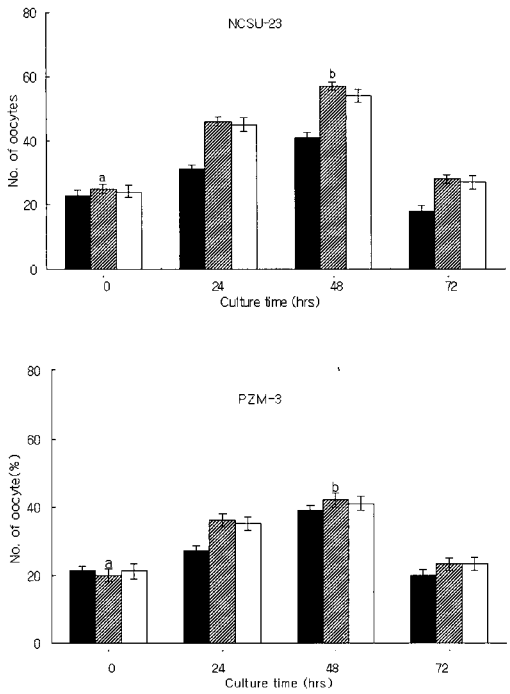

Fig. 3. The IVM rate of oocytes cultured in NCSU-23 and PZM-3 supplemented with 25 or 50 ng/ml β-ME for 0~72 hrs (■ Control, ▨ 25 ng/ml, □ 50 ng/ml).

* ^{ab} Different letters (between ■ and ▨ group) indicate significant differences (p<0.05).

Table 2. *In vitro* maturation rate of oocytes cultured in NCSU-23 with supplementation of hormones

Supplementation of hormone	No. of oocytes examined	No. of oocytes cultured for	
		24 h	48 h
Control	40	21(52.5)	20(50.0)^{a}
PMSG	45	29(64.4)	34(75.6)^{b}
hCG	45	30(66.7)	35(77.8)^{b}
PMSG+hCG	45	32(71.1)	36(80.0)^{b}
hCG+β-E.	44	31(70.5)	38(86.4)^{b}
PMSG+β-E.	46	33(71.7)	39(84.8)^{b}

* β-E. : β-estradiol

** Values with different superscripts within column were significantly different (p<0.05).

higher than cultured in non supplementation of hormones (64.4%). This result was similar to Shalgi *et al.* (1979), Ball *et al.* (1983) and Hensleigh and Hunter (1985) reported that the maturation rate of oocytes cultured in NCSU-23 supplemented with hormones for 48 hrs higher than cultured in non supplementation of hormones.

CONCLUSION

This study was investigated *in vitro* maturation rate of oocytes cultured in maturation media with or without supplementation of EGF, β-ME and hormones.

1. When the oocytes were cultured in TCM-199, NCSU-23 and PZM-3 for 48 hrs, the maturation rates were 25.4±0.70%, 30.4±0.70% and 30.4±2.82%, respectively. The maturation rate of oocytes cultured in TCM-199 was slightly lower than cultured in NCSU-23 and PZM-3 (p<0.05).
2. The IVM rate of oocytes cultured in TCM-199, NCSU-23 and PZM-3 supplementation with 25 ng/ml EGF for 48 hrs were 46.3± 2.8%, 76.6±3.1%, 72.2±2.6%, respectively. When the oocytes were cultured in TCM-199, NCSU-23 and PZM-3 supplemented with 25 ng/ml for 48 hrs, the IVM rate were higher than cultured in none supplementation of EGF (p< 0.05).
3. The IVM rate of oocytes were cultured in TCM-199, NCSU-23 and PZM-3 supplementation with 25 μM/ml β-ME for 24 or 48 hrs were 35.8±0.70%, 34.5±1.41%, 35.5±1.41 %, 32.2±0.70% and 43.9±1.41%, 41.7±1.41%, 44.4±0.70%, 40.6±0.70%, respectively. When the oocytes were cultured in TCM-199, NCSU-23 and PZM-3 supplemented with 25 μM/ml β-ME for 48 hrs, the IVM rate were higher than cultured in non supplementation higher of β-ME (p<0.05).

4. The IVM rate of oocytes cultured in TCM-199 with supplementation of MSG, hCG, PMSG +hCG, hCG+β-estradiol, PMSG+β-estradiol for 48 hrs were 75.6%, 77.8%, 80.0%, 86.4% and 84.8%, respectively, and the IVM rate of oocytes cultured in NCSU-23 supplemented with hormones for 48 hrs higher than cultured in non supplementation of hormones (64.4%).

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