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Synergetic effects of epidermal growth factor and estradiol on cytoplasmic maturation of porcine oocytes

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Summary

This study was conducted to examine the effect of epidermal growth factor (EGF) and 17β-estradiol (E_2) on nuclear and cytoplasmic (male pronuclear formation and early embryo development) maturation of porcine oocytes. Oocytes were aspirated from antral follicles and cultured in modified TCM-199 medium supplemented with 0.57 mM cysteine, 10 IU/ml eCG, 10 IU/ml hCG, with or without EGF and/or E_2. *In vitro* fertilisation of matured oocytes was performed in a modified Tris-buffered medium (mTBM) with frozen-thawed ejaculated spermatozoa. Oocytes were transferred to NCSU-23 supplemented with 0.4% bovine serum albumin at 6 h after *in vitro* fertilisation. Significantly higher ($p < 0.05$) rates of nuclear maturation, pronuclear formation and cleavage (91.7%, 65.2% and 37.3%, respectively) were observed when oocytes were cultured in the medium containing both EGF (10 ng/ml) and E_2 (1 μg/ml) than in the medium supplemented with either EGF or E_2 or without both. Intracellular glutathione concentration in the oocytes cultured in the medium containing both E_2 and EGF was also significantly higher (12.1 pmol per oocyte) than that of oocytes cultured in the medium with E_2 or EGF alone or without both. These findings suggested that EGF and E_2 have a synergestic effect on both nuclear and cytoplasmic maturation of porcine oocytes.

Keywords: EGF, Estradiol, *In vitro* maturation, Porcine oocyte

Introduction

Successful methods for *in vitro* maturation (IVM) and *in vitro* fertilisation (IVF) of porcine oocytes have not been developed although recent studies have significantly improved culture conditions, in which approximately 30% of fertilised oocytes can develop to the blastocyst stage (Abeydeera & Day, 1997*a*, *b*; Abeydeera *et al.*, 1998*a*, *b*; Wang *et al.*, 1997, 1999). Incomplete cytoplasmic maturation and high polyspermic fertilisation of oocytes are two unsolved problems for production of pig embryos *in vitro*. *In vitro* studies have indicated that addition of epidermal growth factor (EGF) to culture medium could improve both nuclear and cytoplasmic maturation in many mammals including pig (Reed *et al.*, 1993; Ding & Foxcroft, 1994; Coskun & Lin, 1995; Wang & Niwa, 1995*a*, *b*, 1997; Singh *et al.*, 1997; Grupen *et al.*, 1997; Abeydeera *et al.*, 1998*a*, 2000; Sirotkin *et al.*, 2000). Illera *et al.* (1998) reported that gonadotropins and porcine follicular fluid (pFF) could be replaced by addition of EGF and insulin-like growth factor I (IGF-I) to maturation media. As EGF was found in the follicular fluid (Westergaard & Anderson, 1989), it was suggested that EGF might participate in regulation of oocyte maturation.

On the other hand, stimulatory effects of estradiol on oocyte maturation have been observed (Moor *et al.*, 1980; Yoshimura *et al.*, 1989). However, such effects were not observed when pig oocytes were matured *in vitro* in a medium containing serum or follicular fluid (Yoshida *et al.*, 1989; Funahashi *et al.*, 1993). It is possible that growth factors and steroid hormones or gonadotropins regulating oocyte maturation through some known and unknown mechanisms individually or together. Although the effects of EGF alone or estradiol alone on the porcine oocyte maturation were examined, addition of both to oocyte maturation

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medium and their effects on nuclear and/or cytoplasmic maturation have not been examined. Also previous studies were conducted in media containing serum or follicular fluid, in which hormones and growth factors are present, and thus the exact effects of the supplementation may be masked. Therefore, in the present study we examined the effects of EGF and/or estradiol-17β (E_2) on nuclear and cytoplasmic maturation of porcine oocytes in a serum-free medium.

Materials and methods

Culture medium

Chemicals were purchased from Sigma Chemical Co. unless stated otherwise. The basic medium, designated TCM-199B (pH 7.4), used for the maturation of oocytes was tissue culture medium (TCM) 199 (with Eagle's salts; Gibco, USA) supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 75 μg potassium penicillin G/ml and 50 μg streptomycin sulfate/ml. This medium was essentially the same as that used by Wang *et al.* (1992, 1994) except that calcium lactate was omitted in the present study. Also 10 IU hCG and 10 IU PMSG/ml were added to the maturation medium before culture. Insemination medium was modified Tris-buffered medium (mTBM) that was exactly the same as our previous report (Wang *et al.*, 1997). Embryo culture medium was North Carolina State University (NCSU) 23 medium (Petters *et al.*, 1993) containing 0.4% bovine serum albumin (BSA; A-8022).

Preparation and culture of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 2 h in 0.9% (w/w) NaCl solution containing 75 μg potassium penicillin G/ml and 50 μg streptomycin sulfate/ml at 30–35 °C. Cumulus–oocyte complexes (COCs) were aspirated from antral follicles 2–5 mm in diameter with an 18 gauge needle fixed to a 10 ml disposable syringe. The COCs were washed three times with Hepes-TL-PVA and three times with maturation medium. Batches of 60 COCs were transferred to maturation medium supplemented with 10 ng/ml EGF and/or 1 μg E_2, which had been previously covered with warm paraffin oil in a polystyrene culture dish (35 × 10 mm) and equilibrated in an atmosphere of 5% CO_2 in air for about 3 h, and cultured at 39 °C for 44 h in the same conditions. After culture, some oocytes (one-third) were fixed to examine nuclear maturation; subgroups of oocytes were inseminated or used for glutathione assay.

Sperm preparation and *in vitro* fertilisation

After culture, oocytes were freed from cumulus cells in the maturation medium containing 0.1% (w/v) hyaluronidase obtained from bovine testis (Type I-S, H-3506), and then washed three times with the insemination medium (mTBM). Thereafter, batches of 40 oocytes were transferred into a 50 μl droplet of mTBM covered with paraffin oil. The dishes were kept in a CO_2 incubator until spermatozoa were added for insemination. For IVF, one 0.1 ml frozen semen pellet, made as described by Wang *et al.* (1991), was thawed at 39 °C in Dulbecco's phosphate-buffered saline (DPBS) containing 1 mg BSA/ml (Fraction V, A-7888) and antibiotics. After three washes, spermatozoa were resuspended with mTBM containing 2 mM caffeine to give a concentration of 1×10^6 cells/ml, and 50 μl of sample was added to 50 μl of the fertilisation drop containing the oocytes. Six hours after insemination at 39 °C in an atmosphere of 5% CO_2 in air, oocytes were removed from fertilisation drops, washed four times in embryo culture medium and cultured in 500 μl of the same culture medium in a four-well culture plate (Nunc, Roskilde) for examination of fertilisation and early development.

Assessment of nuclear maturation, pronuclear formation and cleavage

After 44 h of maturation culture, oocytes were denuded from enclosed cumulus, mounted on slides, fixed for 48–72 h in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid and examined under a phase-contrast microscope at a magnification of ×400. Nuclear stages were classified as germinal vesicle (GV), GV breakdown (GVBD), metaphase I (M-I), metaphase II (M-II) (Wang *et al.*, 1994). For evaluation of fertilisation, 16 h after insemination, oocytes were also mounted on slides, fixed, stained and examined using the method described above. Cleavage was evaluated under a stereomicroscope 48 h after insemination.

Glutathione assay

The glutathione assay was based on the procedures reported by Funahashi *et al.* (1996) and Wang *et al.* (1997). Briefly, after maturation cumulus cells were removed completely from oocytes and the oocytes washed three times with a stock buffer (0.2 M sodium phosphate/l containing 10 mM sodium EDTA, pH 7.2). Five microlitres of stock buffer containing 10–30 oocytes was transferred to a 1.5 ml microfuge tube. The oocytes were ruptured by agitation with a narrow-bore glass pipette, and 5 μl of 1.25 mM phosphoric acid was added to the tube. The tubes containing samples were

kept in a freezer at –20 °C until assay. The concentration of glutathione in oocytes was determined by the 5,5′-dithiobis-(2-nitrobenzoic acid) and glutathione disulfide reductase recycling assay (Anderson, 1985).

Statistical analysis

Four replicate trials for examination of oocyte nuclear maturation, fertilisation and development were conducted. All percentage data were subjected to arc sine transformation before statistical analysis. Comparisons among maturation media containing different kinds of hormones were analysed by ANOVA. Three replicate trials for glutathione content were conducted. The data were expressed as mean ± SEM and analysed by Student's *t*-test.

Results

Compared with control (no EGF or E_2), addition of EGF or E_2 to the maturation medium individually did not increase M-II rates (70.7% to 72.5%; Table 1), male pronuclear formation rates (48.9% to 37.9%; Table 2), cleavage rates (23.6% to 22.7%; Table 3) or intracellular glutathione concentrations (5.3 ± 0.6 to 6.9 ± 0.4 pmol per oocyte; Fig. 1). However, addition of both EGF and E_2 significantly ($p < 0.05$) increased maturation (M-II) rate (91.7%), male pronuclear formation rate (65.2%) and cleavage rate (37.3%). Intracellular glutathione concentration (12.1 ± 1.8 pmol per oocyte) was also significantly ($p < 0.01$) increased compared with other groups.

Table 1 Nuclear maturation of oocytes after 44 h IVM with or without EGF (10 ng/ml) and/or E_2 (1 μg/ml) in the maturation medium

Addition of		Total no. of	No. of oocytes at the stage of		
E_2	EGF	oocytes examined	GV	GVBD–MI	MII(%)
–	–	6	1	12	48 (78.7)[a]
+	–	75	0	22	53 (70.7)[a]
–	+	80	0	22	58 (72.5)[a]
+	+	72	0	6	66 (91.7)[b]

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

Table 2 Pronuclear formation of oocytes after IVM–IVF with frozen-thawed sperm

Addition of		No. of oocytes inseminated	No. (%) of oocytes fertilised	No. of fertilized oocytes with	
E_2	EGF			FPN and sperm head(s) (%)	MPN and FPN (%)
–	–	72	34 (47.2)[a]	15 (44.1)	19 (55.9)[a]
+	–	45	22 (48.9)[a]	11 (50.0)	11 (50.0)[a]
–	+	66	25 (37.9)[a]	14 (56.0)	11 (44.0)[a]
+	+	69	45 (65.2)[b]	15 (33.3)	30 (66.7)[b]

[a,b]Values with different superscripts within the same column are significantly different ($p < 0.05$).
FPN, female pronucleus; MPN, male pronucleus.

Table 3 Development of porcine oocytes after IVM in medium with and/or without E_2 and EGF

Addition of		No. of oocytes cultured	No. (%) of oocytes cleaved	No. (%) of embryos at the stages of		
E_2	EGF			2–3 cell (%)	4–7 cell (%)	≥8 cell (%)
–	–	134	26 (19.4)[a]	7 (26.9)	14 (53.8)	5 (19.2)
+	–	110	26 (23.6)[a]	10 (38.5)	12 (46.2)	4 (15.4)
–	+	154	35 (22.7)[a]	12 (34.3)	14 (40.0)	9 (25.7)
+	+	177	66 (37.3)[b]	17 (25.8)	34 (51.5)	15 (22.7)

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

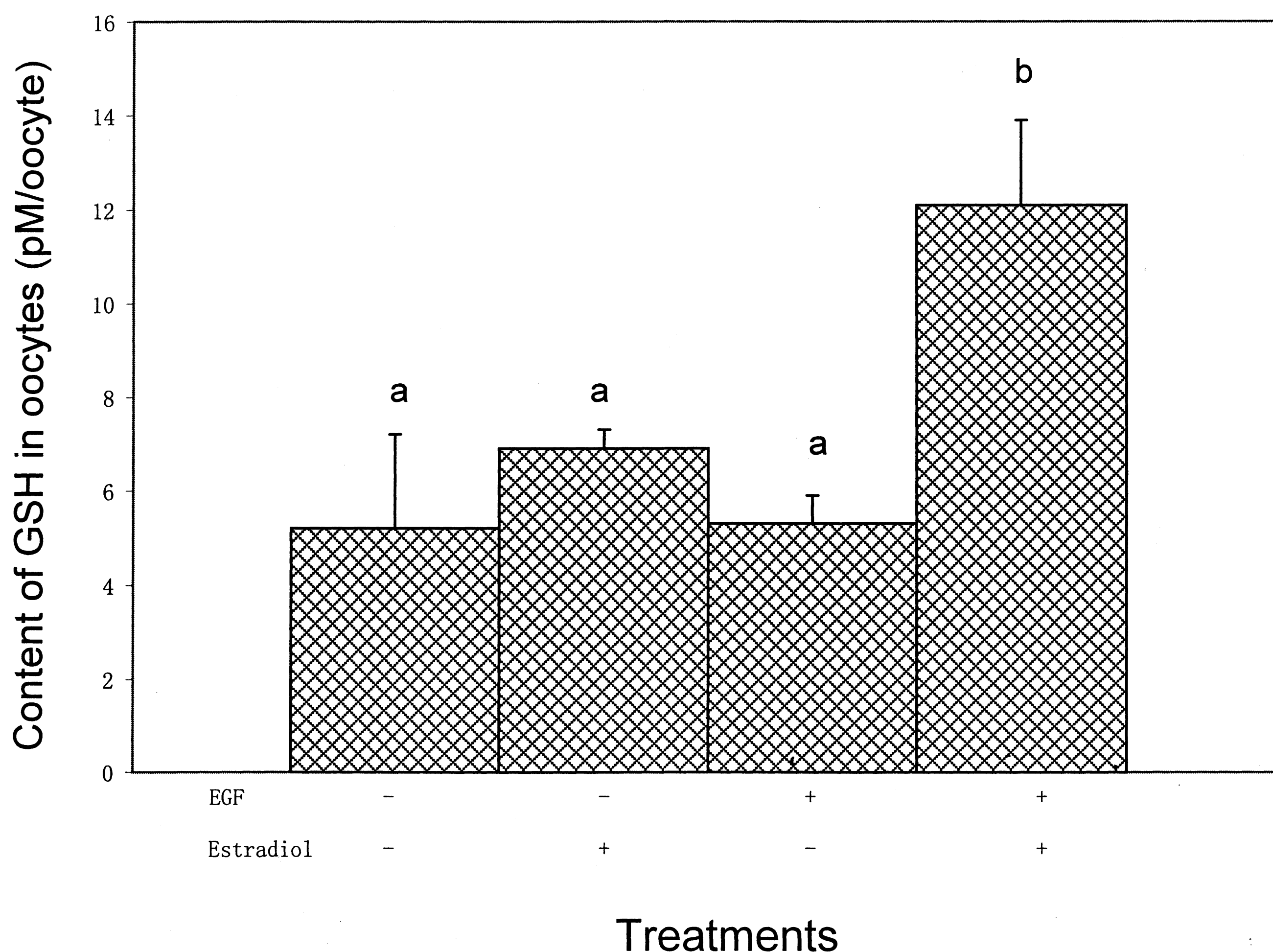

Figure 1 Glutathione (GSH) concentrations in oocytes after 44 h of IVM in medium with or without EGF (10 ng/ml) and/or estradiol (1 µg/ml). Experiments were repeated three times. Columns with different letters are significantly different ($p < 0.05$).

Discussion

The present study indicates that the addition of EGF or E_2 alone does not improve nuclear and cytoplasmic maturation of porcine oocytes in a serum-free medium supplemented with gonadotropins; however, addition of both EGF and E_2 to the medium significantly enhances both nuclear and cytoplasmic maturation, as revealed by increased rates of oocytes reaching M-II, forming male pronuclei and undergoing early development, and by increased intracellular glutathione synthesis.

It was reported that E_2 inhibited GVBD and nuclear maturation in COCs in maturation medium without gonadotropins (Singh *et al.*, 1993; Bing *et al.*, 1999). However, in a medium with cysteamine and gonadotropins, addition of E_2 could improve nuclear maturation (Bing *et al.*, 1999). It seems that gonadotropins are necessary for porcine oocyte to undergo nuclear maturation. Thus we examined the effects of E_2 in the presence of gonadotropins. We found that addition of E_2 alone did not improve nuclear maturation and cytoplasmic maturation. However, when E_2 and EGF were both supplemented in the medium, both nuclear and cytoplasmic maturation were significantly increased. The different results on E_2 from different researchers may be due to different culture media used. In most studies, fetal calf serum (FCS) was added to the maturation media, while the IVM system used in this study was a protein-free system. A protein-free medium has advantages over a serum-containing medium for examination of the effects of an individual component on oocyte maturation.

It has been shown that oocytes require a specific intrafollicular steroid environment for the completion of the full maturation process and that alteration of the steroid profile during maturation leads to gross abnormalities at fertilisation (Moor *et al.*, 1980). Yoshimura *et al.* (1989) also found that addition of E_2 to maturation medium containing gonadotropins and prolactin increased the rates at which rabbit ova develop to morulae and blastocysts. Therefore, the slightly inhibitory effect of E_2 on gonadotropin-induced

nuclear maturation may be of importance in delaying meiotic maturation to allow better synchrony of cytoplasmic maturational processes. In this study, cytoplasmic maturation was verified by male pronuclear formation, early cleavage and intracellular glutathione concentration: all data were increased after addition of both E_2 and EGF to the culture medium. *In vitro* maturation of sheep oocytes also indicated that addition of E_2 to the maturation medium was not required when follicular fluid had already been added to the IVM medium (Guler *et al.* 2000). However, in defined conditions supplementation of E_2 did have a positive effect on early embryo cleavage and blastocyst formation (Guler *et al.*, 2000).

The stimulatory effect of EGF on oocyte maturation was obvious even in the medium without gonadotropin (Ding & Foxcroft, 1994; Wang *et al.*, 1995*a*, *b*). The concentration of EGF in follicular fluid ranged between 0.60 and 2.42 ng/ml, corresponding to approximately 50% of the level in human serum at the time of follicle aspiration (Westergaard & Anderson, 1989). EGF can induce porcine oocyte maturation in both complex media and serum-free media (Reed *et al.*, 1993; Singh *et al.*, 1993, 1997; Ding & Foxcroft, 1994; Wang & Niwa, 1995*a*, *b*; Grupen *et al.*, 1997; Abeydeera *et al.*, 1998*a*; Illera *et al.*, 1998; Sirotkin *et al.*, 2000). However, a stimulatory effect of EGF alone on nuclear or cytoplasmic maturation was not observed in the present IVM system. Abeydeera *et al.* (1998a) found that the presence or absence of EGF during IVM did not affect cumulus expansion, nuclear maturation, fertilisation parameters or cleavage rate, but increased blastocyst development in a concentration-dependent manner. However, Singh *et al.* (1997) found no difference in protein synthesis pattern when pig oocytes were matured in TCM-199-FCS with or without EGF. Our study indicates that nuclear and cytoplasmic maturation of porcine oocytes was increased after addition of both E_2 and EGF, although the mechanism is unknown.

Cytoplasmic maturation as evidenced by pronuclear formation (Wang *et al.*, 1995) and developmental competence to cleavage or blastocyst stage (Abeydeera 1998*a*) of pig oocytes is closely correlated with intracellular glutathione content (Wang *et al.*, 1997; Abeydeera 1998*b*, 2000). Therefore, we examined the glutathione concentration of pig oocytes matured in mTCM-199B supplemented with both EGF and E_2. The result clearly showed that EGF and E_2 significantly improved the synthesis of glutathione in oocytes. A higher glutathione content in oocytes may lead to retention of more glutathione than in those oocytes with a lesser amount of glutathione after fertilisation. Subsequently, embryos with a higher concentration of glutathione can overcome the oxidative stress during *in vitro* culture. it was reported that free oxygen radicals produced by oxygen were harmful to embryo development (Legge *et al.*, 1991) and intracellular glutathione could enhance the ability of the oocyte to eliminate cytotoxic effects of hydrogen peroxide (Nasr-Esfahani *et al.*, 1990).

In conclusion, the present study indicates that supplementation of maturation medium with both EGF and E_2 can produce synergetic effects on porcine oocyte nuclear and cytoplasmic maturation *in vitro* in a protein-free maturation system.

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References

Abeydeera, L.R. & Day, B.N. (1997*a*). Fertilization and subsequent development *in vitro* of pig oocytes inseminated in a modified Tris-Buffered medium with frozen-thawed ejaculated spermatozoa. *Biol. Reprod.* **57**, 729–34.

Abeydeera, L.R. & Day, B.N. (1997*b*). *In vitro* penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. *Theriogenology* **48**, 537–44.

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Rieke, A., Prather, R.S. & Day, B.N. (1998*a*). Presence of epidermal growth factor during *in vitro* maturation of pig oocytes and embryo culture can modulate blastocyst development after *in vitro* fertilization. *Mol. Reprod. Dev.* **51**, 395–401.

Abeydeera, L.R., Wang, W.H., Prather, R.S. & Day, B.N. (1998*b*). Maturation *in vitro* of pig oocytes in protein-free culture media: fertilization and subsequent embryo development *in vitro*. *Biol. Reprod.* **58**, 1316–20.

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Rieke, A., Murphy, C.N., Prather, R.S. & Day, B.N. (2000). Development and viability of pig oocytes matured in a protein-free medium containing epidermal growth factor. *Theriogenology* **54**, 587–97.

Anderson, M.E. (1985). Determination of glutathione and glutathione disulfide in biological samples. In: *Glutamate, Glutamine, Glutathione and Related Compounds* (ed. A. Meister), pp. 548–55. New York: Academic Press.

Bing, Y.Z., Nagai, T. & Rodriguez-Martinez, H. (1999). Effects of cysteamine, FSH and estradiol-17β on *in vitro* maturation of porcine oocytes. *Theriogenology* **55**, 867–76.

Coskun, S. & Lin, Y.C. (1995). Mechanism of action of epidermal growth factor-induced porcine oocyte maturation. *Mol. Reprod. Dev.* **42**, 311–7.

Ding, J. & Foxcroft, G.R. (1994). Epidermal growth factor enhances oocyte maturation in pigs. *Mol. Reprod. Dev.* **39**, 30–40.

Funahashi, H. & Day, B.N. (1993). Effects of the duration of exposure to supplemental hormones on cytoplasmic maturation of pig oocytes *in vitro*. *J. Reprod. Fertil.* **98**, 179–85.

Funahashi, H., Kim, N.H., Stumpf, T.T., Catley, T. & Day, B.N. (1996). Presence of organic osmolytes in maturation medium enhances cytoplasmic maturation of pig oocytes. *Biol. Reprod.* **54**, 1412–19.

Grupen, C.G., Nagashima, H. & Nottle, M.B. (1997). Role of epidermal growth factor and insulin-like growth factor-I on porcine oocyte maturation and embryonic development *in vitro*. *Reprod. Fertil. Dev.* **9**, 571–5.

Guler, A., Poulin, N., Mermillod, P., Terqui, M. & Cognie, Y. (2000). Effect of growth factors, EGF and IGF-I, and estradiol on *in vitro* maturation of sheep oocytes. *Theriogenology* **54**, 209–18.

Illera, M.J., Lorenzo, P.L., Illera, J.C., Petters, R.M. (1998). Developmental competence of immature pig oocytes under the influence of EGF, IGF-I, follicular fluid and gonadotropins during IVM–IVF processes. *Int. J. Dev. Biol.* **42**, 1169–72.

Legge, M. & Sellens, M.H. (1991). Free radical scavengers ameliorate the 2-cell block in mouse embryo culture. *Hum. Reprod.* **6**, 867–71.

Moor, R.M., Polge, C. & Willadsen, S.M. (1980). Effect of follicular steroids on the maturation and fertilization of mammalian oocytes. *J. Embryol. Exp. Morphol.* **56**, 319–35.

Nasr-Esfahani, M.H., Aitken, J.R. & Johnson, M.H. (1990). Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryo developed *in vitro* or *in vivo*. *Development* **109**, 501–7.

Petters, R.M. & Wells, K.D. (1993). Culture of pig embryos. *J. Reprod. Fertil. Suppl.* **48**, 61–73.

Reed, M., Estrada, J.L., Illera, M.J. & Petters, R.M. (1993). Effects of epidermal growth factor, insulin-like factor-I, and dialyzed porcine follicular fluid on porcine oocyte maturation *in vitro*. *J. Exp. Zool.* **266**, 74–8.

Singh, B., Barbe, G.J. & Armstrong, D.T. (1993). Factors influencing resumption of meiotic maturation and cumulus expansion of porcine oocyte-cumulus cell complexes *in vitro*. *Mol. Reprod. Dev.* **36**, 113–9.

Singh, B., Meng, L., Rutledge, J.M. & Armstrong, D.T. (1997). Effects of epidermal growth factor and follicle-stimulating hormone during *in vitro* maturation on cytoplasmic maturation of porcine oocytes. *Mol. Reprod. Dev.* **46**, 401–7.

Sirotkin, A.V., Dukesova, J., Makarevich, A.V., Kubek, A. & Bulla, J. (2000). Evidence that growth factors IGF-I, IGF-II and EGF can stimulate nuclear maturation of porcine oocytes via intracellular protein kinase A. *Reprod. Nutr. Dev.* **40**, 559–69.

Wang, W.H., Niwa, K. & Okuda, K. (1991). *In vitro* penetration of pig oocytes matured in culture by frozen-thawed ejaculated spermatozoa. *J. Reprod. Fertil.* **93**, 491–6.

Wang, W.H., Uchida, M. & Niwa, K. (1992). Effects of follicle cells on *in vitro* penetration of pig oocytes by cryopreserved, ejaculated spermatozoa. *J. Reprod. Dev.* **38**, 125–31.

Wang, W.H., Abeydeera, L.R., Okuda, K. & Niwa, K. (1994). Penetration of porcine oocytes during maturation *in vitro* by cryopreserved, ejaculated spermatozoa. *Biol. Reprod.* **50**, 510–15.

Wang, W.H. & Niwa, K. (1995*a*). Effect of epidermal growth factor and gonadotrophins on cumulus expansion and nuclear maturation of pig oocytes in serum-free medium. *Assist. Reprod. Tech. Andr.* **7**, 41–5.

Wang, W.H. & Niwa, K. (1995*b*). Synergetic effects of epidermal growth factor and gonadotropins on the cytoplasmic maturation of pig oocytes in a serum-free medium. *Zygote* **3**, 345–50.

Wang, W.H., Abeydeera, L.R., Cantley, T.C. & Day, B.N. (1997). Effects of oocyte maturation media on development of pig embryos produced by *in vitro* fertilization. *J. Reprod. Fertil.* **111**, 101–8.

Wang, W.H., Abeydeera, L.R., Han, Y.M., Prather, R.S. & Day, B.N. (1999). Morphologic evaluation and actin filament distribution in porcine embryos produced *in vitro* and *in vivo*. *Biol. Reprod.* **60**, 1020–8.

Westergaard, L.G. & Anderson, C.Y. (1989). Epidermal growth factor in human preovulatory follicles. *Hum. Reprod.* **4**, 257–60.

Yoshida, M., Bamba, K. & Kojima, Y. (1989). Effects of gonadotropins and estradiol-17β on the timing of nuclear maturation and cumulus mass expansion in pig oocytes cultured *in vitro*. *Jpn. J. Anim. Reprod.* **35**, 86–91.

Yoshimura, Y., Hosoi, Y., Iritani, A., Nakamura, Y., Atlas, S.J. & Wallach, E.E. (1989). Developmental potential of rabbit oocytes matured *in vitro*: the possible contribution of prolactin. *Biol. Reprod.* **41**, 26–33.