

Effects of the duration of exposure to hormone supplements on cytoplasmic maturation of pig oocytes *in vitro*

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The effect of hormone supplements on cytoplasmic maturation *in vitro* was examined by incubating oocyte–cumulus complexes in a medium with PMSG (10 iu ml^{-1}), hCG (10 iu ml^{-1}) and oestradiol ($1 \mu\text{g ml}^{-1}$) for various periods and then transferring them to medium without added hormones for the remainder of the maturation period. Exposure of oocyte–cumulus complexes to hormone supplements for 2 h improved only germinal vesicle breakdown and maturation rates compared with complexes not exposed to added hormones. The removal of hormone supplements at 20 h after the start of culture enhanced the ability of oocytes to form male pronuclei 10 to 12 h after insemination. Further, the effects of transfer of intact and oocyctomized oocyte–cumulus complexes to hormone-free medium at 20 h on cumulus expansion were examined. The diameter and morphology of the intact oocyte–cumulus complexes were improved after the removal of oocyte–cumulus cell complexes from hormonal exposure. The responses of oocyctomized oocyte–cumulus complexes to hormone were similar to those of intact oocyte–cumulus complexes with the exception of corona radiata expansion. The results suggest that the removal of hormone supplements from maturation media at 20 h after culture enhanced cytoplasmic maturation and cumulus expansion. Further, cumulus expansion does not appear to depend on intercellular communication between cumulus cells and oocytes. Oocyctomy did influence expansion of the corona radiata during culture.

Introduction

Pig follicular oocyte–cumulus complexes have been cultured in media supplemented with several combinations of hormones: PMSG, hCG and oestradiol (Yoshida *et al.*, 1990, 1992; Wang *et al.*, 1991); FSH, LH, prolactin and oestradiol (Mattioli *et al.*, 1988a); FSH, LH and oestradiol (Zheng and Sirard, 1992); FSH, LH and prolactin (Mattioli *et al.*, 1988b; Nagai and Moor, 1990); FSH and LH (Mattioli *et al.*, 1990, 1991; Galeati *et al.*, 1991); LH and oestradiol (Nagai *et al.*, 1988); FSH (Naito *et al.*, 1988); and LH (Nagai *et al.*, 1990; Mattioli *et al.*, 1991). The addition of hormone supplements into maturation media enhances nuclear maturation and cumulus expansion of the oocyte–cumulus complexes (Meinecke and Meinecke-Tillmenn, 1979; Hillensjo and Channing, 1980; Minato and Toyoda, 1982; Yoshida *et al.*, 1989; Prochazka *et al.*, 1991). In addition, the supplementation of FSH together with follicular fluid (Naito *et al.*, 1988) or the addition of LH with follicular shells (Mattioli *et al.*, 1991) improves cytoplasmic maturation as determined by male pronuclear formation. In control or superovulated pigs, the concentrations of gonadotrophins and steroids in follicular fluid change dramatically during preovulatory oocyte maturation (Moor, 1974; McNatty *et al.*, 1975; Hunter *et al.*, 1976; Ainsworth *et al.*, 1980; Lenton *et al.*, 1988). Oocyte maturation *in vivo* is induced by the preovulatory surge of gonadotrophins via granulosa

cells (Eppig, 1991) and also cumulus expansion can be induced with FSH or LH (Hillensjo and Channing, 1980). Cytoplasmic maturation is considered to require a strictly regulated sequence of hormonal changes during maturation (Osborn and Moor, 1983). The change in hormonal concentration during maturation, therefore, may be of considerable importance for the secretory activity of cumulus cells, a subset of granulosa cells. Consequently, meiotic or cytoplasmic maturation of oocytes may be affected indirectly by the degree and duration of hormonal exposure of cumulus cells.

The present study was undertaken to examine the effects of timed exposure of pig oocyte–cumulus complexes to hormone supplements on the cumulus expansion and male pronuclear formation after *in vitro* fertilization. Furthermore, the effect of intercellular communication between cumulus cells and oocytes on cumulus expansion was determined by culture of intact and oocyctomized oocyte–cumulus complexes.

Materials and Methods

Culture media

The basic medium (mM199) for the manipulation of oocytes and spermatozoa was Medium 199 with Earle's salts (Gibco BRL, Life Technologies Inc., Grand Island, NY) supplemented with $3.05 \text{ mmol D-glucose l}^{-1}$ (Sigma Chemical Co., St Louis, MO), $2.92 \text{ mmol calcium lactate l}^{-1}$ (Sigma), 0.91 mmol sodium

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pyruvate l^{-1} (Sigma), 75 μg potassium penicillin G ml^{-1} (Sigma) and 50 μg streptomycin sulphate ml^{-1} (Sigma). The basic medium for oocyte maturation (OMM199) was mM199 supplemented with hormonal supplements, which were 10 iu PMSG ml^{-1} (Intervet America Inc., DE), 10 iu hCG ml^{-1} (LyphoMed Inc., Rosemont, IL), 1 μg oestradiol ml^{-1} (Sigma) and 10% (v/v) porcine follicular fluid (pFF). The hormone supplements were added according to previous reports (Yoshida *et al.*, 1990; Wang *et al.*, 1991; Yoshida *et al.*, 1992). pFF was used in the present study because in preliminary experiments pFF, rather than fetal calf serum (FCS), was shown to be a better supplement for maturation medium (Funahashi and Day, 1993). pFF was collected with oocyte–cumulus complexes used in this study from superficial follicles, 3–6 mm in diameter, of prepubertal pig ovaries within 4 to 6 h after slaughter. Thereafter, pFF was heat inactivated for 30 min at 56°C, filtered through 1.2 and 0.45 μm syringe filters and stored at -20°C until use.

Preparation and culture of follicular oocytes

Ovaries were collected from prepubertal gilts at a local abattoir. Transportation of ovaries to the laboratory was carried out at 25°C in Dulbecco's phosphate-buffered saline supplemented with 5.54 mmol D-glucose l^{-1} , 0.33 mmol sodium pyruvate l^{-1} , 75 μg potassium penicillin G ml^{-1} and 50 μg streptomycin sulfate ml^{-1} (mDPBS). Oocytes were aspirated through an 18-gauge needle into a disposable 10 ml syringe from follicles 3–6 mm in diameter. Oocyte–cumulus complexes with uniform ooplasm and a compact cumulus cell mass were collected in mDPBS with 2% (v/v) newborn piglet serum and then washed three times with OMM199 pre-gassed in a CO_2 incubator (in an atmosphere of 5% CO_2 in air) at 39°C for more than 3 h. Ten oocyte–cumulus complexes were transferred to a droplet of 100 μl of OMM199 covered with paraffin oil in a culture dish (Falcon, Beckton Dickinson and Co., Lincoln Park, NJ), which were pre-gassed in a CO_2 incubator at 39°C for more than 3 h, and then cultured at 39°C in an atmosphere of 5% CO_2 in air. At 0, 2, 20, 30 and 40 h after the start of culture, the oocyte–cumulus complexes were cultured in OMM199 without PMSG, hCG and oestradiol for 40, 38, 20, 10 and 0 h, respectively.

Sperm preparation

Sperm-rich fractions (15 ml) were collected from a boar by the gloved hand method, and after adding antibiotics (75 μg potassium penicillin G ml^{-1} and 50 μg streptomycin sulfate ml^{-1}) the semen samples were kept at 20°C for 16 h (Cheng, 1985). Thereafter, the semen was washed three times with 0.9% NaCl plus 10 mg BSA ml^{-1} (fraction V, Sigma) by centrifugation (1200 g for 3 min) to remove seminal plasma. At the end of washing, the spermatozoa were resuspended at 2×10^8 cells ml^{-1} in mM199 at pH 7.8 and supplemented with 10% FCS. The sperm suspension was preincubated for 90 min at 39°C in an atmosphere of 5% CO_2 in air.

In vitro fertilization

After 40 h of culture, oocytes were used for *in vitro* fertilization. The ten oocyte–cumulus complexes were washed three

times with mM199 at pH 7.4 supplemented with 10 mmol caffeine sodium benzoate l^{-1} and 10% FCS, and then placed into a droplet of 50 μl of the mM199 under paraffin oil, which had been pre-gassed in a CO_2 incubator at 39°C for more than 3 h. The dish was placed in an incubator at 39°C in 5% CO_2 in air until spermatozoa were added for insemination. Preincubated spermatozoa, 50 μl , were added to 50 μl of medium containing oocytes following dilution from 2×10^8 cells ml^{-1} to 2×10^6 cells ml^{-1} with mM199 at pH 7.8 and supplemented with 10% FCS (final sperm concentration: 1×10^6 cells ml^{-1}). Oocytes were cultured with spermatozoa for 10 to 12 h at 39°C.

Assessment of cytoplasmic maturation

At the end of the culture following insemination, eggs were removed from the droplet and aspirated in 4 ml mDPBS supplemented with 0.1% hyaluronidase to remove cumulus cells and supernumerary spermatozoa from the zona pellucida. Thereafter, eggs were mounted, fixed for 48 h or more in 25% (v/v) acetic alcohol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid and examined under a phase-contrast microscope at $\times 400$ magnification. The meiotic stage of the oocytes was assessed according to Hunter and Polge (1966). Oocytes at the stage of anaphase I to metaphase II were regarded as matured. Oocytes were designated as penetrated when they had at least one swollen sperm nucleus or a male pronucleus and corresponding sperm tail in the vitellus. Those oocytes with more than one swollen sperm nucleus or male pronucleus were considered to be polyspermic.

Measurement of cumulus diameter and assessment of cumulus expansion

The effects of changes in hormonal culture conditions during maturation on cumulus expansion were examined by culturing oocyte–cumulus complexes in OMM199 without hormone supplements (group A), in OMM199 for 20 h with added hormones followed by culture with no hormones (group B) or in OMM199 with hormones (group C) in a droplet covered with warm paraffin oil. Each droplet contained one oocyte–cumulus complex. Cumulus cell expansion was evaluated by measurement of cumulus diameter and assessment of the degree of cumulus expansion after 0, 20, 30 and 40 h of culture. The diameter of a single oocyte–cumulus complex was determined by measuring minimum and maximum diameter with an eye piece micrometer under stereomicroscope at $\times 40$ magnification and by calculating a mean diameter as (minimum diameter + maximum diameter) $\times 2^{-1}$. The degree of cumulus expansion was assessed according to a subjective scoring system from 0 to 4 as described by Vanderhyden *et al.* (1990).

Oocyectomy

The effect of intercellular communication between cumulus cells and oocytes on the enhanced cumulus expansion by removal of added hormones at 20 h was determined by removing oocytes microsurgically from oocyte–cumulus complexes (oocyectomy) after aspiration from follicles and washing with mDPBS supplemented with 2% (v/v) newborn piglet serum. The micromanipulation technique, with minor modification, was the method

Table 1. Effect of duration of exposure of pig oocyte–cumulus complexes to hormonal supplements in maturation medium on male pronuclear formation after fertilization *in vitro*

Duration of culture (h)		Number of oocytes examined	Number of oocytes			Number (%) of polyspermic oocytes ⁶	Number (%) of oocytes with male and female pronuclei ⁶
With hormones ¹	Without hormones ²		GVBD ³ (%)	Matured ⁴ (%)	Penetrated ⁵ (%)		
0	40	93	62 (67) ^a	50 (54) ^a	32 (64) ^a	25 (78) ^a	5 (16) ^a
2	38	81	73 (90) ^b	61 (75) ^b	56 (92) ^b	49 (88) ^{ab}	16 (29) ^{ab}
20	20	106	106 (98) ^c	97 (90) ^c	93 (96) ^b	88 (95) ^b	62 (67) ^c
30	10	120	118 (98) ^c	112 (93) ^c	103 (92) ^b	93 (90) ^{ab}	40 (39) ^b
40	0	111	110 (99) ^c	88 (88) ^c	94 (96) ^b	89 (95) ^b	34 (36) ^b

¹Primary culture medium which was modified M199 contained 10 iu PMSG ml⁻¹, 10 iu hCG ml⁻¹, 1 µg oestradiol ml⁻¹ and 10% (v/v) porcine follicular fluid.

²Secondary culture medium was the primary culture medium without PMSG, hCG and oestradiol.

³Germinal vesicle breakdown.

⁴The oocytes at the stage of anaphase I to metaphase II were regarded as matured.

⁵In matured oocytes.

⁶In penetrated oocytes.

Different superscripts within columns denote significant differences ($P < 0.05$).

described by Buccione *et al.* (1990) and Prochazka *et al.* (1991). Briefly, each oocyte–cumulus complex was held in the end of a holding pipette in 100 µl droplet of mDPBS supplemented with 2% (v/v) newborn piglet serum. A thin aspirating micropipette was then introduced through the ooplasm into the lumen of the holding pipette. After the tip of the aspirating micropipette was drawn back and replaced in the ooplasm, the ooplasm was aspirated into the holding and aspirating pipettes by a burst of negative pressure. Usually 10 to 15 oocyctomized oocyte–cumulus complexes were prepared within 15 min. Each of the oocyctomized and control intact oocyte–cumulus complexes was cultured in a 50 µl droplet of OMM199 for primary 20 h culture and then in a 50 µl droplet of OMM199 without hormone supplements for a second 20 h period. The morphology and diameter of oocyte–cumulus complexes were recorded.

Statistical analysis

Treatment effects on cytoplasmic maturation were determined by examining averages of four replicate trials for statistical differences using χ^2 analysis. A total of four replicate trials were conducted to determine the effects of changes in hormonal conditions on cumulus expansion. The effects of oocyctomy on cumulus expansion were determined by three separate trials. The mean diameter of oocyte–cumulus complexes in each experimental group was expressed as mean \pm SD. χ^2 test and Student's *t* test were used to determine statistical differences in morphology and diameter of oocyte–cumulus complexes, respectively. Probability of $P < 0.05$ was considered to be statistically significant.

Results

Cytoplasmic maturation

There were no differences in the sperm penetration of pig oocytes among replicate trials in the present study. After 10 to

12 h in co-culture with spermatozoa, differences in the ability of pig oocytes to form male pronuclei were related to the time of removal of hormone supplements from maturation medium (Table 1). Male pronuclear formation rate was significantly enhanced ($P < 0.05$) by removing hormone supplements at 20 h after the start of culture (67%). The pronuclear formation rates of oocytes cultured with hormone supplements for 30 h and for the complete duration of culture (39 and 36%, respectively) were not different from eggs exposed to the supplements for only 2 h (29%). Although germinal vesicle breakdown, maturation and sperm penetration occurred when oocyte–cumulus complexes were cultured in a medium without hormone supplements, an initial 2 h exposure of oocyte–cumulus complexes to hormone supplements significantly improved these traits (Table 1). When oocyte–cumulus complexes were exposed to the hormone supplements for more than 20 h, there were no differences in germinal vesicle breakdown, maturation, sperm penetration or polyspermy rates. In all treatment groups, a high percentage (75–95%) of polyspermic penetration was observed (Table 1).

Cumulus expansion

The morphology of cumulus expansion (the degree; 0 to 1) and the mean diameter (260 ± 39 µm at 0 h to 250 ± 85 µm at 40 h) of oocyte–cumulus complexes showed little change during a 40 h culture when the oocyte–cumulus complexes were cultured in a medium without hormone supplements (group A). However, when oocyte–cumulus complexes were exposed to hormone supplements for at least 20 h (group B and C), expansion was enhanced by increased duration of culture (Fig. 1 and Table 2). At 30 and 40 h of culture, the mean diameter (820 ± 247 µm and 1076 ± 433 µm, respectively) of oocyte–cumulus complexes removed from exposure to hormone supplements after 20 h (group B) was larger ($P < 0.05$) than that (620 ± 178 µm and 755 ± 264 µm, respectively) of oocyte–cumulus complexes cultured continuously in the presence of

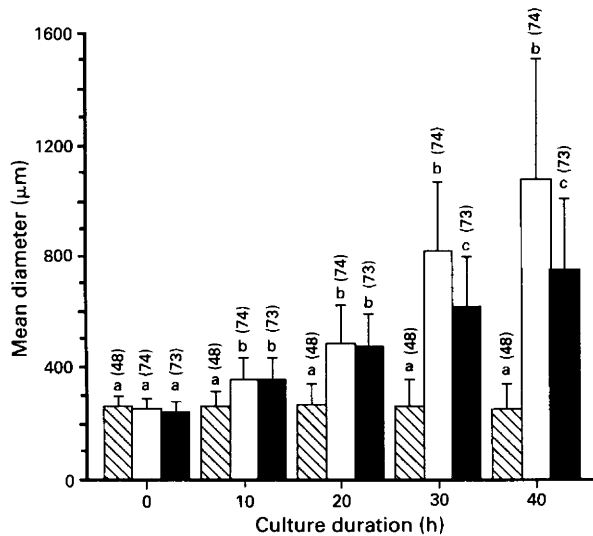


Fig. 1. The effects of duration of exposure of oocyte–cumulus complexes to hormone supplements on cumulus expansion. Individual oocyte–cumulus complexes were cultured in a droplet of hormone-free medium (Group A; ▨) for 40 h, one in medium supplemented with hormones (PMSG, hCG and oestradiol) for first 20 h and then moved to hormone free medium for a further 20 h (Group B; □), and one in medium with the hormones for 40 h (Group C; ■). The diameter of each oocyte–cumulus complex was measured at 0, 10, 20, 30 and 40 h after culture. The bars are given as the means \pm SD from four experiments. Columns with different letter above the bars are significantly different among groups at the same observation point ($P < 0.05$). The numbers in parentheses on top of bars indicate the number of oocyte–cumulus complexes examined.

hormones (group C) (Fig. 1). More oocyte–cumulus complexes developed to the advanced morphological stage in group B than in group C at 30 and 40 h after culture (Table 2), and some (23%, 11 of 48) of the oocyte–cumulus complexes in group B had expanded corona radiata after culture, whereas none of the oocyte–cumulus complexes in group C had expanded corona radiata at that time.

When oocyte–cumulus complexes were cultured in a medium with hormone supplements for 20 h and then without added hormones for 20 h, the mean diameter of the oocyte–cumulus complexes increased with the duration of culture ($391 \pm 99 \mu\text{m}$ at 20 h, $827 \pm 167 \mu\text{m}$ at 30 h and $991 \pm 211 \mu\text{m}$ at 40 h), and there were no significant differences between diameter of oocyte–cumulus complexes (the above) and intact oocyte–cumulus complexes ($436 \pm 106 \mu\text{m}$ at 20 h, $861 \pm 231 \mu\text{m}$ at 30 h and $1176 \pm 360 \mu\text{m}$ at 40 h) (Fig. 2). After 30 h of culture, the morphology of cumulus expansion in the oocyte–cumulus complexes was similar to that of intact oocyte–cumulus complexes (Table 3). However, none of the corona radiata of oocyte–cumulus complexes underwent expansion at 40 h after culture, whereas 17% (9 of 52) of intact oocyte–cumulus complexes had expanded corona radiata.

Discussion

In the present study, we found that removal of hormone supplements (PMSG, hCG and oestradiol) from maturation

medium at 20 h after the start of culture improved the ability of pig oocytes to form a male pronucleus 10 to 12 h after insemination. The exposure of oocyte–cumulus complexes to the supplements for only 2 h enhanced germinal vesicle breakdown and maturation of the oocytes, and nearly all of the oocytes showed meiotic maturation after exposure to hormones for 20 h or longer. These results suggest that there are at least two different phases during *in vitro* maturation, i.e. meiotic maturation is controlled by stimulation from hormone supplements in the primary phase (20 h) and cytoplasmic maturation is enhanced by the absence of some or all of the hormone supplements in the second 20 h. Pig oocytes complete germinal vesicle breakdown *in vivo* and *in vitro* about 20 h after hCG injection (Hunter and Polge, 1966; Ainsworth *et al.*, 1980) or culture (Naito and Toyoda, 1991). It appears therefore that the requirement of hormone supplementation is limited to the germinal vesicle breakdown phase. *In vivo*, concentrations of gonadotrophins and steroids in follicular fluid change dramatically during oocyte maturation (Moor, 1974; McNatty *et al.*, 1975; Hunter *et al.*, 1976; Ainsworth *et al.*, 1980; Lenton *et al.*, 1988), and, in particular, the concentration of oestrogens in prepubertal gilts declines rapidly between 4 and 16 h after hCG injection (Ainsworth *et al.*, 1980). It is possible that these hormonal changes affect cytoplasmic maturation of the oocytes, although we did not examine the effects of removing each hormone supplement on cytoplasmic maturation in the present study. Further, our use of pFF derived from superficial follicles (3 to 6 mm in diameter) of prepubertal gilts, as a supplement for maturation media, would supply not only proteins but also a basal amount of the gonadotrophins and oestradiol. However, these hormonal contents in pFF were not determined. Naito *et al.* (1988) have reported that only 36% of oocytes matured when pig oocytes were cultured for 48 h in pFF which was derived from medium-sized follicles (2–5 mm in diameter) of gilt ovaries.

The addition of follicular fluid with FSH during the *in vitro* maturation period (48 h) enhances male pronuclear development at 18 h after insemination (39 versus 61%; Naito *et al.*, 1988). In the present study, however, the male pronuclear formation rate of oocytes at 10 to 12 h after insemination was very low (36%) when the oocytes were cultured in a medium supplemented with hormones and 10% follicular fluid for 40 h before insemination. The low pronuclear formation rate in the present study may be due to the reduced duration of the culture period for maturation because the male pronuclear formation rate was 59% at 10 to 12 h after insemination when oocyte–cumulus complexes were cultured in the same medium for 50 h before insemination (Funahashi and Day, 1993). Our results therefore suggest that a short exposure to hormones may advance the time of male pronuclear formation. In like manner, the supplementation of follicular shells during maturation *in vitro* (Mattioli *et al.*, 1988a, 1991) improves the rate of formation of a male pronucleus in oocytes at 10 h, as well as at 18 h, after insemination (Moor *et al.*, 1990).

We also found that removal of hormone supplements 20 h after culture enhanced the cumulus expansion of oocyte–cumulus complexes after 30 h of culture compared with that of oocyte–cumulus complexes cultured with hormones for 30 h. Cumulus expansion results when cumulus cells secrete hyaluronic acid which expands the spaces between the cumulus cells (Eppig,

Table 2. Effects of removal of hormone supplements in maturation medium on cumulus expansion of pig cumulus–oocyte complexes

Time of observation (h of culture)	Experimental group ¹	Number of oocytes examined	Degree of cumulus expansion ² (%)					Cumulus expansion index ³
			0	1	2	3	4	
0	A	48	48 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0.00
	B	48	48 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0.00
	C	48	48 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0.00
10	A	48	48 (100)	0 (0) ^a	0 (0)	0 (0)	0 (0)	0.00
	B	48	0 (0)	48 (100) ^b	0 (0)	0 (0)	0 (0)	1.00
	C	48	2 (4)	46 (96) ^b	0 (0)	0 (0)	0 (0)	0.96
20	A	48	46 (96)	2 (4)	0 (0) ^a	0 (0)	0 (0)	0.04
	B	48	0 (0)	8 (17)	40 (83) ^b	0 (0)	0 (0)	1.83
	C	48	0 (0)	7 (15)	41 (85) ^b	0 (0)	0 (0)	1.85
30	A	48	44 (92)	4 (8)	0 (0)	0 (0) ^a	0 (0)	0.08
	B	48	0 (0)	0 (0)	11 (23)	37 (77) ^b	0 (0)	2.77
	C	48	0 (0)	0 (0)	25 (52)	23 (48) ^c	0 (0)	2.48
40	A	48	35 (73)	13 (27)	0 (0)	0 (0)	0 (0) ^a	0.27
	B	48	0 (0)	0 (0)	3 (6)	34 (71)	11 (23) ^b	3.17
	C	48	0 (0)	0 (0)	5 (10)	43 (90)	0 (0) ^a	2.90

¹Cumulus–oocyte complexes were cultured in OMM199 without hormones for 40 h (group A), in OMM199 for 20 h with added hormones followed by culture with no hormones for 20 h (group B) or in OMM199 with hormones for 40 h (group C).

²0, no detectable response; 1, minimum observable response; 2, expansion limited to the outer layer of cumulus cells; 3, cumulus expansion except the corona radiata; 4, maximum degree of expansion; i.e. the cumulus oophorus and corona radiata have undergone expansion.

³Mean degree of cumulus expansion.

Different superscripts within columns at each time of observation denote significant difference ($P < 0.05$).

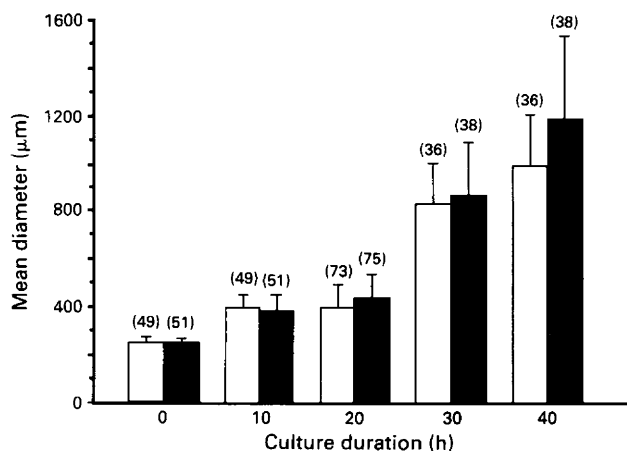


Fig. 2. The effects of oocytectomy on cumulus expansion. Individual intact oocyte–cumulus complexes (■) or oocytectomized oocyte–cumulus complexes (□) were incubated in a droplet of medium supplemented with hormones (PMSG, hCG and oestradiol) for 20 h and then moved into a droplet of hormone-free medium for 20 h. The diameter of each complex was measured at 0, 10, 20, 30 and 40 h after culture. The bars are given as the mean \pm SD from three experiments. There were no significant differences between intact and oocytectomized groups. The numbers in parentheses on top of bars indicate the number of oocyte–cumulus complexes tested.

1991). The degree of cumulus expansion as measured by the mean diameter of cumulus oophorus can therefore be regarded as a marker of the metabolic abilities of cumulus cells. The

present results suggest that the placement of oocyte–cumulus complexes in hormone-free medium at about 20 h after culture enhances the secretory potential of cumulus cells. McNatty *et al.* (1975) suggested that the hormonal changes during maturation may be of considerable importance for secretory activity of the granulosa cells both before and after ovulation. However, Mattioli *et al.* (1988b) reported that, when oocyte–cumulus complexes were cultured in medium conditioned by follicle walls, an active intercellular communication between cumulus cells and oocyte was seen for 30 to 40 h of culture, although decreasing at 32 h after culture, which is similar to that of oocytes matured *in vivo* (Motlik *et al.*, 1986). Although the change of intercellular communication in media supplemented with follicular fluid and hormones has not been examined, it is possible that the intercellular communication is maintained by the metabolic stimulation of cumulus cells caused by the change of hormonal conditions during the second half of maturation culture.

In mice, in which cumulus expansion is stimulated directly by FSH *in vitro*, it is known that oocytectomized oocyte–cumulus complexes fail to show cumulus expansion in response to FSH (Buccione *et al.*, 1990). Since medium conditioned by isolated oocytes induced hyaluronic acid synthesis by isolated cumulus cells (Salustri *et al.*, 1990) and increased the expansion of oocytectomized complexes (Buccione *et al.*, 1990), it is considered that mouse oocytes produce the expansion enabling factor(s), and the oocyte participates in the regulation of cumulus cell function (Buccione *et al.*, 1990). In the present study, when pig oocyte–cumulus complexes were exposed to hormone supplements for the initial 20 h of the culture, oocytectomized

Table 3. Effects of oocytectomy on cumulus expansion of pig cumulus–oocyte complexes

Time of observation (h of culture)	Experimental group	Number of oocytes examined	Degree of cumulus expansion ² (%)					Cumulus expansion index ³
			0	1	2	3	4	
30	Oocytectomy ¹	50	0 (0)	0 (0)	4 (8)	46 (92)	0 (0)	2.92
	Intact	52	0 (0)	0 (0)	5 (10)	47 (90)	0 (0)	2.90
40	Oocytectomy	50	0 (0)	0 (0)	2 (4)	48 (96)	0 (0)	2.96
	Intact	52	0 (0)	0 (0)	4 (8)	39 (75)	9 (17)	3.10

¹Oocyte–cumulus complexes were removed from the oocyte by micromanipulation at the start of culture.

²0, no detectable response; 1, minimum observable response; 2, expansion limited to the outer layer of cumulus cells; 3, cumulus expansion except the corona radiata; 4, maximum degree of expansion; i.e. the cumulus oophorus and corona radiata have undergone expansion.

³Mean degree of cumulus expansion.

oocyte–cumulus complexes did not exhibit expansion of corona radiata 40 h after culture, whereas some of the intact oocyte–cumulus complexes showed full cumulus expansion. Prochazka *et al.* (1991) compared the expansion of oocytectomized oocyte–cumulus complexes with that of intact oocyte–cumulus complexes 24 h after culture and concluded that cumulus expansion of oocyte–cumulus complexes did not require the cumulus expansion-enabling factor from oocytes. Our results, however, indicate that intercellular communication with oocytes is necessary for the expansion of corona radiata during the latter half of maturation, even though expansion of cumulus cells except for corona radiata does not depend on the interaction with the oocytes. On reflection, the failure of corona radiata expansion in the oocyte–cumulus complexes that were cultured with hormones for 40 h may be due to deficient intercellular communication due to hormones. As no more than 23% of oocytes reached expansion grade 4 in any treatment groups in the present study, our culture conditions during *in vitro* maturation appear not to be optimal for the expansion of corona radiata. Further investigations to determine suitable conditions for full expansion of corona radiata may increase the ability of pig oocytes to form a male pronucleus after *in vitro* fertilization.

In the present study, the incidence of polyspermic penetration was very high in all treatment groups. A high incidence of polyspermy in pig oocytes has been observed by many investigators (Nagai *et al.*, 1984; Cheng, 1985; Mattioli *et al.*, 1988a). Mattioli *et al.* (1989) reported that 53% of penetrated oocytes were polyspermic when ten to fifteen oocytes were cultured in 2 ml of fertilization medium containing 1×10^5 spermatozoa ml^{-1} for 6 to 8 h. Our higher incidence of polyspermic entry may be due to a higher sperm concentration (1×10^6 cells ml^{-1}) in a small volume (100 μl) of fertilization medium, or to exposure of oocytes to spermatozoa for longer duration, 10 to 12 h, or to both factors. Rath (1992) reported that there was a correlation between the number of inseminated spermatozoa per oocyte and the incidence of polyspermy. Further, Cheng (1985) found that a longer period (more than 8 h) of incubating oocytes with spermatozoa results in a significant increase in polyspermy. As Wang *et al.* (1991) expressed, high incidence of polyspermy in pig oocytes matured *in vivo* suggests that polyspermy is not due entirely to abnormality of oocytes matured *in vitro*.

In summary, we demonstrated that the transfer of pig oocyte–cumulus complexes from medium supplemented with hormones to hormone-free medium at 20 h after culture enhanced the cytoplasmic maturation of pig oocytes, which was evaluated by the ability of the oocytes to form a male pronucleus after *in vitro* fertilization. Removal of hormones from the culture medium also stimulated cumulus expansion during *in vitro* maturation. Furthermore, expansion of cumulus cells, except for the corona radiata, did not depend on the intercellular communication with oocytes.

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