Cytoplasmic maturation for activation of pig follicular oocytes cultured and arrested at metaphase I

K. Kikuchi¹, T. Nagai², J. Ding³, N. Yamauchi^{2*}, J. Noguchi¹ and Y. Izaike^{1*}

¹Department of Genetic Resources II, National Institute of Agrobiological Resources, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan;

²Department of Animal Production, Tohoku National Agricultural Experiment Station, Shimokuriyagawa, Morioka, Iwate 020-0123, Japan; and ³Oak Brook Fertility Center, 2425 W. 22nd Street, Suite 102, Oak Brook, IL 60523, USA

A large population (62–90%) of pig follicular oocytes can mature to metaphase II after culture for 48 h. However, a proportion (6–22%) remain in an immature stage at metaphase I (metaphase I-arrested). The main objective of this study was to determine whether the cytoplasm of metaphase I-arrested pig oocytes is capable of being activated by sperm penetration or parthenogenetic stimulation. After culture for 48 h, oocytes without a polar body (73% were shown to be at metaphase I after staining) and those with a polar body (94% were at metaphase II) were fertilized *in vitro*. A total of 69% and 62% of the oocytes were activated to form a female pronucleus, respectively, and the rate of polar body extrusion induced by fertilization in the activated oocytes was 90% (the first polar body) and 95% (the second polar body), respectively. When oocytes without and with a polar body were stimulated with an electric pulse, 53% and 81% of the oocytes were activated, respectively. The rate of polar body extrusion in the activated oocytes was 73% (the first polar body) and 79% (the second polar body), respectively. In contrast, young metaphase I oocytes cultured for 24 h had low (6%) or zero activation rate after *in vitro* fertilization or electric pulse stimulation. However, about one-third of the young metaphase I oocytes penetrated by spermatozoa after *in vitro* fertilization responded to electric pulse 12 h after insemination, and almost all (93%) were activated when they were stimulated 24 h after insemination. Patterns of polypeptide synthesis and histone H1 kinase activity were similar in metaphase I-arrested and metaphase II oocytes, and were characterized by increase in a 25 kDa polypeptide and by decrease in kinase activity. Although the first step of meiotic division is impaired, these results indicate that metaphase I-arrested oocytes are mature cytoplasmically.

Introduction

Preliminary studies showed that when pig follicular oocytes are cultured for about 48 h and fertilized *in vitro*, two types of activated oocytes with a female pronucleus are observed after fixation and staining: oocytes with two polar bodies and oocytes with one polar body. Because the second polar body is extruded from an oocyte matured to metaphase II (MII) as the result of normal fertilization, activated oocytes with one polar body may be an experimental artefact or derived from activation of immature oocytes. Three possibilities have been addressed in earlier studies: (i) one polar body may be removed from the specimen during fixation and staining of oocytes; (ii) MII oocytes are activated by penetrating spermatozoa but the second polar body is not extruded; and (iii) immature oocytes, such as oocytes

arrested at metaphase I (MI) (MI-arrested oocytes), are activated by sperm penetration and emit the first polar body. The last hypothesis seems most likely because, under experimental conditions, spermatozoa can penetrate immature oocytes from the germinal vesicle (GV) stage to MII in mice (Iwamatsu and Chang, 1972), hamsters (Barros and Munoz, 1974), rabbits (Overstreet and Bedford, 1974), rats (Niwa and Chang, 1975), dogs (Mahi and Yanagimachi, 1976), cows (Niwa *et al.*, 1991), pigs (Wang *et al.*, 1994) and humans (Overstreet and Hembree, 1976; Overstreet *et al.*, 1980). The penetrated immature oocytes that undergo germinal vesicle breakdown (GVBD) can be activated and form a female pronucleus in rats (Niwa and Chang, 1975), hamsters (Usui and Yanagimachi, 1976) and cows (Chian *et al.*, 1992; Abeydeera *et al.*, 1994). The objectives of the present study were: (i) to confirm the existence of fertilized oocytes with one polar body by direct staining with a fluorescent dye; (ii) to investigate whether the cytoplasm of MI-arrested oocytes is capable of forming a female pronucleus after sperm penetration or parthenogenetic stimulation by electric

*Present address: Department of Animal Reproduction, National Institute of Animal Industry, Norindanchi PO Box 5, Tsukuba, Ibaraki 305-0901, Japan.
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pulse; and (iii) to compare the maturity of oocyte cytoplasm in MI and MII oocytes by analysis of polypeptide synthesis and histone H1 kinase activity.

Materials and Methods

Collection of pig follicular oocytes

Pig follicular oocytes were collected as described by Kikuchi *et al.* (1993). Ovaries were obtained at a local abattoir and transported at 35°C to the laboratory. The ovaries were washed in PBS (Nissui Pharmaceutical Co. Ltd, Tokyo) and placed in medium 199 (with Hanks' salts; Gibco, Life Technologies Inc., Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 20 mmol Hepes l⁻¹ (Dojindo Laboratories, Kumamoto), 100 iu penicillin G potassium ml⁻¹ (Sigma Chemical Co., St Louis, MO) and 0.1 mg streptomycin sulfate ml⁻¹ (Sigma). Follicles 3–6 mm in diameter were punctured with a surgical blade and cumulus–oocyte complexes were collected.

Experiment 1: nuclear changes using different maturation systems

Two culture systems were used for oocyte maturation to obtain a large number of immature oocytes or to compare the behaviour of penetrated sperm heads with that reported in other species. In the first system, approximately 50 cumulus–oocyte complexes were cultured in 35 mm plastic dishes (Falcon No. 1008, Becton Dickinson and Company, Lincoln Park, NJ) containing 2 ml culture medium consisting of medium 199 (with Earle's salts; Gibco) supplemented with 10% FBS and gonadotrophin (NIH-LH-022, 2.5 µg ml⁻¹; NIH-FSH-P2, 2.5 µg ml⁻¹) (m-M199) (Kikuchi *et al.*, 1993). Parietal granulosa cells (5 × 10⁶ cells ml⁻¹, dispersed from the follicular wall) and two everted theca shells were also included. The cumulus–oocyte complexes were cultured at 39°C under 5% CO₂ in air on a mechanical platform with gentle agitation (Nagai *et al.*, 1993). In some experiments, Waymouth MB 752/1 medium (Gibco) supplemented with 10% FBS, 10% (v/v) pig follicular fluid, 2.5 µg FSH ml⁻¹ (Antrin, Denka Pharmaceutical Co. Ltd, Kanagawa) and antibiotics (penicillin G potassium and streptomycin sulfate) was used as the culture medium (m-Waymouth) (Kikuchi *et al.*, 1995a). About ten cumulus–oocyte complexes were cultured in 100 µl maturation medium covered with mineral oil (E. R. Squibb & Sons Inc., Princeton, NJ) in 35 mm plastic dishes at 39°C under 5% CO₂ in air. Some of the oocytes cultured from 24 to 48 h were fixed in acetic alcohol (1:3) for 2–3 days, stained with 1% (w/v) aceto-orcin solution and examined for nuclear status under a phase contrast microscope to confirm the nuclear changes during culture in both media. Four replicated trials were performed in this experiment.

Experiment 2: confirmation of accuracy of whole mount preparation after fixation and staining

The presence of polar bodies in oocytes after *in vitro* fertilization (IVF) was determined by fluorescent DNA

staining of the live oocytes before examination to confirm that the polar bodies could not be removed by fixation with acetic alcohol and staining with aceto-orcin.

IVF of cultured oocytes. After culture in m-M199 for 48 h, IVF was carried out as described by Kikuchi *et al.* (1993). Bracket and Oliphant solution (1975) modified with 10 mg BSA ml⁻¹ (Fraction V, Sigma) and 2 mmol anhydro-caffeine l⁻¹ (Sigma) was used as fertilization medium. Frozen epididymal spermatozoa (Kikuchi *et al.*, 1998) from a Large White boar were thawed and preincubated for 1 h at 37°C under 5% CO₂ in air in a 35 mm plastic dish containing 0.25 ml medium 199 (with Earle's salts) adjusted to pH 7.8 with 0.1 mol NaOH l⁻¹ after supplementation with 2.9 mmol calcium lactate l⁻¹, 3.1 mmol glucose l⁻¹, 25 mmol Hepes l⁻¹ and 10% FBS (Nagai *et al.*, 1988). The concentration of spermatozoa during preincubation was 2.5 × 10⁸ cells ml⁻¹. A proportion of the preincubated spermatozoa was introduced into 100 µl fertilization medium containing about 20 cumulus–oocyte complexes so that the final sperm concentration at fertilization was 1.0 × 10⁶ cells ml⁻¹. Cumulus–oocyte complexes were co-incubated with spermatozoa for 6 h and were then transferred to Brinster's medium for ovum culture II solution (BMOC-II; Brinster, 1965) containing 4 mg BSA ml⁻¹ and cultured for 4 h.

Staining with a fluorescent dye and aceto-orcin solution. Inseminated and cultured oocytes were mounted on a glass slide and covered with a cover slip (whole mount preparation). PBS containing 10 µmol 4',6-diamidino-2-phenylindole l⁻¹ (DAPI, Sigma) was introduced into the space between the glass slide and the cover slip, and oocytes were examined under a fluorescence microscope. This staining method allows the nuclear status of oocytes and penetrated spermatozoa, and the condition of polar bodies in the perivitelline space to be observed. The preparations were immediately transferred into acetic alcohol and fixed, and were then stained and examined for nuclear status as described earlier. Four replicated trials were performed in this experiment.

Experiment 3: IVF and artificial stimulation of immature and mature oocytes

Cultured oocytes were divided into immature and mature groups and were then fertilized *in vitro* or stimulated by an electric pulse to investigate whether both MI and MII oocytes respond to the stimuli of sperm penetration or parthenogenetic stimulation.

Classification of immature and mature oocytes. After culture in m-Waymouth for 24 or 48 h, oocytes were denuded using 150 iu hyaluronidase (Sigma) ml⁻¹ and gentle pipetting. Denuded oocytes were transferred to medium 199 with Hank's salts (described earlier) and then immature and mature oocytes, classified as those without (PB–) or with (PB+) the first polar body, respectively, were collected under a Nomarski differential interference contrast microscope (Kikuchi *et al.*, 1995a). Some of the classified oocytes were fixed, stained and examined to determine the efficacy of this classification. The remaining oocytes were used in the

experiments involving IVF or parthenogenetic stimulation. Three replicated trials were performed in this experiment.

IVF of classified oocytes. After culture for 24 or 48 h, oocytes were classified as PB- or PB+. A proportion of these oocytes was inseminated and cultured before fixation as described for Expt 2. Non-inseminated oocytes were used as a control. Nuclear status was examined after fixation and staining. Three replicated trials were performed in this experiment.

Parthenogenetic stimulation of classified oocytes. Oocytes classified as PB- and PB+ were subjected to parthenogenetic activation. Oocytes were washed three times in the activation solution consisting of 0.3 mol D-mannitol l⁻¹ (Wako Pure Chemical, Tokyo), 0.1 mmol CaCl₂·2H₂O l⁻¹, 0.1 mmol MgCl₂·2H₂O l⁻¹ and 0.2 mg BSA ml⁻¹. The oocytes were then transferred to the hybridizing chamber (FTC-22W, Shimadzu Corporation, Tokyo) containing 50 µl activation solution, and stimulated with a 20 µs pulse at 1.5 kV DC cm⁻¹ using a somatic hybridizer (SSH-10, Shimadzu). Non-stimulated oocytes were used as a control. Stimulated and non-stimulated oocytes were transferred to BMOC-II solution and cultured for 10 h. Oocytes were then fixed, stained and examined for nuclear status. Three replicated trials were performed in this experiment.

Experiment 4: culture of immature oocytes after IVF and response to electric stimulation

The behaviour of sperm nuclei incorporated into the cytoplasm of young MI oocytes and the ability of oocyte cytoplasm to be activated after parthenogenetic stimulation were examined to clarify the differences in the cytoplasm of young MI oocytes and MI-arrested or MII oocytes.

Culture of immature oocytes fertilized in vitro after 24 h culture in maturation medium. After culture for 24 h in m-M199, cumulus-oocyte complexes were inseminated with preincubated boar spermatozoa. After 6 h co-incubation with spermatozoa, some of the oocytes were immediately fixed (6 h after insemination), and the others were cultured in BMOC-II solution for 6, 12, 18, 24 and 30 h and were then fixed (12, 18, 24, 30 and 36 h after insemination, respectively). Nuclear status was examined after staining.

Artificial stimulation of immature IVF oocytes. After IVF and subsequent culture, the oocytes were subjected to an electrical pulse 12 and 24 h after insemination, as described in Expt 3. The oocytes were then incubated in BMOC-II solution for 12 h before fixation and staining for examination of nuclear status. Three replicated trials were performed in this experiment.

Experiment 5: examination of polypeptide synthesis and histone H1 kinase activity

Cytoplasmic indices such as polypeptide synthesis and histone H1 kinase activity in oocytes were evaluated to

compare the cytoplasm of MI (24 h), MI-arrested (48 h) and MII (48 h) oocytes.

Fluorography of [³⁵S]polypeptides synthesized during oocyte culture. After culture in m-M199 for 24 or 48 h, cumulus-oocyte complexes were labelled with [³⁵S]methionine and classified according to their nuclear status after staining with DAPI before preparation for electrophoresis and autoradiography (Ding *et al.*, 1992a,b). Briefly, cumulus-oocyte complexes were incubated in labelling solution containing [³⁵S]methionine at a final concentration of 1.85 × 10⁷ Bq ml⁻¹ (Amersham) for 3 h at 39°C (see Moor *et al.*, 1981). The cumulus-oocyte complexes were then washed in [³⁵S]methionine-free labelling medium and cultured transiently (15 to 20 min) in medium containing 10 µmol DAPI l⁻¹. Cumulus cells were then removed from the oocytes and nuclear status was determined under a fluorescence microscope. Oocytes were recovered individually and placed in groups according to their nuclear configurations. A group of 1–3 oocytes of the same configuration was washed briefly in a solution of 10 mmol Tris l⁻¹ and 0.1 mmol EDTA l⁻¹, transferred in a small volume (< 4 µl) of this solution to a 0.5 ml microtube, lyophilized, and stored at -70°C until required for gel electrophoresis. Oocytes were prepared for protein analysis and polypeptides were separated using 8–15% linear gradient SDS-PAGE (Moor *et al.*, 1981).

Histone H1 kinase activity. After culture in m-Waymouth for 24 and 48 h, oocytes were denuded by hyaluronidase treatment, stained with DAPI and classified as GV, MI or MII stage under a fluorescence microscope (Kikuchi *et al.*, 1995b). The oocytes were washed three times in the assay solution consisting of 60 mmol β-glycero phosphate l⁻¹, 30 mmol p-nitro-phenyl phosphate l⁻¹, 25 mmol Mops l⁻¹ (pH 7.2), 15 mmol EGTA l⁻¹, 15 mmol MgCl₂ l⁻¹ and 0.1 mmol sodium vanadate l⁻¹ (Wako) for the assay of histone H1 kinase activity (Naito and Toyoda, 1991). Ten oocytes were placed separately into plastic tubes containing 5 µl solution, frozen at -70°C to break the oocyte membrane and stored at the same temperature until the assay was performed. The histone H1 kinase assay was carried out as described by Dominko and First (1996). The reaction mixture contained 5 µl of the sample solution, which was thawed in a solution containing 1 µl of 1.0 mg histone H1 ml⁻¹ (Sigma), 1 µl [γ-³²P]ATP (3.7 × 10⁸ Bq ml⁻¹, Amersham), 5 µl kinase buffer (containing 80 mmol β-glycero phosphate l⁻¹, 20 mmol ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid l⁻¹, 15 mmol MgCl₂ l⁻¹ and 1 mmol dithiothreitol l⁻¹) and protein kinase inhibitors (1 mmol sodium ortho vanadate l⁻¹, 10 µg aprotinin ml⁻¹ and 10 µg pepstatin A ml⁻¹) at room temperature just before the assay. The mixture was incubated for 30 min at room temperature. The reaction was stopped by the addition of 10 µl of 2 × electrophoresis sample buffer containing 100 mmol dithiothreitol ml⁻¹. The mixture was then subjected to 10% SDS-PAGE (Laemmli, 1970), and phosphorylated histone bands were visualized after autoradiography. The intensity of the bands was analysed using an image densitometer (Bio-Rad, Model GS-700). Three replicated trials were performed in this experiment.

Table 1. Nuclear status in pig oocytes after culture for 24–48 h in m-M199 or m-Waymouth medium

Medium	Culture (h)	Number of oocytes examined	Number of oocytes at					
			Germinal vesicle	Germinal vesicle breakdown	Metaphase I	Anaphase/telophase I	Metaphase II	Degenerated
m-M199	24	57	3 (5 ± 3) ^{ab}	25 (44 ± 21) ^a	28 (49 ± 20) ^{ab}	0 (0 ± 0) ^a	0 (0 ± 0) ^a	1 (2 ± 1)
m-M199	30	74	7 (9 ± 3) ^{ac}	6 (8 ± 5) ^b	41 (55 ± 14) ^a	9 (12 ± 8) ^b	9 (12 ± 9) ^a	2 (3 ± 3)
m-M199	36	57	1 (2 ± 3) ^{ab}	0 (0 ± 0) ^b	9 (16 ± 4) ^c	0 (0 ± 0) ^a	46 (81 ± 3) ^{bc}	1 (2 ± 3)
m-M199	42	53	1 (2 ± 3) ^{ab}	0 (0 ± 0) ^b	8 (15 ± 1) ^c	0 (0 ± 0) ^a	42 (79 ± 6) ^b	2 (4 ± 2)
m-M199	48	63	0 (0 ± 0) ^b	1 (2 ± 2) ^b	4 (6 ± 3) ^c	0 (0 ± 0) ^a	57 (90 ± 5) ^d	1 (2 ± 2)
m-Waymouth	24	69	12 (17 ± 3) ^c	10 (15 ± 7) ^b	42 (61 ± 5) ^a	0 (0 ± 0) ^a	0 (0 ± 0) ^a	5 (7 ± 5)
m-Waymouth	30	113	10 (9 ± 1) ^a	4 (3 ± 2) ^b	76 (67 ± 3) ^a	11 (10 ± 5) ^{ab}	7 (6 ± 5) ^a	5 (4 ± 3)
m-Waymouth	36	123	8 (7 ± 3) ^a	2 (2 ± 1) ^b	31 (25 ± 2) ^{bc}	21 (17 ± 8) ^{ab}	59 (48 ± 5) ^e	2 (2 ± 2)
m-Waymouth	42	103	6 (6 ± 1) ^{ab}	0 (0 ± 0) ^b	22 (21 ± 2) ^{bc}	6 (6 ± 3) ^{ab}	62 (60 ± 0) ^e	7 (7 ± 5)
m-Waymouth	48	126	9 (7 ± 2) ^{ab}	1 (1 ± 1) ^b	28 (22 ± 2) ^{bc}	6 (5 ± 3) ^{ab}	78 (62 ± 2) ^{ce}	4 (3 ± 3)

Percentages are expressed as mean ± SEM.

^{a-c}Within columns percentages with different superscripts are significantly different by Duncan's multiple range test ($P < 0.05$).

Table 2. Pig follicular oocytes fertilized *in vitro* and stained with DAPI and aceto-orcein

Oocyte examination	Number of oocytes examined	Percentage of maturing oocytes ^a	Percentage of penetrated oocytes	Percentage of activated oocytes ^b						
				Total	One polar body		Two polar bodies			
					Total	En ^c	MPN ^c	Total	En ^c	MPN ^c
DAPI	122	121 (99 ± 1)	90 (74 ± 4)	85 (70 ± 3)	20 (16 ± 4)	12 (10 ± 3)	8 (7 ± 2)	65 (53 ± 6)	31 (25 ± 8)	34 (28 ± 7)
Aceto-orcein	122	121 (99 ± 1)	90 (74 ± 4)	85 (70 ± 3)	20 (16 ± 4)	11 (9 ± 2)	9 (7 ± 3)	65 (53 ± 6)	25 (21 ± 7)	40 (33 ± 7)

Percentages are expressed as mean ± SEM.

^aOocytes that underwent germinal vesicle breakdown.

^bActivated oocytes with a female pronucleus.

^cOocytes with enlarged or swollen sperm heads (En) and at least one male pronucleus (MPN).

Statistical analysis

All data, except for the culture of immature oocytes fertilized *in vitro* after 24 h culture in maturation medium (Expt 4) and fluorography of [³⁵S]polypeptides synthesized during oocyte culture (Expt 5), were subjected to ANOVA using general linear model procedures (SAS). Nuclear status, sperm penetration and oocyte activation were analysed by Duncan's multiple range test after transformation using *arcsin* of percentage (Snedecor and Cochran, 1967).

Results

Experiment 1

Nuclear status in oocytes cultured in m-M199 and m-Waymouth for 24–48 h is summarized (Table 1). In both maturation media, GVBD had already started in the oocytes that were cultured for 24 h. However, oocytes cultured in m-M199 showed a significantly higher proportion of GVBD ($P < 0.05$) earlier in the culture (24 h) than those cultured in m-Waymouth. The percentage of nuclear maturation to MII in oocytes cultured in m-M199 for 36–48 h was significantly higher ($P < 0.05$) than that in oocytes cultured in m-Waymouth. However, a proportion of cultured oocytes remained at

immature stages such as GV, GVBD and MI after culture for 48 h. The percentage of immature oocytes was not different in oocytes cultured for 30–48 h in both media. A total of 2–7% degenerated oocytes was observed with the same incidence in each of the cultures.

Experiment 2

Examination after *in vitro* fertilization and staining with DAPI of 122 oocytes cultured in maturation medium for 48 h revealed that 70% of the oocytes were putatively activated and had a female pronucleus (Table 2). When those oocytes were re-examined after fixation with acetic alcohol and staining with aceto-orcein, 92% (78/85) of the oocytes produced results that were in agreement with DAPI staining. No difference in the number of polar bodies was observed in the two examinations. However, 8% (7/85) of the results were not in agreement with sperm nuclear changes. In these oocytes, male pronuclei were mistakenly identified as penetrated (enlarged or swollen) sperm heads because their morphology is similar under the fluorescence microscope. With the two methods of examination, 24% (20/85) of the activated oocytes were found to have only one polar body (Fig. 1).

Fig. 1. Whole mount preparation of a pig oocyte fertilized *in vitro*. The oocyte was stained with 4',6-diamidino-2-phenylindole (DAPI) (a,c) and after examination was immediately fixed in acetic alcohol, stained with aceto-orcein and re-examined (b,d). Images (a) and (b) show one focal plane and (c) and (d) show a second focal plane. In this activated oocyte, a pronucleus (determined as a female pronucleus) (Pn), four penetrated or enlarged sperm heads (E1–E4) and a polar body (determined as the first polar body) (Pb) were observed. Scale bars represent 10 μm.

Experiment 3

The efficacy of classification of immature or mature oocytes by observation of the first polar body is shown (Table 3). After culture for 24 h, all oocytes were classified as PB– and no PB+ oocytes were present. Examination after fixation and staining showed that all oocytes were at an immature stage such as GV, GVBD and MI stages (16, 8 and 72%, respectively). A total of 238 oocytes were examined after culture for 48 h, and 33% (79/238) and 67% (159/238) were classified as PB– and PB+, respectively. Microscopic evaluation

of oocytes revealed that 78% (62/79) of PB– oocytes and 3% (5/159) of PB+ oocytes were at MI, while 13% (10/79) of PB– oocytes and 94% (149/159) of PB+ oocytes were at MII.

The results of IVF of PB– and PB+ oocytes are summarized (Table 4). After culture in maturation medium for 24 h and insemination, 69% of oocytes had been penetrated. However, only 9% (5/56) of the oocytes were activated and had formed a female pronucleus. The remaining penetrated oocytes were not activated: almost all were at MII and incorporated spermatozoa had slightly decondensed heads. After culture in maturation medium for 48 h and IVF, 62% of PB+ oocytes

Table 3. Efficiency of classification of pig oocytes on the basis of the presence of a polar body

Culture (h)	Oocyte ^a	Total number of oocytes examined	Percentage of oocytes at the stage of					
			Germinal vesicle	Germinal vesicle breakdown	Metaphase I	Anaphase/ Telophase I	Metaphase II	Degenerated
24 ^b	PB-	160	25 (16 ± 2) ^c	13 (8 ± 1) ^c	115 (72 ± 6) ^c	0 (0 ± 0)	0 (0 ± 0) ^c	7 (4 ± 4)
48	PB-	79	7 (9 ± 2) ^d	0 (0 ± 0) ^d	62 (78 ± 3) ^c	0 (0 ± 0)	10 (13 ± 6) ^c	4 (5 ± 2)
48	PB+	159	4 (3 ± 2) ^d	0 (0 ± 0) ^d	5 (3 ± 2) ^d	0 (0 ± 0)	149 (94 ± 4) ^d	1 (1 ± 1)

Percentages are expressed as mean ± SEM.

^aCultured oocytes were denuded and classified as PB- (no polar bodies) or PB+ (with the first polar body).

^bAfter culture for 24 h, all oocytes were classified as PB-, no PB+ oocytes were present.

^{c,d}Within columns percentages with different superscripts are significantly different by Duncan's multiple range test ($P < 0.05$).

had been penetrated, of which all were activated and had formed a female pronucleus, and 95% had both first and second polar bodies (Fig. 2a). Most of the first polar body contained scattered chromatin, while in the second polar body condensed chromatin was observed. Of the PB- oocytes, 69% were penetrated and all were activated, and 90% emitted only one polar body (Fig. 2b). All of the PB+ and PB- oocytes cultured without spermatozoa were not activated.

The results of artificial stimulation of oocytes with an electric pulse are shown (Table 5). PB- oocytes that had been cultured for 24 h were not activated to form a female pronucleus when stimulated with an electric pulse. In contrast, in oocytes cultured for 48 h and stimulated artificially, 53% of PB- oocytes were activated, 73% of which emitted a first polar body, and a significantly higher percentage (81%) of PB+ oocytes were activated, of which 79% emitted both first and second polar bodies. The morphology of the polar bodies in both types of activated oocyte was similar to those of IVF oocytes.

Experiment 4

The results of IVF and culture of immature oocytes that had been cultured previously for 24 h are shown (Table 6). The percentage of oocytes penetrated by spermatozoa was 88% (275/314). When oocytes were fixed 6 h after insemination, 77% of the penetrated oocytes were still at MI and heads of penetrating spermatozoa were still condensed (Fig. 3a). By 12 h after insemination, 45% of penetrated oocytes had reached MII, in which most of the sperm heads were enlarged or swollen (Fig. 3b), and 24% had been activated by penetrated spermatozoa, of which 50% contained a female pronucleus. Formation of a male pronucleus was observed in two of the five oocytes in which a female pronucleus had formed. Between 18 and 24 h after insemination, 65–66% of the oocytes had not been activated by penetrated spermatozoa and were arrested in MII, while 18–25% were activated and most contained both female and male pronuclei. At 18 h after insemination, some of the enlarged sperm heads in the penetrated oocytes (MII) became condensed into a small mass (50%) (Fig. 3c,d) or transformed into metaphase chromosomes (16%) (Fig. 3e). Some of the condensed sperm heads were incorporated into the maternal metaphase plate (Fig. 3c) and others were associated with the spindle (Fig. 3d). At 24 to 36 h after

insemination, in 3–44% of the penetrated oocytes (MII), the sperm heads had condensed to a small mass. The incidence of transformation of sperm chromatin to metaphase chromosomes in MII oocytes increased from 21% at 24 h after insemination to 36% at 36 h after insemination.

When inseminated oocytes were cultured for 12 h after insemination and treated with an electrical pulse, only 35% were activated and formed a female pronucleus after a further 12 h in culture (Table 7). However, when oocytes were stimulated at 24 h after insemination, most were activated (93%, $P < 0.05$). The percentage of male pronucleus formation in oocytes stimulated at 24 h after insemination was higher (77%, $P < 0.05$) than that in oocytes stimulated at 12 h after insemination (35%).

Experiment 5

Changes in the pattern of polypeptide synthesis in pig oocytes at MI (24 and 48 h culture) and MII (48 h culture) are shown (Fig. 4). Oocytes cultured for 48 h were characterized by an increase in the synthesis of a 25 kDa polypeptide irrespective of the nuclear status (MI or MII). Synthesis of 25 kDa polypeptide in MI oocytes after 24 h culture was much lower compared with MI oocytes cultured for 48 h.

Results of histone H1 kinase activity are summarized (Fig. 5). Some of the oocytes remained at the GV stage after culture for 24 or 48 h (Table 1). Histone H1 kinase activity in GV stage oocytes remained at basal values regardless of the duration of the culture period. Histone H1 kinase activity in oocytes at MI after culture for 24 h was significantly higher ($P < 0.05$) than that in oocytes cultured for 48 h. However, histone H1 kinase activity in MI-arrested oocytes was significantly higher ($P < 0.05$) than that in oocytes that had matured to MII.

Discussion

In the present study, the nuclear status of oocytes was examined after maturation in two culture systems: m-M199 (non-static culture with follicle shells) and m-Waymouth (static culture with pig follicular fluid). Expt 1 showed that the incidence of nuclear maturation to MII was higher using m-M199 (79–90%) after culture for 36 to 48 h compared with m-Waymouth (48–62%). Although there was no difference in

Table 4. *In vitro* fertilization of pig oocytes cultured and classified on the basis of the presence of a polar body

Cultrure (h)	Oocyte ^a	Insemination	Total number of oocytes examined	Percentage of unfertilized oocytes	Percentage of penetrated oocytes	Number of activated oocytes ^b						Percentage of degenerated oocytes	
						One polar body			Two polar bodies			Polyspermy (%) ^d	MPN ^c (%) ^d
						Total (%)	MPN ^c (%) ^d	Polyspermy (%) ^d	Total (%) ^d	MPN ^c (%) ^d	Polyspermy (%) ^d		
24	PB-	+	81	24 (30 ± 2) ^e	56 (69 ± 1) ^e	5 (6 ± 2) ^e	4 (80 ± 17) ^e	1 (20 ± 17) ^e	0 (0 ± 0) ^e	1 (20 ± 17) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	1 (1 ± 1)
24	PB-	-	52	52 (100 ± 0) ^f	0 (0 ± 0) ^f	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0)
48	PB-	+	102	31 (30 ± 12) ^e	70 (69 ± 11) ^e	70 (69 ± 11) ^f	63 (90 ± 2) ^e	58 (83 ± 8) ^f	37 (53 ± 23) ^f	6 (9 ± 2) ^e	5 (7 ± 3) ^e	1 (1 ± 1)	1 (1 ± 1)
48	PB-	-	104	104 (100 ± 0) ^f	0 (0 ± 0) ^f	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0)	0 (0 ± 0)
48	PB+	+	157	59 (38 ± 13) ^e	97 (62 ± 14) ^e	97 (62 ± 14) ^f	5 (5 ± 3) ^e	4 (4 ± 2) ^e	2 (2 ± 1) ^e	82 (85 ± 3) ^f	51 (53 ± 7) ^f	1 (1 ± 1)	1 (1 ± 1)
48	PB+	-	134	132 (98 ± 2) ^f	0 (0 ± 0) ^f	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0) ^e	0 (0 ± 0)	2 (1 ± 1)

Percentages are expressed as mean ± SEM.

^aOocytes were cultured in maturation medium for 24 or 48 h and classified on the basis of the presence or absence of a polar body (PB+ or PB-). The oocytes were then fertilized *in vitro* for 5 h and cultured for a further 5 h.^bActivated oocytes with a female pronucleus that emitted one polar body or both the first and second polar bodies (two polar bodies).^cOocytes with at least one male pronucleus (MPN).^dPercentages of the total number of activated oocytes.^eWithin columns percentages with different superscripts are significantly different by Duncan's multiple range test ($P < 0.05$).

Fig. 2. Morphology of polar bodies after *in vitro* fertilization (IVF) of mature and immature pig oocytes cultured for 48 h. Cultured oocytes were classified as PB+ and PB- (with or without the first polar body, respectively), fertilized *in vitro* and examined under a phase-contrast microscope after fixation and staining with aceto-orcein. (a) Two polar bodies were observed after IVF of PB+ oocytes. The first polar body (1PB), in which the chromosome was scattered, and the second polar body (2PB), in which condensed chromatin was observed. The second polar body was recognized because of the association with the spindle (not in focus). Both single female (FPN) and male (MPN) pronuclei were observed. A sperm tail associated with the MPN is not in focus. (b) One polar body (1PB) associated with the spindle (S) was observed. This polar body had condensed chromatin and was similar to that of the second polar body of PB+ oocytes after fertilization and extrusion of two polar bodies. Scale bars represent 10 μ m.

the percentage of immature oocytes at GV, GVBD and MI stages in the two media, there was a tendency for more oocytes to arrest at MI after culture in m-Waymouth compared with m-M199. Therefore, in some experiments in the present study, oocytes were cultured in m-Waymouth to obtain a large number of oocytes arrested at an immature stage such as MI.

Expt 2 revealed that when non-classified oocytes cultured

for 48 h were fertilized *in vitro*, two types of activated oocytes were produced: oocytes with one polar body and oocytes with two polar bodies. Examination under a fluorescence microscope after staining with DAPI enabled observation of oocyte and penetrated sperm nuclear status, and polar bodies within the perivitelline space. With this method, there is no possibility of detachment of polar bodies from oocytes during staining. The specimens were also fixed with acetic

Table 5. Artificial activation of pig follicular oocytes classified at 24 or 48 h of culture on the basis of the presence of a polar body

Culture (h)	Oocyte ^a	Stimulation	Total number of oocytes examined	Percentage of inactivated oocytes	Number of activated oocytes ^b				Percentage of degenerated oocytes
					Total (%)	One polar body ^c (%) ^d	Two polar bodies ^c (%) ^d	Others ^c (%) ^d	
24	PB-	+	111	104 (94 ± 5) ^e	0 (0 ± 0) ^e	0	0	0	7 (6 ± 5)
24	PB-	-	20	16 (80 ± 8) ^e	0 (0 ± 0) ^e	0	0	0	4 (20 ± 8)
48	PB-	+	70	29 (41 ± 5) ^f	37 (53 ± 5) ^f	27 (73 ± 10) ^e	5 (14 ± 5) ^e	5 (14 ± 11)	4 (6 ± 2)
48	PB-	-	33	33 (100 ± 0) ^g	0 (0 ± 0) ^e	0	0	0	0 (0 ± 0)
48	PB+	+	154	28 (18 ± 5) ^h	124 (81 ± 5) ^g	11 (9 ± 3) ^f	98 (79 ± 7) ^f	15 (12 ± 3)	2 (1 ± 1)
48	PB+	-	64	63 (98 ± 3) ^g	0 (0 ± 0) ^e	0	0	0	1 (1 ± 3)

Percentages are expressed as mean ± SEM.

^aOocytes were cultured in maturation medium for 24 or 48 h and classified on the basis of the presence or absence of a polar body (PB+ or PB-). Oocytes were then fertilized *in vitro* for 5 h and cultured for a further 5 h.

^bActivated oocytes with a female pronucleus.

^cActivated oocytes with the first body (one polar body) or the first and second polar bodies (two polar bodies). Some of the activated oocytes were fragmented or lacked polar bodies (Others).

^dPercentages of the total number of activated oocytes.

^{e-h}Within columns percentages with different superscripts are significantly different by Duncan's multiple range test ($P < 0.05$).

alcohol, stained with aceto-orcein and examined under a phase-contrast microscope, the standard method for oocyte examination used in many laboratories. Almost all (92%) of the penetrated oocytes showed extrusion of the same number of polar bodies as seen after DAPI staining. These results confirm that, after IVF of pig oocytes, activated oocytes with one polar body and a female pronucleus are not the result of fixation or staining. Therefore, the oocytes with two polar bodies are considered to be the result of normal fertilization of matured oocytes at MII. However, oocytes with one polar body may be the result of penetration of MI oocytes. Two possibilities exist for the latter: (i) an oocyte at MII is fertilized, but the second polar body is not extruded; and (ii) an oocyte arrested at MI is fertilized and the first polar body is extruded.

Cultured oocytes were classified as immature and mature and were fertilized *in vitro* or stimulated with an electrical pulse (Expt 3) to confirm which hypothesis is acceptable. Of those oocytes classified as PB- (without a polar body) after culture for 48 h, 78% were MI oocytes and 13% were MII. Of those oocytes classified as PB+ (with the first polar body), 94% were MII oocytes. The 13% error rate (MII) for PB- may be the result of the difficulty of finding a polar body in the dark ooplasm, or extrusion of the polar body immediately after selection. Use of a fluorescence microscope after staining with a fluorescent dye appears to be a reliable method for observing a polar body. However, the dye itself or UV irradiation may have harmful effects on the oocyte. Therefore, this method is not suitable for oocyte classification for IVF or electric stimulation. Nevertheless, the procedure adapted in the present study had a significant effect on selection of oocytes. IVF and parthenogenesis experiments using both PB- and PB+ oocytes cultured for 48 h confirm

that MI-arrested oocytes can be activated to form a female pronucleus, as did MII oocytes when they were penetrated by spermatozoa and emitted a first polar body. These results indicate that MI-arrested oocytes have relatively mature cytoplasm and meiotic division can be resumed by stimuli such as sperm penetration or an electrical pulse, as suggested in mouse oocytes (Eppig *et al.*, 1994; McConnell *et al.*, 1995; Polanski, 1995). However, unlike activated MII oocytes, activated MI oocytes did not complete the second meiosis. Therefore, the genome of activated MI-arrested oocytes is maintained in the diploid state. In light of these observations, it is important to consider the ploidy of fertilized oocytes and to discuss genome size because less attention has been given to the number of polar bodies after IVF. It is also interesting that there are morphological differences in the first polar body between activated MI-arrested and MII oocytes. The appearance of the first polar body in activated MI-arrested oocytes is very similar to that of the second polar body in activated MII oocytes. The morphology of the polar bodies, especially chromatin condensation, may be controlled by the condition of ooplasm, as the extent of cytoplasmic maturation, just before extrusion of the polar body.

When immature oocytes cultured for 24 h were inseminated (Expt 4), spermatozoa penetrated the ooplasm and the heads of most of the penetrating spermatozoa were transformed. The sperm heads first 'enlarged' and then 'condensed' (mouse, Iwamatsu and Chang, 1972; hamster, Barros and Munoz, 1974; rabbit, Overstreet and Bedford, 1974; rat, Niwa and Chang, 1975; dog, Mahi and Yanagimachi, 1976; cow, Niwa *et al.*, 1991; pig, Wang *et al.*, 1992), and finally transformed to 'metaphase chromosomes' (mouse, Clarke and Masui, 1986; cow, Abeydeera and Niwa, 1992; pig, Wang and Niwa, 1997) as the subsequent culture

Table 6. Nuclear changes in pig oocytes after *in vitro* fertilization at 24 h of culture, and morphological changes of penetrating sperm heads

Culture after insemination (h) ^a	Total number of oocytes examined	Percentage of penetrated oocytes at the stage of ^b																			
		Metaphase I				Anaphase/Telophase I				Metaphase II with one polar body ^c				Female condensed chromatin with two polar bodies ^c				Female pronucleus with two polar bodies ^c			
		Total		Cn	En	Total		Cn	En	Total		Cn	En	Total		Cn	En	Total		En	MPN
6	39	37 (94)	30 (77)	27 (69)	3 (8)	5 (13)	4 (10)	1 (3)	0 (0)	-	-	-	-	2 (5)	2 (5)	-	-	0 (0)	-	-	
12	42	37 (88)	1 (2)	1 (1)	-	7 (17)	-	7 (17)	19 (45)	1 (2)	18 (43)	-	3 (7)	5 (12)	5 (12)	3 (7)	3 (7)	5 (12)	3 (7)	2 (5)	
18	38	34 (89)	1 (3)	1 (1)	-	0 (0)	-	-	25 (66)	-	-	19 (50)	6 (16)	1 (3)	1 (3)	1 (3)	-	7 (18)	-	7 (18)	
24	75	70 (93)	2 (3)	2 (3)	-	0 (0)	-	-	49 (65)	-	-	33 (44)	16 (21)	0 (0)	0 (0)	-	-	19 (25)	3 (4)	16 (21)	
30	81	66 (83)	1 (1)	1 (1)	-	0 (0)	-	-	41 (51)	-	-	19 (23)	22 (27)	0 (0)	0 (0)	-	-	24 (30)	7 (9)	17 (21)	
36	39	31 (79)	2 (5)	2 (5)	-	0 (0)	-	-	15 (38)	-	-	1 (3)	14 (36)	0 (0)	0 (0)	-	-	14 (36)	3 (8)	11 (28)	

^aInseminated oocytes were examined every 6 h after insemination.
^bOocytes were penetrated by spermatozoa and developed to the stage of metaphase I, anaphase or telophase I, condensed female chromatin or female pronucleus. In the activated oocytes, sperm heads were condensed (Cn), enlarged or swollen (En), condensed into a small mass (Cs), metaphase chromosome (Mc) or male pronucleus (MPN) (see Fig. 3).
^cOocytes with the first polar body (one polar body) or the first and second polar bodies (two polar bodies).

Fig. 3. Sperm heads penetrating immature (metaphase I) pig oocytes. Oocytes were cultured for 24 h, fertilized *in vitro* and subsequently cultured for up to 36 h (see Table 6). Arrows indicate tails of the penetrating spermatozoa. Heads of penetrating spermatozoa were condensed (Cn) at 6 h after insemination (a), but became enlarged or swollen (En) at 12 h after insemination (b). (c,d,e) At 18 h after insemination, some sperm heads recondensed into a small mass (Cs) or transformed into metaphase chromosomes (Mc). Some of the recondensed heads were incorporated into the maternal metaphase plate (Mp) and others were associated with the spindle (S). In (d) one recondensed head is visible; another is out of focus. Scale bars represent 10 μ m.

was prolonged. Clarke and Masui (1986) reported that transformation of sperm heads to metaphase chromosomes in mouse MI oocytes is regulated by a maturation or M-phase promoting factor (MPF; Masui and Markert, 1971). In the present study, metaphase chromosomes were present in pig oocytes that had proceeded to MII, in which high MPF

activity is observed (Naito and Toyoda, 1991). However, after 48 h culture, MI-arrested or MII oocytes form a well developed male pronucleus after sperm penetration. These results emphasize the difference in maturity of the cytoplasm between young MI oocytes and MI-arrested or MII oocytes. It is interesting that the nuclear status of penetrated

Fig. 4. Changes in the pattern of polypeptide synthesis during culture of pig oocytes. Oocytes were sampled after 24 and 48 h in culture and then labelled with [³⁵S]methionine for 3 h. After labelling, the nuclear status of oocytes was examined by staining with 4',6-diamidino-2-phenylindole (DAPI) and oocytes were classified as metaphase I (MI) and metaphase II (MII). Oocytes were then subjected to SDS-PAGE. Lane 1: MI oocyte after 24 h culture; lane 2: MI oocyte after 48 h culture; lane 3: MII oocytes after 48 h culture. The arrow indicates a band of 25 kDa polypeptide in lanes 2 and 3.

immature oocytes changes from MI to MII in the same proportion as observed in non-inseminated immature oocytes. Some of the condensed sperm heads were incorporated into the oocyte metaphase plate, as if they were maternal chromosomes, or became associated with the spindle. These results indicate that young MI oocytes were not activated by sperm penetration, although some were activated spontaneously and formed both female and male pronuclei as the duration of the culture period increased. Penetrated oocytes were activated easily compared with unpenetrated oocytes cultured for the same duration. The reason for this is unclear, although the possibility that the spermatozoon contains a factor that promotes oocyte activation (Swann, 1990) must be considered. When inseminated oocytes were stimulated, the percentage of

Fig. 5. Activity of histone H1 kinase in pig oocytes after culture for 24 or 48 h. Oocytes at germinal vesicle stage (GV), immature oocytes at metaphase I (MI) and mature oocytes at metaphase II (MII) were identified using fluorescence microscopy. Histone H1 kinase activity is presented as mean $\pm$ SEM. Bars with different letters are significantly different ($P < 0.05$).

activation (female and male pronucleus formation) was increased at 24 h after insemination. The ability of penetrated oocytes to be activated also seems to be enhanced as cytoplasmic maturation progresses, as occurs in oocytes cultured for 48 h under conditions that promote normal maturation.

Ding *et al.* (1992b) reported that synthesis of a 25 kDa polypeptide increased between MI (28 h in culture) and MII (32–47 h). In addition, the transition of 25 kDa protein to 22 kDa protein is associated with oocyte activation and pronuclear formation after penetration of oocytes by spermatozoa (Ding *et al.*, 1992a). Expt 5 revealed that the pattern of protein synthesis in MI-arrested oocytes after 48 h culture was similar to that in MII oocytes cultured for the same duration, and was different from that in MI oocytes cultured for 24 h. This observation is in agreement with a report on mouse oocytes (Eppig *et al.*, 1994). Expts 3 and 4 also confirm that cytoplasmic maturation in immature oocytes arrested at MI has already occurred and they respond to sperm penetration or parthenogenetic activation, as do oocytes at MII. These results indicate that cytoplasmic maturation of pig oocytes depends not on nuclear status but on the duration of culture. Although the function of the 25

Table 7. Activation after electrical pulse of pig oocytes cultured for 24 h and fertilized *in vitro*

Culture after electrical pulse (h after insemination) ^a	Number of oocytes examined	Percentage of oocytes penetrated	Percentage of activated oocytes ^b		
			Total	Penetrated oocytes	With MPN ^c
12	17	16 (94 $\pm$ 5)	6 (35 $\pm$ 6) ^d	6 (35 $\pm$ 6) ^d	6 (35 $\pm$ 6) ^d
24	30	26 (87 $\pm$ 2)	28 (93 $\pm$ 4) ^e	24 (80 $\pm$ 5) ^e	23 (77 $\pm$ 2) ^e

Percentages are expressed as mean $\pm$ SEM.
^aInseminated oocytes were stimulated with an electric pulse 12 or 24 h after insemination.
^bActivated oocytes with a female pronucleus.
^cMPN, a male pronucleus.
^{d,e}Within columns percentages with different superscripts are significantly different by Duncan's multiple range test ($P < 0.05$).

kDa polypeptide is unclear, it appears to play an important role in cytoplasmic maturation in both MI-arrested oocytes and oocytes that have matured to MII. Funahashi *et al.* (1995) proposed that cytoplasmic glutathione concentration reflects the degree of cytoplasmic maturation in pig oocytes matured *in vitro* because it is correlated with the ability to form a male pronucleus (Yoshida, 1993). The 25 kDa polypeptide reported here is different from glutathione, a major intercellular free thiol. In addition, the modification of the 25 kDa polypeptide to a 22 kDa polypeptide is a dephosphorylation process (Ding *et al.*, 1992a). It is unclear whether there is any association between the amount of 25 kDa polypeptide and cytoplasmic glutathione.

The mechanism of arrest at MI remains to be elucidated. Cytostatic factor (CSF), a homologue of the *c-mos* proto-oncogene product (Sagata *et al.*, 1989), promotes MII arrest in unfertilized frog oocytes (Masui and Markert, 1971). Furthermore, Hirao and Eppig (1997) reported that *c-mos* participates in sustaining MI arrest in oocytes from crossbred mice (LT strain). However, the function of *c-mos* in pig oocytes arresting at MI has not been reported. The phenomenon of oocyte arrest appears to be similar to that induced by CSF in MII oocytes, even though in arrested oocytes the nuclear status is MI. It will be necessary to determine the relationship between physiological arrest at MI and the function of CSF in pig oocytes using molecular approaches in future studies.

One of the well known factors that regulates oocyte maturation is MPF, which can be measured as histone H1 kinase activity (Arion *et al.*, 1988; Labbe *et al.*, 1988a,b, 1989). Naito and Toyoda (1991) reported that during *in vitro* maturation of pig oocytes, histone H1 kinase activity is high in both MI and MII oocytes. However, in some culture media, histone H1 kinase activity is higher in MI oocytes than in MII oocytes (Naito *et al.*, 1992). In the present study, MI oocytes cultured for 24 h showed higher histone H1 kinase activity compared with MII oocytes cultured for 48 h, and activity in oocytes arrested at MI was intermediate. These results are in agreement with the proposal that histone H1 kinase activity is closely related to the ability of oocytes to be activated when they are matured and aged *in vitro* (Kikuchi *et al.*, 1995a). A decrease in histone H1 kinase activity appeared to occur in both MI and MII oocytes when the culture period was extended to 48 h. However, the extent of the decrease may vary depending on the nuclear status of oocytes. Indeed, the sensitivity to parthenogenetic stimulation of MII oocytes cultured for 48 h was greater (81% activated oocytes) than that of MI-arrested oocytes (53%), whereas no MI oocytes were activated after culture for 24 h. These results indicate that a decrease in histone H1 kinase activity is also important for cytoplasmic maturation in MI-arrested oocytes and in oocytes that have matured to MII after culture in maturation medium.

In conclusion, in pig follicular oocytes cultured for a period that was long enough to produce MII oocytes, some of the oocytes arrested at MI. The MI-arrested oocytes, which were immature with regard to nuclear status, had relatively mature cytoplasm that was able to promote oocyte activation after sperm penetration or parthenogenetic stimulation. The relationship between cytoplasmic maturation and a 25 kDa

polypeptide or a decrease in histone H1 kinase activity in the mechanism of oocyte arrest at MI requires clarification.

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