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Distribution of Cortical Granules in Porcine Oocytes Inseminated In Vitro at Various Times of Culture for Maturation In Vitro

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Abstract: *The distribution of cortical granules (CGs) during in vitro maturation of porcine oocytes was investigated with a confocal laser microscope. The incidence of exocytosis of CGs in oocytes inseminated at various maturation stages was also investigated to determine the dependency of the block of polyspermy on the zona reaction in porcine oocytes matured in vitro. CGs were synthesized but not yet transported beneath the oolemma at 18 h of culture (GVBD stage). CGs transported beneath the oolemma were found at 30 h of culture (at stages metaphase I to anaphase I). From 24 h to 36 h of culture, when a dramatic change in the pattern of distribution of CGs was observed, oocytes were inseminated. Although there was no difference among the groups in the rate of oocytes penetrated, the rate of polyspermy decreased as the culture period of the oocytes was prolonged to more than 30 h. It can be concluded from the results that the oocytes in which cortical granules have been transported beneath the oolemma can react to some degree against excessive penetration of spermatozoa, but polyspermy could not be avoided completely. This may reflect the incomplete amount of CGs synthesized during culture or the incompleteness of exocytosis of CGs in the oocytes matured in vitro.*

Key words: *Cortical granules, IVM, IVF, Porcine oocyte*

Although *in vitro* fertilization of porcine oocytes matured *in vitro* has been successful, efficiency in obtaining piglets after transfer of embryos needs to be improved [1–4]. The main cause of this failure is attributed to incomplete cytoplasmic maturation. During maturation, many changes take place in the oocytes, such as a change in the protein profile [5]. Changes in the amount and the distribution of cortical granules (CGs) were also observed [6].

In mammals there are two mechanisms whereby polyspermy is blocked: the zona reaction and the plasma membrane block. Oocytes in golden hamster, dog, sheep and human depend primarily on the zona reaction, whereas rat and mouse oocytes depend on the plasma membrane block, and rabbit oocytes depend on both [7]. Since supplementary spermatozoa have been observed in the zona pellucida but not in the perivitelline space of porcine oocytes fertilized *in vivo*, porcine oocytes are thought to block polyspermy primarily by the zona reaction.

Although a few spermatozoa have been seen in the porcine ampula during fertilization *in vivo* [8], a relatively large number of spermatozoa have been used for insemination to gain high penetration rates. This causes a high rate of polyspermy, possibly because many spermatozoa seem to be able to penetrate an oocyte prior to completion of the block. The delayed exocytosis and/or incomplete dispersion of CG materials to the perivitelline space during sperm penetration seemed to be critical in porcine oocytes matured and fertilized *in vitro* [6, 9]. In human oocytes fertilized *in vitro*, polyspermy is attributed to the incompleteness of the reaction [10].

In this study, to determine the dependency on the zona reaction for blocking polyspermy in porcine oocytes matured *in vitro*, the incidence of exocytosis of CGs in oocytes inseminated at various maturation stages was investigated by confocal laser microscopy.

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Materials and Methods

Medium

The handling medium was 20 mM Hepes-buffered (pH 7.4 in air) Waymouth's medium MB 752/1 (Flow Laboratory, USA) supplemented with 1 mg/ml polyvinylalcohol (PVA, MW 30,000–70,000; Sigma, USA) and antibiotics. The maturation medium was almost the same as that described by Yoshida *et al.* [11]: Waymouth's medium MB 752/1 supplemented with 10% (V/V) fetal bovine serum (Filtron, Australia), 10% (V/V) pig follicular fluid (collected from follicles 3–5 mm in diameter derived from immature gilt, filter-sterilized, and stored at −80°C until use), 10 IU/ml PMSG (Sankyo Co., Ltd., Japan), 10 IU/ml hCG (Teikoku Hormone Mfg. Co., Ltd., Japan), 1 mM L-glutamine and antibiotics. Sperm washing saline (9.6 mg/ml NaCl) was supplemented with 1 mg/ml PVA and antibiotics, and its pH was adjusted to 7.4 with NaOH. The sperm preincubation medium consisted of modified Tyrode's solution [12] containing 16.7 mM sodium lactate, 1 mM sodium pyruvate, 5 mM D-glucose, 2 mM $CaCl_2$ and 5 μg/ml kanamycin sulfate, and pH was adjusted to 7.8. Since a high penetration rate had been obtained by spermatozoa preincubated in the medium without protein, BSA was substituted for PVA [13]. The fertilization medium was the same as the preincubation medium, except that the concentration of $CaCl_2$ was increased to 4.6 mM and 2 mM caffeine was added, and pH was adjusted to 7.6.

In vitro maturation of oocytes

Ovaries of prepubertal gilts were collected just after slaughter, and they were transported in a thermos from a local slaughterhouse to the laboratory within 2 h of slaughter. After several washings with sterile warm saline, follicular oocytes were collected by aspiration with an 18-G needle attached to a 5-ml syringe and pooled in 10-ml test tubes set in a water bath (37°C) for settling. About 3 ml of the lower portion of the collected fluid containing cumulus-oocyte complex (COC) was transferred to a petri dish. Oocytes with packed cumulus cells and dense cytoplasm were collected under a dissection microscope and washed twice with the handling medium. Selected COCs were washed with the maturation medium, then incubated in droplets (200 μl for each 20 COCs) of maturation medium under mineral oil (Squibb, USA) in plastic dishes (Falcon 1008; Becton Dickinson, USA) at 39°C under an atmosphere of 5% CO_2 in air for various lengths of time, according to the experiment.

In vitro fertilization

The concentrated portion of freshly ejaculated semen was collected from a boar by the hand glove method and stored in the dark for 18–42 h at 15°C. About 1 ml of semen was washed twice with the washing medium by centrifugation (10 min at 800 g), and after a further two washings with the preincubation medium, spermatozoa were preincubated in the preincubation medium at a concentration of 1×10^8 cells/ml under an atmosphere of 5% CO_2 in air at 37°C for 2 h. After preincubation, spermatozoa were washed with the fertilization medium (10 min at 700 g) and co-cultured with the COCs in droplets (100 μl for each 10 COCs) of the fertilization medium for 6 h. In an experiment to determine the appropriate duration of co-culture, spermatozoa were co-cultured with COCs for 2–12 h. The final concentration of spermatozoa was adjusted to 1×10^6 cells/ml. After co-incubation with spermatozoa, oocytes were washed and incubated in droplets of the culture medium for 4 h.

Light microscopy of oocytes

For observation of the features of nuclei in oocytes maturing *in vitro*, collected COCs (0 h of culture) and COCs cultured for 6–48 h were used. Cumulus cells were dispersed by pipetting in the handling medium containing 0.1% hyarulonidase (Sigma, USA). After several washings, oocytes were fixed with acetic alcohol (1:3) for 3–4 days and stained with 1% aceto-orcein for observation of the configuration of nuclei under a phase-contrast microscope. Observed configurations of nuclei were classified according to the criteria described by Hunter and Polge [14] and Motlik and Fulka [15]. For the observation of inseminated oocytes, oocytes were processed in the same way except for hyarulonidase treatment at the end of culture.

Observation of distribution of cortical granules in oocytes

The distribution of CGs in the oocytes was observed according to the method reported by Yoshida *et al.* [9]. Briefly, after washing oocytes with PBI [16], cumulus cells surrounding oocytes were removed by treatment with 0.1% (w/v) hyaluronidase (Sigma, USA). After washing with PBI three times, oocytes were fixed in 5% paraformaldehyde PBI for 30 min at room temperature. Zonae pellucidae of inseminated oocytes were removed by treatment with acidic Tyrode's solution (pH 2.5) prior to fixation. Fixed oocytes were stored in PBI for a maximum of 4 days at 4°C. On the day of observation, the specimens were pretreated with 0.1% triton X-100 for 5 min, then incubated in PBI

containing 100 μg/ml FITC-conjugated Peanut agglutinin (FITC-PNA, VECTOR, USA) for 30 min. After washing with PBI 5 times, the oocytes were mounted in glycerol spotted on a slide glass, then squashed lightly with a cover glass to fix the position of the specimen. Distribution of CGs on the cross section (1 μm thickness) of the oocytes was observed under a confocal laser microscope (Bio-Rad MRC-500). As a negative control, oocytes without treatment with FITC-PNA were used each time.

Statistical analysis of data

Differences in rates of oocytes among classes were tested by chi square analysis. Differences in the mean number of spermatozoa which penetrated an oocyte were tested by Student's *t*-test.

Results

As shown in Table 1, oocytes that underwent germinal vesicle break down (GVBD; matured further than the early diakinesis stage) were observed from 18 h of culture, and almost all of the oocytes (89.5%) completed GVBD by 24 h of culture. The percentage of oocytes in metaphase I reached the maximum at 24–30 h of culture, and a significantly larger number of oocytes matured to metaphase II by 30 h of culture.

Oocytes could be classified into three types according to the distribution patterns of CGs: Type I, scattered fluorescence in the cytoplasm (Fig. 1-a); Type II, monolayer of fluorescence beneath the oolemma (Fig. 1-b); and Type III, only a little fluorescence in the cytoplasm (Fig. 1-c). The changes in the distribution of CGs in oocytes during culture for maturation *in vitro* were observed. As shown in Table 2, Type II oocytes were observed from 18 h of culture, and the number of such oocytes reached a high level at 30 h of culture.

To determine the optimal duration of insemination, oocytes cultured for 48 h were co-cultured with preincubated spermatozoa at a final concentration of 1 × 10^6 cells/ml for 2–12 h. The results are shown in Table 3. As previously reported [17], the number of penetrated oocytes increased as the duration of insemination became longer, but the number of monospermic oocytes decreased as the duration of insemination became longer. The estimated efficiency (% of monospermic oocytes multiplied by penetration rate) was highest when the oocytes were inseminated for 6 h (35% of inseminated oocytes were monospermic). To obtain monospermic oocytes at a relatively high rate in this study, the duration of insemination was set to 6 h in subsequent experiments.

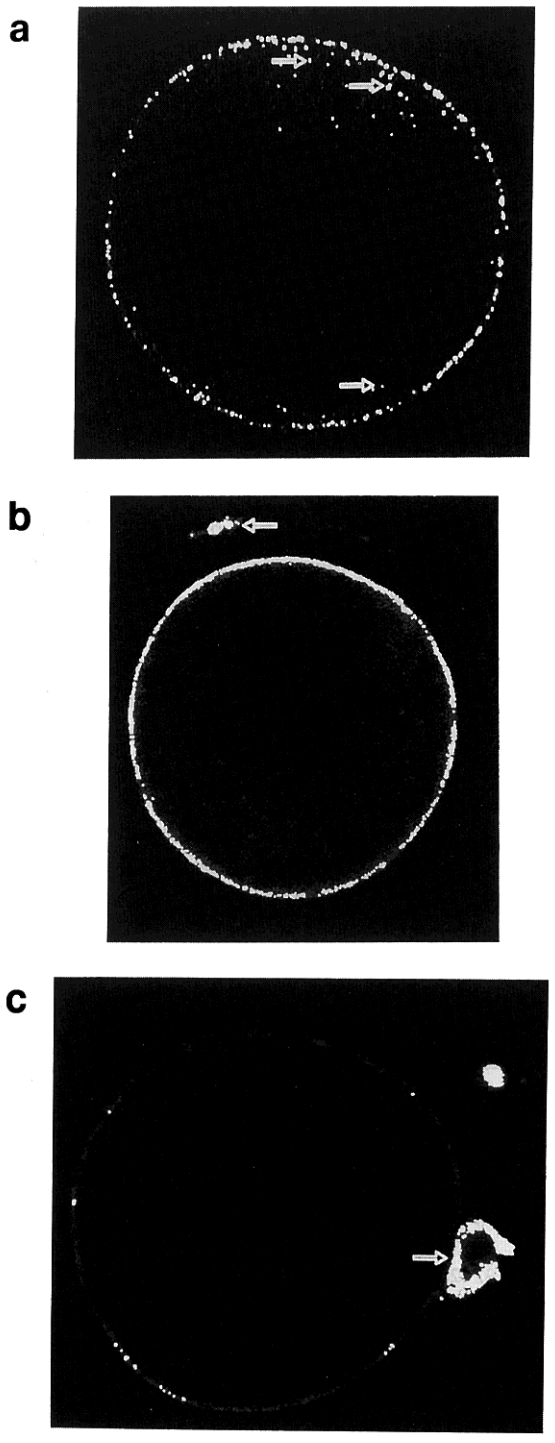
Oocytes cultured for various lengths of time (24–36 h) were inseminated, and then the distribution of CGs and sperm penetration were observed. The distribution patterns of CGs were the same as those of maturing oocytes except for one distribution pattern in which fluorescence was observed partially in the peripheral region of the cytoplasm (Type IV, Fig. 2). Those Type-IV oocytes observed after insemination could be regarded as oocytes penetrated by spermatozoa because Type-IV oocytes were not observed without insemination (Table 2). The results are shown in Tables 4 and 5. There was no significant difference in the rates of penetrated oocytes that were cultured for various times before insemination. Nevertheless, when oocytes cultured for 24 h were inseminated with spermatozoa, only 19% of the penetrated oocytes had decondensed sperm heads and about 90% of the oocytes were polyspermy, and half of the oocytes were judged to be types I and II. As

Table 1. Changes in the nuclear features of porcine oocytes cultured *in vitro*

Culture time (h)	No. of oocytes examined	Nuclear feature (%)				
		GV 1-4*	ED-LD	M I	A I-M II	A II-T II
0	119	108 (90.8)^{a}	11 (9.2)^{a}	0 (0.0)^{a}	0 (0.0)^{a}	0 (0.0)^{a}
6	134	127 (94.8)^{a}	5 (3.7)^{a}	0 (0.0)^{a}	2 (1.5)^{a}	1 (0.7)^{ab}
12	111	106 (95.5)^{a}	3 (2.7)^{ab}	0 (0.0)^{a}	1 (0.9)^{a}	1 (0.9)^{ab}
18	116	70 (60.3)^{b}	37 (31.9)^{c}	5 (4.3)^{a}	3 (2.6)^{a}	1 (0.8)^{ab}
24	133	14 (10.5)^{c}	55 (41.4)^{c}	40 (30.1)^{b}	24 (18.0)^{b}	0 (0.0)^{a}
30	113	10 (8.8)^{c}	14 (12.4)^{a}	32 (28.3)^{b}	51 (45.1)^{c}	6 (5.3)^{b}
36	111	15 (13.5)^{c}	2 (1.8)^{a}	13 (11.7)^{c}	64 (57.7)^{c}	7 (6.3)^{ab}
42	142	21 (14.8)^{c}	6 (4.2)^{a}	16 (11.3)^{c}	68 (47.9)^{c}	32 (22.5)^{c}
48	164	18 (11.0)^{c}	5 (3.0)^{ab}	21 (12.8)^{c}	73 (44.5)^{c}	47 (28.7)^{c}

*GV1-4, germinal vesicle stage 1-4; ED, early diakinesis; LD, later diakinesis; M I, metaphase I; AI, anaphase I; M II, metaphase II; A II, anaphase II; T II, telophase II. abc: Significantly different ($P<0.01$).

the duration of the culture of oocytes increased to 30–36 h, the number of oocytes with decondensed sperm heads and the number of oocytes judged to be type IV increased, and the number of polyspermy oocytes decreased.



Discussion

The distribution of CGs was determined from the pattern of fluorescence of FITC-PNA in the oocytes. In type I oocytes, CGs were thought to be synthesized but not yet transported beneath the oolemma. The CGs might be transported beneath the oolemma, and these oocytes were judged to be type II. Wang *et al.* [18] also showed that CG migrates to the cortex of the oocyte and forms a monolayer by 26 h of maturation *in vitro*. Among type III oocytes, there may be some in which there were too few CGs to be detected under a confocal laser microscope. It was probable that the activity of the synthesis of CG declined after culture for more than 24 h in these oocytes, and the percentage of such oocytes was 10–16%. Considering the increase in the number of type II oocytes in this study, it seems that transportation of CGs started at 18 h of culture (GVBD stage) and was completed by 30 h of culture (at the metaphase I stage). A similar time course of transportation of CGs in *in vivo* oocytes was observed electron microscopically by Cran and Cheng [19], who showed

Table 2. Distribution of cortical granules in porcine oocytes cultured *in vitro*

Culture time (h)	No. of oocytes examined	Distribution of cortical granules* (%)		
		Type I	Type II	Type III
0	49	53.1[a]	0.0[a]	46.9[a]
18	44	56.8[a]	15.9[b]	27.3[ab]
24	49	36.7[a]	46.9[c]	16.3[b]
30	47	17.0[b]	68.1[c]	14.9[b]
36	48	8.3[b]	77.1[d]	14.6[b]
42	46	6.5[b]	82.6[d]	10.9[b]

*Oocytes were typed according to the distribution of cortical granules. See text and Fig. 1. abc: Significantly different ($P<0.01$).

Fig. 1. Distribution of cortical granules in oocytes cultured *in vitro*. Cortical granules were labeled with FITC-PNA and detected by conforcal laser microscopy. Details are described in Materials and Methods. The pattern of distribution of cortical granules in oocytes was judged to be one of three types: Type I, in which there were CGs in the cytoplasm (arrows) and beneath the oolemma (a); Type II, in which all CGs detected were beneath the oolemma (in some case, fluorescence was detected in the remaining cumulus cells (arrow)) (b); and Type III, in which only a few CGs were detected (in some cases, fluorescence was detected in the first polar body (arrow)) (c).

Table 3. Penetration of oocytes by preincubated boar spermatozoa at various insemination times

Duration of insemination (h)	No. of oocytes inseminated	No. of GVBD oocytes (%)	No. of oocytes penetrated (%)	Mean no. of spermatozoa penetrated	% of monospermic oocytes
2	52	50 (96.2)	5 (9.6)[a]	1.0 ± 0.0[d]	100[a]
4	49	45 (91.8)	18 (36.7)[b]	1.3 ± 6.9[d]	77.8[a]
6	62	61 (98.4)	54 (87.1)[c]	2.2 ± 1.4[e]	40.7[b]
8	53	51 (96.2)	48 (90.6)[c]	2.2 ± 1.2[e]	31.3[b]
10	64	62 (96.9)	54 (84.4)[c]	2.1 ± 1.3[e]	27.8[b]
12	52	51 (98.1)	43 (82.7)[c]	2.8 ± 1.7[f]	20.9[b]

abc: Significantly different (P<0.01). def: Significantly different (P<0.05).

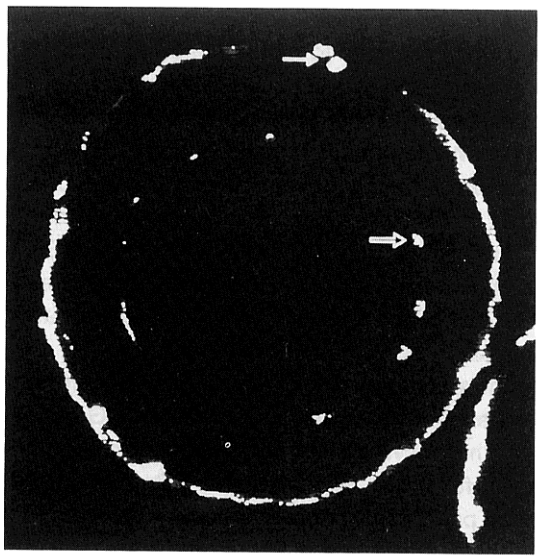


Fig. 2. Distribution of cortical granules in oocytes cultured and fertilized *in vitro*. Oocytes cultured for 24–36 h were inseminated with preincubated spermatozoa prior to detecting CGs. Details are given in Materials and Methods. Another pattern of distribution of CGs (Type IV) was detected in addition to the three types shown in Fig. 1. In Type IV oocytes, fluorescence was detected as clusters in the peripheral region of the cytoplasm (blank arrow). When the zona pellucida was not removed, spermatozoa attached to the zona (white arrow) as well as zona pellucida were also detected. In the experiment, zonae pellucidae were removed to avoid these signals.

Table 4. Distribution of cortical granules in porcine oocytes fertilized *in vitro*

Culture time of oocyte (h)	No. of oocytes examined	No. of oocytes observed and typed* (%)			
		Type I	Type II	Type III	Type IV
24	12	5 (41.7)[a]	1 (8.3)[a]	2 (16.7)[a]	4 (33.3)[a]
30	16	0 (0.0)[b]	0 (0.0)[a]	4 (25.0)[a]	12 (75.0)[b]
36	14	0 (0.0)[b]	4 (28.6)[b]	0 (0.0)[b]	10 (71.4)[ab]

*Oocytes were typed according to the distribution of cortical granules. See text and Fig. 2. ab: Significantly different (P<0.05).

Table 5. Penetration of preincubated boar spermatozoa to oocytes cultured for various times

Culture time of oocytes (h)	No. of oocytes inseminated	No. of oocytes penetrated (%)	No. of oocytes with decondensed sperm heads (%)	Mean no. of spermatozoa penetrated	% of monospermic oocytes
24	25	21 (84.0)[a]	4 (19.0)[a]	2.2 ± 1.4[a]	9.5[c]
30	23	21 (91.3)[a]	9 (42.9)[a]	4.0 ± 1.9[b]	14.3[cd]
36	26	22 (84.6)[a]	12 (54.6)[b]	3.4 ± 1.6[b]	27.3[d]

ab: Significantly different (P<0.05). cd: Significantly different (P<0.01).

that transportation of CGs in the oocytes *in vivo* started at the GVBD stage and was completed at the metaphase II stage (30 h post injection of hCG). Yoshida *et al.* [9] also reported that the time courses of transportation of CGs in oocytes matured *in vivo* and those matured *in vitro* were comparable. It remains unclear whether the slight difference between our results and the results of Cran and Cheng concerning the time of completion of CGs transportation is due to the difference in the source investigated. Another possible explanation for the inconsistency between nuclear configuration and distribution of CGs is that cytoplasmic maturation, including change in the distribution of CGs, might not accord with nuclear maturation.

From 24 h to 36 h of culture, when dramatic change in the rates of Types I- and II-oocytes was observed, oocytes were inseminated. It is thought that CGs were exocytosed after penetration by the spermatozoon and then the distribution of CG materials changed to type IV. In this study, to remove accessory spermatozoa from the zonae pellucidae and perivitelline space, zona pellucida were removed and the oocytes were washed by pipetting prior to the observation of CGs. It has been reported that the contents of CGs dispersed to the perivitelline space after exocytosis [20], but those substances could be removed after zona removal. It is thought that the fluorescence observed in Type IV oocytes indicated the presence of CGs or their contents left in oocytes after penetration by the sperm. Cran and Cheng [6], Wang *et al.* [18], and Hyttle *et al.* [21] also reported that CG materials remained after sperm penetration in porcine and bovine oocytes. It remains to be clarified microscopically whether CG materials are left after exocytosis in porcine oocyte as is observed in mouse oocytes, where part of the exocytosed CG contents remains on the egg surface and aggregates gradually to form large clumps [22]. But it was deduced from the change in the distribution of fluorescence that most of CGs were exocytosed after sperm penetration. No Type III oocytes were observed after insemination of oocytes cultured for 36 h. As mentioned above, it was highly probable that Type III oocytes observed during maturation *in vitro* (Table 2) had already had a decline in their ability to synthesize some proteins as well as CG and had started to degenerate. Such oocytes might be unconsciously eliminated from the observation after insemination or prolonged culture for 46 h.

A comparison of the results shown in Tables 2 and 4 indicates that Type-II oocytes change to Type-IV oocytes after insemination, so that in oocytes classified as Type II, CGs can be expected to be exocytosed by 10 h of postinsemination, whereas in oocytes classified as Types I, exocytosis of CGs can be expected to be negligible because few CGs were present beneath the oolemma in those oocytes. In fact, the number of penetrated oocytes with decondensed sperm heads was smaller in the oocytes cultured for 24 h than in those cultured for 30–36 h, and the competence of activation by sperm penetration is thought to have increased as the duration of culture was increased from 24 h to 36 h. Therefore, the incidence of the block of polyspermy can be expected to be higher in oocytes cultured for 30–36 h than in oocytes cultured for 24 h. Indeed, while the number of penetrated oocytes was the same in all groups, the rate of polyspermy decreased as the culture period was prolonged to more than 30 h. Furthermore, the monospermic rate in oocytes inseminated at 30–36 h of culture was higher than that in the oocytes cultured for 24 h. Wang *et al.* [18] reported that CGs exocytosis had occured after 7-h insemination and a subsequent 11 to 12 h of culture. Nevertheless, it can be concluded from our results that the oocytes in which CGs have been transported beneath the oolemma (Type II oocytes) can react against the excess penetration of spermatozoa by 10 h post insemination.

From a comparison of the number of spermatozoa which penetrated oocytes that differed in CG exocytosis ability (the oocytes cultured for 24 h and the oocytes cultured for 36 h), exocytosis of CGs plays a role in blocking polyspermy of porcine oocytes matured *in vitro*. Nevertheless, polyspermy could not be avoided completely, and the cause of polyspermy may be inadequacy of the amount of CGs synthesized during culture or the incompleteness of exocytosis of CGs as observed in Type IV eggs. Considering the number of spermatozoa which penetrated at 2–12 h of insemination (monospermic rate in Table 3), the blocking of extra sperm penetration in the oocytes matured *in vitro* seemed to start a few hours after sperm penetration and it might have remained unchanged thereafter. From 4 h to 6 h of insemination, the majority of spermatozoa might obtain the ability to penetrate oocytes and many spermatozoa might penetrate an oocyte before the oocyte became protective from extra sperm penetration. In the experiment comparing the number of spermatozoa penetrating oocytes matured *in vitro* and *in vivo* by *in vitro* and *in vivo* fertilization [23], the mean number of spermatozoa that penetrated oocytes *in vitro* significantly exceeded 1.0, regardless of whether the oocytes were matured *in vitro* or *in vivo*, but all of the oocytes matured and fertilized *in vivo* were monospermy. The polyspermy observed in *in vitro* fertilization of pig oo-

cytes may be intrinsically inevitable, without mimicking the oviductal environment [23, 24].

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