

## ***In Vitro* Fertilization and Development of Porcine Oocytes Matured in Follicular Fluid**

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**Abstract.** This study was conducted to assess the fertilization and development of porcine oocytes matured in a solo follicular fluid (pFF) using different *in vitro* culture systems and insemination periods. Cumulus-oocyte complexes (COCs), follicular cells (FCs), and pFF were collected from the follicles of ovaries. The pFF was used as a maturation medium (MpFF) after supplementation with follicle stimulating hormone (FSH) and antibiotics. The COCs were matured in a 15 ml test tube containing 3.5 ml of MpFF with FCs ( $5.2 \times 10^6$  cells/ml; rotating culture system) or 2 ml of MpFF without FCs in a 35-mm petri dish (static culture system) for 44 to 48 h. After maturation culture, oocytes were co-incubated with frozen-thawed spermatozoa for 5 h and then cultured for 7 days. The total mean rates of sperm penetration, normal fertilization, male pronucleus (MPN) formation, cleavage, and development to the blastocyst stage of oocytes after insemination were significantly higher ( $P < 0.01$ ) in the rotating culture system than in the static culture system. In conclusion, compared with the static culture system, the rotating culture system is adequate for the production of developmentally competent porcine oocytes when MpFF is used as a maturation medium.

**Key words:** Embryonic development, Follicular fluid, *In vitro* maturation, Pigs, Rotating culture

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**F**or the successful production of pigs by *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT), it is necessary to obtain a large number of high-quality *in vitro* matured oocytes. Cumulus oocyte complexes (COCs) collected from ovarian follicles in ovaries are cultured in a maturation medium and used for subsequent IVF and SCNT. In pigs, supplementation of porcine follicular fluid (pFF) with *in vitro* maturation (IVM) medium has been reported to have beneficial effects on *in vitro* nuclear maturation of oocytes and their subsequent IVF and embryonic development [1–3]. In general, various concentrations of pFF have been added into maturation medium based on NCSU or tissue culture medium (TCM) solution [4–6]. However, there has been only 1 report concerning the *in vitro* culture (IVC) of porcine oocytes matured in 100% pFF and fertilized *in vitro*, and the report showed the improved development of IVM/IVF oocytes up to the 8-cell stage after 120 h of IVF, but they did not observe the blastocyst formation [7]. If oocytes matured in only follicular fluid—without standard media—could develop to the blastocyst stage after IVF, it may not only decrease the cost of preparation of IVM media but also resemble the *in vivo* conditions of oocytes in follicles.

Piglets derived from IVM/IVF oocytes were first produced by non-static culture using co-culture with follicular cells (FCs) [8]. In our previous study, we also found that when porcine oocytes

were matured in a solo pFF supplemented with FCs, both the static and non-static (rotating) culture systems supported the meiotic competence of the oocytes and their subsequent male pronucleus (MPN) formation after IVF [9]. An early study on IVM/IVF in cattle showed that oocytes matured *in vitro* by non-static culture with FCs could develop to the blastocyst stage at a higher rate than those matured by static culture [10]. Since little information is available on the development of oocytes matured in only pFF without standard media and then fertilized with spermatozoa, it would be worthwhile to compare spermatozoa fertilization and the developmental competence of oocytes obtained by static and non-static culture as IVM systems.

However, a high incidence of polyspermic penetration remains the main obstacle to the production of a large number of porcine IVF embryos. The incidence of polyspermy seems to be related to oocyte cytoplasmic maturation, which affects MPN formation and its subsequent development [11, 12].

The objectives of the present study were to examine whether pFF as a solo IVM medium can be useful for the production of developmentally competent porcine oocytes. We compared the effects of 2 IVM culture systems on the fertilization and development of resultant IVM oocytes after IVF, in which oocytes were matured using either a static culture system in a petri dish or a rotating culture system in a test tube. To increase the efficiency of pFF as a solo IVM medium, we investigated the fertilization and development of oocytes matured by each culture system.

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## Material and Methods

### *Oocyte collection and IVM*

Porcine ovaries were obtained from prepubertal cross-bred gilts (Landrace and Large White breeds) at an abattoir and transported to the laboratory at 35 C in Dulbecco's phosphate-buffered saline (PBS, Nissui Pharmaceutical, Tokyo, Japan). The ovaries were washed several times in PBS, and the follicles (3 to 6 mm in diameter) were aspirated using a 10-ml syringe with an 18-gauge needle. Large clusters of FCs were removed from pFF by filtration through a 212- $\mu$ m mesh screen (Tokyo Screen, Tokyo, Japan). The filtered pFF containing COCs was transferred to a 90-mm petri dish (BD Falcon, Franklin Lakes, NJ, USA); subsequently, only COCs with several layers of cumulus cells were collected and used for IVM. After collection of the COCs, pFF was transferred into a test tube (15 ml, Corning Incorporated, Corning, NY, USA) and then centrifuged at  $1,800 \times g$  for 15 min. The supernatant of follicular fluids was collected and used as a basal medium for oocyte maturation. At the same time, small clusters of FCs were collected from the centrifuged tubes and used for culture. The maturation medium (MpFF) consisted of pFF (basal medium) supplemented with 0.12 AU/ml FSH (Kyoritsu Seiyaku, Tokyo, Japan) and gentamicin (50  $\mu$ g/ml; Sigma Chemical, St. Louis, MO, USA), and was used for IVM after filtration through a 0.22- $\mu$ m filter.

To compare the effects of the 2 maturation culture systems on the fertilization and development of oocytes after IVF, about 30–50 COCs were cultured in 2 ml of MpFF in a 35-mm petri dish (Falcon) covered with paraffin oil (Paraffin Liquid; Nacalai Tesque, Kyoto, Japan) (static culture system) or 3.5 ml of MpFF in a 15-ml test tube (Corning, Corning, NY, USA) (rotating culture system) with a vented screw cap (Vented Screw Cap; BD Falcon, Franklin Lakes, NJ, USA). In our previous study [9], we found that the addition of FCs to pFF in the rotating culture system promoted nuclear maturation of porcine oocytes and MPN formation after IVF. In contrast, when the static culture system was used for IVM, the addition of FCs to pFF was found to be detrimental to oocyte maturation. Therefore, in the rotating culture system, the COCs were cultured in MpFF supplemented with FCs ( $5.2 \times 10^6$  cells/ml) in a 15-ml test tube. The tube was rotated at 10 rpm using a rotating machine (Hodate Shokai, Tokyo, Japan) in an incubator (Hirasawa O<sub>2</sub> CO<sub>2</sub> incubator, Tokyo, Japan) during maturation culture. The COCs were cultured for 44 to 48 h in an atmosphere of 5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub> at 38.5 C.

### *IVF and IVC of IVM oocytes*

IVF was carried out as described previously [4]. In brief, the fertilization medium used was a modified Pig-FM [13] supplemented with 2 mM caffeine and 5 mg/ml bovine serum albumin (BSA; Fraction V, Sigma). The same batch of frozen semen from the epididymis of a single boar (Landrace) was used for these experiments. Frozen-thawed spermatozoa were preincubated for 15 min at 37 C in a TCM 199 with Earle's salts (Invitrogen, Carlsbad, CA, USA) adjusted to pH 7.8 [14]. A portion (10  $\mu$ l) of the preincubated spermatozoa was introduced into 90  $\mu$ l of Pig-FM containing about 20 COCs surrounded by expanded cumulus cells. The final sperm concentration was adjusted to  $1 \times 10^5$  cells/ml. The COCs were co-incubated with spermatozoa at 38.5 C under 5% CO<sub>2</sub> for 5 h. After co-incubation of

the gametes, the oocytes were freed from the surrounding cumulus cells and attached spermatozoa, and then transferred into porcine zygote medium (PZM-5; IFP, Yamagata, Japan) [15] covered with paraffin oil (Nacalai Tesque) in an atmosphere of 5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub> at 38.5 C. The presumptive zygotes were cultured in PZM-5 for 7 days. The percentages of embryos cleaved at or beyond the 2-cell stage and those that developed into blastocysts were assessed under a stereomicroscope at 48 h and 7 days after insemination, respectively. At the end of culture, an embryo with clear blastocoels was defined as a blastocyst.

### *Assessment of fertilization*

At 10 h after IVF, some presumptive zygotes were mounted on a glass slide and fixed with a solution of acetic acid:ethanol (1:3 v/v) for 48 to 72 h. The fixed zygotes were stained with acetic-orcein (1% orcein in 45% acetic acid) and examined under a phase-contrast microscope. We assessed the following fertilization parameters: (1) total sperm penetration rate, calculated from the proportion of whole oocytes having a single female pronucleus and a single or multiple penetrated sperm nuclei or MPNs; (2) normal fertilization rate, calculated from the proportion of monospermic penetration oocytes having female and male pronuclei; (3) polyspermic fertilization rate, calculated from the proportion of oocytes having a single female pronucleus and multiple penetrated sperm nuclei or MPNs; and (4) MPN formation rate, calculated from the proportion of whole oocytes with MPNs.

### *Statistical analysis*

The mean values of oocytes fertilized, embryos cleaved, and embryos developed to the blastocyst stage were analyzed using an analysis of variance in SAS (SAS for Windows, version 9.1; SAS Institute Japan, Tokyo, Japan). Differences with  $P < 0.05$  were considered significant.

## Results

As shown in Table 1, the total mean rates of sperm penetration, normal fertilization, and MPN formation of oocytes after IVF were significantly higher ( $P < 0.01$ ) in the rotating culture system than those in the static culture system, whereas the rates of polyspermic fertilization of oocytes did not differ between the 2 groups ( $P > 0.05$ ).

As shown in Table 2, the rates of oocytes that cleaved and developed to the blastocyst stage after IVF were significantly higher ( $P < 0.01$ ) in the rotating culture group than in the static culture group.

## Discussion

To our knowledge, this is the first report demonstrating that using pFF solely as a maturation medium for porcine oocytes successfully promotes IVF of resultant IVM oocytes and their subsequent development to the blastocyst stage after IVC. Our results agree with those of previous reports that demonstrated that the presence of pFF in maturation culture media promotes nuclear maturation of porcine oocytes and subsequent formation of the MPN [3, 5, 16–18]. Moreover, this finding may support the hypothesis that one or more follicular factors derived from granulosa or theca

**Table 1.** Effects of maturation culture system on the fertilization of porcine oocytes matured in porcine follicular fluid for 44 to 48 h following *in vitro* fertilization\*

Maturation culture system	No. of oocytes examined	Oocyte fertilization			
		No. (%) of oocytes penetrated	No. (%)** of oocytes normally fertilized	No. (%)*** of oocytes with polyspermic fertilization	No. (%)**** of oocytes with MPNs
Static	91	51 (56.0 ± 2.8) <sup>a</sup>	16 (32.6 ± 5.2) <sup>a</sup>	22 (43.4 ± 2.2) <sup>a</sup>	29 (58.2 ± 5.6) <sup>a</sup>
Rotating	86	61 (71.1 ± 3.5) <sup>b</sup>	27 (44.5 ± 2.4) <sup>b</sup>	27 (44.4 ± 3.9) <sup>a</sup>	43 (70.9 ± 3.2) <sup>b</sup>

\* Percentages are expressed as the mean ± SEM. Six replicated trials were carried out. \*\* Monospermic fertilization with female and male pronuclei and two polar bodies. Percentages were calculated by dividing the number of oocytes with normal fertilization by the total number of sperm-penetrated oocytes. \*\*\* Percentages were calculated by dividing the number of oocytes with polyspermic fertilization by the total number of sperm-penetrated oocytes. \*\*\*\* MPN, male pronucleus. Percentages were calculated by dividing the number of oocytes with MPNs by the total number of sperm-penetrated-oocytes. <sup>a-b</sup> Values with different superscripts in the same column are significantly different (P<0.05).

**Table 2.** Effects of maturation culture system on the development of porcine oocytes matured in porcine follicular fluid for 44 to 48 h following *in vitro* fertilization (IVF)\*

Maturation culture system	No. of oocytes examined	No. (%) of embryos developed to**	
		≥2-cell	Blastocyst
Static	132	76 (58.0 ± 2.4) <sup>a</sup>	6 (4.4 ± 1.4) <sup>a</sup>
Rotating	128	98 (74.7 ± 3.4) <sup>b</sup>	14 (11.6 ± 1.6) <sup>b</sup>

\* Percentages are expressed as the mean ± SEM. Six to eight replicated trials were carried out. \*\* The cleavage and blastocyst formation of embryos were assessed 48 h and 7 days after IVF, respectively. <sup>a-b</sup> Values with different superscripts in the same column are significantly different (P<0.05).

cells and accumulated in follicular fluid support the developmental competence of oocytes [3, 5]. The presence of follicular fluid in the IVM medium can potentiate the stimulatory action of FSH on cumulus expansion, nuclear maturation, sperm penetration, and MPF after IVF [5, 6, 19, 20]. Therefore, most maturation media used for porcine oocyte culture in current porcine research contain pFF; however, the concentrations of pFF in the media have varied (10–20%) [4, 5, 21]. Naito *et al.* [7] reported that porcine oocytes matured in 100% pFF had improved developmental competence up to the 8-cell stage after 120 h of IVF, but they did not observe blastocyst formation. In the present study, we found that pFF as a solo maturation medium supports the ability of resultant matured oocytes to be fertilized *in vitro* with spermatozoa, form a MPN and develop to the blastocyst stage after IVC, irrespective of the maturation culture system used. These findings indicate that pFF can be used as a solo and simple maturation culture medium for *in vitro* production of blastocysts in pigs.

Naito *et al.* [18] reported that when porcine oocytes were matured only in pFF without standard media using the static culture system, about 80% of the oocytes formed MPNs after IVF. However, we observed that 45–58% of oocytes matured in the static culture system formed MPNs after IVF. The reason for the discrepancy in the rates of MPN formation is not clear, but the different IVF methods used may be one reason, as the sperm penetration rate (56.0–71.1%) was lower in our IVF system compared with the result obtained by Naito *et al.* [18] (88%).

We found that the rotation culture system provided IVM oocytes with significantly higher rates of sperm penetration, normal fertilization, MPN formation after IVF, cleavage, and development to the blastocyst stage after IVC compared with oocytes produced using the static culture system. It has been suggested that the non-static system is beneficial because (1) attachment of FCs to the bottom of culture dishes is prevented and (2) steroids secreted from FCs are rapidly and evenly dispersed in the medium, thereby preventing high concentrations of steroids from accumulating locally around the oocytes [25]. It has been suggested that both deficient and excessive steroid environments during oocyte maturation result in a poor ability of resultant IVM oocytes to induce normal MPN formation after IVF [21, 22]. Therefore, the steroids secreted from the somatic cells in the rotating culture system may be adequate for promotion of the cytoplasmic maturation of oocytes related to the formation of a normal MPN and embryonic development after IVF and IVC [12].

Compared with the previous studies using the same IVF system except for IVM using a synthetic medium [23, 24], the rates of pronuclear formation and blastocyst formation were slightly low in this study. Moreover, compared with a different IVM/IVF/IVC system reported by Yoshioka *et al.* [25], both of the penetration and blastocyst formation rates were low. Thus, it may be possible to improve the rotating culture system using MpFF, determining if there are any physiologically active substances in MpFF that are insufficient or superabundant. Furthermore, there was such a little difference between oocytes matured in MpFF and synthetic media in terms of the blastocyst formation rates that MpFF may be used not only as a labor-saving medium, but also as a model for *in vivo* maturation of COCs.

In conclusion, these results indicate that porcine oocytes matured in pFF as a solo maturation culture medium can form an MPN after IVF and then develop into blastocysts. Furthermore, the rotating culture system is adequate for the production of developmentally competent porcine oocytes when the oocytes are matured in a solo pFF medium.

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