For 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 polyspermy 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 the developmental competence of oocytes obtained by static and non-static culture as IVM systems.

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.
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-μm mesh 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 × 10^6 cells/ml) in a 15-ml test tube. The tube was rotated at 10 rpm using a rotating machine (Hodate Shokai, Japan) and gentamicin (50 μg/ml; Sigma Chemical, St. Louis, MO, USA), and was used for IVM after filtration through a 0.22-μm filter.

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.

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 granulose 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*

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

* 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. *–† 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)*

<table>
<thead>
<tr>
<th>Maturation culture system</th>
<th>No. of oocytes examined</th>
<th>No. (%) of embryos developed to**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥2-cell</td>
</tr>
<tr>
<td>Static</td>
<td>132</td>
<td>76 (58.0 ± 2.4)*</td>
</tr>
<tr>
<td>Rotating</td>
<td>128</td>
<td>98 (74.7 ± 3.4)†</td>
</tr>
</tbody>
</table>

* 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. *–† Values with different superscripts in the same column are significantly different (P<0.05).

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 MpFF 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 MpFF medium.

References


