In-vitro Culture with a Tilting Device in Chemically Defined Media During Meiotic Maturation and Early Development Improves the Quality of Blastocysts Derived from In-vitro Matured and Fertilized Porcine Oocytes

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Abstract. Under physiological conditions, mammalian oocytes and embryos appear to be stimulated not only chemically but also mechanically, such as by compression, shear stress and/or friction force in the follicle and female reproductive tract. The present study was undertaken to examine the effects of kinetic culture with a tilting device in chemically defined media during in vitro maturation (IVM) of porcine oocytes and in vitro culture (IVC) following in vitro fertilization (IVF) on the early developmental competence and quality of blastocyst. After culture in a chemically defined medium, modified porcine oocyte medium (mPOM) containing gonadotropins and dibutyryl cAMP for 20 h, the mean diameter of the cumulus-oocyte complexes (COCs) was larger in the tilting culture than in the static controls, whereas the diameter of the oocytes did not differ. When culture of the COCs was continued additionally in a fresh medium without gonadotropins and dibutyryl cAMP for 24 h, the incidences of oocytes completing GVBD and developing to the metaphase-II stage did not differ between the tilting and static culture systems. Furthermore, the sperm penetration after IVF and developmental competence of the oocytes to the blastocyst stage were not different between the tilting and static systems during IVM and IVC. However, tilting culture during both IVM and IVC had a significant positive effect on the number of cells per blastocyst (P<0.05). These observations indicate that tilting culture during IVM and IVC in chemically defined media improves the quality of blastocyst, as determined by the number of cells per blastocyst, without any effects on penetrability and developmental competence.

Key words: Inclining device, in vitro culture, in vitro fertilization, Oocytes, Pig

Porcine cumulus-oocyte complexes (COCs) have been successfully cultured for in vitro maturation (IVM), and oocytes have also been fertilized and developed to the cleavage and blastocyst stages in vitro [1]. Although many piglets have been produced following IVM, in vitro fertilization (IVF) and in vitro culture (IVC) [2, 3], the quality of embryos produced in vitro is still poor, and the developmental competence remains an area requiring further research [3, 4]. Furthermore, a successful IVM-IVF-IVC system utilizing chemically defined media has been recently developed to produce blastocysts and piglets [5, 6]. Development of an efficient system for in-vitro production of embryos in chemically defined media would have an advantage in research to clarify the mechanism or effects of various materials during IVM, IVF and/or IVC.

In general, oocytes and embryos are cultured statically in dishes or well plates in CO₂ or 3-gas incubators. Under physiological conditions in vivo, however, oocytes and embryos appear to be exposed to various mechanical stimuli, such as compression, shear stress and friction force, from a possible change of hydrostatic pressure in follicles and the kinetics of oviductal epithelial cells. Intrafollicular pressure is known to change to some extent during late follicular development and ovulation in several mammalian species [7–10]. In the oviduct, both motion of the epithelial microvilli and the active contractile pattern of the smooth muscle are also known to occur for transportation of oocytes and embryos [11]. In fact, some somatic cells have sometime been cultured under kinetic culture conditions [12, 13].

Although there have been a few reports that have used a non-static culture system during IVM [14, 15], the detailed and precise effects of a non-static culture system on IVM of oocytes and early development to the blastocyst stage following IVF are still unclear. Recently, we demonstrated that the advantage of the tilting embryo culture system is demonstrated with frozen-thawed 2-cell mouse embryos and 3- to 11-cell human embryos [16]. The number of cells per blastocyst was improved significantly by using the tilting culture system, whereas the number was also affected by the volume of culture medium per embryo, and the system did not affect the incidence of embryos that developed to the blastocyst stage [16]. However, it is still unknown if use of the tilting culture system during IVM improves the embryonic developmental competence and/or quality of embryos following IVF. Furthermore, the advantage of the tilting embryo culture system has not
examined in domestic animals.

In the present study, we examined the effects of the tilting culture system during IVM of porcine COCs and/or IVC following IVF of oocytes on early development to the blastocyst stage and the quality of blastocysts, as determined by the number of cells per blastocyst. To make the effect of the tilting culture system striking in the present study, we used chemically defined culture media during IVM and IVC.

**Materials and Methods**

**Chemicals and culture media**

Potassium chloride, KH₂PO₄, MgCl₂·6H₂O, CaCl₂·2H₂O, sodium citrate and citric acid were purchased from Ishizu Pharmaceutical (Osaka, Japan), sodium chloride and paraffin liquid were obtained from Nacalai Tesque (Kyoto, Japan) and eCG (Serotropin) and hCG (Gonatropin) were purchased from ASKA Pharmaceutical (Tokyo, Japan). Unless specified, other chemicals were purchased from Sigma Aldrich Japan (Tokyo, Japan).

The medium used for collecting and washing COCs was modified TL-HEPES-PVA medium composed of 114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO₃, 0.34 mM KH₂PO₄, 10 mM Na-lactate, 0.5 mM MgCl₂·6H₂O, 2 mM CaCl₂·2H₂O, 10 mM HEPES, 0.2 mM Na-pyruvate, 12 mM sorbitol, 0.1% (w/v) polyvinyl alcohol, 25 μg/ml gentamicin and 65 μg/ml potassium penicillin G. The basic IVM medium used was a BSA-free chemically defined medium, POM (Research Institute for the Functional Peptides, Yamagata, Japan) supplemented with 50 μM beta-mercaptoethanol (mPOM). This IVM medium supports successful development to the blastocyst stage following IVF [5] and piglet production [6].

All media (except modified TL-HEPES-PVA) were equilibrated at 39°C in an atmosphere of 5% CO₂ in air overnight prior to use (only PGM-tac4 was equilibrated under paraffin liquid).

**Culture systems**

As a kinetic culture, we used a tilting culture system (SW-1, STREX, Osaka, Japan) consisting of an electrical device with a power cord that was designed to be used in a humidified incubator (Fig.1a) [16]. A 4-well culture plate (NUNC #176740, Thermo Fisher Scientific, Roskilde, Denmark) was set on the tilting plate of the device in a CO₂ incubator. The tilting plate was controlled to incline 20 degrees, to maintain the inclination for 1 min and then to turn conversely to an inclination 20 degrees for 12 sec (Fig. 1b). As a control static culture system, the same culture plate (NUNC #176740) was conventionally put on the same shelf of a CO₂ incubator for culture.

**Preparation and culture of COCs**

Ovaries were collected from slaughtered prepubertal gilts at a local abattoir and transported to the laboratory in 0.9% NaCl containing 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate. Using an 18-gauge needle and a disposable 10-ml syringe, COCs were aspirated from antral follicles (3 to 6 mm in diameter) on the surfaces of ovaries and washed three times with modified TL-HEPES-PVA medium at room temperature (25°C) [2]. Forty to fifty COCs with uniform ooplasm and a compact cumulus cell mass were washed three times with IVM medium. These complexes were subsequently cultured in 500 μl of the same medium supplemented with gonadotropins (10 iu eCG/ml and 10 iu hCG/ml) and 1 mM dibutyryl cyclic adenosine 3’,5’-monophosphate (dbcAMP) in a 4-well culture plate by the static or tilting culture methods for 20 h at 39°C in an atmosphere of 5% CO₂ in air. The COCs (mean diameter: 268.3 ± 2.5 μm) were then randomly transferred to 500 μl of the IVM medium without gonadotropins and dbcAMP after being washed three times with the same medium and cultured by the static or tilting culture methods for an additional 24 h [2, 17]. After IVM culture, oocytes were stripped of cumulus cells by pipetting with 0.1% (w/v) hyaluronidase and eval-

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**Fig. 1.** (A) Image of the entire SW-1 tilting device. (B) A culture plate on the tilting device (the rear) and control culture plate (static culture; the front) in an incubator. The plate of the device continued to move in the same manner at an inclination of 20 degrees, maintained that inclination for 1 min and then switched to an inclination 20 degrees conversely for 12 sec. The length of the side of the culture plate is 65 mm.
uated for nuclear maturation. Some of the oocytes were washed with modified TL-HEPES-PVA three times, mounted, fixed for 48 h or more in 25% (v/v) acetic acid-alcohol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid and then examined under a phase-contrast microscope at ×400 magnification.

Measurement of COC and oocyte diameters

Digital photo images were taken of COCs at ×100–200 magnifications with a stage micrometer (Nikon, Tokyo, Japan) 20 h after the start of culture for IVM in the presence of gonadotropins and dbcAMP. On a monitor screen, the largest and smallest diameters of each COC (except for a few COCs in which the diameters could not be determined from the images) were examined with Photoshop CS2 (Adobe Systems, San Jose, CA, USA). The mean diameter of each COC was calculated from the largest and smallest diameters. After measurement, COCs were denuded by pipetting with 0.1% (w/v) hyaluronidase. The mean diameter of the oocytes (excluding the zona pellucida) was also determined using the same method.

Preparation of fresh boar spermatozoa and in vitro fertilization

Semen-rich fractions (30 to 50 ml) were collected from three Berkshire boars by the glove-hand method at a local experimental station and were diluted 4 times with modified Modena solution [18]. The diluted semen samples were transported to the laboratory within 2 h of collection. After washing once by centrifugation at 750 g for 3 min, spermatozoa were resuspended at a concentration of 1 × 10^6 cells/ml in modified Modena solution containing 5 mM cysteine and 20% (v/v) boar seminal plasma. Diluted spermatozoa were used for IVF after storage overnight at 15 C (the sample was cooled down from room temperature to 15 C for 4 h and then kept at the same temperature). Just before use, stored spermatozoa were placed at room temperature for 15 to 20 min, washed three times by centrifugation at 750 g for 3 min with modified TL-HEPES-PVA solution and then resuspended at a concentration of 1 × 10^6 cells/ml in PGM-tac4.

After dilution of sperm suspension to 1 × 10^6 cells/ml with PGM-tac4, fifty microliters of the diluted suspension was added in the same volume of PGM-tac4 containing thirty denuded oocytes (final sperm concentration was 5 × 10^5 cells/ml). The oocytes were co-cultured with spermatozoa in 100 μl droplets of PGM-tac4 under paraffin oil for 8 h at 39 C in an atmosphere of 5% CO2 in air. At 8 h after insemination, the oocytes were incubated in 500 μl of PZM-5 in a 4-well culture plate by the static or tilting culture methods for 7 days at 39 C in an atmosphere of 5% CO2 in air. Cleavage and blastocyst formation of the oocytes were examined at 2 and 7 days after the start of culture, respectively. The blastocysts (with a compacted cell mass containing a blastocoel) were stained with 5 μg/ml bisbenzimide (Hoechst 33342) to determine the number of nuclei by using an epifluorescent microscope.

Experimental design

In the first experiment, the mean diameters of COCs and oocytes were measured 20 h after the start of static and tilting culture in the presence of gonadotropins and dibutyryl cAMP. Furthermore, following static and tilting culture for IVM with gonadotropins and dibutyryl cAMP for 20 h and then without them for 24 h, the meiotic progression of the oocytes was compared between the culture systems.

In the second experiment, after static and tilting culture for IVM, these oocytes were co-cultured with spermatozoa under static conditions for 8 h. At the end of culture, sperm penetration in these oocytes was examined. Some oocytes in each group (static and tilting cultures) were divided into two groups, and then culture was continued for IVC under static and tilting culture conditions for 7 days (2 × 2 groups during IVM and IVC).

Statistical analysis

All COCs were randomly distributed within each experimental group, and each experiment was repeated 4 or 5 times. Statistical analyses of results were used for treatment comparisons and carried out by one-way or two-way analysis of variance (ANOVA) using the JMP 5.0 (SAS Institute, Cary, NC, USA) program. If the P value was smaller than 0.05 in ANOVA, the Tukey-Kramer HSD test was utilized using the same program. All data were expressed as means ± SEM. P<0.05 was considered to be statistically significant.

Results

After culture in a chemically defined medium containing gonadotropins and dbcAMP for 20 h, the mean diameter of the COCs was significantly larger (P<0.01) in the tilting culture than in the static controls (Table 1 and Fig. 2). However, the diameters of the oocytes excluding the zona pellucida in the tilting culture did not differ from those of the static controls (P=0.22).

Following culture for IVM in a chemically defined medium with gonadotropins and dbcAMP for 20 h and then without them for 24 h, the incidences of oocytes completing GVBD and meiotic progress to the metaphase-II stage did not differ (P=0.29 and P=0.27, respectively) between the tilting and static culture systems (Table 2).

Following IVF (mean penetration rates were 65.4 ± 8.4% in static IVM and 72.0 ± 12.9% in tilting IVM; monospermy rates for the number of oocytes examined were 39.3 ± 5.0% in static IVM and 41.9 ± 7.5% in tilting IVM) in a chemically defined medium, there were no effects of tilting culture during IVM and IVC on cleavage rate and the incidence of embryos developing to the blastocyst stage (Table 3). However, significant differences were found in the effect of tilting culture during IVM (P=0.047) and IVC (P<0.01) on the number of cells per blastocyst. The mean number of cells per blastocyst was highest when the tilting system was adopted during both IVM and IVC (P<0.01).

Discussion

Developmental competence to the blastocyst stage following IVM-IVF of porcine oocytes has been improved significantly by modification of the culture medium and supplements during IVM [19]. Recently, we succeeded in producing piglets following embryo transfer of blastocysts derived from IVM-IVF-IVC in gonadotropin-free chemically defined media [6]. Our final goal is...
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development of an efficient IVM-IVF-IVC system using chemically defined media. However, culture is currently performed conventionally under static culture conditions. For further breakthrough improvements in the efficiency of blastocyst formation and/or blastocyst quality following IVM-IVF-IVC, introduction of other techniques might be required to the chemically defined system. In the present study, we tried to apply the tilting culture system to the chemically defined system and found that kinetic culture with a tilting culture device during IVC did not affect the incidence of blastocyst formation but did significantly improve the quality of the produced blastocysts as determined by the number of cells per blastocyst. These results were consistent with a recent report that the tilting culture system during IVC significantly improved the quality of frozen-thawed mouse and human embryos, as determined by cell number per blastocyst [16]. In the oviduct, early embryos are exposed to various mechanical stimuli, such as shear stress by a tubal fluid flow, compression by peristaltic tubal wall movement and kinetic friction force with microvilli of the epithelial cells [11]. Therefore, these faint mechanical stimulations during early development under physiological conditions should be beneficial to maintain the quality of embryos. However, the mean number of cells per blastocyst in the current study appears to be still lower than those when oocytes are cultured in POM supplemented with porcine follicular fluid [5]. Yoshioka et al. have also demonstrated that blastocysts derived from oocytes matured in POM supplemented with polyvinyl alcohol contain a smaller number of cell, as compared with those from oocytes matured in the presence of porcine follicular fluid [5]. This means that further modifications of media may be still required even after application of a tilting culture system to chemically defined IVM and IVC. Although we did not add porcine follicular fluid into the maturation medium because our final goal was to develop an efficient chemically defined IVM-IVF-IVC system, to obtain more blastocysts from IVM-IVF-IVC, supplementation of follicular fluid should be beneficial.

Table 1. Mean diameter of COCs and oocytes after culture statically or kinetically for in-vitro maturation for 20 h

<table>
<thead>
<tr>
<th>Culture styles</th>
<th>No. of oocytes</th>
<th>Mean diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>COCs</td>
</tr>
<tr>
<td>Static</td>
<td>29</td>
<td>284.2 ± 8.2</td>
</tr>
<tr>
<td>Tilting</td>
<td>32</td>
<td>357.5 ± 11.3</td>
</tr>
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</table>

1 COCs were cultured in the presence of 10 IU/ml eCG, 10 IU/ml hCG and 1 mM dibutyryl cAMP in mPOM. 2 Mean diameter was calculated from the maximum and minimum diameters of COCs and oocytes after they were denuded. 3 Static: the culture plate was placed on a shelf in a CO2 incubator. Tilting: the culture plate was placed on the tilting culture device in a CO2 incubator (see Fig. 1). Data are presented as means ± SEM from four replicated experiments. Values with different superscripts within a column are significantly different (P<0.05).

Fig. 2. COCs after culture by static culture (the upper part) and by the tilting culture system (the lower part) for 20 h. These COCs were cultured in modified POM containing gonadotropins and 1 mM dbcAMP at 39°C in an atmosphere of 5% CO2 in air. The black bar shows 250 μm.
physical stimulation of the cumulus cell mass by cell number per blastocyst. During final follicular development, COCs were combined with others, the tilting culture during IVM and used denuded oocytes for IVF, the other researchers inseminated COCs without removing the cumulus cells. Therefore, the status of the cumulus mass surrounding the oocyte after non-static culture could be different from that of the static control, and consequently might be beneficial for sperm penetration and pronuclear formation. In fact, we found here a difference in cumulus expansion of COCs even 20 h after the start of IVM.

In the current study, the P value for the effect of tilting culture during IVC on the cell numbers per blastocyst was much lower (less than 0.0001) than that for the effect of tilting culture during IVM (0.0474; Table 3). Therefore, tilting culture during IVC beneficially contributes to the quality of the blastocysts as compared with that during IVM. During preimplantation development, in fact, embryos suffer various physical stresses, such as shear stress, compression and kinetic friction force, in the oviducts [11], whereas COCs receive only compression during final follicular development and ovulation [7–10]. Embryos, rather than COCs, may be sensitive to beneficial physical stress. On the other hand, excessive shear stress appears to be detrimental to preimplantation embryos. Shear stress at 1.2 dynes/cm² has been known to induce stress-activated protein kinase phosphorylation that precedes and causes apoptosis in mouse embryos [26]. According to a recent study, the calculated shear stress during tilting culture in the present study was 7.0–15 × 10⁻³ dynes/cm² in the medium [16]. Thus, tilting culture with a suitable shear stress of around 7.0–15 × 10⁻³ dynes/cm² is beneficial in improving the quality of blastocysts, as determined by the mean cell number per blastocyst.

In conclusion, the current observations indicate that tilting culture during IVM and in vitro culture following IVF in chemically defined media improves the quality of blastocyst without any effects on the developmental competence, although the embryos may be more sensitive to the beneficial shear stress as compared with COCs.
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Acknowledgments

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