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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

Takayuki KOIKE[1], Koji MATSUURA[2,3], Keiji NARUSE[3] and Hiroaki FUNAHASHI[1]

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[1]Department of Animal Science, Graduate school of Natural science and technology, Okayama University, Tsushima-Naka, Kita-Ku, Okayama, 700-8530, Japan

[2]Research Core for Interdisciplinary Sciences, Okayama University, Tsushima-Naka, Kita-Ku, Okayama, 700-8530, Japan

15 [3]Department of Cardiovascular Physiology, Graduate school of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Shikata, Kita-Ku, Okayama, 700-8558, Japan

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Two figures and three tables

Correspondence should be to H Funahashi; Email: hirofun@cc.okayama-u.ac.jp

Abstract

In the physiological condition, mammalian oocytes and embryos appears to be stimulated not only chemically but also mechanically, such as 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 the in vitro culture (IVC) following in vitro fertilization (IVF) on the early developmental competence and the quality of blastocysts. After culture in a chemically defined IVM medium, modified porcine oocyte medium (mPOM) containing gonadotropins and dibutyryl cAMP for 20 h, the mean diameter of cumulus-oocyte complexes (COC) was larger in tilting culture than static controls, whereas the diameter of oocytes did not differ. When the COC were continued culture additionally in a fresh medium without gonadotropins and dibutyryl cAMP for 24 h, the incidences of oocytes completing GVBD and developed to the metaphase-II stage did not differ between tilting and static culture systems. Furthermore, the developmental competence of the oocytes to the blastocyst stage in a chemically defined medium following IVF was also not different between tilting and static systems. However, tilting culture during both IVM and in-vitro culture following IVF significantly affected positively the number of cells in a blastocyst ($p<0.05$). These observations indicate that tilting culture during IVM and the in vitro culture following IVF in chemically defined media improves the quality of blastocyst, as determined the number of cells per blastocyst, without any effects on the developmental competence.

1. Introduction

Porcine cumulus-oocyte complexes (COC) have been successfully cultured for in vitro maturation (IVM), and the oocytes has also been fertilized and developed to the cleavage and blastocyst stages in vitro [1]. Although many piglets have been produced following these 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 remain the place to be revised [3,4].

In general, oocytes and embryos have been cultured statically in dishes or well plates in CO_2 or 3-gases incubators. In the physiological conditions in vivo, however, oocytes and embryos appears to be exposed to various mechanical stimuli, such as compression, share stress and friction force, from a change of hydrostatic pressure in follicles and the microvilli of oviductal epithelial cells. Intrafollicular pressure is known to change to some extent during late follicular development and ovulation in several mammalian species [5-8]. In the oviduct, both motion of the epithelial microvilli and active contractile pattern of the smooth muscle are also known to occur for the transportation of oocytes and embryos [9].

Although there were a few reports used non-static culture system during IVM [10,11], the detailed and precise effects of non-static culture system on IVM of oocytes and the early development to the blastocyst stage following IVF is still unclear. Recently, advantage of tilting embryo culture system is demonstrated with frozen-thawed 2-cell mouse embryos and 3-11-cell human embryos [12]. By using the tilting embryo culture system, the cell number in a blastocyst improves significantly, although the system does not affect the incidence of embryos developed to the blastocyst stage [12]. However, it is still unknown if the tilting culture system during IVM improves the embryonic developmental competence and/or the 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 effect of tilting culture system during IVM of porcine COC and/or IVC following IVF of the oocytes on early development to the blastocyst stage and the quality of blastocysts, as determined by the number of cells per blastocyst. We demonstrated that the tilting culture system both during IVM of porcine COC and during IVC following IVF of the

oocytes improved in the quality of blastocysts without any reduction of developmental competence of the embryos.

2. Materials and methods

2.1. Chemicals and Culture Media

Potassium chloride, KH_2PO_4, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, sodium citrate and citric acid were purchased from Ishizu Pharmaceutical Co., Ltd (Osaka, Japan). Sodium chloride and paraffin liquid were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Unless specified, other chemicals were purchased from Sigma Aldrich Japan K.K. (Tokyo, Japan).

The medium used for collecting and washing COC was modified TL-HEPES-PVA medium composed of 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 mM KH_2PO_4, 10 mM Na-lactate, 0.5 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, 0.2 mM Na-pyruvate, 12 mM sorbitol, 0.1% (w/v) polyvinylalcohol, 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 a successful development to the blastocyst stage following IVF [13] and piglet production [14]. The chemically define media for IVF and in vitro development to the blastocyst stage [13] were PGM-tac4 (Research Institute for the Functional Peptides, Yamagata, Japan) and PZM-5 (Research Institute for the Functional Peptides, Yamagata, Japan), respectively. All media (except modified TL-HEPES-PVA) were equilibrated at 39 C in an atmosphere of 5% CO_2 in air overnight prior to use (only PGM-tac4 was under paraffin liquid).

2.2. Culture systems

For kinetic inclination culture, we used a tilting culture system, SW-1 (STREX, Inc., Osaka, Japan) which was an electrical device with a power cord that was designed to be used in a humidified incubator [12]. A 4-well culture plate (NUNC #176740, Thermo Fisher Scientific, Inc., Roskilde, Denmark) was set on the tilting plate of the device in a CO_2 incubator. The tilting plate

was controlled to incline 20 degree, to keep the inclination for 1 min and then to turn conversely to the inclination of 20 degree for 12 sec. As control static culture system, the same culture plate
105 (NUNC #176740) was conventionally put on the same shelf of a CO_2 incubator for culture.

2.3. Preparation and culture of COC

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
110 streptomycin sulphate. Using an 18-gauge needle and a disposable 10-ml syringe, COC were aspirated from antral follicles (3 to 6 mm in diameter) on the surface of ovaries, and washed three times with modified TL-HEPES-PVA medium at room temperature (25 C) [2]. Forty to fifty COC 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
115 supplemented with gonadotropins (10 iu eCG/ml and 10 iu hCG/ml) and 1mM dibutyryl cyclic adenosine 3',5'-monophosphate (dbcAMP), in a 4-well culture plate (NUNC #176740) by static or tilting culture methods for 20 h at 39°C in an atmosphere of 5% CO_2 in air. The OCCs were then transferred to 500 μl of the IVM medium without gonadotropins and dbcAMP after washing three times with the same medium and cultured by static or tilting culture methods for an additional 24 h
120 [2,15]. After IVM culture, oocytes were stripped of cumulus cells by pipetting with 0.1% (w/v) hyaluronidase and evaluated 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 400x magnification.
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2.4. Preparation of fresh boar spermatozoa, in vitro fertilization

Semen-rich fractions (30 to 50 ml) were collected from totally three Birkshire boars by glove-hand method at a local experimental station and were diluted 4 times with modified Modena solution [16]. The diluted semen samples were transported to the laboratory within 2 h of collection.
130 After washing once by centrifugation at 750 *g* for 3 min, spermatozoa was re-suspended at a concentration of 1 x 10^8 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 hours and then was kept at the same temperature). Just before use, stored spermatozoa were placed at room temperature for 15
135 to 20 min, washed three times by centrifugation at 750 *g* for 3 min with modified TL-HEPES-PVA solution and then re-suspended at a concentration of 1×10^8 cells/ml in PGM-tac4.

After dilution to 1×10^6 cells/ml with PGM-tac4, fifty micro-liter of diluted sperm suspension was inseminated in the same volume of PGM-tac4 (final sperm concentration was 5×10^5 cells/ml). Thirty denuded oocytes were co-cultured with spermatozoa in 100 μl droplet of PGM-tac4 under
140 paraffin oil for totally 8 h at 39 C in an atmosphere of 5% CO_2 in air. At 8 h after insemination, oocytes were incubated in 500 μL of PZM-5 in a 4-well culture plate by static or tilting culture methods for 7 days at 39 C in an atmosphere of 5% CO_2 in air. Cleavage and blastocyst formation of the oocytes were examined on 2 and 7 days after the start of culture, respectively. The blastocysts were stained with 5 μg/ml bis-benzimide (Hoechst 33342) to determine the number of
145 nuclei by using an epi-fluorescent microscope.

2.5. Experimental design

To determine the effect of tilting culture during IVM, COC were taken digital photo images at x100-200 magnification with a stage micrometer (Nikon, Tokyo, Japan) 20 h after the start of
150 culture for IVM in the presence of gonadotropins and dbcAMP. On a monitor screen, the largest and smallest diameters of some COC were examined with Photoshop CS2 (Adobe Systems Inc., San Jose, CA, USA). The mean diameter of each COC was calculated from the largest and smallest diameters. After the measurement, COC were denuded by pipetting with 0.1% (w/v) hyaluronidase. The mean diameters of the oocytes were also measured with the same method. At
155 the end of culture for IVM (totally 44 h after the start of culture), furthermore, some COC were denuded by pipetting with 0.1% (w/v) hyaluronidase, and meiotic progression of the oocytes were examined.

At 2 and 7 days following the start of culture for IVC, the incidences of IVF embryos cleaved and developed to the blastocyst stage were determined respectively. The embryos developed to
160 the blastocyst stage were examined the number of nuclei.

2.6. *Statistical analysis.*

All COC were randomly distributed within each experimental group and each experiment was repeated 4 or 5 times. All percentage data were subjected to arc-sine transformation before statistical analysis. 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, Inc., Cary, NC) program. If the P value was smaller than 0.05 in ANOVA, Tukey-Kramer's HSD test was followed using the same program. All data were expressed as means ± SEM. $P \leq 0.05$ was considered to be statistically significant.

3. Results

The mean diameter of cumulus-oocyte complexes (COC) was significantly larger ($P<0.01$) in tilting culture than static controls after culture in a chemically defined medium containing gonadotropins and dibutyryl cAMP for 20 h (Table 1). However, the diameter of oocytes in tilting culture did not differ from static controls ($P=0.22$).

Following culture for IVM in a chemically defined medium with gonadotropins and dibutyryl cAMP for 20 h and then without those for 24 h, both 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 tilting and static culture systems (Table 2).

Following IVF (mean penetration rate was 68.7 ± 5.5% and monospermy rate in the number of oocytes examined was 41.0 ± 1.8%) in a chemically defined medium, there were not any effects of tilting culture during IVM and IVC on cleavage rate and the incidence of embryos developing to the blastocyst stage (Table 3). However, significances 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. Mean number of cells in a blastocyst was highest when the tilting system was adopted during both IVM and IVC ($P<0.01$).

190 **4. Discussion**

Developmental competence to the blastocyst stage following IVM-IVF of porcine oocytes has been improved significantly by modification of culture medium and the supplements during IVM [17]. Recently, we have succeeded to produce piglets following embryo transfer of blastocysts derived from IVM-IVF-IVC in gonadotropin-free chemically defined media [14].
195 However, these cultures have been performed conventionally in a static culture condition. For further breakthrough improvement in the efficiency of blastocyst formation and/or the 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 tilting a culture device during IVC did not
200 affected the incidence of blastocyst formation, but significantly improved the quality of produced blastocysts as determined by the cell number of blastocyst. These results are 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 the cell number per blastocysts [12]. In the oviduct, early embryos are exposed to various mechanical stimuli, such as share stress by a
205 tubal fluid flow, compression by peristaltic tubal wall movement and kinetic friction force with mirocvilli of the epithelial cells, from a change and the microvilli of oviductal epithelial cells [9]. Therefore, these faint mechanical stimulations during early development in physiological conditions should be beneficial to maintain the quality of embryos.

In the present study, tilting culture of COC during IVM also improved cumulus expansion
210 (Table 1) and the cell numbers in blastocysts following IVF and IVC (Table 3). For successful IVM of porcine oocytes, active communication between oocyte and surround cumulus cells appears to be required during IVM, especially the early half period following gonadotropic stimulation [18]. Stimulation of cumulus cells mass with gonadotropins transiently increases the intracellular cAMP level of porcine oocytes and the transient intracellular change induces the meiotic resumption and
215 progression [19]. Secretions from oocytes, such as growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) [20], are also known to regulate the function of cumulus cells [21] and improve the developmental competence [22]. In the current study, the tilting culture

during IVM stimulated cumulus expansion and beneficially affected the quality of blastocysts, as determined by the cell number per blastocyst. During final follicular development and around the
220 ovulation, COC are known to exposed mechanical compression by changes of hydrostatic pressure in follicles [5-8]. Physical stimulus of cumulus cell mass seems to increase the secretion from cumulus cells and affected the interaction of cumulus-cumulus and/or cumulus-oocyte, since secretion of hyaluronic acids and disconnection of cumulus-cumulus tight junction are contained in the cumulus expansion. Therefore, a moderate mechanical stress during IVM may stimulate the
225 communication ability of cumulus cells, and consequently appears to improve the quality of blastocysts.

In the current study, however, the P value about the effect of tilting culture during IVC on the cell numbers in blastocysts was much lower (less than 0.0001) than that about the effect of tilting culture during IVM (0.0474; Table 3). Therefore, the tilting culture during IVC beneficially
230 contributes to the quality of the blastocysts, as compared with that during IVM. During preimplantation development, in fact, embryos suffer various physical stress, such as shear stress, compression and kinetic friction force, in the oviducts [9], whereas COC receive only compression during final follicular development and ovulation [5-8]. Therefore, embryos, rather than COC, may be sensitive to the beneficial physical stress. On the other hand, excessive shear stress appears
235 to be detrimental for preimplantation embryos. Shear stress at 1.2 dynes/cm^2 has been known to induce stress-activated protein kinase phosphorylation that preceded and caused apoptosis in mouse embryos [23]. According to a recent study, calculated shear stress during tilting culture in the present study was 7.0-15 x 10^{-3} dynes/cm^2 in the medium [12]. Thus, suitable shear stress around 7.0-15 x 10^{-3} dynes/cm^2 by tilting culture is beneficial to improve the quality of blastocysts,
240 as determined by the mean cell number per blastocyst.

In conclusion, 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 embryos may be more sensitive to the beneficial shear stress, as compared with COC.

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Acknowledgments

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Legend of figures

Figure 1. A culture plate on the tilting device (the rear) and control culture plate (static culture; 325 the front) in an incubator. The plate of the device continued the same movements that was inclined 20 degrees, kept the inclination for 1 min, and then inclined 20 degrees conversely for 12 sec. The side length of the culture plate is 65 mm.

Figure 2. COC after cultured by static culture (the upper part) and by tilting culture system (the 330 lower part) for 20 h. These COC were cultured in modified POM containing gonadotropins and 1 mM dbcAMP at 39 ^{o}C in an atmosphere of 5% CO_2 in air. The black bar shows 250 μm.

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Table 1. Mean diameter of COC and the oocyte after cultured statically or kinetically for in-vitro maturation for 20 h[1]

Culture Styles[3]	No. of oocytes Examined	Mean diameter (μm)[2] of	
		COC	oocyte
Static	29	284.2 ± 8.2^a	132.2 + 1.3
Tilting	32	357.5 ± 11.3^b	134.4 + 1.2

[1] COC 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 maximum and minimum diameters of COC and the oocytes after denuded.

[3] Static: culture plate was placed on a shelf in a CO_2 incubator, Tilting: culture plate was placed on the tilting culture device in a CO_2 incubator (see Figure 1).

Data are given as mean $\pm$ SEM from four replicated experiments.

Values with different superscripts within column are significantly different ($P < 0.05$).

Table 2. Meiotic progress of oocytes cultured for IVM[1] in static and kinetic culture systems

Culture styles[2]	No. of oocytes examined*	%[3] oocytes completing GVBD	%[3] M-II oocytes
Static	83	91.7 $\pm$ 2.3	88.1 $\pm$ 3.4
Tilting	88	96.0 $\pm$ 3.0	90.3 $\pm$ 1.3

[1] COC were cultured in the presence of 10 IU/ml eCG, 10 IU/ml hCG and 1 mM dibutyryl cAMP in mPOM for 20 h and then continued culture in the absence of those in fresh mPOM for further 24 h.

[2] Static: culture plate was placed on a shelf in a CO_2 incubator, Tilting: culture plate was placed on the tilting culture device in a CO_2 incubator (see Figure 1).

[3] Percentage based on the total number of oocytes examined.

Data are given as mean $\pm$ SEM from four replicated experiments.

Table 3. Effect of tilting culture during IVM and IVC on early development of IVM-IVF oocytes

	Culture styles[1]				Significance of effects within culture styles (P value)	
During IVM	Static	Static	Tilting	Tilting		
During IVC	Static	Tilting	Static	Tilting	during IVM	during IVC
No. of eggs examined	144	98	156	148	-	-
%[2] of eggs cleaved	72.8 ± 7.2	59.4 ± 5.9	65.7 ± 5.2	76.6 ± 1.6	0.3662	0.8198
%[2] of blastocyst formation	28.3 ± 5.4	24.9 ± 4.1	29.1 ± 4.0	25.8 ± 3.2	0.8486	0.4496
Cells/blastocyst	30.4 ± 2.1	34.1 ± 1.4	30.1 ± 1.4	44.3 ± 2.4	0.0474	<0.0001

[1] Static: culture plate was placed on a shelf in a CO_2 incubator, tilting: culture plate was placed on the inclining culture device in a CO_2 incubator (see Figure 1).

[2] Percentage based on the total number of eggs examined.

Data are given as mean ± SEM from four to five replicated experiments.