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Modification of the medium volume and gel substrate under *in vitro* culture conditions improves growth of porcine oocytes derived from early antral follicles

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Abstract. This study compared the effects of different volumes of culture medium for the *in vitro* growth of oocytes derived from porcine early antral follicles (EAFs). Oocyte granulosa cell complexes (OGCs) were collected from EAFs (0.5–0.7 mm in diameter) and individually cultured for 14 days. When OGCs were cultured in 1 ml of medium with or without polyacrylamide gel (PAG), the presence of PAG supported granulosa cell (GC) proliferation and oocyte growth. When OGCs were cultured in 0.2 or 1 ml of medium on PAG, the number of GC in the OGC culture and the developmental ability of the oocytes cultured *in vitro* were significantly higher for the 1 ml of culture medium group than for the 0.2 ml group. In conclusion, a combination of a large volume of culture medium with PAG improved the growth and developmental ability of the oocytes cultured *in vitro*, which were comparable to the oocytes collected from large antral follicles.

Key words: Early antral follicle, Granulosa cell, Oocyte growth

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Ovaries contain a greater number of growing small follicles than large antral follicles. Although the culture conditions for oocyte growth have been improved in recent decades, the developmental competence of oocytes cultured *in vitro* remains low, which restricts the source of oocytes for embryo production to those enclosed in large antral follicles. Oocytes are surrounded by granulosa cells (GCs), and factors such as the interaction between oocytes and GCs and number of GCs are important for oocyte growth and follicle development [1, 2]. Therefore, research on *in vitro* culture conditions for oocyte granulosa cell complexes (OGCs) derived from early antral follicles (EAFs) has focused on how to retain the three-dimensional (3D) structures of OGCs concomitant with the active proliferation of GCs during the culture period; for example, culturing OGCs in a v-shape plate [3–5], OGCs in alginate hydrogels [6–9], or culturing OGCs in polyvinylpyrrolidone (PVP)-containing medium [10]. In addition, we have reported that culturing porcine OGCs on a polyacrylamide gel (PAG) as a substrate (gel culture system) enhanced GC proliferation, maintained the 3D structure of the OGCs, and improved the developmental competence of oocytes cultured *in vitro* [11]. In this culture system, GC numbers increased from 7,000 immediately following collection from EAFs to 160,000 at the end of the culture period [11, 12]. Intriguingly, we found that the number of GCs surrounding oocytes is closely related to the diameter, lipid content, and ATP content of oocytes cultured *in vitro* [13]. This relationship between GC number and oocyte quality was also observed for bovine oocytes cultured *in vitro* [14]. Furthermore, Munakata *et al*. found that the number of GCs in follicles (3–5 mm in diameter) was closely related to the ability of the enclosed oocytes to develop to the blastocyst stage [15]. Considering our previous report showing that the average number of GCs in porcine antral follicles (AFs: 3–5 mm in diameter) was 639,794 ± 15,348 [15] and could be increased to 160,000 using the current *in vitro* culture system [13], we hypothesized that further proliferation of the GCs might improve oocytes developmental capacity. A fundamental factor determining the cell number in closed culture environments is the volume of the culture medium. Therefore, the present study investigated the volume of culture medium appropriate for *in vitro* oocyte growth. We also examined whether a large volume of medium combined with the gel culture system could increase GC number, and thus improve the developmental ability of *in vitro* cultured oocytes to levels similar to those of oocytes collected from large antral follicles.

When OGCs were cultured in 1 ml medium with or without PAG (Fig. 1), the rate of antrum formation did not differ between the two culture conditions (P > 0.05, Fig. 2-A). The OGCs developed in 1 ml of medium on the gel substrate contained about 400,000 cells and the oocytes had larger diameters compared with those cultured without the gel substrate (Fig. 2-B, C). When OGCs were cultured in 0.2 or 1 ml of medium on a PAG (Fig. 1), the OGCs cultured in 1 ml-conditions were larger than those cultured in the 0.2 ml system (Fig. 3-A and B). Antrum formation was similar between the two culture conditions; however, the number of GCs in OGC cultures differed markedly (Fig. 4-A and B). No difference in the final sizes of the oocytes between the two culture conditions was observed (Fig. 4-C). When oocytes cultured *in vitro* were subjected to activation

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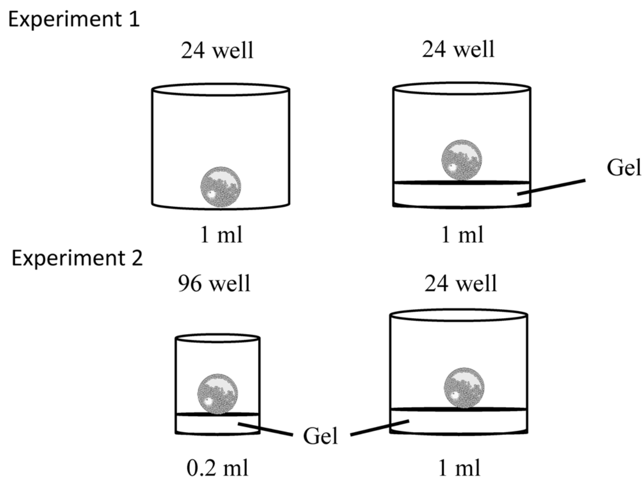

Fig. 1. Schematic design of the *in vitro* culture of oocyte granulosa cell complexes (OGCs). OGCs were individually cultured with or without a gel in 0.2 ml or 1 ml of *in vitro* growth (IVG) medium.

followed by *in vitro* maturation culture, the developmental rate to the blastocyst stage was significantly higher for oocytes cultured in 1 ml of medium compared with those developed in 0.2 ml of medium (Table 1). We further compared the difference between the oocytes cultured in the two culture conditions. The lipid content, as measured by Nile red or the cytoplasmic color, was reported to be associated with high developmental competence [16, 17] In addition, the lipid content reflects the developmental ability of oocytes cultured *in vitro* [13]. The amount of lipid in oocytes tended to be greater in those cultured in 1 ml of medium; however, the difference was not significant compared with those cultured in 0.2 ml of medium (Fig. 4-D).

The developmental rate to the blastocyst stage was 17.5 ± 5.8%, which was comparable to that of oocytes derived from antral follicles (average of the data from 11 trials 13.3 ± 2.3%; average cell number of the blastocysts = 40.2 ± 4.6). GCs play a role in oocyte growth via the close interaction between the two cells. Granulosa cells of OGCs actively metabolize medium components, for example, the glucose concentration in the medium of OGCs was reduced by half during a 4-day culture period [18]. The medium volume and cellular proliferation are a classical topic in cell research; however, the optimal medium and cell volume has been rarely discussed in

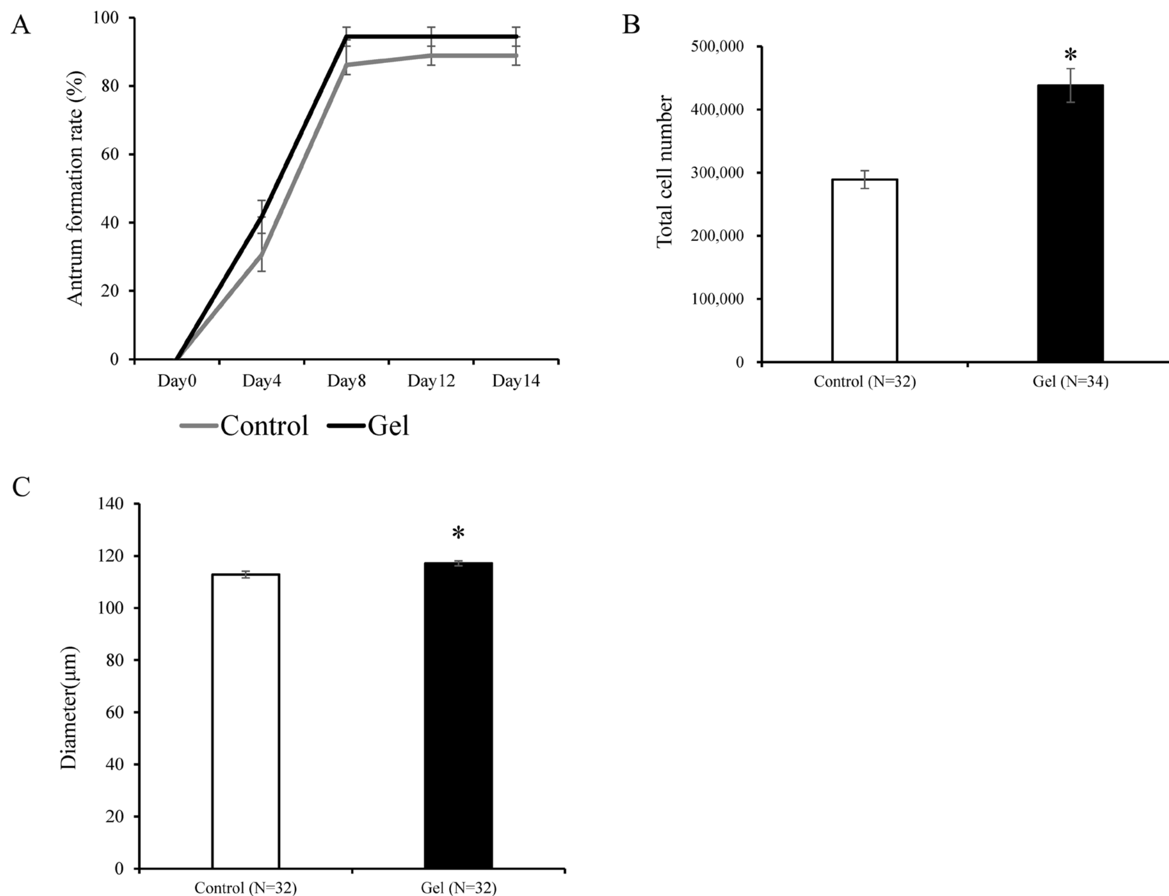

Fig. 2. Antrum formation (A), granulosa cell number of oocyte granulosa cell complexes (OGCs) (B) and diameter of oocytes (C) developed in 1 ml of medium with or without a gel culture system. Data are presented as means ± standard error of the mean (SEM) of four replicates. * $P < 0.05$.

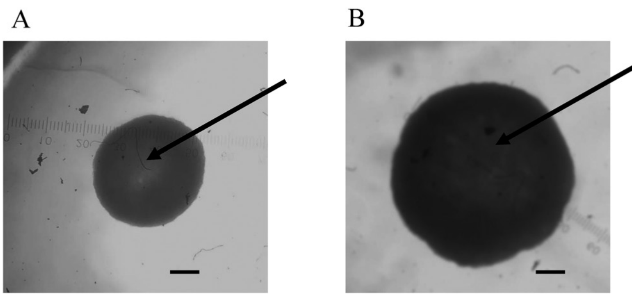

Fig. 3. Representative oocyte granulosa cell complexes (OGCs) that were individually cultured in 0.2 ml (A) or 1 ml (B) medium. Arrows indicates the antrum. Bars represent 200 µm.

recent literature. It is generally accepted that high cell concentrations result in cell dysfunction because of a lack of metabolites and oxygen, while a large volume of medium supports cellular homeostasis and alters gene expression [19]. Therefore, to maintain cellular metabolic functions, the provision of oxygen and nutrients are crucial factors [20, 21]. In this context, a study of the culture of hepatocyte multilayers on polydimethylsiloxane membranes showed that oxygen provision to the cells is a key factor for long-term cell culture [22]. In the present study, using a large volume of medium combined with PAG as a culture substrate greatly enhanced GC proliferation. We have demonstrated that when OGCs were cultured in 0.2 ml medium with the gel substrate, the gel culture substrate itself enhanced GC proliferation and oocyte growth, leading to high developmental ability to the blastocyst stage [12]. Consistent with this report, 1 ml of medium without gel provided less support for GC proliferation and oocyte growth compared with culture with the gel. The results of this study suggested that gel culture substrates combined with a large medium volume are beneficial to support GC proliferation, which might reflect the provision of sufficient energy and oxygen to the OGCs.

GC number is reported to be associated with developmental competence of enclosed oocytes [14], and a high number of GCs is associated with the lipid and ATP contents in oocytes cultured *in vitro* [13]. Furthermore, a high lipid content in oocytes is a marker of

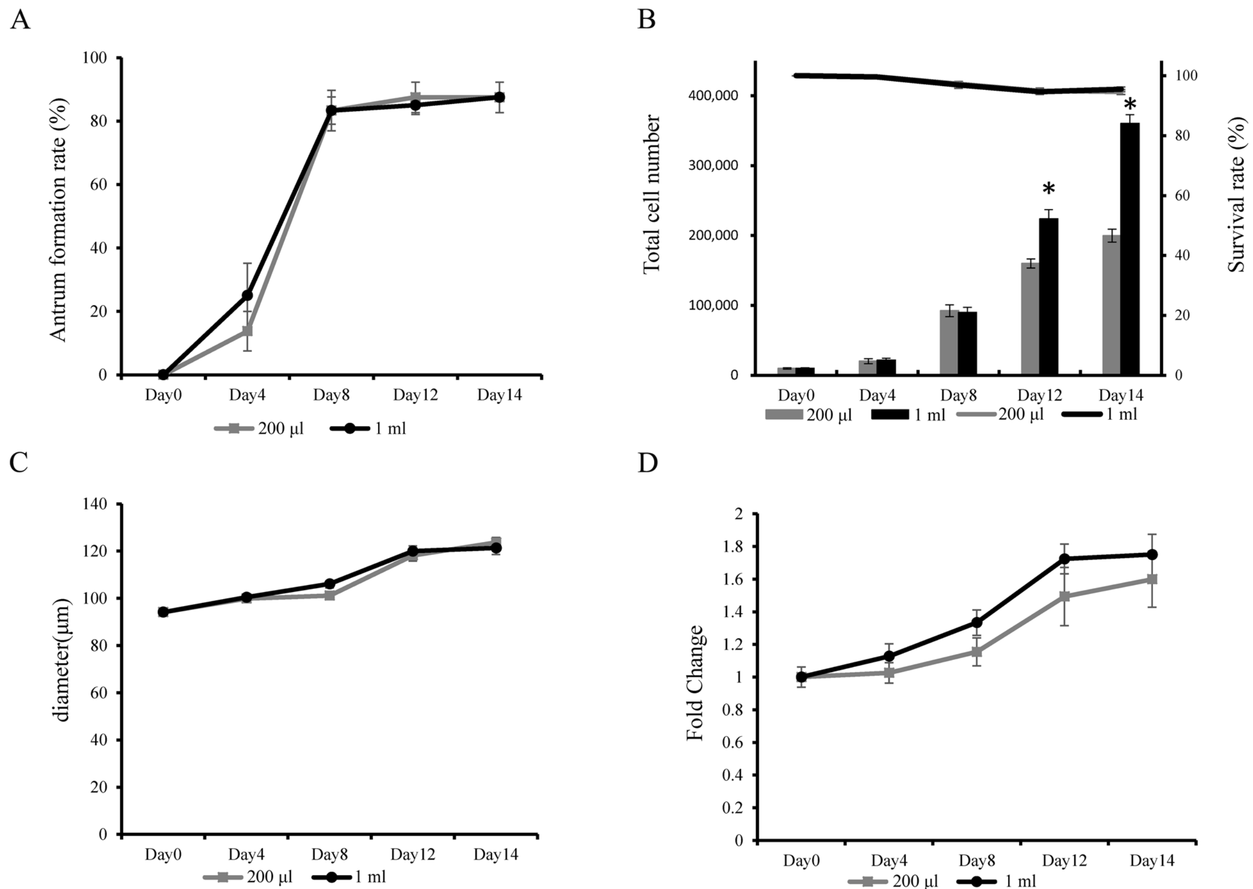

Fig. 4. Antrum formation (A), granulosa cell number of oocyte granulosa cell complexes (OGCs) (B), diameter of oocytes (C), and relative lipid content in oocytes. OGCs were individually cultured 0.2 ml or 1 ml of medium on a gel culture substrate for 14 days. Data are presented as means ± standard error of the mean (SEM) of four replicates. * $P < 0.05$.

Table 1. Effect of medium volume on the developmental competence of oocyte cultured *in vitro*

Medium volume (ml)	No. of replicates	No. of OGCs	No. of oocytes	No. of blastocysts	Rate of blastulation (%)	Total cell number
0.2	5	75	67	4	5.9 ± 2.8	30.0 ± 2.0
1	5	75	65	10	17.5 ± 5.8 *	44.2 ± 3.5 *

Data are presented as means ± standard error of the mean (SEM) of five replicates. * $P < 0.05$. OGCs, oocyte granulosa cell complexes.

high competency [17]. We therefore addressed the effect of medium volume on the lipid content in oocytes. The oocytes developed in a large volume of culture medium had a high lipid content; however, the difference compared with oocytes cultured in a lower volume was not significant. Therefore, more research is needed to elucidate the molecular mechanism underlying the beneficial effect of the culture conditions on oocyte competence. However, the present study showed that the rate of development to the blastocyst stage of cultured oocytes was similar to that of cells collected from antral follicles. This indicated that using the current culture condition or further modifications of the culture volume; highly competent oocytes could be obtained from early antral follicles in large animals.

In conclusion, given the optimal culture conditions, including gel culture substrate and a higher amount of culture medium, pig oocyte cultured *in vitro* could achieve a similar developmental ability to the blastocyst stage to those collected from large antral follicles.

Methods

Chemicals and media

All reagents were purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise stated. To culture OGCs, we used α-minimal essential medium (MEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 mM taurine, 0.1 mAU/ml follicle-stimulating hormone (Kawasaki Mitaka, Tokyo, Japan), 2% polyvinylpyrrolidone-360 (Sigma-Aldrich), 2 mM hypoxanthine (Sigma-Aldrich), 1% insulin transferrin selenium (Gibco, Paisley, UK), 1 µg/ml 17β-estradiol, 3 mg/ml bovine serum albumin (BSA), and antibiotics. The *in vitro* maturation (IVM) medium was Medium 199 (Gibco), supplemented with 10% porcine follicular fluid (pFF), 0.5 mM L-cysteine, 0.9 mM sodium pyruvate, 1 mM L-glutamine, 10 ng/ml epidermal growth factor, 5% fetal calf serum, 10 IU/ml equine chorionic gonadotropin (ASKA Pharma, Tokyo, Japan), and 10 IU/ml human chorionic gonadotropin (Fuji Pharma, Tokyo, Japan). *In vitro* culture of embryos and oocyte activation was conducted in porcine zygote medium 3 (PZM3) [23]. *In vitro* culture of OGCs and oocyte maturation was performed at 38.5°C under atmospheric conditions of 5% CO_2 and 95% air, whereas *in vitro* embryo culture was performed at 38.5°C in an atmospheric conditions of 5% O_2, 5% CO_2, and 90% N_2. The pFF used for the IVM medium was aspirated from AFs (3–5 mm in diameter) of one hundred gilts and centrifuged (10,000 × *g*) for 20 min. The resulting supernatants were collected, sterilized, and stored at –20°C until use.

Ovaries collection and collection of oocytes from EAFs

Ovaries were collected from prepubescent gilts at a local slaughterhouse and transported to the laboratory (at approximately 35°C, in phosphate-buffered saline (PBS) containing antibiotics) within 1 h. The ovarian cortical tissues were excised from the ovarian surface under a stereomicroscope, and OGCs were collected from EAFs (0.5–0.7 mm in diameter).

In vitro growth (IVG) of oocytes derived from EAFs

OGCs collected from EAFs were individually transferred to a well containing 0.2 ml medium (in 96-well plates, Becton Dickinson) or 1 ml medium (in 24-well plates, Thermo Fishers Scientific) and cultured for 14 days (Fig. 1). The bottom of the culture wells were set with or without a PAG sheet, and the OGCs were cultured on the gel if present. Half of the medium was replaced with fresh medium, and antrum formation was examined every of 4 days.

Preparation of 0.3% PAG sheet

PAG constituted of N-methylenebisacrylamide, water, acrylamide, ammonium peroxodisulfate, and N,N,N',N'-tetramethylethylenediamine, and was prepared according to the general method for western blot analysis. Details on PAG preparation for culture of OGCs derived from EAFs have been previously reported [11].

Collection of cumulus cells and oocyte complexes (COCs)

COCs were aspirated from antral follicles (3–5 mm in diameter) using 21G needles connected to a syringe and oocytes surrounding the thick cumulus cells were picked up under a stereo microscope (Olympus, Tokyo, Japan). The COCs were subjected to *in vitro* maturation.

In vitro maturation, activation, and in vitro culture (IVC)

After IVG (14 days), OGCs with an antrum cavity were subjected to IVM for 48 h. In addition, COCs collected from antral follicles (3–5 mm diameter) were subjected to IVM. After IVM, oocytes were denuded from the surrounding GCs, and parthenogenetically activated in IVC medium containing 10 µg/ml ionomycine for 5 min, and incubated for 5 h in PZM3 containing 10 µg/mL cytochalasin B and 10 µg/mL cycloheximide at 38.5 °C. After activation, the embryos were cultured for 8 days in culture medium and the rate of blastulation and the total cell number of blastocysts was determined. Blastocysts were fixed in 4% paraformaldehyde, mounted onto microscope slides using ProLong Gold antifade reagent with 2-(4-amidonophenyl)-1H-indole-6-carboxamidine (DAPI; Invitrogen, OR, USA), and observed to count the total blastocyst cell number using a fluorescence digital microscope (Keyence).

Measurement of number and survival rate of GCs surrounding oocytes cultured in vitro

After IVG, GCs were enzymatically dispersed (Accumax; Innovative Cell Technologies, San Diego, CA, USA), and the total GC number was calculated based on the volume and concentration of the cellular suspension using a hematocytometer. In addition, the cell survival rate was determined using trypan blue staining.

Measurement of oocyte diameter

The diameter of the ooplasm was measured (X and Y-axis with a 90° angle) under a digital microscope (Keyence). Averages of the X and Y-axis values were calculated as the diameter of the oocytes.

Measurement of lipid in oocytes

Oocytes denuded from OGCs were fixed in 4% paraformaldehyde followed by staining with 10 µg /ml Nile Red (Wako, Osaka, Japan) for 10 min. After washing, the oocytes were mounted on a microscope slide with pro-long gold antifade reagent with DAPI (Invitrogen). Fluorescence images of lipids were captured using a fluorescence digital microscope (Keyence), and the fluorescence intensity was measured using the Image-J software (NIH, Bethesda, MD, USA).

Experimental design

In this study, each set of 20 OGCs derived from EAFs was allocated to 1 ml of medium, with or without PAG, for 14 days, and antrum formation, granulosa cell number, and oocyte diameters were then examined. Every four days during IVG, four OGCs were randomly picked up and used for experimentation. In the next experiment, OGCs were cultured in 0.2 or 1 ml of medium on a PAG for 14 days, and oocytes and GCs were subjected to experimentation. These experiments were repeated four times. In the next experiment, 15 OGCs were cultured in 0.2 or 1 ml of medium on a PAG for 14 days, and oocytes cultured *in vitro* were subjected to IVM, parthenogenetic activation (PA), and IVC to examine their developmental competence to the blastocyst stage. This experiment was repeated five times.

In the last experiment, COCs were collected from antral follicles (3–5 mm in diameter). These oocytes were subjected to IVM- and PA, followed by IVC to determine the ability of the oocytes to develop to the blastocyst stage.

Data analysis

All data were analyzed using analysis of variance (ANOVA) followed by post hoc Tukey's test. Percentages were arcsine transformed before analysis. Values less than 0.05 were considered significantly different.

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