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# In Vitro Culture of Pig Oocytes Collected from Early Antral Follicles

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**Abstract:** Pig early antral follicles (0.5–1.0 mm in diameter) containing oocytes approximately 100 μm in diameter were collected from ovaries. The intact early antral follicles, oocyte-cumulus-granulosa cell complexes (OCGs), and oocyte-cumulus complexes (OCs) dissected from the follicles were embedded in collagen gels, cultured in Waymouth's medium containing 5% fetal calf serum and 0.05 mg/ml sodium pyruvate for 8 days, and the oocyte morphology was examined. During the culture, follicular cells grew out of the complexes and spread into the collagen gels. All of the recovered oocytes had been detached from cumulus cells in the complexes after the culture. Although no significant increase in diameter was observed, 7% of the oocytes showed normal morphology in the cultured follicles, as did 53% in the OCGs and 47% in the OCs. Before culturing, oocytes from early antral follicles had decondensed filamentous chromatin or thicker stringy chromatin distributed throughout the germinal vesicle (GV0 stage). In the cultured follicles, OCGs and OCs, 0% (0/42), 16% (7/43) and 12% (5/43) of the oocytes, respectively, were at the GVI-MI (the first metaphase) stage, and 2% (1/42), 21% (9/43) and 10% (4/43) of the oocytes, respectively, formed female pronuclei. These results suggest that 37% (16/43) and 21% (9/43) of oocytes in OCGs and OCs, respectively, survived the 8-day culture and advanced their meiotic stage.

**Key words:** Collagen gel, Early antral follicle, In vitro culture, Pig oocyte.

A small population of pig oocytes with a diameter of 30 μm in primordial follicles begin growing and ultimately reach a size of 120 μm. During this oocyte growth, the surrounding granulosa cells proliferate, and the follicles increase in size. In turn, selected fully grown oocytes

resume meiosis, reach the second metaphase, and are then ovulated under the influence of gonadotropins.

Mouse oocytes grow to their final size in culture when they maintain junctional communication with surrounding granulosa cells [1]. In the pig, Hirao et al. [2] reported that oocytes at the mid-growth phase (70–90 μm) in preantral follicles grew to their final size and matured to the second metaphase. Mouse oocytes have been seen to nearly reach their final size in early antral follicles [3], but pig oocytes in the early antral follicles are still in their growing phase, and do not have meiotic competence [4, 5]. As early antral follicles develop into large antral follicles 4–6 mm in diameter, pig oocytes reach 120 μm and acquire meiotic competence.

In this study, we used 100 μm-diameter oocytes from early antral follicles, and examined three culturing methods based on the previously reported collagen gel embedding method [2]. It has been reported that mouse oocytes cultured in dissected intact secondary follicles [6–8] and in oocyte-granulosa cell complexes [9, 10] can grow to their final size and acquire meiotic competence. We cultured the intact early antral follicles, oocyte-cumulus-granulosa cell complexes, and oocyte-cumulus complexes of the pig in collagen gels for 8 days, and examined the survival rates and nuclear morphology of the cultured oocytes.

## Materials and Methods

*Collection of growing oocytes from early antral follicles*

Ovaries were obtained from cross-bred gilts slaughtered at a local abattoir. Following three washes with Dulbecco's phosphate-buffered saline containing 0.1% polyvinylalcohol, early antral follicles with diameters of 0.5–1.0 mm were dissected from the ovarian cortices in Waymouth's medium (pH 7.2; Waymouth 752/1MB; Sigma Chemical Co., St Louis, MO, U.S.A.) containing 1 mg/ml bovine serum albumin (Intergen Co., NY,

U.S.A.), 0.05 mg/ml sodium pyruvate, 0.336 mg/ml NaHCO₃, 0.1 mg/ml kanamycin (Sigma Chemical Co.), and 5 mg/ml HEPES. Since an antrum was plainly observed in the follicles larger than 0.4 mm in diameter under a dissection microscope, follicles with diameters of 0.5–1.0 mm were collected and used as early antral follicles in subsequent experiments. Connective tissues surrounding the follicles were stripped off with forceps. Healthy early antral follicles, in which no granulosa cells were detached from the follicle wall, were selected under a dissection microscope. From some of the follicles, oocyte-cumulus complexes with parietal granulosa cells (OCGs) and oocyte-cumulus complexes (OCs) were collected with fine forceps. Each of the follicles and complexes was washed in the medium and transferred into a 10 $\mu$l drop of HEPES-buffered Waymouth's medium under paraffin oil, where the diameters of the oocytes (excluding zona pellucida) were measured to the nearest 1 $\mu$m by means of an ocular micrometer (Nikon, Tokyo, Japan).

*Culture of oocytes*

The culture of the oocytes was based on the method described by Hirao *et al.* [2]. Follicles, OCGs and OCs containing growing oocytes of 90–110 $\mu$m in diameter were embedded in collagen gels. The collagen mixture consisted of 0.3% acid collagen solution (Cellmatrix Type I: Nitta Gelatine, Tokyo, Japan), 10 times-concentrated Waymouth's medium, and 0.05 N sodium hydroxide solution containing 22 mg/ml NaHCO₃ and 47.7 mg/ml HEPES at a ratio of 8:1:1. The mixture was placed on the bottom of a Petri dish (#1008: Falcon, Becton Dickinson Labware, Lincoln Park, NJ, U.S.A), and the follicles, OCGs and OCs were transferred into the mixture with a small volume of the medium. The gel was put into an incubator at 39°C for 20 min. After gelatinization, 4 ml of the culture medium was poured on the gel, and the oocytes were cultured at 39°C in a humidified atmosphere of 5% CO₂ and 95% air for 8 days. The culture medium was Waymouth's medium containing 5% fetal calf serum (Bio Whittaker, Walkersville, ML, U.S.A) and 0.05 mg/ml sodium pyruvate. On every third day, half of the volume of the culture medium was replaced by fresh medium.

*Examination of the oocytes*

After the culture, the collagen gels were torn with fine forceps, and the oocytes were recovered. The oocytes were then denuded completely by pipetting, and transferred into 10 $\mu$l drops of HEPES-buffered Waymouth's medium. Oocytes that showed evidence

of cytoplasmic or other forms of degeneration were excluded from further analysis. Oocytes showing normal morphology were regarded as surviving oocytes, and their diameter was measured. They were then mounted on slides, fixed in acetic ethanol (1:3), stained with 1% aceto-orcein, and examined for their nuclear morphology under a differential interference microscope. The precise nuclear stage was determined on the basis of changes in the configuration of chromosomes and the nuclear membrane [11]. The results for all replicates were pooled and analyzed. Statistical differences in the mean diameter of the oocytes were analyzed by Student's *t*-test. The other values were analyzed by the chi-square test and by Fisher's exact test. A probability of less than 0.05 was considered statistically significant.

## Results

From one ovary, 15–20 healthy small antral follicles of 0.5–1.0 mm in diameter were collected. The mean diameters of the oocytes as categorized into three groups were as follows: 99.9 ± 4.7 $\mu$m (n=42) in the follicles (Fig. 1a); 100.3 ± 4.1 $\mu$m (n=43) in the OCGs (Fig. 1b); and 100.0 ± 3.6 $\mu$m (n=43) in the OCs (Fig. 1c) (Table 1). Follicular cells grew out from the periphery of the cultured follicles and spread into the collagen gels during the culture, although we could not distinguish between thecal cells and granulosa cells under the inverted microscope (Fig. 1d). Granulosa cells of OCGs also spread into the collagen gels (Fig. 1e). In the cultured follicles and OCGs, oocytes were still surrounded by the somatic cells after 8 days. In OCs, cumulus cells spread into the collagen gels, and oocytes did not maintain the spherical structure and changed their form to oval after 8 days (Fig. 1f).

All of the oocytes that were recovered from the follicles, OCGs, and OCs had been detached from cumulus cells in the complexes. The cytoplasm had shrunken in 93% of the oocytes in the follicles, 47% of those in the OCGs, and 53% of those in the OCs, and these oocytes were classified as degenerated oocytes (Table 1). We regarded the rest of the oocytes, which showed normal morphology under the inverted microscope, as surviving oocytes, and measured their diameter. The mean diameters of the oocytes in the cultured follicles, OCGs and OCs were 98.3 ± 2.5 (n=3), 99.6 ± 6.2 (n=23) and 98.2 ± 8.0 $\mu$m (n=20), respectively, and no significant differences between the means were observed. The mean diameters of the oocytes in each group showed no significant increase after 8 days.

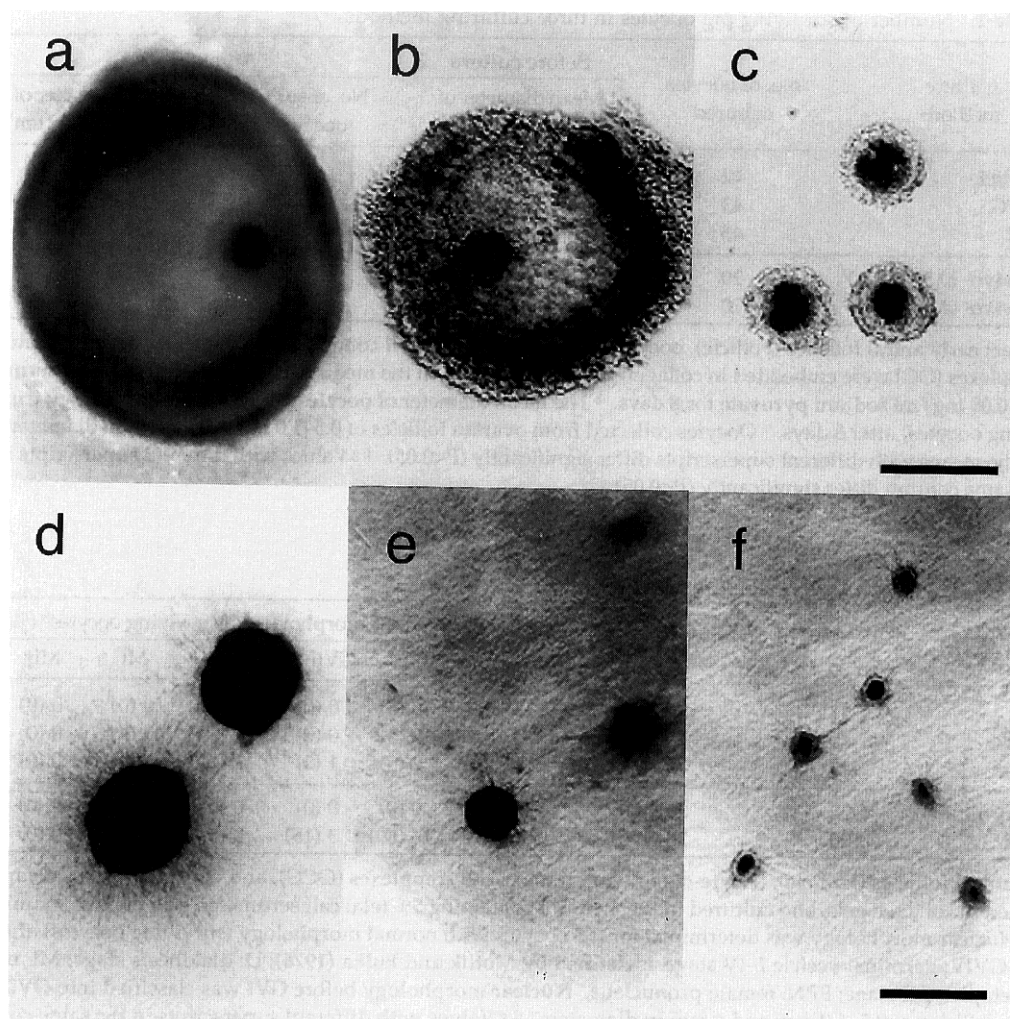In the oocytes collected from early antral follicles

**Fig. 1.** Pig early antral follicles (a), oocyte-cumulus-granulosa cell complexes (OCGs, b) and oocyte-cumulus complexes (OCs, c) were collected from early antral follicles. The follicles (d), OCGs (e) and OCs (f) were cultured for 8 days in collagen gels. The scale bars in a-c and d-f represent 200 μm and 500 μm, respectively.

with a diameter of 0.5–1.0 mm, decondensed filamentous chromatin or thicker stringy chromatin was distributed throughout the germinal vesicle (GV0 stage) (Table 2). On the other hand, a condensing chromatin ring was formed around the nucleolus in the oocytes from 4 mm follicles, showing a morphology of "GV I"-stage oocytes as characterized by Motlik and Fulka [11]. After 8 days, 2 surviving oocytes were at the GV0 stage in the follicles, 7 in the OGCs, and 11 in the OCs. Five oocytes in the OCGs and two in the OCs had undergone germinal vesicle breakdown at the diakinesis (D) or first metaphase (MI) stage. In all three groups, some oocytes had one or more female pronuclei. These oocytes did not have the first polar body.

## Discussion

The pig oocytes from 0.5–1.0 mm early antral follicles had a mean diameter of 100 μm, and those from 4 mm follicles had a mean diameter of 120 μm. Hirao *et al.* [2] reported that growing pig oocytes with diameters of 70–89.5 μm in preantral follicles grew to their final size in culture. In their experiment, 31–43% of the oocytes were recovered after 16 days of culture. When intact early antral follicles were cultured for 8 days in the present study, only 7% of oocytes had normal morphology, and the rest of the oocytes had degenerated. Whereas Hirao *et al.* [2] used 0.2–0.3 mm follicles in

**Table 1.** Number of surviving pig oocytes in three culturing methods

| Culture method[a] | No. of oocytes cultured | Before culture | After culture | |
|---|---|---|---|---|
| | | Mean diameter of oocytes ± SD ($\mu$m) | No. of surviving oocytes (%) | Mean diameter of oocytes ± SD[b] ($\mu$m) |
| Follicle | 42 | 99.9 ± 4.7[d] | 3 (7)[f] | 98.3 ± 2.5[d] |
| OCG | 43 | 100.3 ± 4.1[d] | 23 (53)[g] | 99.6 ± 6.2[d] |
| OC | 43 | 100.0 ± 3.6[d] | 20 (47)[g] | 98.2 ± 8.0[d] |
| in vivo[c] (0.5–1 mm) | 20 | 100.9 ± 4.9[d] | | |
| in vivo[c] (4 mm) | 20 | 119.8 ± 1.6[e] | | |

[a] Intact early antral follicles (Follicle), oocyte-cumulus-granulosa cell complexes (OCG), and oocyte-cumulus complexes (OC) were embedded in collagen gels, and cultured in the medium containing 5% fetal calf serum and 0.05 mg/ml sodium pyruvate for 8 days. [b] The mean diameter of oocytes with normal morphology (surviving oocytes) after 8 days. [c] Oocytes collected from ovarian follicles of 0.5–1.0 mm and 4 mm in diameters. [d,e] The means with different superscripts differ significantly (P<0.05). [f,g] Values with different superscripts in the same column differ significantly (P<0.05).

**Table 2.** Nuclear morphology of cultured pig oocytes for 8 days

| Culture method[a] | No. of oocytes cultured | No. of surviving oocytes (%) | Nuclear morphology of surviving oocytes[b] (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | GV0 | GVI | GVII–IV | D | MI | MII | FPN |
| Follicle | 42 | 3 (7)[e] | 2 (5)[d] | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (2)[d] |
| OCG | 43 | 23 (53)[f] | 7 (16)[de] | 2 (5)[d] | 0 (0) | 4 (9) | 1 (2) | 0 (0) | 9 (21)[e] |
| OC | 43 | 20 (47)[f] | 11 (26)[e] | 2 (5)[d] | 1 (2) | 1 (2) | 1 (2) | 0 (0) | 4 (10)[de] |
| in vivo[c] (0.5–1 mm) | 20 | 20 (100) | 20 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| in vivo[c] (4 mm) | 20 | 20 (100) | 1 (5)[de] | 16 (80)[e] | 3 (15) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

[a] Intact early antral follicles (Follicle), oocyte-cumulus-granulosa cell complexes (OCG), and oocyte-cumulus complexes (OC) were embedded in collagen gels, and cultured in the medium containing 5% fetal calf serum and 0.05 mg/ml sodium pyruvate for 8 days. [b] Nuclear morphology was determined for the oocytes with normal morphology (surviving oocytes) after 8 days of culture, GVI–GVIV: germinal vesicle I–IV stage as defined by Motlik and Fulka (1976); D: diakinesis stage; MI: metaphase I stage; MII: metaphase II stage; FPN: female pronucleus. Nuclear morphology before GVI was classified into GV0. [c] Oocytes collected from follicles of 0.5–1.0 mm and 4 mm in diameters. [d,e] Values with different superscripts in the same column differ significantly (P<0.05).

their study, we used much large 0.5–1.0 mm follicles, and it is possible that oxygen and nutrients in the culture medium could not reach the enclosed oocytes. Hirao et al. [2] collected preantral follicles by collagenase digestion, and found that the collected follicles contained no thecal cells. In contrast, when we dissected early antral follicles under a dissection microscope we found that the follicles contained thecal cells. Since we collected early antral follicles mechanically, the thecal cells could decrease oocyte viability during the culture period.

About half of the oocytes in the OCGs and OCs had normal morphology after 8 days of culture, but all of the oocytes in the complexes were denuded, and no increase in diameter was observed. In ovarian follicles, there are gap junctions between the oocyte and surrounding cumulus granulosa cells [12, 13]. Amino acids

and nutrients for the oocyte are transferred from granulosa cells to the oocytes through the junctions. In the cultured OCGs and OCs, it is thought that granulosa cells had become degenerated and the junctions were disrupted, and so, in turn, the oocytes failed to grow. When naked growing mouse oocytes are co-cultured with fibroblasts or in a fibroblast conditioned medium [14, 15], the oocytes survive and acquire meiotic competence, although they do not grow. The surviving pig oocytes in the OCGs and OCs were kept in conditions similar to those for the denuded mouse oocytes.

The chromatin configuration in the oocyte nucleus changes during oocyte growth [4, 16]. In the oocytes collected from early antral follicles that were 0.5–1.0 mm in diameter, decondensed filamentous chromatin or thicker stringy chromatin was distributed throughout the germinal vesicle. On the other hand, a condensing chro-

matin ring was formed around the nucleolus in the oocytes collected from 4 mm follicles. The filamentous or stringy chromatin configuration did not change in 16% (7/43) of the oocytes in cultured OCGs, or in 26% (11/43) of those in cultured OCs, but 16% (7/43) of the oocytes in cultured OCGs were at the GVI-MI stage, as were 12% (5/43) of those in cultured OCs. Furthermore, 21% (9/43) of OCG oocytes and 10% (4/43) of the OC oocytes formed female pronuclei. Because the female pronucleus is formed by decondensation of condensed chromosomes, it is thought that the oocytes resumed meiosis and the chromosomes became condensed during the culture period. As no first polar bodies were observed in the oocytes, it is possible that the oocytes resumed the first meiosis and reached the diakinesis or first metaphase stage when chromosome decondensation started to form the female pronucleus. Nevertheless, these results suggest that a total of 37% (16/43) of OCG oocytes and 21% (9/43) of OC oocytes survived the 8-day culture and advanced their meiotic stage, and that the cultured oocytes partially acquired meiotic competence by virtue of an autonomous program. Canipari *et al.* [14] concluded that growing mouse oocytes have an autonomous chronological program for the acquisition of meiotic competence, since the denuded oocytes resume meiosis spontaneously during the culture period without any growth.

Oocytes in ovarian follicles remain at the prophase in the first meiosis. After gonadotropic stimulation, they resume meiosis, reach the second metaphase, and stop again. In the present study, because we did not add any meiosis-arresting substances to the culture medium, some oocytes resumed meiosis spontaneously. The addition of hypoxanthine or IBMX, which inhibit cAMP-phosphodiesterase, is necessary to maintain the meiotic arrest of cultured oocytes at the prophase [17, 18]. We do not know why some cultured oocytes formed a female pronucleus without emission of the first polar body. The cytostatic factor, first reported in frog oocytes by Masui and Markert [19], is now known to be the product of proto-oncogene c-mos, and MOS protein is believed to participate in the maintenance of metaphase arrest in the mouse oocytes [20, 21]. Recently, mRNA of c-mos has been detected in pig oocytes [22]. It is thought that the production of c-mos protein is not sufficient to maintain the cultured pig oocytes at the metaphase.

## Acknowledgement

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