Effect of Droplet Size and Number of Oocytes Examined on Mouse Oocyte Quality in In Vitro Maturation

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Abstract: *The number of cumulus–oocyte complexes (COCs) in a droplet is known to affect oocyte maturation rates. In this study, we investigated whether droplet size or the number of COCs examined in in vitro maturation (IVM) affect mouse oocyte maturation, fertilization, or early embryonic development. Moreover, we aimed to determine the optimal, practical culture condition of IVM that could be achieved without changing the culture medium composition. The droplet sizes used were 20, 50, 100, and 200 μl, and the numbers of COCs examined were 1, 5, 10, 20, and 50. The groups with oocyte maturation rates exceeding 75% were treated with in vitro fertilization and in vitro culture. Compared with in vivo-matured oocytes, the 20 COCs/100-μl group did not exhibit significant differences in developmental competence and quality. We also observed progression to blastocysts in the 5-COC group, but the zygotes were likely to form multiple pronuclei. The results indicate: (1) droplet size and numbers of COCs examined in IVM affect oocyte quality; (2) the 20 COCs/100-μl condition effectively stimulated maturation in all experiments in this study; and (3) culture of 5 COCs in IVM can also produce blastocysts.*

Key words: *Oocyte quality, In vitro maturation, Early embryonic development, Culture condition, Mouse*

Introduction

Over many years, oocyte culture conditions in *in vitro* maturation (IVM) have been developed in order to meet the requirements as do the conditions *in vivo* [1, 2]. However, IVM has many drawbacks and oocytes matured *in vitro* have lower fertility than those matured *in vivo* [3]. Therefore, IVM conditions need to be further improved.

At our laboratory, various numbers of cumulus–oocyte complexes (COCs), e.g. 20 COCs, are cultured in 100-μl droplets of culture medium for IVM. The number of COCs in a droplet affects the oocyte maturation rate. This is because cumulus cells and oocytes supply each other with paracrine and autocrine factors, a mechanism that is essential for oocyte development [4]. The results of a previous study suggest that high paracrine and autocrine activities will be achieved in cultures with more than 20 COCs [4]. However, it is unclear whether culture with more than 20 COCs does enhance oocyte quality. Moreover, COCs utilize many nutrients in the droplets during IVM. Thus, if oocyte density in the droplets islow, COCs can utilize more nutrients, and their quality may be improved; however, this supposition also requires confirmation.

In this study, our objective was to investigate whether droplet size or number of COCs examined affect oocyte maturation, fertilization, or early embryonic development. In groups with the same droplet size, we investigated whether differences in the number of COCs influenced oocyte quality. In groups with the same number of COCs, we determined whether differences in droplet size influenced the quality. First, we investigated the effects of different IVM conditions on oocyte maturation, fertilization, and early embryonic development. Second, we evaluated total cell numbers (TCNs) and mRNA expression in blastocysts to examine the effects of different culture conditions on early embryonic development. In addition, we analyzed the mRNA expression of *glucose transporter-1* (*Glut-1*) and *desmocollin III* (*Dc III*), which are involved in metabolism, compaction, and cavitation in blastocysts [5].

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Furthermore, based on those results, we deduced the optimal, practical IVM condition that could be achieved without changing the culture medium composition.

Materials and Methods

Oocyte collection

Follicle development was stimulated by intraperitoneal injection of each mouse with 5 IU of pregnant mare serum gonadotropin (PMSG, Serotropin; Asuka, Tokyo, Japan). COCs for IVM were harvested from mice 46–48 h after the injection of PMSG by puncturing the large antral follicles with a 26-gauge needle. COCs were collected in Leibovitz's L-15 medium (Invitrogen, Grand Island, NY) containing 0.1% polyvinyl alcohol (PVA; Sigma, St. Louis, MO) and 4 mM hypoxanthine (Sigma).

Moreover, *in vivo*-matured oocytes were collected as a control for *in vitro* fertilization (IVF) and *in vitro* culture (IVC) experiments. Forty-eight hours after PMSG administration, superovulation was stimulated by intraperitoneal injection of each mouse with 5 IU of human chronic gonadotropin (hCG, Puberogen; Yell, Tokyo, Japan). Oviducts were removed 13–14 h after hCG administration, and COCs were collected in Leibovitz's L-15 medium containing 0.1% PVA and 4 mM hypoxanthine.

In vitro maturation

The culture medium used for maturation was Waymouth's MB752/1 medium (Invitrogen) containing 5% FCS (Sankyo Kagaku, Tokyo, Japan), 0.23 mM pyruvic acid (Nacalai Tesque, Kyoto, Japan), 75 mg/l of penicillin G (Meiji Seika, Tokyo, Japan), and 50 mg/l of streptomycin sulfate (Meiji Seika). This medium was designated as the basal medium. To examine FSH-induced meiotic maturation, 4 mM hypoxanthine and 100 IU/l of FSH (Sigma) were added to the basal medium. COCs were cultured for 18 h in culture medium overlaid with paraffin liquid (Nacalai Tesque) in a humidified atmosphere of 5% CO_2 in air at 37°C. IVM was performed using different numbers of COCs per droplet and different sizes of the culture medium droplet. The experimental groups were as follows (number of COCs/droplet size): 1/20, 5/20, 10/20, 20/20, 50/20, 1/50, 5/50, 10/50, 20/50, 50/50, 1/100, 5/100, 10/100, 20/100, 50/100, 1/200, 5/200, 10/200, 20/200, and 50/200 μl.

Evaluation of the degree of cumulus expansion

Eighteen hours after the start of culture, the degree of cumulus expansion was evaluated. Optical images of COCs were captured using DP2-BSW software (Olympus, Tokyo, Japan). Ratios of the degree of expansion were calculated as the diameter of the COC divided by the diameter of the oocyte as measured by Motic Images software (Shimadzu, Tokyo, Japan). In the 1-COC groups, 1 COC was examined in each experiment, and the experiment was performed 10 times (total of 10 COCs). In the 5-COC groups, 2 COCs were randomly selected and examined in each experiment, and the experiment was performed 5 times (total of 10 COCs). In the 10-, 20-, and 50-COC groups, 1 central COC and 3 marginal COCs in the mass were selected and examined in each experiment, and the experiment was performed 5 times (total of 5 central and 15 marginal COCs).

Immunostaining of oocytes

Immunostaining of oocytes was performed as described previously [6]. COCs were collected 18 h after the start of culture. Oocytes were removed from the cumulus cells with 0.1% hyaluronidase (Sigma). After the oocytes had been washed 3 times with PBS containing 0.1% PVA (PBS–PVA), they were fixed with 2% paraformaldehyde (Sigma) in Dulbecco's PBS(−) containing 0.1% PVA and 0.2% Triton X-100 (Wako) at room temperature for 40 min. The oocytes were washed 3 times in PBS–PVA containing 1% BSA (Sigma) (PBS–PVA–BSA) and stored in PBS–PVA–BSA overnight. For immunodetection of microtubules, the oocytes were incubated with anti-α-tubulin antibody (Sigma) at a dilution of 1:500 in PBS–PVA–BSA for 40 min at room temperature. After the oocytes had been washed 3 times with PBS–PVA–BSA, they were incubated with anti-mouse Alexa Fluor 488 IgG (Molecular Probes, OR) at a dilution of 1:200 in PBS–PVA–BSA for 30 min at room temperature. The oocytes were washed again 3 times with PBS–PVA–BSA, and oocyte nuclei were labeled with 10 μg/ml of propidium iodide (PI) (Sigma) for 60 min at room temperature. After the oocytes had been washed another 3 times with PBS–PVA–BSA, they were mounted on glass slides. Staining with Alexa Fluor 488 and PI generated green and red fluorescent signals, respectively. The samples were viewed using a Bio-Rad MRC-1024 confocal scanning laser microscope (Tokyo, Japan). The 1- and 5-COC groups experiments were repeated 20 and 4 times, respectively. The 10-, 20-, and 50-COC groups experiments were repeated 3 times for all oocytes.

In vitro fertilization and culture

IVF–IVC was performed as described previously [6]. Spermatozoa were preincubated for 2–3 h in 400-μl droplets of human tubal fluid (HTF) medium to allow capacitation. The final concentration was 700 spermatozoa/μl. After IVM, metaphase II (MII) oocytes were washed 3 times with HTF medium and placed into 200-μl HTF droplets with spermatozoa for 4 h. After IVF, the oocytes were washed 5 times with potassium simplex optimized medium (KSOM) and cultured in 100-μl KSOM droplets. The zygotes with 2pronuclei were selected after 6 h of IVF and cultured for 5 days in KSOM droplets. Experimental groups were selected based on the result of IVM (maturation rate > 75%). *In vivo*-matured oocytes were used as a control. In the 5-COC groups, the IVM experiments were performed 4 or 5 times, and all MII oocytes were examined in each IVF experiment. Moreover, in the 50-COC groups, MII oocytes were divided in half in each IVM experiment and examined twice per IVF experiment. These experiments were repeated 3 times. We examined different numbers of COCs per droplet and droplet sizes in the IVM experiments, but we examined similar numbers of oocytes and same droplet sizes to reduce the influence of IVF-IVC conditions as follows: in the IVF experiments, 15 to 21 MII oocytes were examined per experiment and in the IVC experiments, 11 to 19 zygotes were examined per experiment.

Count of total cell numbers of blastocysts

Blastocysts were collected on day 5 after the start of IVC, including hatching or hatched blastocysts. After the blastocysts were washed 3 times with PBS–PVA, they were fixed with 2% paraformaldehyde (Sigma) in Dulbecco's PBS(−) containing 0.1% PVA and 0.2% Triton X-100 at room temperature for 10 min. The blastocysts were washed again 3 times with PBS–PVA and then incubated with 5 μg/ml of Hoechst 33342 (Sigma) for 10 min at room temperature. After 3 additional washes with PBS-PVA, the blastocysts were mounted on glass slides. Hoechst 33342 generated a blue signal. TCNs of blastocysts were counted using an ultraviolet fluorescent microscope.

Semi-quantitative RT-PCR analysis of blastocysts

RT-PCR analyses of blastocysts were performed as described previously [6]. In addition to *Glut-1* and *Dc III*, we analyzed glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) as an intrinsic control because *G3PDH* is known as a housekeeping gene and its expression is not influenced by derepressors such as cytokines or phorbol esters [7]. Blastocysts were collected on day 5 after the start of IVC, including hatching or hatched blastocysts. Total RNA extraction and DNA synthesis were performed using Cells-to-cDNA™ II (Ambion, Austin, TX) for 5 blastocysts per experiment per group. PCR was performed using rTaq polymerase (Takara, Shiga, Japan). Each primer was designed according to published mouse cDNAs. For *Glut-1*, the sense (5′-ACTATCCCAGCAACCCTCCT-3′) and antisense (5′-AAACTCCTCCAGGGACTGGT-3′) primers generated a 177-bp fragment. For *Dc III*, the sense (5′-CTTGCAGTGAACATCGAGGA-3′) and antisense (5′-AGGCGAGCAGCTGTATCATT-3′) primers generated a 218-bp fragment. For *G3PDH*, the sense (5′-CCACTCTTCCACCTTCGATG-3′) and antisense (5′-GAGGGAGATGCTCAGTGTTG-3′) primers generated a 225-bp fragment. The amplification conditions were as follows: 94°C for 5 min; 35 cycles of 20 s each for denaturation at 94ºC, annealing (58ºC for *Glut-1*, 56ºC for *Dc III*, and 58ºC for *G3PDH*), and extension at 72ºC; and final extension at 72ºC for 3 min. The DDBJ/EMBL/GenBank accession numbers for mouse *Glut-1*, *Dc III*, and *G3PDH* cDNA sequences are NM_011400, NM_007882, and NM_008084, respectively. The expression of both mRNAs was detected by densitometric analysis. The expression levels of *Glut-1* and *Dc III* were normalized by the levels of *G3PDH*.

Statistical Analysis

Each experiment was performed 3 or more times per group per experiment. When 3 or more groups were compared, the data were analyzed by one-way ANOVA followed by Dunnett's test or the Tukey-Kramer test. The control group was compared with the other groups using Dunnett's test. All groups were compared with each other using the Tukey-Kramer test. All data are expressed as means ± SEM. A *P* level of less than 0.05 was considered statistically significant.

Results

Oocyte maturation rates in IVM

To determine whether different IVM conditions affect oocyte maturation rates, we performed IVM under different conditions (Fig. 1). The maturity of the oocytes was evaluated by observing the first polar body emission, spindle formation, and chromosome alignment. Oocytes were evaluated 18 h after the start of culture. The rates in the 20-μl group were lower than in the other groups, whereas those in the 50- and 100-μl

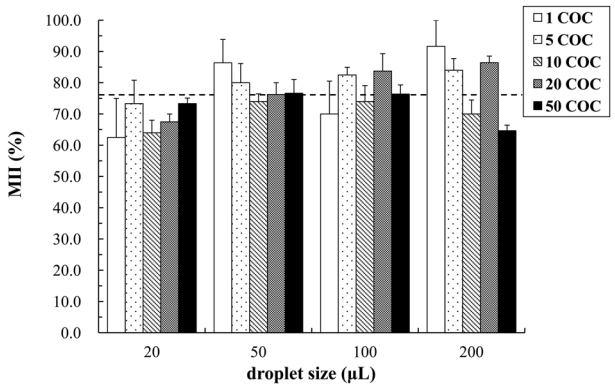

Fig. 1. The oocyte maturation rates in IVM. COCs were cultured in the presence of hypoxanthine and FSH for 18 h in 20-, 50-, 100-, and 200-μl droplets of culture medium. The numbers of COCs examined were 1, 5, 10, 20, and 50. The dotted line indicates a maturity rate of 75%. Values are the means ± SEM of more than 3 replicates.

groups were higher. The oocyte maturation rates in the 200-μl group, which exceeded 75%, were higher than those in the 20/100-μl group. In the 1-COC groups, although their maturation rates varied, the rates in the 50- and 200-μl groups were higher. In the 5- and 20-COC groups, the maturation rate increased with increasing droplet size. In the 10-COC group, the rates for all droplet sizes were less than 75%. In the 50-COC group, the rate was approximately 75%, excluding the 50/200-μl group that had the second lowest maturation rate among all the experimental groups. The conditions of IVM were set to achieve theoretical maturity rates of 75% or more in this experiment. We made an exception for the 1-COC group because the average rate fluctuated greatly throughout the experiments. This is because the maturation rate in the 1-COC group was either 0% or 100%. Hence, although some oocytes matured in cultures with 1 COC, this condition is not practical for IVM in instances where several oocytes can be collected. Therefore, the experimental groups for IVF–IVC were as follows: 5/50, 20/50, 50/50, 5/100, 20/100, 50/100, 5/200, and 20/200 μl.

Cumulus expansion in different IVM conditions

We observed differences of cumulus expansion among the different IVM conditions (Figs. 2A, B). As shown in Fig. 2A, the degree of cumulus expansion was not significantly different in the 1-COC group. Furthermore, only that in the 5/100-μl group was significantly different from those in the other 5-COC

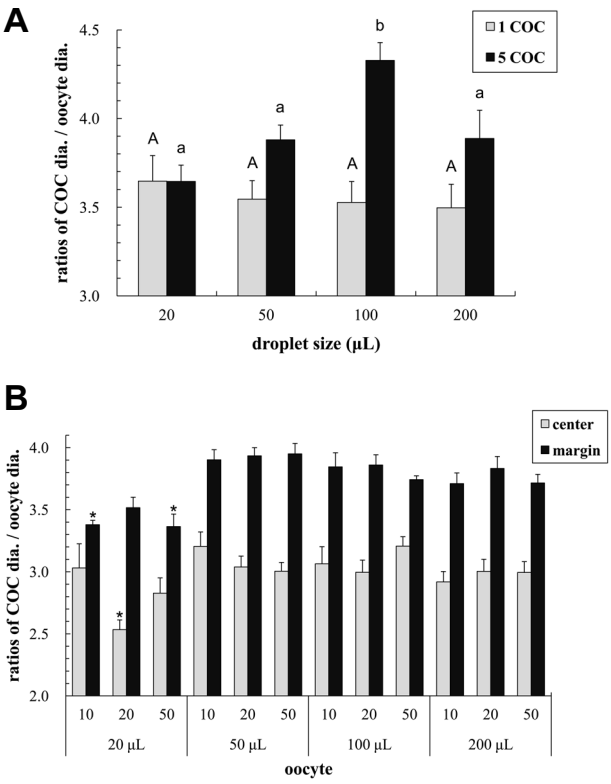

Fig. 2. Differences in the cumulus expansion of each group after 18 h of IVM. (A) Degree of cumulus expansion in the 1- and 5-COC groups. Values are the means ± SEM for 10 COCs per group. Data were analyzed by one-way ANOVA followed by the Tukey-Kramer test. $^{A,\ a-b}$Values with different superscripts were significantly different within the same COC number group ($P < 0.05$). (B) Differences in the degree of cumulus expansion between central and marginal COCs. Values are the means ± SEM of 5 replicates each for 1 central COC and 3 marginal COCs per experiment (total of 20 COCs). Data were analyzed by one-way ANOVA followed by Dunnett's test; the control is the 20/100-μl group (*, $P < 0.05$).

groups ($P < 0.05$). As shown in Fig. 2B, the cumulus cells of central COCs expanded less than those of marginal COCs in all groups. The degree of cumulus expansion in the 20/20-μl (center), 10/20-μl, and 50/20-μl groups (margin) showed significant differences from the control ($P < 0.05$).

Effects of different IVM conditions on IVF and IVC

To test whether different IVM conditions affect early embryonic development, we performed IVF–IVC with oocytes matured under different IVM conditions. These results are shown in Table 1. In the 5-COC group,

Table 1. Effects of different IVM conditions on fertilization and culture

Size	N/drop	n	2PN (% ± SEM)	Multi PN (% ± SEM)	2-Cell (% ± SEM)	Blastocyst (% ± SEM)
Control		51	96.1 ± 3.3	2.0 ± 1.7	94.1 ± 5.0	88.2 ± 2.7
50 μl	5	65	75.6 ± 12.4	14.6 ± 8.6	68.3 ± 11.2	39.0 ± 2.8*
	20	60	86.0 ± 11.1	4.7 ± 3.7	81.4 ± 9.8	44.2 ± 9.7*
	50	75	54.9 ± 12.5*	7.8 ± 2.0	51.0 ± 9.1*	37.3 ± 3.2*
100 μl	5	70	76.7 ± 0.9*	15.0 ± 1.3*	65.0 ± 6.4*	30.0 ± 3.0*
	20	60	94.0 ± 6.3	4.0 ± 4.2	94.0 ± 6.3	60.0 ± 14.3
	50	75	87.2 ± 6.3	10.6 ± 5.5	83.0 ± 8.3	44.7 ± 3.7*
200 μl	5	75	72.6 ± 5.6*	16.1 ± 1.5*	72.6 ± 5.6*	48.4 ± 10.0*
	20	60	80.4 ± 7.8	9.8 ± 2.0	78.4 ± 7.0	47.1 ± 6.3*

Values are the means of 3 replicates. The rates were calculated as each number divided by the number of MII oocytes per experiment. Data were analyzed by one-way ANOVA followed by Dunnett's test (*, $P < 0.05$; significantly different from control). Size, droplet size; N/drop, number of COCs examined per droplet in IVM; n, the number of oocyte examined; 2PN, zygotes forming 2pronuclei; Multi PN, zygotes forming multiple pronuclei.

zygotes were likely to form multiple pronuclei, especially those in the 5/100-μl and 5/200-μl groups ($P < 0.05$). Only the 20/100-μl group did not exhibit a significant difference in blastocyst formation from the control ($P > 0.05$).

Effects of different IVM conditions on TCNs of blastocysts after IVF and IVC

Table 2 displays TCNs of blastocysts cultured under different IVM conditions. TCNs in the 50-COC group were small. TCNs in the 5/100-μl and 20/100-μl groups were higher than in the other groups; furthermore, in the other groups, there were no significant differences from the control ($P > 0.05$).

RT-PCR analysis of blastocysts developed under different IVM conditions

We analyzed the mRNA expression of *Glut-1* and *Dc III* at the blastocyst stage. Blastocysts were collected on day 5. As shown in Fig. 3, *Glut-1* mRNA was expressed at higher levels in the 20/50-μl and 20/100-μl groups than in the *in vivo* group. In addition, *Dc III* mRNA was expressed at higher levels in the 20/50-μl, 20/100-μl, and 5/200-μl groups than in the *in vivo* group. Thus, in the 20/50-μl and 20/100-μl groups, relatively higher gene expression was observed.

Table 2. Effects of different IVM conditions on TCNs of blastocysts after IVF and culture

Size	N/drop	No. of blastocysts examined	Total cell number (mean ± SEM)
Control		16	75 ± 6.7
50 μl	5	10	52 ± 5.2*
	20	10	41 ± 2.1*
	50	12	42 ± 5.6*
100 μl	5	10	66 ± 4.5
	20	11	62 ± 5.6
	50	10	40 ± 5.3*
200 μl	5	10	43 ± 3.0*
	20	18	52 ± 4.4*

These experiments were repeated 3 times. Data were analyzed by one-way ANOVA followed by Dunnett's test (*, $P < 0.05$; significantly different from control). Size, droplet size; N/drop, number of COCs examine per droplet in IVM.

Discussion

This study demonstrated that different IVM conditions affect oocyte quality in similar culture medium compositions.

The oocyte maturation rates in the 20-μl groups were lower than those in the other groups, and the droplet color changed from red to yellow in the 20/20-μl and 50/20-μl groups. This is because Waymouth's MB 752/1 medium contains phenol red. High densities of oocytes require large amounts of nutrient and oxygen, which 20-μl droplets would not provide in sufficient amounts. Therefore, we believe that oocyte maturation was arrested or delayed in the 20-μl groups. In the 10-COC groups (especially the 100- and 200-μl groups), oocyte maturation rates were lower than those in the 5- and 20-COC groups. However, the percentages of oocytes undergoing germinal vesicle breakdown were virtually identical (5-COC: 94.0 ± 2.0%; 10-COC: 89.0 ± 2.6%; 20-COC: 90.4 ± 1.3%). From this result, we infer that oocyte maturation was delayed in the 10-COC groups.

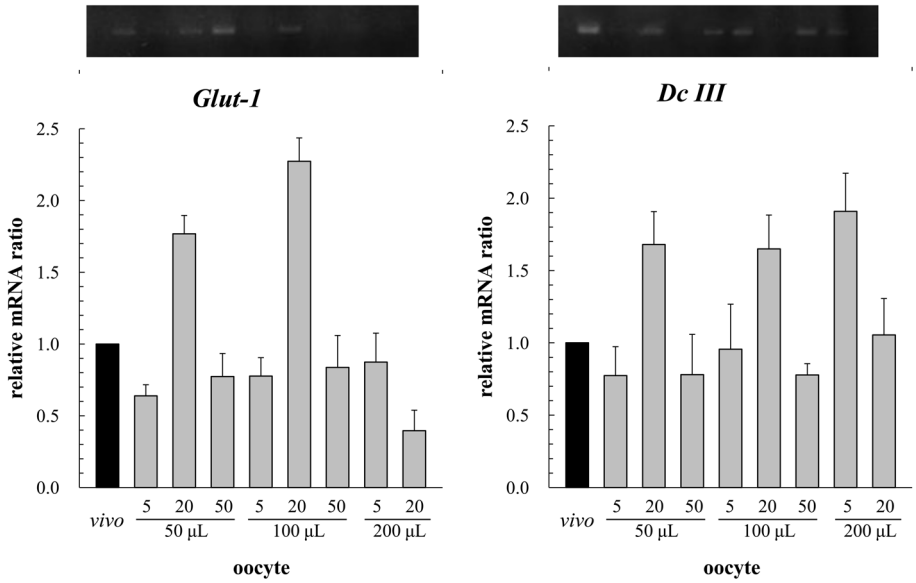

Fig. 3. RT-PCR analysis of *Glut-1* and *Dc III* mRNA expression levels of each group. Blastocysts were collected on day 5 after the start of *in vitro* culture, including hatching or hatched blastocysts. Values are the means ± SEM of 3 replicates. The expression of both mRNAs was detected by densitometric analysis. The expression levels of *Glut-1* and *Dc III* were normalized by the levels of *G3PDH*. The ratio of mRNA expression was calculated based on the level of the control group expression.

Meanwhile, the differences in the rates between the 10-COC groups and 5-COC groups, or 10-COC groups and 20-COC groups were approximately 10% or less; a rate of 10% is a difference of 1 oocyte in the 10-COC groups. Therefore, we interpret this result as meaning that there were no significant differences between the 10-COC groups and the 5- and 20-COC groups. In the 200-μl groups, COCs were scattered throughout the 200-μl droplet. Therefore, we postulate that paracrine function was weaker in the 200-μl groups. In addition, in the 50/200-μl group, the number of dead oocytes was large (3 or 4 dead oocytes per droplet; 7.3 ± 0.7%). It is known that factors secreted by dead oocytes harm other living oocytes and this would explain why the maturation rate in the 50/200-μl group was the second lowest among the experimental groups.

In vivo, oocyte maturation and cumulus expansion are stimulated by gonadotropin [8], and cumulus expansion is required for normal ovulation rates to occur [9, 10]. Moreover, *in vitro*, cumulus expansion is stimulated by FSH [11–13]. It is thought that COCs in the smaller oocyte density groups can more readily utilize FSH. However, in 1- and 5-COC groups (excluding the 5/100-μl group), the degree of cumulus expansion was not significantly different from those in other groups. Therefore, we suggest that cumulus expansion is associated with not only FSH, but also with certain paracrine factors. As shown in Fig. 2B, the degree of expansion of central COCs was smaller than that of marginal COCs. We suggest that this is because central COCs cannot sufficiently utilize FSH, nutrients, and oxygen due to competition from the marginal COCs, and central COCs have limited space for cumulus cells to expand in.

In IVF–IVC, 2pronuclei formation and 2-cell formation rates were not different from those of the control, but blastocyst formation rates were significantly lower ($P < 0.05$), excluding the results of the 20/100-μl group. This result suggests that different IVM conditions affect oocyte fertility and developmental competence. Although 5-COC groups are assumed to be unsuited for IVM because only culture with a certain number of COCs produced blastocysts in past studies, in the present study, we were able to produce blastocysts in the 5-COC groups. However, the zygotes in these groups were likely to form multiple pronuclei. Furthermore, multiple pronuclei formation rates did not depend on droplet size, suggesting a paracrine function effect. Kawamura *et al.* reported that early embryonic development in IVM was improved by culture with brain-

derived neurotrophic factor, which is one of the paracrine factors produced by cumulus cells [14]. Hence, we propose that multiple pronuclei formation occurred in the 5-COC groups because of a delay in cytoplasmic maturation due to the absence of paracrine factors.

TCNs of blastocysts increase during the period of blastocyst expansion and hatching [15]. Therefore, blastocysts exhibit high developmental competence and enhanced pregnancy rates when their TCNs are high. The 5/100-μl and 20/100-μl groups did not exhibit significant differences from the control ($P < 0.05$), that is, they might have had high developmental competence similar to *in vivo*-matured oocytes.

Glucose cannot support the development of early embryos until the 4-cell stage [16]. However, after the 8-cell stage, the substrate preference of embryos switches from pyruvate to glucose. This change may depend on the competence of oocytes to take up glucose [17], in which *Glut-1* has a role [18]. *Dc III* is a transmembrane glycoprotein that belongs to the cadherin family of cell adhesion receptors and plays a role in the proper compaction and differentiation of these tissues during embryonic development [19]. For these reasons, *Glut-1* and *Dc III* appear to be important for early embryonic development. Therefore, we analyzed the expression of their mRNAs. The groups in which blastocysts expressed *Glut-1* and/or *Dc III* mRNA produced high-quality oocytes in IVF–IVC. Thus, there is a possibility that *Glut-1* and *Dc III* mRNA expression levels are correlated with the rates of embryo development. Furthermore, in the 20/50-μl and 20/100-μl groups, *Glut-1* and *Dc III* mRNAs were expressed more highly than in the control group. Therefore, in these 20/50- and 100-μl groups, we believe that the state of the blastocysts was closer to those observed *in vivo* than those in the other groups. We suggest that at the high levels of *Glut-1* and *Dc III* mRNAs expression in these 2 groups were correlated with the oocyte number in IVM. In the 20-COC group, excluding the 200-μl group, the expression levels of both mRNAs were stronger than in the control group. As mentioned above, oocyte maturation requires paracrine and autocrine activity [4]. Thus, the 20/50-μl and 20/100-μl groups might have had a favorable balance of these activities as manifested by the high expression levels of these genes observed in these groups.

From our experiments, it is clear that different IVM conditions affect oocyte maturation, fertilization, and early embryonic development. The 20/100-μl group, IVM condition was found to be the optimal condition in this study.

The most significant finding of this study was that culture with 5 COCs could produce blastocysts, even though it was thought that only culture with a great number of COCs could produce blastocysts. However, this culture condition needs improvement. For example, in the 5/100-μl group, multiple pronuclei formation was likely. In addition, although TCNs of blastocysts were large, *Glut-1* mRNA expression was low to undetectable, that is, the expression of mRNAs related to blastocyst quality was low. Hence, both TCNs and mRNA expression need to be analyzed to evaluate blastocyst quality.

In this study, we demonstrated that (1) differences in droplet size and COC numbers affected oocyte maturation in IVM, fertilization, and early embryonic development; (2) the 20 COCs/100-μl condition effectively stimulated maturation in all experiments; and (3) culture with 5 COCs in IVM can produce blastocysts.

Further studies will be required to improve the culture conditions to inhibit the formation of multiple pronuclei when using 5 COCs or fewer and to enhance the quality of *in vitro*-matured oocytes to levels similar to that of *in vivo*-matured oocytes.

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