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The Relationship between the Level of Progesterone Secreted from Cumulus Cells and Oocyte Developmental Competence in In Vitro Matured Human Cumulus Oocyte Complexes

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Abstract: *Developmental competence of in vitro matured human oocytes is dependent on the morphology of the cumulus-oocyte complex (COC) just after collection from the follicle. We postulated that COCs categorized as having poor morphology (two or fewer less than two layers of cumulus cells) would not secrete a sufficient amount of matulation factors, resulting in low developmental competence of the matured oocytes. In the present study, the level of progesterone secreted from good morphology COCs with three or more layers of cumulus cells, 39.2 ± 12.8 ng/ml (n=31), was significantly higher than that secreted from poor morphology COCs (9.65 ± 1.34 ng/ml, n=22). The addition of 20 ng/ml progesterone to in vitro maturation culture of the poor morphology group significantly improved the fertilization ability of the oocytes. The rates of development to the morula and blastosyst stages were also increased by progesterone, however the differences were not significant. In conclusion, the secreted level of progesterone during in vitro maturation of human COCs was dependent on the number of cumulus cells attached to oocyte. When an oocyte is surrounded by two or fewer 2 layers of cumulus cells, the addition of progesterone to FSH- and hCG-containing medium appears to be a useful method for obtaining an oocyte with a high developmental competence.*

Key words: *Cumulus-oocyte complex, Developmental competence, Progesterone, Human IVM, Cumulus cells*

Received: July 7, 2011
Accepted: September 5, 2011
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Introduction

During the ovulation process, the LH surge directly acts on granulosa cells, but not on cumulus cells, inducing luteinization of granulosa cells, cumulus expansion and oocyte nuclear maturation. Granulosa cells are known to produce prostaglandin E2 (PGE2) and progesterone as a consequence of induced transcription of prostaglandin synthase 2 (*Ptgs2*), P450scc (*Cyp11a1*) and 3-*β* hydroxysteroid dehydrogenase (*Hsd3b1*) [1]. The receptor for PGE2 (EP2) that is expressed on cumulus cells is the one of G protein coupling receptors and stimulates adenylcyclase activity, resulting in the accumulation of cyclic AMP (cAMP) in the cells [2, 3]. The increase in cAMP is involved in the induction of hyaluronan synthesis to form cumulus expansion via the PKA or p38MAP kinase dependent pathway [4]. The progesterone secreted from granulosa cells also activates cumulus cells in a receptor-dependent manner [5]. When anti-progesterone neutrizing neutral antibody was injected to eCG+hCG-primed rats, ovulation and meiotic resumption of oocytes were significantly suppressed [6], suggesting that progesterone activates the receptors on cumulus cells and plays an important role during the ovulation process.

In *in vitro maturation* of oocytes, cumulus-oocyte complexes (COCs) are recovered from antral follicles, and are then cultured in a suitable medium [7]. The addition of FSH supports the acquisition of the developmental competence of oocytes after *in vitro* fertilization [8, 9]. The positive effects of FSH are dependent on cumulus cells since FSH receptors (FSHR) are expressed on cumulus cells but not on the oocyte [10]. Since FSHR is

Table 1. Characteristics of the 63 cycles

	P(–)	P(+)	total
No. of cycles with an aspiration	30	33	63
No. of oocytes with an aspiration	53	69	122
Age of the patient	32.4 ± 0.8	31.2 ± 0.7	31.8 ± 0.5
Day of aspiration	8.8 ± 0.4	8.2 ± 0.5	8.5 ± 0.3

P(–) ; COCs were cultured without exgeneous progesterone.
P(+) ; COCs were cultured with exgeneous progesterone.

constitutively expressed in cumulus cells, the addition of FSH dramatically increases cAMP and this higher level is maintained during the maturation process [11]. Thus, the cAMP dependent pathway is fully activated in cumulus cells by exogenous FSH, resulting in the production of hyaluronan matrix and oocyte maturation. Progesterone production is also induced by FSH in cumulus cells [12]. When porcine COCs were cultured with FSH and progesterone receptor antagonist, cumulus expansion was deficient and the rate of blastocyst formation was significantly lower than that of oocytes cultured without an antagonist [13, 14]. Also, Yamashita *et al*. (2003) reported that the level of progesterone secreted by COCs in culture medium was raised when higher numbers of COCs were cultured in a well [15]. When one COC was cultured with FSH in a well, meiotic resumption was delayed, and this suppression was overcome by the addition of progesterone to the FSH-containing medium. Thus, the number of cumulus cells in a well is correlated to the level of secreted progesterone in the culture medium, and the amount of progesterone secreted by cumulus cells is a limited factor for cumulus cell function and oocyte maturation in *in vitro* maturation of oocytes.

Using human COCs, we recently determined the relationship between the morphology of COCs before *in vitro* maturation and oocyte developmental competence after *in vitro* maturation [16]. The results clearly showed that the maturation rate was not affected by the different morphology of COCs, however the rate of blastocyst formation after *in vitro* fertilization was significantly lower in the poor morphology group (COCs with two or fewer layers of cumulus cells) than that in oocytes surrounded by three or more layers of cumulus cells. From these results, we postulated that the cultivation of COCs categorized as having poor morphology would not produce a sufficient amount of progesterone, resulting in the low developmental competence of the matured oocyte. Although Hasegawa *et al*. reported that during the ovulation process, the progesterone receptor is transiently expressed in cumulus cells of human COCs in an IVF program [17], there is little information about the role of progesterone in *in vitro* maturation of human oocytes of COCs, the level of progesterone secreted by COCs and the receptor expression in COCs according to morphology. In this study, in order to try and answer the above questions, we categorized COCs into a good morphology group (three or more layers of cumulus cells) and poor morphology group (three or more layers of cumulus cells). Each COC was cultured with or without progesterone and then used for *in vitro* fertilization. We also analyzed the level of progesterone secreted from each category of COC, and the expression level of the progesterone receptor (*Pgr*).

Materials and Methods

Patients' selection and treatment protocol

Infertile patients who visited St. Luke Clinic, Oita, Japan to undergo diagnostic and/or therapeutic laparoscopy for pelvic endometriosis or pelvic adhesive lesions with regular menstrual cycles were offered IVM treatment. A total of 63 patients with a mean age of 32.4 ± 0.8 (range, 24–45) yr consented to participation in this study during the period from March, 2004 through June, 2005 (Table 1).

For women receiving FSH-priming, purified urinary FSH preparation (Fertinorm P, Serono International SA, Switzerland, with the declared ratio of FSH:LH/1:0.00035) was injected intramuscularly at a fixed dose of 150 IU daily for 3 consecutive days, starting from 4 days before laparoscopy, as described in our previous study [16]. Follicle aspiration was performed laparoscopically around the mid to late follicular phase of the cycle from several follicles of 5–10 mm in diameter.

COCs were recovered by follicle aspiration with a 17G needle (Cook IVF. Indiana, USA). The collection medium was HEPES-buffered TCM199 (Gibco BRL. Grand Island, NY) supplemented with 3% (W/V) heparin (Mochida Pharmacentical Co. Tokyo, Japan), 0.3% (W/V) polyvinylpyrrolidone (Sigma Chemical Co. St. Louis, MO) and 4 mM hypoxanthine (Sigma Chemical Co. St Louis, MO).

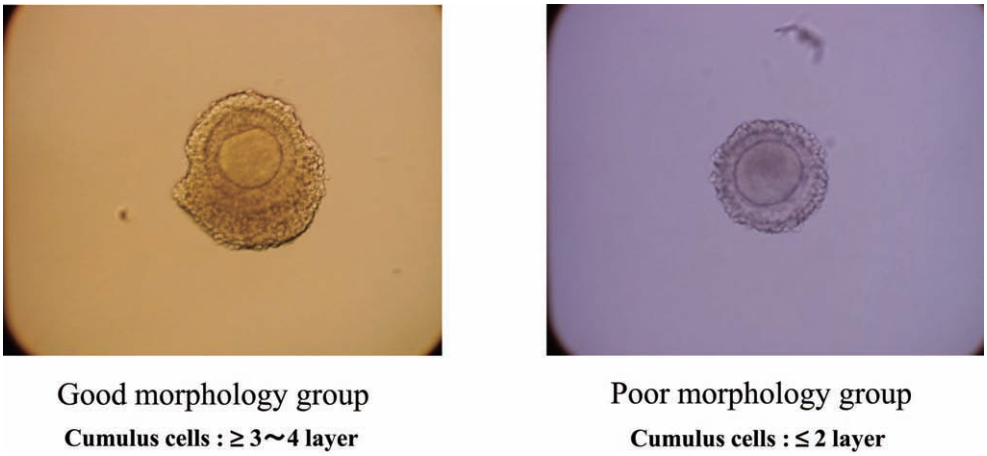

Fig. 1. Morphological grading of cumulus-oocyte complexes (COCs) just after collection from follicles. COCs were classified according to the number of cumulus cell layers: good morphology was group defined as COCs with three or more more than 3 layers of cumulus cells, poor morphology as two or fewer layers of cumulus cells.

In vitro maturation and in vitro fertilization

The morphological appearance of COCs was observed immediately after harvest using a phase-contrast microscopy (DIAPHOT 300; Nikon Co., Tokyo, Japan). COCs were categorized into 2 groups. The good morphology group COCs had three or more layers of cumulus cells surrounding the oocyte, and the poor morphology group COCs two or fewer layers of cumulus cells (Fig. 1).

The COCs were individually cultured in a well of a microculuture plate for 44 h under a humidified 6% CO_2, 4% O_2, plus 90% N_2 gas phase at 37 °C in 150 µl of culture medium containing 200 mIU/ml FSH preparation (Fertinorm P, Serono International SA, Switzerland) and 1 IU/ml hCG preparation (Profasi: Serono International SA, Switzerland), as described in our previous study [15]. Some COCs were cultured with FSH, hCG and 20 ng/ml of progesterone (Sigma). The maturation medium was TCM199 (Gibco) supplemented with 10% (V/V) inactivated human male serum and 0.6 mM cystein (Sigma).

After the 44 h of culture, the progesterone levels in the culuture medium were analayzed using specific assay kits. The oocytes were then denuded to examine extrusion of the first polar body, and all metaphase II (MII) oocytes were inseminated with the patients' male pertners' sperm by means of intracytoplasmic sperm injection (ICSI). The injected oocytes were individually cultured in a 30 µl droplet of Blast Assist System 1 (BAS1, Medicult, Jyllinge, Denmark) under mineral oil (Fuso Pharmaceutical Industries Ltd, Osaka, Japan) until 72 h after ICSI; then they were transferred to a second culture medium, BAS2 (Medicult) and culutured for an additional 96 h.

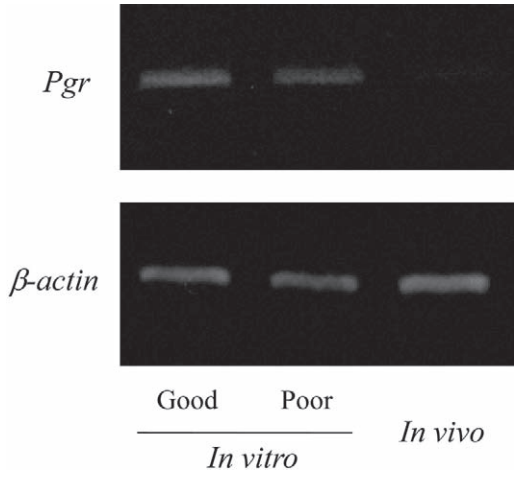

Fig. 2. The expression of *Pgr* mRNA in cumulus cells of COCs which were cultured with FSH+hCG for 44 h, or matured in *in vivo*.

The detection of Pgr mRNA in cumulus cells of COCs by RT-PCR

After the 44 h culture of COCs, cumulus cells were separated from the oocytes and washed three times in PBS. Total RNA was extracted from the cumulus cells using the SV Total RNA Isolation System (Promega. Madison, WI), according to the instruction manual, and dissolved in nuclease-free water. The final RNA concentrations were determined by absorbance using a spectrophotometer. To collect the RNA from *in vivo* matured COC's, the cumulus cells were derived from *in vivo* ma-

Table 2. Primers used for the determination of human *Pgr* and *β-actin* mRNA by RT-PCR

mRNA	Primer sequence	Predicted product size
Pgr	F: 5'-AGC CCA CAA TAC AGC TTC GAG-3'	254 bp
	R: 5'-TTT CGA CCT CCA AGG ACC AT-3'	
β-actin	F: 5'- CTA CAA TGA GCT GCG TGT GG-3'	450 bp
	R: 5'-TAG CTC TTC TCC AGG GAG GA-3'	

tured COCs after hyaluronidase treatment in an *in vitro* fertilization program [16].

RT-PCR was performed according to a coupled one-step procedure using the Access RT-PCR System (Promega) with some modifications [17]. Briefly, 5 ng of total RNA was reverse transcribed at 48 °C for 45 min, denatured at 94 °C for 2 min, and amplified for 33 (*β-actin*) or 35 cycles (*Pgr*) of denaturation at 94 °C for 30 sec, primer annealing at 56 °C (*β-actin*) or 60 °C (*Pgr*) for 1 min, and an extension at 68 °C for 1 min, with a final extension step of 7 min at 68 °C. The amplified products were analyzed by electrophoresis on 2% agarose gels. *β*-actin was used as a control for reaction efficiency and to control for variations in concentrations of mRNA in the original RT reaction. The primer sequences and predicted product sizes are were given in Table 2. The amplified cDNAs were directly sequenced as described in our previous study [16] to verify their authenticity.

Experimental design

In Experiment 1, COCs were categorized into two groups (good morphology and poor morphology), and then each COC was cultured with FSH and hCG. After 44-h culture, the cultured medium was collected to analyze the level of progesterone secreted by COCs. The matured oocytes were fertilized by ICSI, and the collected cumulus cells were used for RT-PCR analysis to detect the expression of *Pgr* mRNA.

Experiment 2 was conducted to examine the effects of adding progesterone to the medium on the meiotic maturation and the developmental competence of oocytes categorized as poor morphology group. Each COC was cultured with FSH, hCG and/or 20 ng/ml of progesterone for 44 h. In our previous study, using porcine COCs, the addition of 20 ng/ml progesterone improved the oocyte maturation when each porcine COC was cultured with FSH and LH [15]. The matured oocytes were used for ICSI.

Statistical analysis

Data were compared using the with the use of Chi-squared test or Student's *t*-tests, and were judged as statistically significant when the *P*-value was <0.05.

Results

Experiment 1

COCs just after collection from follicles were categorized by their morphology (good or poor). Each COC was individually cultured with FSH and hCG for 44 h and then the secreted level of progesterone was analyzed. The mean level of progesterone in the good morphology group was 39.2+/−12.8 ng/ml, and was significantly higher than that in poor morphology group (9.65+/−1.34 ng/ml).

The expression of *Pgr* mRNA was detected in cumulus cells of COCs in both morphology groups. However, the PCR product was not detected in cumulus cells of *in vivo* matured COCs.

About 70% of oocytes in the good morphology group reached the MII stage, a rate that was higher than that in the poor morphology group (59.1%), but the difference was not significant. The percentage of successful morula observed in the poor morphology group (13.6%) was significantly lower than that in the good morphology group (38.7%). About 30% of oocytes developed to the blastocyst stage in the good morphology group (Fig. 3).

Experiment 2

To examine whether the addition of progesterone to FSH- and hCG-containing medium assisted the oocytes categorized as poor morphology group to acquire developmental competence, each COC that had two or fewer layers of cumulus cells was cultured with FSH, hCG and progesterone. The MII rate and fertilization rate were improved by the addition of progesterone, and the-difference in fertilization rate was significant. The percentages of oocytes developing to the morula stage and the blastocyst stage were also higher in the progesterone treatment group, however the difference was not significant (Fig. 4-1). In contrast, when each COC categorized as good morphology was cultured with FSH, hCG and

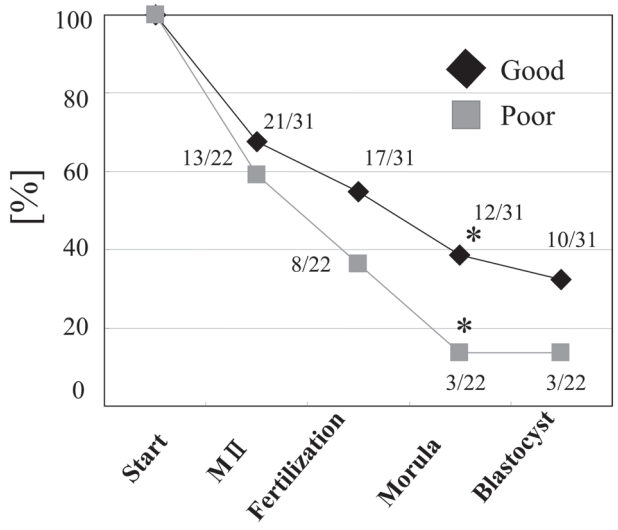

Fig. 3. Rates of maturation, fertilization and embryogenesis of human cumulus-enclosed oocytes matured *in vitro* according to cumulus-oocyte complex (COCs) morphology
◆good morphology group, ■poor morphology group
Superscripts indicate statistically significant differences (**P<0.05*).

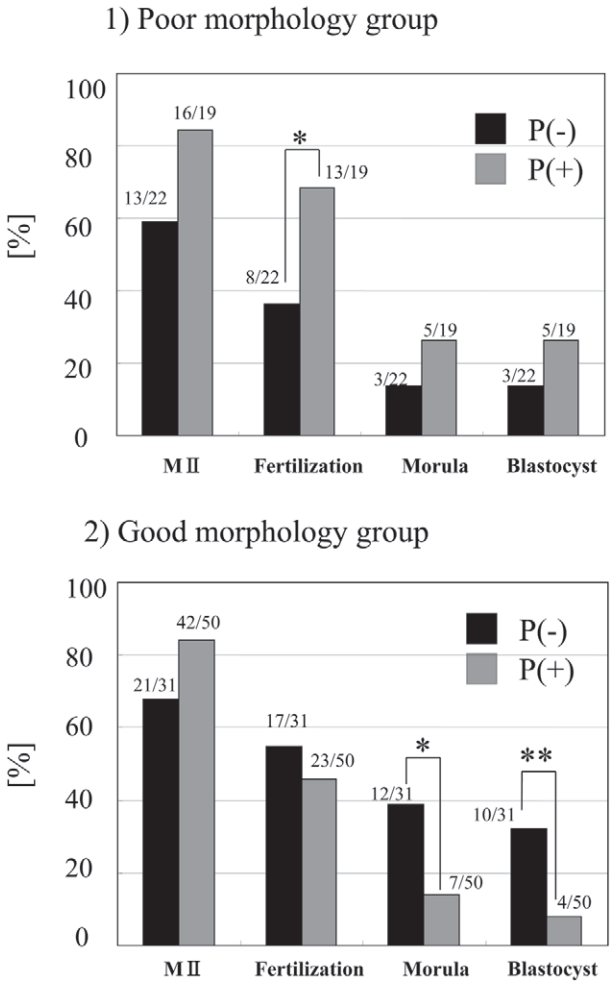

Fig. 4. Comparison of rates of maturation, fertilization and embryogenesis of human cumulus-enclosed oocytes matured with or without exgeneous progesterone (P).
1) Poor morphology COCs were cultured with FSH, hCG and/or 20 ng/ml of progesterone. After 44 h, the cumulus cells were stripped and examined for the emission of polar body. Mature oocytes were used for ICSI. P (–) ; COCs were cultured without exgeneous progesterone. P (+) ; COCs were cultured with exgeneous progesterone. Superscripts indicate statistically significant differences (**P<0.05).*
2) Good morphology COCs were cultured with FSH, hCG and/or 20 ng/ml of progesterone. After 44 h, the cumulus cells were stripped and examined for the emission of polar body. Mature oocytes were used for ICSI. P (–) ; COCs were cultured without exgeneous progesterone. P (+) ; COCs were cultured with exgeneous progesterone. Superscripts indicate statistically significant differences (**P<0.05, **P<0.01).*

progesterone, the percentages of oocytes developing to the morula and the blastocyst stages were significantly decreased as compared with the COCs cultured without progesterone, suggesting that a high amount of progesterone negatively regulates oocyte developmental competence (Fig. 4-2).

Discussion

Progesterone is converted from cholesterol by two enzymes, P450scc and 3*β*HSD. The expressions of these genes are detected in cumulus cells during the ovulation process [18]. Yamashita *et al*. reported that cumulus cells have a cholesterol synthesis pathway, and the *de-novo* synthesized synthesis cholesterol was used for progesterone production [19]. In cumulus cells, the cholesterol uptake pathway is also activated during the ovulation process, since the scavenger receptor type B1, that is one of the HDL receptors, is expressed in cumulus cells during the ovulation process [20]. Thus, progesterone is produced by cumulus cells and then secreted into follicular fluid *in vivo* or culture medium *in vitro*.

In this study, progesterone accumulation in culture medium was detected when human COCs were individually cultured with FSH and hCG. Since the level of progesterone is dependent on the number of cumulus cells surrounding the oocyte, a significantly higher level of progesterone was observed in the culture medium used for COCs categorized as good morphology than that in the

medium used for the poor morphology group. Almost the same levels of *Pgr* mRNA were detected in cumulus cells in both of the good and poor morphology goups, however significant differences were found in the developmental competence to the morula stage after ICSI, suggesting that the progesterone secreted by COCs acts in cumulus cells to enhance the developmental competence of human oocyte. It has been reported that the role of progesterone in oocyte maturation is different among the species. In mice, progesterone receptor knockout mice showed impaired ovulation, however, when mature oocytes were recovered from their follicles, the oocytes had developmental competence after *in vitro* fertilization [5]. On the other hand, when rat follicular enclosed oocytes were cultured with LH and a progesterone synthesis inhibitor, aminoglutethimide, the oocytes reached the MII stage, but the fertilization rate was significantly suppressed by the inhibitor [21]. Mori *et al*. also reported that neutral anti-progesterone antibody delayed meiotic progression to the MII stage in rat oocytes *in vivo* [22]. Also, in porcine COCs, progesterone synthesis inhibitor or progesterone receptor antagonist reduced the numbers of oocytes resuming meiosis, and the developmental competence of matured oocytes [14, 23]. From these reports and the present results of Experiment 1, we concider that the addition of progesterone to gonadotropin-containing medium improved the fertilization and development to the blastocyst stage of human COCs, especially in the poor morphology group.

It is notable that in Experiment 2 of this study, when human COCs from the poor morphology group were individually cultured with FSH, hCG and 20 ng/ml progesterone for 44 h, the fertilization rate was significantly increased as compared with that of oocytes cultured without the addition of progesterone. This fertilization difference may be partially due to the addition of progesterone increasing the number of oocytes developing to the MII stage. The development to the morula and the blastosyst stage was also up-regulated by progesterone, though the difference was not significant. Thus, in human oocytes as well as in rats and pigs, progesterone acts on COCs to regulate oocyte developmental competence *in vitro*, indicating that the addition of progesterone to *in vitro* culture medium is beneficial in human IVM techniques.

Unfortunately, the addition of progesterone significantly decreased the numbers of oocytes developing to the morula and blastocyst stages when good morphology COCs were cultured. Since the COCs in the good morphology group produced significantly higher amounts of progesterone than those in the poor morphology group, it is possibile that their cumulus cells were stimulated too strogly by progesterone. We showed that progesterone suppresses cell proliferation and induces cell differentiation in cumulus cells of porcine COCs [24]. The progesterone receptor (PGR) has two splicing variants, type A and type B, and their role is completely different in the uterus and mammalian glands [25–27]. In cumulus cells of porcine COCs, type B PGR is expressed immediately after culture with FSH, then the expression pattern changes to type A PGR [13, 14, 28]. The activation of type B PGR does not induce cell differentiation, whereas type A PGR plays an important role in the expression of the LH receptor and cumulus expansion [28]. Although the present study demonstrated the expression of PGR by RT-PCR using primers for recognizing the common region of the *Pgr* gene, further studies to clarify the dose effects of progesterone and its mechanisms in more detail are required to develop maturation medium useful for human COCs. Neverthless, our results of negative effects of progesterone on COCs from the good morphology group suggest that the number of COCs in each well or the volume of medium in each well is an important factor in the full maturation of oocytes in IVM treatment.

In conclusion, human COCs produce progesterone in a cumulus cells number dependent manner. The progesterone secreted may be involved in the acquisition of developmental competence by an oocyte, but a high level of progesterone also suppresses the acquisition of developmental competence. When a human oocyte surrounded by two or fewer layers of cumulus cells is used for IVM, the addition of progesterone to FSH- and hCG-containing medium should result in higher percentages of good quality oocytes. However, further studies to determine the dose level of progesterone and the time point at which to add it are required for its development for clinical use in human IVM.

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