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Effects of Granulosa Cells, Cumulus Cells, and Oocyte Density on In Vitro Fertilization in Women

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The objective of this study was to determine if in vitro fertilization (IVF) rate is affected by the diameter of oocyte–cumulus complex (OCC), by coculturing oocytes with autologous granulosa cells, or by increasing lhe oocyte density in culture medium. Women with previous fertility problems underwent the IVF program. In study 1, the diameter of OCC was graded on retrieval on a scale of 0–3: grade 0 = no cumulus at all; grade 1 = diameter of 75–100 μm; grade 2 = diameter of 125–150 μm; and grade 3 = diameter of 200–225 μm. In study 2, oocytes were cocultured with autologous granulosa cells. In study 3, oocytes were cocultured in groups of one, two, or four. OCCs with a grade >1.5/3 resulted in a greater (*P* = 0.04) proportion of embryo / oocyte than did OCCs with a grade < 1.5/3 (0.91 ± 0.05 vs 0.68 ± 0.10; mean ± SEM). Coculturing oocytes with autologous granulosa cells did not affect (*P* = 0.42) the proportion of embryo/oocyte (0.63 ± 0.11 vs 0.74 ± 0.07 in controls). Coculturing oocytes in groups of two or four in culture drop did not affect (*P* = 0.37 and *P* = 0.38, respectively) the proportion of embryo/oocyte (0.63 ± 0.07 vs 0.73 ± 0.08 in controls, and 0.73 ± 0.08 vs 0.63 ± 0.08 in controls, respectively). In conclusion, coculturing oocytes with autologous granulosa cells or increasing the oocyte density from 1 to 2 or 4 oocytes/culture drop, in the context of our study, did not affect rate of IVF and embryo formation. The diameter of OCC at retrieval may give some indication regarding its future fertilization and development. This diameter varies with the type of ovarian stimulation and the patient's age. This variation in diameter does not correlate with oocyte maturity.

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Key Words: Granulosa; cumulus; coculture; oocyte; in vitro fertilization.

Introduction

In vitro fertilization (IVF) and embryo transfer have become a commonly practiced treatment for a variety of causes of infertility. However, there is great frustration at low fertilization rate, in some patients, and a very low implantation rate per embryo generally. In bovine, embryo transfer results in a much higher pregnancy rate. A systematic study of factors that affect embryo formation is needed so that we may, step by step, improve our IVF results. Many studies in human IVF are not prospectively randomized, and therefore, we have committed ourselves to a systematic prospective study of a variety of factors and conditions.

An extensive amount of literature is now developing in the interaction of the oocyte with the granulosa cells that surround the oocyte (cumulus cells) or the rest of follicular granulosa cells. The subject matter has recently been reviewed by three groups *(1–3)*, and it is therefore unnecessary to elaborate here in detail. Suffice it to say that the oocyte influences the cumulus cells, and cumulus cells may have important roles in fertilization, embryonic development, and achievement of pregnancy. A systematic investigation of such interactions in the context of human IVF is just starting. Often in IVF, the oocyte is stripped off its cumulus cells just after retrieval or 24 h later. Oocytes are cultured in granulosa-free medium. Are these good practices in the context of low pregnancy rates in human IVF vs embryo transfer in domestic animals?

In this article, we report studies of three factors pertaining to oocyte–granulosa and oocyte–oocyte interaction. At the time of oocyte retrieval, the diameter of cumulus oophorus can be easily measured, which is noted to be very variable. Is this a predictor of subsequent embryo formation (2–4 cells)? There have been studies in experimental animals, farm animals, and human IVF of the effect of addition of granulosa cells to the culture system. Therefore, we performed a controlled prospective study of such a factor on embryo development. Similarly, we addressed the

question of whether coculturing embryos together may favorably or adversely affect early embryo development (to two- to four-cell stage).

Results

In study 1A, oocyte-cumulus complexes (OCCs) with diameter grading >1.5 resulted in 0.91 ± 0.05 embryo formation (number of embryos divided by number of oocytes), whereas OCCs with diameter grading <1.5 resulted in an embryo formation of 0.68 ± 0.10 ($P = 0.04$; Table 1). The ratio of number of OCCs with diameter grading >1.5 to total number of OCCs was calculated for each of the 20 women. The ratio varied from 0.00 to 1.00, with a mean of 0.57 ± 0.31 (SD) and a median of 0.57.

In study 1B, OCCs with diameter grading <1.5 showed 0.81 ± 0.04 mature oocytes (number of mature oocytes divided by total number of oocytes), whereas OCCs with diameter grading >1.5 showed 0.77 ± 0.06 mature oocytes. No difference was noted ($P = 0.65$; Table 2).

In study 1 C, OCC diameter grading was smaller in Fertinorm than in clomiphene citrate treatment ($P = 0.03$; Table 3). Peak serum estradiol 17β (E_2) was higher both in Fertinorm and Pergonal treatments than in clomiphene citrate treatment ($P < 0.05$). Number of retrieved oocytes was greater in Fertinorm than both in Pergonal and clomiphene citrate treatments ($P < 0.005$).

The OCC diameter grading was negatively correlated to the age of women ($r = –0.49$; $P = 0.0003$), but uncorrelated to the peak E_2 ($r = –0.21$; $P = 0.14$).

In study 2, the effect of addition of ,granulosa cells was studied. In the groups with the addition of granulosa cells, the ratio of two- to four-cell embryos over the number of oocytes was 0.63± 0.11, whereas in the control group (no granulosa cells added), it was 0.74 ± 0.07 ($P = 0.42$; Table 4).

In study 3A, when oocytes were cultured individually, the ratio of two- to four-cell embryos over the number of oocytes was 0.73 ± 0.08, whereas when two oocytes were cultured together, the ratio was 0.63 ± 0.07 ($P = 0.37$; Table 5). In study 3B, coculturing of four oocytes together resulted in embryo to oocyte ratio of 0.73 ± 0.08, whereas culturing of oocytes individually resulted in a ratio of 0.63 ± 0.08 ($P = 0.38$; Table 6). The simple statistics for the variables in study 3C are shown in Table 7. In study 3C, the embryo density adversely affected the embryo cell number (slope = –0.10; $P = 0.03$) and the ratio of good-quality embryos (slope = –1.31; $P = 0.0005$). The average diameter of the retrieved OCC did not affect the embryo cell number (slope = –0.07; $P = 0.86$), but favorably affected the ratio of good-quality embryos (slope = 5.77; $P = 0.05$).

When comparing the number of oocytes in each of the above three studies, one noted a difference in the average number of oocytes/patient. In study 1, all consecutive patients could be included. In study 2, some patients were excluded because their oocyte aspirates contained very few granulosa cells. Because of this, they could not be included in a study where we needed a large number of granulosa cells. In. studies 3A and 3B, there was a need for a minimum of three oocytes/woman to compare one vs two oocytes cocultured, and five oocytes/woman to compare one vs four oocytes cocultured. Therefore, patients with <3 or 5 oocytes, respectively, aspirated were excluded in these groups.

The figure "number of embryos per oocyte" was on a per-woman basis, calculated by the number of embryos observed at 48 h postretrieval divided by the number of oocytes allotted to the particular study subgroup. It is of note that among the three studies, the average number of oocytes retrieved was 3.6, 6.0, and 9.6, respectively. The embryo formation was 0.77, 0.72, and 0.67, respectively. This may mean that more oocytes formed results in a greater preponderance of immature oocytes. However, our study was not designed to address this issue, which has been addressed by other investigators.

Table 1

In Vitro Formation of Two- to Four-Cell Good-Quality Embryos from Retrieved OCCs with Different Diameter Gradings in 20 Women in Study 1A

	OCC grade[a]		
Variable	≤ 1.5/3	> 1.5/3	*p*
No. oocytes/woman[b]	1.6 ± 0.30	2.0 ± 0.31	
No. embryos/woman[b]	1.0 ± 0.23	1.8 ± 0.27	
No. embryos/oocyte[b]	0.68 ± 0.10	0.91 ± 0.05	0.04

[a]OCCs were graded according to diameter on a scale of l to 3; grade 1 = 75–100 μm; grade 2 = 125–150 μm; and grade 3 = 200–225 μm.

[b]Values are means ± SEM.

Table 2

Status of Nuclear Maturity of Oocytes Obtained from OCCs with Different Diameter Gradings in 27 Women Scheduled for ICSI in Study 1B

	OCC grade[a]		
Variable	≤ 1.5/3	> 1.5/3	*p*
No. oocytes/woman[b]	6.0 ± 0.68	2.6 ± 0.35	
No. mature oocytes/woman[b]	4.9 ± 0.55	2.1 ± 0.36	
% mature oocytes/woman[b]	0.81 ± 0.04	0.77 ± 0.06	0.65

[a]OCCs were graded according to diameter on a scale of 1 to 3; grade 1 = 75–100 μm; grade 2 = 125–150 μm; and grade 3 = 200–225 μm.

[b]Values are means ± SEM.

Discussion

Better embryonic development (to two- to four-cell stage) in humans will probably be achieved by a laborious

Table 3

Effect of Ovarian Stimulation Types on OCC Diameter Grading, Peak Serum Estradiol17J3 (E_2), and Number of Retrieved Oocytes in Study 1C

Variable	Stimulation type Clomiphene citrate	Fertinorm	Pergonal
n	16	20	15
OCC diameter grading[a,b]	1.8 ± 0.09[c]	1.5 ± 0.08[d]	1.7 ± 0.09[c,d]
Peak serum E_2 (pmol/L)[b]	2,425 ± 682[c]	5,998 ± 608[d]	4,449 ± 695[d]
No. oocytes[b]	2.4 ± 0.7[c]	7.3 ± 0.6[d]	4.2 ± 0.7[c]

[a]OCCs were graded according to diameter on a scale of 1 to 3; grade 1 = 75–100 μm; grade 2 = 125–150 μm; and grade 3 = 200–225 μm.
[b]Values are means ± SEM.
[c,d]Means that do not share a common superscript letter within rows differ ($p < 0.05$).

Table 4

Effect of Coculturing OCCs with Autologous Granulosa Cells on In Vitro Formation of Two- to Four-Cell Good-Quality Embryos in 15 Women in Study 2

Variable	Treatment Control	Granulosa coculture	*p*
No. OCCs	49	40	
No. oocytes/woman[a]	3.3 ± 0.37	2.7 ± 0.37	
No. embryos/woman[a]	2.4 ± 0.34	1.9 ± 0.42	
No. embryos/oocyte[a]	0.74 ± 0.07	0.63 ± 0.11	0.42

[a]Values are means ± SEM.

Table 5

Effect of of Coculturing OCCs in Groups of Two Per Culture Drop on In Vitro Formation of Two- to Four-Cell Good-Quality Embryos in 18 Women in Study 3A

Variable	No. OCCs/culture drop 1	2	*p*
No. OCCs	68	103	
No. oocytes/woman[a]	3.8 ± 0.38	5.7 ± 1.46	
No. embryos/woman[a]	2.7 ± 0.39	3.6 ± 1.14	
No. embryos/oocyte[a]	0.73 ± 0.08	0.63 ± 0.07	0.37

[a]Values are means ± SEM.

Table 6

Effect of Coculturing OCCs in Groups of Four Per Culture Drop on In Vitro Formation of Two- to Four-Cell Good-Quality Embryos in Seven Women in Study 3B

Variable	No. OCCs/culture drop 1	4	*p*
No. OCCs	38	32	
No. oocytes/woman[a]	5.4 ± 1.13	4.6 ± 0.57	
No. embryos/woman[a]	3.4 ± 0.84	3.3 ± 0.42	
No. embryos/oocyte[a]	0.63 ± 0.08	0.73 ± 0.08	0.38

[a]Values are means ± SEM.

Table 7

Characteristics of Pronuclear Zygotes Cultured Together After Fertilization in 81 IVF cycles in Study 3C

Variable	Simple statistics Mean	SD	Min.	Max.
OCC diameter grading[a]	1.59	0.30	1.00	3.00
Oocyte density	4.41	2.32	2.00	12.00
Embryo cell number	3.25	0.96	2.00	8.00
% Good-quality embryos	97	8.1	67	100

[a]OCCs were graded according to diameter on a scale of 1 to 3; grade 1 = 75–100 μm; grade 2 = 125–150 μm; and grade 3 = 200–225 μm.

and systematic prospective randomized study of various factors. In this article, we have presented three such studies: First, we demonstrated that the diameter of OCC is positively correlated to development of good-quality embryos. It was very easy to measure the diameter of OCC at the time of oocyte retrieval. Therefore, this simple and harmless method can be used to classify oocytes. We demonstrated that there is the same maturity of such oocytes between the two groups with smaller or larger OCC diameter. We have also shown in human that the diameter of OCC has an effect on two- to four-cell stage development regardless of oocyte maturity. There is no comparable study in human literature. Saito et al. *(4)* denuded oocytes of their cumulus before fertilization, but conducted a study of coculture with autologous cumulus cells after fertilization. They noted better embryo development in the coculture group vs control (no cumulus) group.

What is the structural basis of variation in OCC diameter? Is this owing to the size of the oocyte, the number of cumulus cells, cumulus cell expansion, or denudation of some of the cumulus cells in the process of extraction, or related to postmaturity? Our visual observation of numerous oocytes indicated that probably all the above four factors are important. There have been a number of studies

attempting to correlate the appearance of OCC with oocyte maturity. Most centers refer to the work of Veeck *(5)* in this respect. This whole issue was reviewed and further scrutinized by Hammitt et al. *(6)*, who evaluated the importance of tedious training of embryologists vs developing expertise in the above correlation. Marrs et al. *(7)* published a diagram of variation in OCC appearance when they showed that increase in size is related to cell number and cumulus expansion. We are considering another detailed morphological study (for counting and size measurement) may damage the oocytes. Mansour et al. *(8)* showed that it is better to leave the oocytes with cumulus intact for the whole of 48–72 h in IVF culture.

The ratio of oocytes with large OCC diameter to total number of oocytes varied widely (from 0 to 1) among women in study 1A. This variation may be related to many factors. We noted that the OCC diameter changed with the type of ovarian stimulation. Treatment with clomiphene citrate to induce superovulation produced OCCs with a larger diameter in comparison to a much more vigorous ovarian stimulation with the use of Fertinorm (a fairly pure follicle-stimulating hormone preparation). When many more oocytes were produced by stimulation with Fertinorm, these oocytes had a smaller diameter of OCC. We were also very interested to note that age had an inverse relationship with OCC diameter. This appears to be a novel observation concerning why there is a lower pregnancy rate with older women. The Fertinorm preparation with no luteinizing hormone (LH) content resulted in the smallest OCC diameter grading, higher peak serum E_2, and highest number of oocytes.

In study 3C, the larger average diameter of OCC, the higher the good-quality embryo development, once again establishing a relationship between the diameter of OCC and good embryo development. Mansour et al. *(8)* fertilized the oocytes retrieved for IVF treatment. Twenty-four hours after retrieval, they randomly assigned the oocytes into two groups: oocytes were either left with cumulus intact or stripped off their cumulus. Observing the oocytes on d 3 postfertilization, they noted that the ones with cumulus intact had a significantly better development. Van Blerkom *(3)* identified three patterns of cumulus appearance in culture medium. These patterns were not related to fertilization or the appearance of the embryo, but surprisingly, correlated very much with pregnancy rate.

Second, coculture with autologous granulosa cells made no difference to two- to four-cell stage development. Dirnfeld et al. *(9)* conducted a somewhat different study by coculturing all oocytes with autologous granulosa cells after fertilization, and found improvement in comparison to a previous IVF cycle. However, this was not a prospectively randomized study, which may explain the difference between the two studies reported by us and Dirnfeld et al. *(9)*. Plachot et al. *(10)* performed a similar prospectively randomized study in 10 patients who had one or more previous total fertilization failures or low fertilization rate. Half the oocytes from each patient were assigned to coculturing with autologous granulosa cells, and the other half were used as control. They found no significant difference between the two groups, an observation that is consistent with ours. It is conceivable that there may be an optimal amount of granulosa cells that may help early embryo development and a larger amount may be detrimental. We used a relatively large amount of granulosa cells, but did not observe a detrimental effect. The prospective study of Plachot et al. *(10)* also came to the same conclusion, and therefore, we can be reasonably assured that coculturing with granulosa cells has neither a beneficial nor a detrimental effect on two- to four-cell stage embryo development.

Third, we investigated coculturing of 2 oocytes or 4 oocytes vs 1 in the same volume of fluid and with the same number of spermatozoa. This was neither beneficial or detrimental to two- to four-cell stage embryo development. Therefore, in circumstances when there are a large number of oocytes, these can be placed together. These observations differ from those reported in experimental animal IVF *(11,12)*. We were interested to know if further increasing the number oocytes per fixed volume may increase the chance of good embryo development. In study 3C, increasing the number of fertilized oocytes (from 2 to 12) in a fixed volume did not improve formation of good-quality embryos. Therefore, it is unlikely that fertilized oocytes have a complementary effect on each other's development in the setting of human IVF. A much more elaborate study of changing the volume and keeping the number of fertilized oocytes fixed is being contemplated by us.

In conclusion, we have first discovered a noninvasive method of predicting oocyte competence by assessing the diameter of the OCC. This supports numerous other observations that cumulus and oocytes interact during the course of fertilization and early embryo development. The diameter of the OCC seems independent of oocyte maturity, but is related to the patient's age and the type of ovarian stimulation. Second, we have demonstrated that coculturing with autologous granulosa cells does not improve oocyte development. Third, we demonstrated that oocyte culture as 1, 2, or 4/culture drop has no beneficial or detrimental effect on development to two- to four-cell stage embryo. When coculturing 2–12 fertilized oocytes in a fixed volume, there was no benefit from increasing the fertilized oocyte density. The reports from experimental animals that coculturing has a beneficial effect were not substantiated in the context of human IVF practice. These studies are part of a prospective randomized evaluation of factors affecting embryo fertilization and development.

Materials and Methods

Ovarian Stimulation, Oocyte Retrieval, Oocyte Culture, and In Vitro Insemination

Three studies were conducted in women with previous fertility problems undergoing an IVF program at the Toronto Fertility Sterility Institute. The patients were treated with a fairly standard protocol *(13)* consisting of ovarian down-regulation and controlled ovarian stimulation using human menopausal gonadotropin, followed by transvaginal oocyte retrieval under ultrasound guidance. The gonadotropins used were Fertinorm (Serono Canada) or Pergonal (Serono Canada). In one group of patients, only clomiphene citrate was used to stimulate the ovaries. Standard oocyte retrieval, culture, and insemination *(13)* were performed with some minor modification as described before *(14)*.

Study Design

In the first part of study 1 (study 1A), OCCs were identified at the time of retrieval in 20 women. They consisted of the oocyte surrounded by layers of granulosa cells, the cumulus oophorus. The diameter of the OCC was measured under a dissecting microscope using a micrometer, and the OCCs were graded as follows: grade 0 = no cumulus at all; grade 1 = diameter of 75–100 μm; grade 2 = diameter of 125–150 μm; and grade 3 = diameter of 200–225 μm. This visual grading of oocytes according to the diameter of OCC is a very easy and quick task to perform and has no potential harm to the oocyte. As each oocyte is placed in a culture well, its grade is recorded beside it. This diameter grading was correlated with formation of 2–4 cell embryos 48 h after retrieval and exposure of oocytes to spermatozoa.

In a separate series of observations (study 1B) in 27 women scheduled for intracytoplasmic sperm injection (ICSI), the cumulus was stripped off the oocytes by incubating in 0.01% hyaluronidase solution for 45 s prior to the ICSI procedure, and the status of nuclear maturity of oocytes was determined under a dissecting microscope. Oocytes that had extruded the first polar body were identified as mature (metaphase II) oocytes. Correlation was made between the diameter of OCC and the status of maturity.

In study 1C, women received either clomiphene citrate only ($n = 16$), Fertinorm ($n = 20$), or Pergonal ($n = 15$) for ovarian follicular stimulation. The retrieved OCCs were graded and averaged for each woman. OCC grade, serum concentration of estradiol-17β prior to hCG injection (peak serum E_2), and number of oocytes retrieved were compared among the three types of stimulation. Correlation also was made between OCC grade and age, and between OCC grade and peak E_2.

In study 2, the OCCs retrieved from 15 women were randomly allocated at the time of identification, in approximately equal numbers within women, to two treatments: (1) untreated control ($n = 49$) and (2) granulosa coculture (OCCs cocultured with autologous granulosa cells; $n = 40$). When oocytes are aspirated in an IVF cycle, the follicular fluid contains varying quantities of loose granulosa cells. In each patient, these granulosa cell aggregates were identified at the time of oocyte retrieval and placed together in HTF culture medium under oil. They were washed off any red blood cells and other materials by being passed through one or several culture media. From this pool, granulosa cell aggregates were randomly added to one-half of the oocytes retrieved. The approximate number of granulosa cells added to each oocyte was $20–30 \times 10^6$ cells. The oocytes were randomly allotted to each treatment, i.e., each containing oocytes with high or low OCC diameter. It is to be noted that the granulosa cells did not contain any cumulus type of granulosa cells. The diameter of OCC was not measured, but since we are dealing with a large number of oocytes, it can be assumed that the random process resulted in equal distribution between the two treatment groups.

In study 3, the OCCs retrieved from 18 women were cultured randomly as 1 OCC/culture drop (control; $n = 68$), or 2 OCCs/culture drop ($n = 103$) in approximately equal numbers within women (study 3A). The OCCs retrieved from another seven women were cultured randomly as 1 OCC/culture drop ($n = 38$), or 4 OCCs/culture drop ($n = 32$) in approximately equal numbers within women (study 3B). In all studies except study 3C, no attempt was made to visualize formation of pronuclei in order to avoid exposure of oocytes/embryos to light and other external factors. At 48 h after oocyte retrieval, oocytes were examined under a dissecting microscope with a ×50 magnification to detect two- to four-cell good quality embryos, i.e. embryos with equal-size blastomeres with no or minor fragments. It is to be noted that allotment of oocytes to each treatment group was random. Therefore, each presumably had a similar allotment of oocytes with large or small cumulus diameter.

Study 3C was conducted to determine if embryo density (total number of embryos in a fixed volume of the culture medium) affected embryo development. Eighty-one IVF cycles were studied. The diameter of OCC at the time of retrieval was assessed. The OCCs were incubated with the sperm, and fertilization was assessed on the next day as judged by the presence of two pronuclei. All fertilized oocytes from the same patient were incubated together in 100 μL culture medium for another day. At this time, the quality and the cell number of embryos were assessed. Ratio of good-quality embryos (suitable for transfer or cryopreservation) over total number of embryos was calculated. Also the average embryo cell number was calculated by adding up all the cells and dividing this by the total embryo number.

Statistical Analyses

In all experiments, data were analyzed by Student's *t*-test using SAS *(15)*, except in study 1C, where analysis of variance using the general linear models procedure of SAS was

used *(15)*, and in study 3C, where analysis of multiple regression using SAS was used *(15)*. The difference among treatment means was assumed to be significant at the level of a p value < 0.05. It is to be noted that in all studies except studies 1C and 3C, oocytes from the same woman were allotted to different groups, i. e., in each woman there were control and study groups.

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