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Failure of human oocyte release at ovulation

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Among 150 patients admitted for ovum aspiration, in vitro fertilization, and embryo transfer in Perth, Western Australia, 14 were found to have had at least one ovulated follicle at the time of laparoscopy. Based upon ultrasound estimation of follicle diameter 24 hours previously, ovulation occurred in 6 of 22 follicles < 1.7 cm in mean diameter and in 16 of 19 follicles > 1.7 cm in mean diameter. When the ruptured follicles were flushed with heparinized fertilization medium, oocytes were collected in 13 of the 22 dispersed follicles. Twelve oocytes developed pronuclei 16 hours after the addition of spermatozoa, and 11 cleaved to the 4-cell stage within 44 hours of insemination. Oocytes were recovered from 6 of 11 patients stimulated with clomiphene alone and from all 3 patients stimulated with clomiphene supplemented with human menopausal gonadotropin.

These observations suggest that oocyte release and follicle rupture are not necessarily synonymous events and that the incidence of retained oocytes in ovulated follicles following stimulation with clomiphene or clomiphene plus human menopausal gonadotropin could be on the order of 60%. Fertil Steril 41:827, 1984

The primary role of follicle growth is to develop a mature oocyte capable of fertilization and normal embryonic development. Ovulation is triggered by rising levels of luteinizing hormone (LH) at the midpoint of the cycle in response to rising serum estrogen levels produced by the growing follicle.[1] The midcycle LH surge has four functions, namely, the maturation of the cumulus mass and its detachment from the granulosa layer, the initiation of meiosis by the oocyte, the luteinization of granulosa tissue, and the commitment of the follicle to imminent rupture within 26 to 32 hours.[1]

Evidence of ovulation can be detected in the simplest form by a thermal shift in basal body temperature. At a clinical level, elevated luteal progesterone levels, disappearance of the follicle on ultrasound, and the detection in the serum or urine of elevated LH levels are all presumptive indications that ovulation has occurred or is imminent.[1] The only event that cannot easily be discerned, except by pregnancy, is the expulsion of the oocyte with follicle rupture. To date, there has been virtually no information available on the inevitability of this event. Just as an LH surge and ovulation are not necessarily synonymous terms, as illustrated by luteinized unruptured follicles,[2] oocyte expulsion and follicle rupture may also not be equivalent events. One of us (J. L. Y.) has recently reported that oocytes could be retained within ruptured follicles in humans.[3]

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In this article, material is presented confirming that oocytes may be collected from recently dispersed follicles and that the extent of ovum recovery may be on the order of 60%.

MATERIALS AND METHODS

The 14 women in the study were drawn from a larger group of patients being treated for infertility by in vitro fertilization and embryo transfer (IVF-ET). Together they form a small group of patients who presented with dispersed follicles at the time of laparoscopy. The nature of the infertility was as follows: Eight patients had absent or nonfunctional fallopian tubes. One of these had anovulatory cycles; one had endometriosis; and four had idiopathic infertility. Follicle growth was stimulated in all patients with 150 mg clomiphene citrate (CC) (Clomid, Merrell Dow Pharmaceuticals, Inc., Cincinnati, OH) on days 2 to 6 of the menstrual cycle. Three women received additional stimulation in the form of three ampules of human menopausal gonadotropin (hMG) (Pergonal, Serono, Rome, Italy) on days 6 through 8.

Follicle growth was monitored by daily early morning ultrasound tracking, cervical score, and serum hormonal assessment comprising 17β-estradiol (E_2), progesterone, and LH. Because daily follicle tracking by ultrasound was preferred, 3-hour urine assessments toward the midpoint of the cycle to define the onset of the LH surge were not attempted. Five patients were monitored by all of the above criteria but were unable to be monitored daily for LH. Generally, when the serum E_2 concentration was on the order of 1500 pmol/l per leading follicle and the average diameter of leading follicles was at least 1.8 cm, the women were admitted to the hospital, and the ovulation process was initiated by a single intramuscular injection of 5000 IU of human chorionic gonadotropin (hCG) given between 10:00 P.M. and 1:00 A.M. A blood sample was taken prior to the hCG injection for determination of whether a spontaneous LH surge had already begun. One patient whose daily morning LH value showed a significant rise of > 2 standard deviations above the mean basal level was admitted for ovum collection the following morning without supplementation with hCG. In the 13 remaining patients who received hCG stimulation, the LH value at the time of triggering was at basal levels in two cases, had risen significantly in six cases, and was unknown in five cases. Ovum aspiration, which was normally timed for 35 to 36 hours after hCG, was moved forward to 32 hours in the six cases where an endogenous LH rise prior to hCG administration was demonstrated. Laparoscopy was usually performed on either day 13 or 14 of the cycle.

Ovulation was determined at the time of laparoscopy by direct visual recognition of a dispersed follicle on the surface of the ovary. All follicles were observed to be intact the previous day by ultrasound. Both ruptured and unruptured follicles were aspirated using a double-needle system. This comprised a 16-gauge aspirating needle fitted with a 23-gauge flushing needle soldered to one side along its full length. The follicular liquor of intact follicles was drained, and the follicle was refilled with flushing medium and subsequently aspirated. The process was repeated until oocyte recovery was confirmed. This was usually in the initial aspirate (45% of follicles) or in the first flushing (45%). With ruptured follicles, the needle was inserted, where possible, through the ruptured stigma and filled with 5 to 10 ml of tissue culture medium, and the fluid was aspirated. This process was repeated up to four times. The flushing medium used was a modified Tyrode's solution[4] containing 2% to 5% heat-inactivated homologous maternal serum and 40 U/ml sodium heparin.

Oocytes in their cumulus matrix that were identified from among the follicle debris were washed twice with flushing medium and twice in the medium without heparin before incubation in 1 ml fertilization medium. This was either modified Tyrode's or Ham's F-10 medium containing between 7.5% and 15% heat-inactivated homologous serum. Culture was carried out in 5-ml plastic tissue culture tubes (Falcon #2003, Falcon Plastics, Oxnard, CA) in an environment containing 5% CO_2, 5% O_2, and 90% N_2. Oocytes were incubated for 3 to 5 hours after laparoscopy before the addition of spermatozoa. One milliliter of semen was diluted to 5 ml with fertilization medium and centrifuged at $200 \times g$ for 15 minutes. The pellet was washed once more as above and then overlaid with 4 ml fresh fertilization medium. A 1-ml sample was collected from the top of the overlay 15 minutes later and adjusted to a concentration of 1×10^6 motile spermatozoa/ml. An aliquot of this enriched motile sample was added to each fertilization tube with a sperm concentration of between 0.5 and 2.0×10^5 spermatozoa/ml.

Gametes were incubated overnight and examined after dissection of the coronal coat, 16 to 20 hours after insemination, for the presence of two pronuclei in the cytoplasm of the oocyte and, where possible, two polar bodies. Embryos were washed once in fertilization medium and transferred to a similar tube containing the same medium without spermatozoa. Embryos were then cultured overnight and transferred to the patient via the cervical route into the uterus, 40 to 44 hours after insemination, when most embryos were at the 4-cell stage.

Follicle size is normally estimated at the time of laparoscopy; but because this estimation was impossible with ruptured follicles, follicle size data were drawn from ultrasound estimations made 24 hours prior to aspiration (day −1). Ultrasound investigations were not made on the day of laparoscopy. Associating follicle size at ultrasound and at laparoscopy was uncomplicated in all but two patients. In these women, the ruptured follicle was assumed to have been the largest follicle seen on ultrasound examination. Follicle size was recorded as the mean of three separate estimations of follicle diameters in different planes and was performed using real time ultrasonic sector scanning (Diasonics Real Time Scanner DRF1, Nuclear Enterprises, Milpitas, CA).

RESULTS

The 14 cases in this report have been drawn from over 150 laparoscopy attempts for IVF-ET carried out at PIVET Laboratory, Cambridge Hospital, Perth. They involve a small group of patients who started ovulation, as indicated by having at least one dispersed follicle on aspiration. Based upon the last estimation of serum LH, it was calculated that rupture would have preceded laparoscopy by no more than 6 hours.[1]

In 4 of the 14 women, all follicles observed the previous day by ultrasound had dispersed. Oocytes were retrieved from three of these patients. In all, seven ruptured follicles were observed, and six oocytes were obtained after flushing. Aspiration of fluid in the pouch of Douglas failed to produce any oocytes. In the remaining ten patients, the ovaries contained some ruptured and some intact follicles of varying size. Oocytes were recovered from ruptured follicles in six patients and intact smaller follicles in nine patients.

The relationship between follicle size and ovulation is detailed in Table 1. Only one follicle of eight with a diameter (on day −1) of < 1.5 cm was found to have ruptured. Five of 14 follicles with a diameter of between 1.5 and 1.7 cm had ruptured. However, 16 of 19 follicles with a diameter of 1.7 cm or greater were found to have dispersed at the time of aspiration. In all, 22 of 41 follicles were found to have ruptured. Thus, even though the average number of follicles per patient was 2.9, only 1.5 follicles per patient were found to have ovulated. Whether the remaining smaller intact follicles would have dispersed in the ensuing period is not known.

Table 1. *Relationship Between Follicle Size, Incidence of Ovulation, and Oocyte Recovery*

Follicles	Ovulated follicles		Nonovulated follicles		Total follicles (ova)
	No. of follicles	No. of ova recovered	No. of follicles	No. of ova recoverd	
< 1.5	1	1	7	5	8 (6)
1.5–1.6	5	3	9	4	14 (7)
1.7–1.8	1	0	1	1	2 (1)
1.9–2.0	3	2	1	1	4 (3)
2.1–2.2	4	3	0	0	4 (3)
2.3–2.4	5	4	0	0	5 (4)
> 2.4	3	0	1	1	4 (1)
Total	22	13	19	12	41 (25)

Flushing of the 22 ruptured follicles produced a total of 13 mature oocytes. Nine oocytes were obtained from 12 follicles with a diameter of between 1.9 and 2.4 cm, but no oocytes were obtained from 3 follicles with a diameter > 2.4 cm. Aspiration of the ruptured follicles yielded little if any fluid (< 1 ml), but most flushings were contaminated with some blood and granulosa tissue.

Of the 13 oocytes recovered from ruptured follicles, 12 fertilized in vitro and 11 developed to normal 4-cell embryos before transfer (Table 2). Even though the sample size is small, there appeared to be little difference in the fertilization and developmental characteristics of oocytes obtained from large and small ruptured follicles. In contrast, fewer than expected oocytes were collected after aspiration and flushing of the remaining follicles, which at the time of laparoscopy had failed to ovulate (53%, Table 1). Oocytes retrieved from the intact follicles exhibited reduced fertilization rates (50%) and embryo development (50%, Table 2), suggesting some degree of follicle immaturity.

In 6 of the 11 patients who received CC alone, flushing of dispersed follicles resulted in retrieval of at least one oocyte. In all, 17 follicles from this

Table 2. *Fertilization and Development of Oocytes Recovered from Ruptured and Intact Follicles*

Type of follicle	Follicle size	No. of oocytes	No. of pronuclear embryos	No. of cleaving embryos
	cm			
Ovulated	< 1.7	4	4	3
	≥ 1.7	9	8	8
Nonovulated	< 1.7	9	5	3
	≥ 1.7	3	1	1

group were flushed, and 9 oocytes were collected, for a recovery rate of 53% (Table 3). Of the three patients receiving CC plus hMG supplementation who were found to have started ovulation, all had retained oocytes in at least one dispersed follicle (recovery rate, 80%). In the one patient in whom fluid was collected on a natural endogenous surge without hCG stimulation and who had ovulated at laparoscopy, an oocyte was retrieved from the single follicle present. Oocytes were retrieved from four follicles from two women who possessed basal levels of LH prior to hCG induction, but who had started ovulation by laparoscopy. Similarly, oocytes were collected from four of six patients who had begun an LH surge prior to the trigger being administered and from two of the five patients whose serum LH levels were unknown at the time of hCG injection. As indicated in Table 1, most patients possessed a combination of large (> 1.8 cm) and medium growing (1.5 to 1.8 cm) follicles. The average E_2 concentration the morning of hCG injection was 4963 pmol/l. In seven patients, the E_2 levels were still rising; five had plateaued; and in one patient, the E_2 level was falling.

DISCUSSION

The results of this study provide information in two areas. First, in the stimulated cycles employed here where ovulation was triggered by hCG, follicles with a diameter > 1.6 cm near the time of the ovulation trigger have the potential to ovulate at a rate > 80% determined by comparing the ultrasonic features of the previous day to the laparoscopy findings. Ovulation was observed, however, with follicles < 1.6 cm, but at a reduced rate (27%). Since ovulation can occur over a period of 4 to 6 hours,[1] more of the small follicles may have ruptured given more time. The data, therefore, provide some clinical guidelines toward the relationship between ovulation and follicle size in stimulated cycles.

Second, the data indicate that the probability of an ovulating follicle failing to expel the oocyte could be on the order of 60% in patients receiving this stimulation regimen. Our ovum collection rate in normal periovulatory follicles is on the order of 90% using the double-lumen needle described. The rate of ovum retention may, in fact, be slightly higher, because some retained oocytes may not have been retrieved. One alternative argument to the concept of ovum retention could be that on entering a ruptured follicle, the oocyte was obtained from an adjoining follicle, which was also pierced. This was considered unlikely because the control over the position of the needle tip is high, and because the characteristic yellow follicle fluid (indicative of entering another follicle) was never noted in any ruptured follicle flushes.

These results imply that release of the oocyte at the time of follicle rupture may not always occur. The oocytes collected from dispersed follicles exhibited normal fertilization and developmental characteristics and therefore were considered mature. This suggests that such mature oocytes were entrapped by some means within the follicle which thereby prevented their release. The condition has previously been briefly described in the form of two case histories where the term "ovum retention" was applied.[3] In this report, we present data indicating 9 of 14 women had such ovulation malfunction. At a clinical level, these data may be interpreted to suggest that as many as six of ten follicles generated by CC with or without hMG may fail to expel the oocyte. Whether this

Table 3. *Relationship Between Follicle Stimulation, Ovulation Induction, and Oocyte Retention*

Treatment	No. of patients	No. of patients with retained oocytes	No. of oocytes recovered/ruptured follicles
Follicle stimulation			
CC	11	6	9/17
CC + hMG	3	3	4/5
Ovulation induction			
Nil	1	1	1/1
hCG with basal LH	2	2	4/4
hCG with rising LH	6	4	4/7
hCG with unknown LH	5	2	4/10

rate proves to be consistent remains to be evaluated, but nevertheless it represents an event of considerable magnitude. There is, of course, no way to assess whether in time the retained oocytes would have been released. We consider this to be relatively unlikely given the totally depressed appearance of the follicle. Whether following the release of serum factors into the newly formed corpus luteum may aid in its final release is not known. The relationship between the results of this study using patients receiving various stimulants for follicle growth and for ovulation induction and natural, unstimulated cycles is not known, and no attempt is made to equate the two. Furthermore, whether the cycles of patients in this group are representative of all CC-induced cycles is also unclear, because considerable variation exists in the time and dose of CC administration. All patients in this study were stimulated with high doses (150 mg daily) from day 2 for 5 days.

Normally, the oocyte within the cumulus mass should be free-floating at the time of ovulation. The parameters involved in the detachment of the oocyte from the follicle wall and in ovulation are, however, not necessarily the same. Three factors have been suggested to be involved in the rupture of the follicle wall. These include, first, enzyme activity, most likely the activation of serum plasminogen by plasminogen activator[5] thought to be synthesized by granulosa tissue under the influence of follicle-stimulating hormone (FSH).[6] A role for plasmin could be to weaken the connective tissue around the follicle. Prostaglandins have also been implicated in ovulation, because the addition of inhibitors of prostaglandin synthesis such as indomethacin can inhibit ovulation, leading to entrapped oocytes.[7] The third factor which may lead to follicle rupture is the layer of norepinephrine-sensitive smooth muscle which surrounds mammalian follicles.[8] The contraction of this smooth muscle layer by catecholamines or by prostaglandins of a periovulatory follicle may cause the rupture of the follicle wall at an area previously weakened by enzyme action. The only factor common to both follicle dispersal and cumulus maturity may be prostaglandins.[7] We have observed follicle rupture on two separate occasions during laparoscopy for ovum pickup. In both cases, the release of the fluid has been more an explosive event than a passive oozing of the follicular fluid. It is thought that the ensuing release of follicular fluid through the ovulation hole carries the oocyte-bearing cumulus mass. If, however, the follicle reaches the point of imminent rupture without a concomitant dissociation of the cumulus mass from the wall of the follicle, the oocyte within its cumulus matrix could be retained.

In mice and rats, the hormone responsible for inducing the preovulatory changes of the cumulus cells, including expansion and dissociation from the follicle wall, is thought to be FSH, rather than LH.[9] In the mouse, Eppig[10] has proposed that the high levels of glycosaminoglycan molecules inhibit the capacity of FSH to stimulate the synthesis of hyaluronic acid by the oocyte/cumulus complex until after exposure of the follicle to LH. It is the synthesis of these molecules which leads to the expansion of the cumulus matrix and the dissociation from the granulosa wall. LH may overcome this inhibition by a reduction in the degree of sulfation of the glycosaminoglycan molecules.[11, 12] Why FSH appears incapable of stimulating cumulus expansion until after the follicle has been stimulated with LH is not known.[10]

In the pig, the synthesis of sulfated glycosaminoglycans is stimulated by FSH.[13] This activity appears maximal in small follicles and diminishes the follicle size. LH and progesterone both act to suppress this activity. Possibly, in stimulated cycles, sustained FSH exposure by the use of CC or by hMG injection during the middle to late growth phase of the follicle may lead to the synthesis of higher concentrations of glycosaminoglycans in the follicle. High levels could mean a delay in the response of the cumulus cells to LH or hCG, delaying the maturation of cumulus expansion, sufficiently so to put the rate of development of the ovulatory cumulus mass out of phase with that of the follicle. Dekel and co-workers[12] have furthermore reported that in the rat the intracellular mucification and tissue dissociation characteristics of the cumulus mass can occur over differing time regimens, depending on whether ovulation was induced by endogenous LH secretion or by hCG injection.

Whatever the mechanism preventing oocyte expulsion, the concept of ovum retention is interesting, in that it represents another form of ovulation disorder. Unlike the luteinized unruptured follicle, where a follicle fails to respond appropriately to the ovulation trigger[2] but the oocyte may be free-floating, in the retained oocyte condition the follicle responds by dispersing, but the oocyte/cumulus oophorus does not. Ovum retention im-

plies that the response of the follicle to an ovulation trigger (either LH or hCG) which leads to follicle rupture could operate on a different time frame from that of the maturation of the oocyte and its surrounding cellular matrix.

At a clinical level, ovulation can be assessed by various means, but oocyte release is confirmed only by pregnancy. It is of interest that one application of IVF technology, i.e., ovum aspiration, has demonstrated the frequency with which oocytes may fail to be released. A high proportion of dispersing follicles in stimulation cycles which fail to expel the oocyte may explain some effects observed with CC. In particular, there are many reports of a good response to CC in terms of follicle numbers but a poor response in terms of multiple pregnancies.[14] The results of this study suggest that one reason could be ovum retention. It would therefore be of value to compare the occurrence of ovum retention in unstimulated cycles with that in the stimulated cycles employed for IVF-ET.

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