Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 4184–4188, July 1983
Physiological Sciences

Decline of follicular oocyte maturation inhibitor coincident with maturation and achievement of fertilizability of oocytes recovered at midcycle of gonadotropin-treated women

(estrogen/follicular fluid/progesterone/atresia)

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Communicated by Roy Hertz, December 13, 1982

ABSTRACT **To examine whether a decline in follicular oocyte maturation inhibitor (OMI) is associated with attainment of oocyte maturation and fertilizability, OMI was measured in follicular fluid (FF) of 39 follicles of 20 normal women given human menopausal gonadotrophin and human chorionic gonadotropin to induce follicular growth and maturation. Oocytes were aspirated per laparoscope, the fluid was saved, and the egg was observed, incubated, and inseminated with the husband's sperm. Concepti that developed to the 4- to 8-cell stage were transferred to the uterus and the women were followed for pregnancy. OMI activity in each FF was measured by using cultured cumulus-enclosed porcine oocytes (30–40 oocytes per FF sample). Estrogen, progesterone, and Δ^4-androstenedione were measured in FF by radioimmunoassay. The FF of 13 preovulatory follicles yielding oocytes that were mature and fertilizable had significantly less OMI activity (mean ± SEM) (0.58 ± 0.10 unit/ml) compared to follicles yielding immature oocytes (2.8 ± 0.56 units/ml; $n = 9$), atretic oocytes (5.5 ± 2.5 units/ml; $n = 7$), or preovulatory oocytes with fractured zonae (1.9 ± 0.63 units/ml; $n = 7$). The estrogen concentration (mean ± SEM) of prevulatory follicles yielding mature fertilizable eggs or mature eggs with fractured zonae was greater (396 ± 34 ng/ml; $n = 20$) compared to follicles yielding immature or atretic eggs (203 ± 59 ng/ml; $n = 9$ and 97 ± 47 ng/ml; $n = 7$, respectively; $P < 0.05$). Progesterone concentration (mean ± SEM; ng/ml) of FF was generally elevated in all preovulatory follicles (635 ± 53) compared to immature or atretic follicles (230 ± 64 and 76 ± 17, respectively; $P < 0.05$). It may be concluded that in normal follicle maturation there is a decline in OMI in the follicle containing an oocyte that becomes mature and fertilizable. There is also an increase in estrogen, progesterone, and follicle size. It is also possible to have an abnormal follicle maturation when there is an increase in size as well as FF, estrogen, and progesterone, but without a decline in OMI—a situation which can lead to production of a nonfertilizable oocyte.**

It is well documented that the human oocyte completes its meiotic maturation within the dominant preovulatory follicle within 30–48 hr prior to ovulation (1, 2). It is believed that the preovulatory gonadotropin surge brings this maturation about; yet the mechanism is poorly understood. Oocyte maturation only occurs within the dominant mature follicle and not in the adjacent less mature follicles. Several investigators in this laboratory have partially purified an oocyte maturation inhibitor (OMI) from immature porcine follicles, which may play a role in keeping the oocyte in the immature dictyate state (3–6). Whether a decline in OMI at the time of ovulation is the cause of oocyte maturation is not known. Furthermore, the mechanisms responsible for choosing the dominant follicle to be ovulated are not well understood. The purpose of this study was to measure changes in OMI in preovulatory human follicles and to correlate them with oocyte maturation and fertilizability, size of the follicle, and steroid levels in the follicle. In this study we observed that attainment of a mature fertilizable oocyte under the influence of a gonadotrophic stimulus was associated with a decline in follicular OMI.

MATERIALS AND METHODS

Patients. A total of 39 follicles was sampled from 20 women, aged 23 to 37 yr, who were part of an *in vitro* fertilization program at the Department of Obstetrics and Gynecology, Eastern Virginia Medical School (Norfolk, VA). The patients had normal menstrual cycles and normal reproductive function except that they had occluded both fallopian tubes or had related disorders. Once daily each patient was examined clinically, a blood sample was taken and assayed for estrogen, and the ovaries were examined by ultrasound (7–10). Women were given 150 international units of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the form of Pergonal to induce follicular maturation. Ten thousand international units of human chorionic gonadotropin (hCG) were given to induce oocyte maturation as described (7–10). Oocytes were recovered from all visible follicles present 36 hr after hCG administration and were subjected to *in vitro* fertilization (7–10). Follicular fluid (FF) was diluted in 4 ml of Dulbecco's phosphate-buffered saline (Dulbecco's P_i/NaCl; in g/liter: NaCl, 8; $CaCl_2$, 0.1; KCl, 1; KH_2PO_4, 0.2; $MgCl_2$, 0.047; Na_2HPO_4, 1.15), saved, and frozen. The state of maturity of the oocytes and their fertilizability was noted.

FF Steroid Radioimmunoassay. The diluted FF samples were given a numerical code, frozen, and shipped to Baltimore, where they were assayed for steroids and OMI without revealing the status of the oocytes until after all assays were completed. Steroids—estrogen, progesterone, and Δ^4-androstenedione—were measured in the FF to give an index of the state of maturity (11) or atresia, or both. FF estrogen content was measured by radioimmunoassay after extraction with diethyl ether as described (12–14), FF progesterone content was measured by radioimmunoassay after extraction with petroleum ether as described (12, 13), and FF Δ^4-androstenedione content was

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Abbreviations: OMI, oocyte maturation inhibitor; FF, follicular fluid; Dulbecco's P_i/NaCl, Dulbecco's phosphate-buffered saline; hCG, human chorionic gonadotropin; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

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measured by radioimmunoassay after extraction with diethyl ether as described (13, 14).

OMI Bioassay. OMI activity was measured in each FF sample after dilution of each to 25% or less (vol/vol) in Dulbecco's P_i/NaCl. Each sample was mixed with additional glucose (5 mM), L-lactate (to 1 mM), Na pyruvate (0.03 mM), and gentamicin (50 μg/ml) added immediately prior to assay. A pool of porcine FF that served as a source of standard OMI was prepared by aspirating 1- to 8-mm porcine follicles by using ovaries obtained at the slaughterhouse (generously provided by the National Institutes of Health under the auspices of Gabe Bialy).

On the day of the OMI assay, the standard porcine FF (25%), porcine serum (25%), human serum (25%), made up in Dulbecco's P_i/NaCl, as well as three to eight samples of human FF were each added to four replicate culture wells of a Falcon microtest plate and were incubated for 32 hr with 15 cumulus-enclosed oocytes per well (total, 40–60 oocytes per group) recovered from 1- to 2-mm porcine follicles as described (3, 4, 15). In every third assay, a dose–response curve of porcine FF (10%, 20%, 25%, and 50%) was run. Porcine serum served as a control fluid for the porcine FF dose–response curve. The standard porcine FF serum, pooled human serum, and FF were diluted in Dulbecco's P_i/NaCl/0.4% bovine serum albumin (previously adjusted to pH 7.4) containing 5 mM glucose, 1 mM L-lactate, 0.03 mM pyruvate, and gentamicin at 50 μg/ml and were filtered through a 0.22-μm filter. The media were equilibrated in humidified 5% CO_2 in air for 1–2 hr before addition of oocytes to the culture well. The pH of equilibrated media and FF was measured (glass electrode) and the osmolality was measured with a vapor pressure osmometer (Wescor). The osmolarity of diluted human FF was between 260 and 320 mosM and was not further adjusted because we have found that porcine oocytes mature equally well in media adjusted to osmolarities of 260 and 320 mosM (6). The pH of 35 of the 40 equilibrated dilute human FF samples was between 7.2 and 7.7. However, the pH of five samples was ≤7.1. In four instances, the low pH was associated with follicular atresia, identified by a FF Δ^4-androstenedione/estrogen ratio of >2, by using the criteria of McNatty *et al.* (11, 16). The cumulus-enclosed oocytes used for these assays were viable (>98%) according to dye-exclusion test with 0.066% trypan blue in Hanks' balanced salts. The oocyte cultures were incubated at 37°C in humidified 5% CO_2 in air for 30–32 hr. After 24 hr the number of cumuli exhibiting outgrowth was evaluated as described (17).

At the end of the 30-hr incubation the oocytes were recovered from culture, stripped of cumulus cells, fixed for 3 hr in acetic acid/ethanol, 1:3 (vol/vol), and stained with acetoorecin as described (15, 17, 18). The porcine oocytes were evaluated for nuclear maturation and classified as originally described by Hunter and Polge (19). Oocytes having an intact germinal vesicle were considered immature. Oocytes in metaphase I and metaphase II as well as anaphase and telophase were considered mature. Degenerated oocytes were also counted and usually represented <5% of total oocytes recovered unless stated specifically. After 30–32 hr of incubation the maturation rate ranged from 40% to 70% in 25% human or porcine serum. The OMI activity was estimated by expressing the oocyte maturation as % inhibition of oocyte maturation compared to 25% human serum and by relating that activity to the activity of the 25% standard porcine FF included in that assay. We have defined 1 unit of OMI activity as that activity in 1 ml of a standard porcine FF. Examples of four dose–response curves of porcine FF run on different batches of ovaries are shown in Fig. 1. The % inhibition of oocyte maturation exerted by the 25% standard porcine FF was 37 ± 4% in eight assays (26% coefficient of variation). Within each assay significance of inhibition of oocyte maturation was evaluated by χ^2 (4). In the case of comparisons between units of OMI activities between groups, data were compared by Duncan's multirange test after one-way analysis of variance.

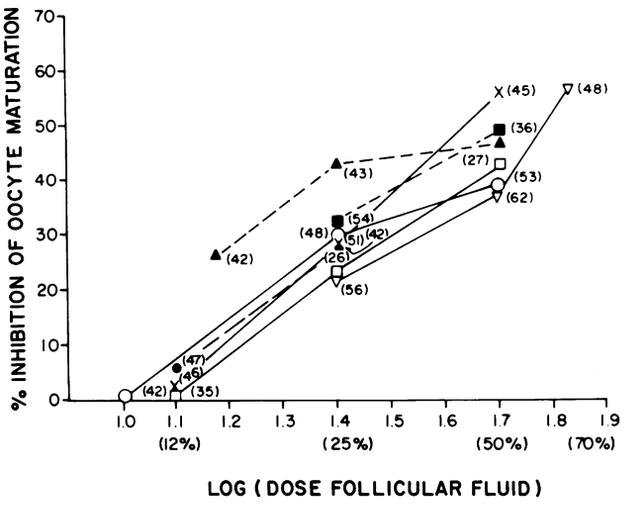

FIG. 1. Assay of FF OMI: dose–response curve of inhibitory action of porcine FF and human FF upon porcine oocyte maturation. The FF was diluted in Dulbecco's P_i/NaCl/0.4% bovine serum albumin/0.03 mM Na pyruvate/2.5 mM L-lactate. The number of oocytes used to estimate each point is shown in parenthesis. Human or porcine serum did not cause inhibition of oocyte maturation. Values shown are samples assayed on different days. ×——×, 8/20/82 pig FF; □——□, 4/7/82 pig FF; ○——○, 5/13/82 pig FF; ▽——▽, 5/26/82 pig FF; ▲---▲, 5/26/82 human FF pool 1; ■---■, 4/7/82 human FF pool 2; ●---●, 8/20/82 human FF pool 3.

RESULTS

Maturity and Fertilizability of Recovered Oocytes. In this series of 20 patients, 6 became pregnant with a single pregnancy. All of the patients responded to the Pergonal treatment and had elevated serum estrogen (mean ± SEM) on the day after the day of hCG administration (designated day 1), which was 748 ± 245 pg/ml (n = 5) in the patients who later became pregnant and 515 ± 52 pg/ml (n = 14) in the patients who later did not become pregnant. Before Pergonal treatment the serum estrogen was 20–40 pg/ml. Oocytes recovered at laparoscopy were classified into five groups (Table 1): group I, mature (having a polar body and a mucified cumulus) and fertilizable, later resulting in a pregnancy; group II, mature and fertilizable, not resulting in a pregnancy when later transferred back to the patient; group III, immature with and without germinal vesicles based on morphology and compact cumulus; group IV, atretic compact appearance with condensed ooplasm with irregular outlined darkened ooplasm, abnormal, nonfertilizable; and group V, preovulatory with a fractured zona, nonfertilizable. Details of the appearance of human oocytes can be found elsewhere (7, 8).

Correlation of Oocyte Status with FF Steroid Levels and Level of FF OMI. Patient data have been grouped according to the five groups of oocytes described above and the respective values for OMI, estrogen, progesterone, Δ^4-androstenedione, and FF volume are summarized in Table 1. In the case of atretic oocytes, follicles were also included in this category, with a Δ^4-androstenedione/estrogen ratio of >5; oocytes are atretic according to McNatty *et al.* (i.e., they had <75% of their normal complement of granulosa cells) (11, 16).

It is clear that OMI concentrations in the fluids of groups I and II composed of mature oocytes were significantly lower than the levels of other groups, suggesting that oocyte matur-

Table 1. Concentrations of preovulatory human FF, estrogen, progesterone, Δ^4-androstenedione, and OMI activity correlated with oocyte maturation, fertilizability, and ability to result in a pregnancy

Group		Follicles,	Follicle volume,	OMI,	FF steroids				
No.	Oocyte status	no.	ml	units/ml	E, ng/ml	P_4, ng/ml	Δ^4-A, ng/ml	Δ^4-A/E ratio	P_4/E ratio
I	Preovulatory (pregnant)	6*	3.1 ± 0.49^a	0.86 ± 0.16^{cd}	383 ± 60^{ab}	551 ± 40^a	111 ± 42^a	0.28 ± 0.08^a	1.74 ± 0.27^a
II	Preovulatory (nonpregnant)	7	2.3 ± 0.42^a	0.58 ± 0.10^d	473 ± 75^a	597 ± 77^a	76 ± 11^a	0.17 ± 0.02^c	1.41 ± 0.19^a
III	Immature	9	1.0 ± 0.2^b	2.8 ± 0.56^{ab}	203 ± 59^{cd}	230 ± 64^b	319 ± 150^b	5.03 ± 2.36^b	1.16 ± 0.30^a
IV	Atretic†	7	0.78 ± 0.15^b	5.51 ± 2.5^a	97 ± 47^d	76 ± 17^b	839 ± 257^c	41.9 ± 31^a	1.48 ± 0.58^a
V	Preovulatory‡	7	2.4 ± 0.5^a	1.9 ± 0.63^{bc}	330 ± 26^{abc}	733 ± 119^a	116 ± 30^a	0.36 ± 0.08^c	2.27 ± 0.35^a

FFs were aspirated from all large follicles of women volunteering for *in vitro* fertilization. Each patient was given Pergonal and hCG to induce follicular growth and maturation. Oocytes were recovered from the largest visible follicles, fertilized with the husband's sperm, allowed to develop to the 4- to 8-cell stage, and transferred back into the patient. Values for FF components are statistically different from each other ($P < 0.05$) if they have a different letter superscript when using analysis of variance and Duncan's multirange test. E, estrogen; P_4, progesterone; Δ^4-A, Δ^4-androstenedione; Δ^4-A/E, Δ^4-androstenedione:estrogen ratio; P_4/E, progesterone:estrogen ratio.

*The number of follicles was five for OMI and the P_4/E ratio.

†Includes fluids with acid pH, low E, Δ^4-A/E ratio of >5, and eggs with atretic appearance.

‡With oocytes with fractured zonae pellucidae.

ation is associated with a decline in OMI. These two groups of follicles yielding preovulatory oocytes also had higher concentrations of FF estrogen in comparison with follicles of group III (immature) and group IV (atretic). Group V, consisting of preovulatory follicles with preovulatory eggs with cracked zonae, which were not fertilizable, was especially interesting because it had FF progesterone and estrogen concentrations comparable to preovulatory follicles yielding fertilizable oocytes (groups I and II). If the progesterone concentration is divided by the estrogen concentration a rough "luteinization index" for that follicle can be calculated. It is 2.27 for group V compared to 1.74 and 1.41 for groups I and II, respectively. The elevated progesterone concentration in group V suggests that the oocytes may have been harvested too late and that they had been in the mature state too long and showed signs of degeneration. The OMI concentration was not as low as it was in the first two groups, which yielded mature fertilizable oocytes (1.9 ± 0.63 vs. 0.86 or 0.58 units/ml). The Δ^4-androstenedione/estrogen ratio was consistently low in the preovulatory follicles (groups I, II, and V) and was elevated in the atretic and immature follicles (groups III and IV), as would be expected.

Inverse Relationship Between OMI and Follicular Size and Estrogen and Progesterone Concentration. There was a consistent inverse relationship between the size of the follicle in milliliters (volume of FF aspirated) and the log of the OMI concentration of the FF ($P < 0.05$). The coefficient of correlation was 0.342 and the equation of the regression was $y = -0.428 + 2.02$. Estrogen concentration, a good measure of follicle maturation, was inversely related to follicular OMI concentration. The coefficient of correlation between the estrogen content of FF and estrogen was −0.4309 and the equation for the regression was $y = -101 + 353$ ($P < 0.05$). A similar inverse rela-

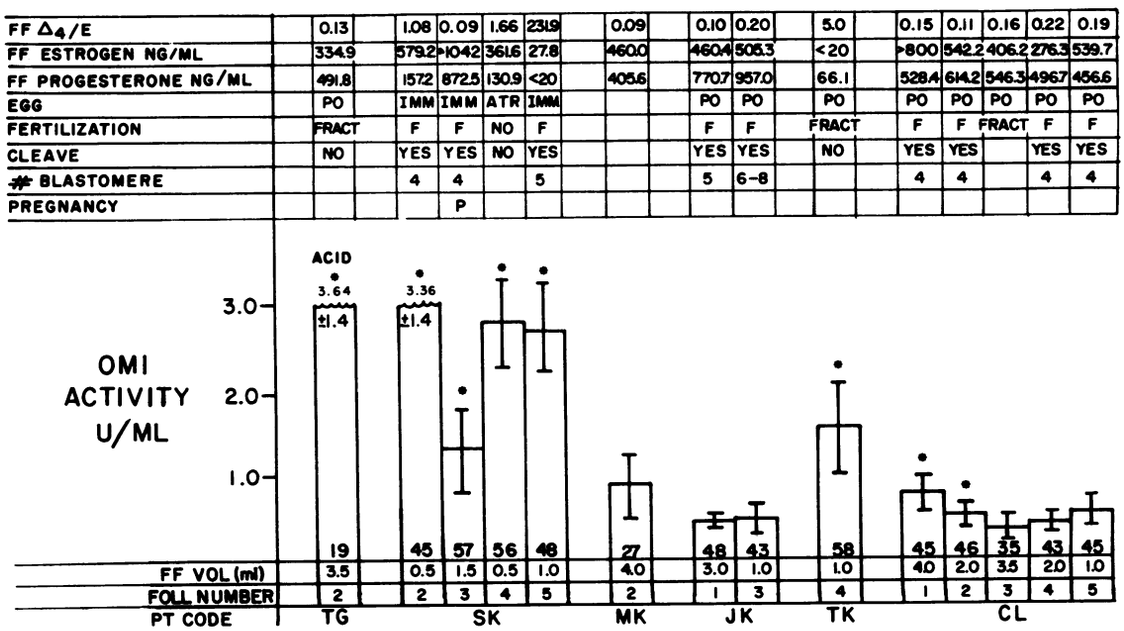

FIG. 2. Levels of OMI activity (bottom portion), steroids (top portion), as well as the status of the egg and its ability to become fertilized, cleave, and result in pregnancy in individual human FFs harvested from 6 of 20 representative patients treated with human menopausal gonadotropin and hCG as described in the text. Values are grouped by patient and the volumes of fluid recovered are given at the bottom. Oocyte designations: IMM, immature with compact cumulus; PO, preovulatory; ATR, atretic (degenerated abnormal); FRACT, preovulatory with fractured zona pellucida. Fertilization designations: NO, nonfertilizable; F, fertilization occurred; P, pregnancy occurred. Steroid designations: Δ^4/E, Δ^4-androstenedione/estrogen ratio. The numbers in the bars designate the number of oocytes used in the OMI bioassay. The asterisks at the top of the bars designate that the inhibition of oocyte maturation was significant ($P < 0.05$).

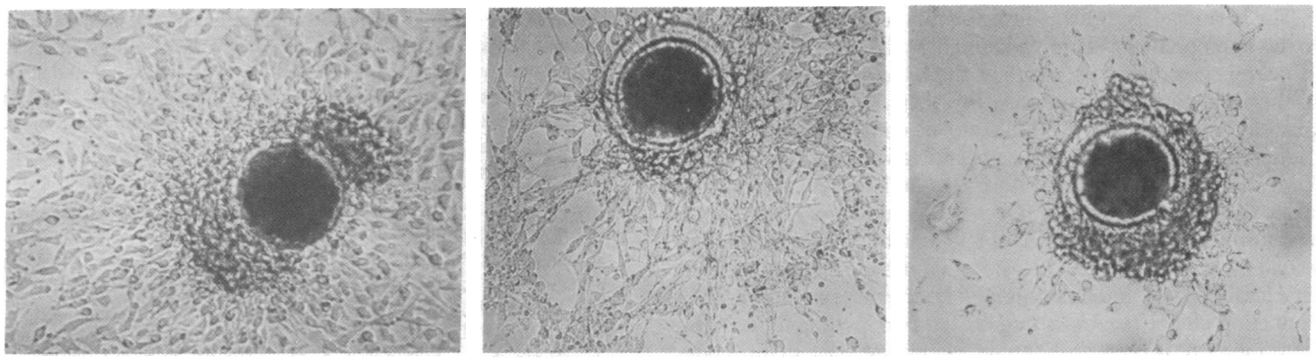

FIG. 3. Appearance of cultured cumulus-enclosed porcine oocytes after culture for 30 hr in the presence of 25% human serum (*Left*), 25% preovulatory human FF having no OMI activity (*Center*), and 25% human FF harvested from an immature follicle. Note that the immature FF prevents the cumulus cells from growing out, which is in contrast to serum and preovulatory FF.

tionship existed between OMI and follicular progesterone concentrations with $r = -0.378$, $m = -135$, and $b = 493$ ($P < 0.05$).

Correlation of Concentrations of Steroids and OMI of Individual Follicles Obtained at the Same Time from the Same Patient. If the steroid and OMI concentrations of fluids of a series of follicles obtained from the same patient at the same time were compared to each other, an inference as to the selection of the dominant follicle(s) may be obtained (Fig. 2). Morphology of cultured porcine cumulus-enclosed oocytes grown in the presence of selected fluids is shown in Fig. 3. Furthermore, the steroid and OMI concentrations can also be correlated with the status of the oocyte in that follicle, making it possible to predict which factors contribute to the production of a mature follicle and an oocyte that is fertilizable. As summarized in Fig. 2, which represents about one-third of the patients used in this study chosen in alphabetical order, in cases in which more than one follicle was examined, there was a tendency for one or more follicles to be "dominant" over the others in terms of estrogen concentrations and oocyte maturation and size (this was true especially in the case of SK). In most cases this follicle consistently had lower OMI levels compared to the others. There was also a tendency for this follicle to have higher estrogen concentrations compared to the others (4 of 5 instances). There were instances (CL and JK) in which two to five equally dominant follicles with comparable estrogen and OMI concentrations were present. These dominant follicles also had low Δ^4-androstenedione/estrogen ratios compared to adjacent follicles. This increased Δ^4-androstenedione/estrogen ratio in the adjacent follicle could reflect the fact that these follicles were more immature than the dominant one(s) or were in a state of atresia. In instances in which an atretic oocyte was recovered the adjacent follicle can clearly be said to be atretic. Such follicles invariably had elevated Δ^4-androstenedione/estrogen ratios (>2.0).

The group of preovulatory follicles yielding mature oocytes with fractured zonae invariably had Δ^4-androstenedione/estrogen ratios characteristic of healthy preovulatory follicles (except TK, who had a FF Δ^4-androstenedione/estrogen ratio of 5.8, indicating that this follicle was probably atretic) but had high progesterone concentrations, indicating that they were probably harvested too late. Addition of FF, especially recovered from follicles containing immature eggs, inhibited cumulus cell outgrowth (Fig. 3).

It would appear that to yield a mature fertilizable oocyte a follicle must have decreased OMI concentrations compared to immature follicles, while at the same time it must have greater ability to secrete estrogen and progesterone. Therefore, the requirements in this population of patients to obtain a viable fertilizable oocyte are when the OMI level is <0.86 unit/ml, estrogen concentration is >473 ng/ml, the Δ^4-androstenedione/estrogen ratio is <0.3, and the progesterone/estrogen ratio is 1.2–1.5.

DISCUSSION

These results demonstrate that oocyte maturation in FSH/LH-hCG-pretreated women is associated with a decline in OMI in the FF. Although these data do not prove that the decline in OMI *per se* is responsible for oocyte maturation, they are suggestive of such. The decline in OMI occurring in the preovulatory follicle in response to LH/hCG could be due to initiation of granulosa cell luteinization, as reflected by an increase in FF progesterone. We have shown that granulosa cells harvested from large porcine follicles secrete low levels of OMI in culture, whereas granulosa cells from small immature follicles secrete more OMI (20). Granulosa cells from large preovulatory primate and porcine follicles that have been exposed to the LH surge can, as a result, start to luteinize, as determined by a rise in FF levels of progesterone and an increased potential of cultured granulosa cells to secrete progesterone and luteinize morphologically (21, 22).

The finding that there is generally a low OMI concentration in large follicles and those that contain more estrogen would again be supportive of the concept that as the follicle matures there is less OMI and progressively more estrogen. Furthermore, the present observation of high follicular estrogen in the presence of a mature oocyte would rule out estrogen as being the physiological OMI, as suggested by McGaughey (23). The decline in OMI in the ripe preovulatory follicle could also be due to the increased blood flow to that follicle, which occurs as a result of LH/FSH action. Because OMI is small (<2,000 daltons) it could be easily cleared from the follicular antrum.

The group of oocytes that had cracked zonae and were nonfertilizable (group V) and came from steroidogenically defined preovulatory follicles were obviously defective. It is possible that the OMI level, which was higher than in the other groups of preovulatory follicles yielding fertilizable oocytes (groups I and II), may not have declined in a timely fashion to permit normal maturation to occur in the follicle. Alternately, the follicles may have luteinized prematurely without giving the oocytes enough time to mature.

From the data it is possible to ascertain the FF environment that is compatible with yielding a mature fertilizable oocyte. The follicle can contain from 2 to 4.5 ml of fluid and the OMI concentrations should be <1 unit/ml, the estrogen concentration should be 300–500 ng/ml, the progesterone/estrogen ratio should be 1.58, and the Δ^4-androstenedione/estrogen ratio should be <0.3.

The hormonal factors that bring about these local FF changes to this appropriate level are undoubtedly pituitary LH and FSH. However, it is clear that not all of the follicles respond equally to the same peripheral stimulus. In a majority of patients it appeared that 1 out of 2 or 3 follicles was dominant and had the higher concentration of estrogen and lower concentration of OMI. The unequal response to FSH and LH is an interesting observation and is not new (see refs. 20 and 24–26 for review) and forms the basis for choice of a follicle to ovulate each menstrual cycle. The plausible local regulating mechanism may lie in a local follicular luteinization inhibitor, which can inhibit FSH induction of LH receptors in cultured porcine granulosa cells. This inhibitor is present in immature but not large mature porcine follicles (20, 26, 27). In other studies we have examined luteinization inhibitor in some of these same human follicles and find it to be essentially absent in the preovulatory follicles containing mature oocytes (12 follicles). Interestingly, an inhibitor of LH receptor induction and progesterone secretion was present in four immature follicles having an immature oocyte and low estrogen levels (unpublished data). From such studies it should be added that, in addition to the criteria mentioned above for a follicle to yield a mature fertilizable egg, it must also have a decline in luteinization inhibitor.

Others have reported on the success of recovering mature oocytes from preovulatory follicles of untreated and treated women (28–30). Interestingly, the steroid levels reported in human fluid recovered from preovulatory follicles of untreated women (refs. 13, 31; unpublished data) are higher than those reported here for Pergonal-treated women. The peripheral serum levels of steroids of these and other similar patients are well within the normal range (refs. 9, 10; unpublished data). Because these follicles yield fertilizable oocytes there is no reason to believe that the lower follicular steroid levels are indicative of an abnormal follicle.

It is of interest in these studies that it was possible to recover an apparently immature-appearing oocyte from a follicle with steroid and OMI levels characteristic of a mature preovulatory follicle that can be matured in culture, fertilized, and result in a normal pregnancy (patient SK, Fig. 2). Apparently, the oocyte had partly matured *in vivo* but the final maturation step had not had time to take place. Two additional immature oocytes from partially mature follicles in the same patient (follicles having fluids with lower than the typical preovulatory level of progesterone and higher than preovulatory levels of OMI) also matured *in vitro* and became fertilized and cleaved. Because all three fertilized eggs from this patient were transferred back to the patient it is impossible to ascertain which one actually implanted and initiated the pregnancy. It would then appear that the oocyte does not have to be within the follicle for the whole maturation process to render it fertilizable, although it has been thought that the mammalian oocyte must remain within the follicle until extrusion of the first polar body to be rendered fertilizable (32). Further studies will be required to determine the minimal time required for an oocyte to partly mature within the follicle to render it able to mature *in vitro* and be fertilizable. Because the time required for germinal vesicle breakdown is a rather long time in the primate and pig (≈20–24 hr) it is quite possible to aspirate an immature-appearing egg that is actually 18 hr toward germinal vesicle breakdown with only 2 hr left of maturation before visible germinal vesicle breakdown occurs.

The able technical assistance of Valerie Evans, Patricia Gagliano, and Lucinda Veeck is acknowledged. We thank Ashish Deshpande and Jack Vallejo for their diligent help in obtaining porcine ovaries and aspirating porcine follicles. The able help of Ms. Gail Williams in preparing this manuscript is gratefully acknowledged. This work was supported in part by National Institutes of Health Grants HD08834 and HD14696 and by U.S. Department of Agriculture–Binational Agricultural Research and Development Grant 1-122-79. C.-Q.L. was supported by a World Health Organization traineeship.

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