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# Increasing Progesterone Secretion and 3β-Hydroxysteroid Dehydrogenase Activity of Human Cumulus Cells and Granulosa-lutein Cells Concurrent with Successful Fertilization of the Corresponding Oocyte

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**In many studies it has been documented that the induction of multiple follicular growth in humans results in an asynchrony between the degree of cumulus mucification, oocyte meiotic maturation, fertilizability, and follicular cell progesterone ($P_4$) secretion. The present study was carried out on oocytes enclosed in fully mucified cumulus. Thus, oocyte fertilizability was correlated to human cumulus cell (hCC) and human granulosa-lutein (G-L) cell competence for $P_4$ secretion in culture. In the G-L cells, $P_4$ secretion and percentage of cells manifesting 3β-hydroxysteroid dehydrogenase (3β-HSD) activity increased concurrently with the period of culture. In the hCC, however, $P_4$ secretion decreased concurrently with elongation of the culture period, whereas the percentage of 3β-HSD-positive cells increased. In hCC corresponding to the fertilized oocytes, $P_4$ accumulation in culture medium was 1.9-fold ($P < 0.001$) and 1.6-fold ($P < 0.02$) higher on days 0–3 and 3–5 of culture, respectively, as compared to $P_4$ accumulation in hCC of unfertilized oocytes. Also, in hCC corresponding to the fertilized oocytes, the degree of 3β-HSD activity was found to be significantly higher shortly after aspiration and after either 3 or 5 days, compared to hCC of unfertilized oocytes. In the G-L cells pooled from all follicles yielding mature cumulus–oocyte complexes, $P_4$ accumulation and percentage of 3β-HSD-positive cells increased concurrently with the increase in percentage of fertilized eggs of each individual woman. These results indicate that in stimulated cycles, follicles yielding mature cumulus–oocyte complex, oocyte fertilizability, and G-L cell or hCC competence for $P_4$ secretion are correlated and synchronous.**

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## INTRODUCTION

Administration of FSH/LH to women as part of treatment for *in vitro* fertilization/embryo transfer (IVF/ET) results in multiple follicular growth of a nonhomogeneous population. Thus, these follicles do not manifest homogeneous responses to the administration of HCG in terms of oocyte meiotic maturation, cumulus mucification, and the expected change in the steroidogenic pattern from estrogen-producing to progesterone ($P_4$)-producing cells. Furthermore, an asynchrony between the degree of oocyte meiotic maturation and cumulus mucification has been reported in women subjected to massive FSH/LH treatment [1, 2]. However, in follicles yielding mature cumulus–oocyte complexes (COC), oocyte fertilizability and subsequent cleavage are correlated to cumulus culture morphology and steroidogenic pattern of the cumulus cells [3]. Furthermore, the steroidogenic pattern of human granulosa-lutein (G-L) cells and the steroid concentration in follicular fluid (FF) are altered in relation to the

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degree of mucification/maturation of the corresponding cumulus mass [4–7] and in relation to the cumulus culture morphology [8]. The type and rate of steroid biosynthesis is dependent on many factors, including intracellular cholesterol availability, degree of proper enzyme activity, etc. [9–11]. The main factor that initially determines $P_4$ synthesis in human cumulus cells (hCC) and human G-L cells as part of corpus luteum formation, in response to the endogenous LH surge or exogenous HCG administration, is the tremendous increase in the activity of various enzymes such as side-chain-cleavage cytochrome *P*-450 ($P$-$450_{SCC}$) and 3$\beta$-hydroxysteroid dehydrogenase (3$\beta$-HSD) [12–14]. The 3$\beta$-HSD activity can easily be determined by a histochemical approach [15]. Thus, in the present study the correlation between oocyte fertilizability and degree of 3$\beta$-HSD activity in the corresponding hCC and G-L cells as well as the rate of $P_4$ secretion were evaluated.

## EXPERIMENTAL

### *Subjects*

Twenty-seven women admitted to the IVF/ET program at Rambam Medical Center who had normal menstrual cycles and whose husbands had been proven fertile were the subjects of this study. Their age ranged between 30 and 35 years. Multiple follicular growth was stimulated by daily treatment with either 50 mg/day clomiphene citrate (Ikaclomin; Ikapharm, Kfar Saba, Israel) combined with 150 IU/day human menopausal gonadotropin (Pergonal; Teva, Petah Tiqva, Israel) or 150 IU/day human FSH (Metrodin; Teva) and 150 IU/day Pergonal. These treatment protocols resulted in the development of $6.7 \pm 0.5$ follicles/woman. All women received 10,000 U HCG (Chorigon; Teva) 36 h before aspiration of oocytes.

### *G-L cell cultures*

The G-L cells were isolated only from follicles containing COC characterized as mature at the time of aspiration, according to criteria detailed earlier by Bar-Ami *et al.* [3]. Immediately after follicular content was aspirated, the COC were removed and cultured under proper conditions for fertilization, and the remaining follicular aspirates were pooled and then centrifuged at 300 *g* for 10 min to recover the G-L cells in the pellet. The G-L cells were suspended in culture medium, which consisted of Ham's F-10 medium (Gibco, Grand Island, NY) buffered with 25 mM sodium bicarbonate (Gibco) and 10 mM Hepes (Gibco) and enriched with 1 mM glutamine (Gibco), 5% inactivated fetal calf serum (Gibco), and streptomycin (50 IU/ml)/penicillin (50 $\mu$g/ml) solution (Gibco). Naturally the aspirated G-L cells contained red blood cells (RBC), which were removed by allowing them to sediment in 60-mm Petri dishes for 20 min, and the floating clumps of G-L cells were collected with a Pasteur pipette. This procedure was repeated two or three times until the preparation of G-L cells was relatively clear of RBC. Ten thousand viable G-L cells/200 $\mu$l were plated in 96-well plates (Falcon, Oxnard, CA); cell viability was assessed using 0.04% trypan blue (w/v) for the dye-exclusion test [16].

The G-L cells were cultured for 7 days in a humidified 5% $CO_2$–95% air incubator at 37°C. On days 3 and 5, medium was replaced with fresh medium, then collected and frozen at −20°C for later steroid radioimmunoassay (RIA). Sometimes cells were cultured in the presence of 40 ng/ml HCG (CR 121, kindly provided by the Center for Population Research of the National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD) or 20 $\mu$M forskolin (Sigma Chemical Co., St Louis, MO).

### *Oocyte and sperm coculture*

Human COC were collected 36 h after HCG administration, usually between 0800 and 1200 h; at this time oocyte fertilizability is greatest, and ovulation rarely occurs [17, 18]. The complexes were placed immediately in 1 ml of insemination medium, which consisted of Ham's F-10 medium buffered with 25 mM sodium bicarbonate and supplemented with 2 mM lactic acid (Calbiochem-Behring, La Jolla, CA), streptomycin (Sigma), penicillin (Sigma), and 10% human cord serum. Preparation of human cord serum was as described previously [3]. Each single COC was cultured in one organ-culture dish (Falcon) at 37°C in 5% $CO_2$–95% air. Fresh semen obtained by masturbation was divided into 2-ml aliquots. Semen (2 ml) was mixed with Ham's F-10 medium (8 ml) and centrifuged at 200 *g* for 10 min. The supernatant was decanted, and the pellet was resuspended in 5 ml medium and recentrifuged. After removal of the supernatant, 1 ml of insemination medium was added without disturbing the pellet. One hour later, the media containing the motile sperm were collected and counted in a Makler chamber. One hundred thousand motile sperm were added to each culture dish within 4–6 h after recovery of the COC. Between 18 and 20 h after addition of sperm, the remaining corona cells attached to the oocytes were mechanically removed to view fertilization, which was marked by the appearance of two pronuclei in the ooplasm. The oocytes or zygotes were transferred to a new dish filled with fresh medium. The dishes containing the hCC were cultured for 2 additional days (3 days' total) to evaluate the steroid secretion of each individual cumulus mass. Culture was continued for another 4 days, with a medium change on day 5. All the conditioned media, i.e. media collected after days 0–3, 3–5, and 5–7, were stored at −20°C for further $P_4$ RIA.

Sometimes, in cases where the husband's sperm had failed to fertilize any of his wife's oocytes in at least two previous trials by regular IVF, IVF/ET was attempted

by micromanipulation of the oocytes. Thus, following 4–6 h incubation, each three complexes were placed in a single organ culture dish filled with 1 ml of a medium composed of a synthetic tubal fluid (HTF; Irvine Scientific, Santa Ana, CA) supplemented with 1 mg/ml bovine testis hyaluronidase (Sigma). hCC removal was assisted mechanically by repeated pipetting with a Pasteur pipette, within about 1–2 min at room temperature.

The naked oocytes were washed twice in 1.5 ml hyaluronidase-free HTF and immediately placed in 1.5 ml fresh HTF medium supplemented with 10% human cord serum, three oocytes per dish. These oocytes were later subjected to micromanipulation as described by Cohen *et al.* [19]. Usually the hCC are discarded, since they have no clinical application. Thus, permission was obtained to utilize these cells for the present study.

The HTF-hyaluronidase solution containing the removed hCC was collected from all dishes into a conical test tube and centrifuged at 300 *g* for 10 min at room temperature. The HTF-hyaluronidase solution was discarded and the cell pellet washed again by centrifugation in fresh Ham's F-10 culture medium. After discarding the Ham's F-10 medium, cells were suspended in 2 ml culture medium, and $2 \times 10^4$ viable cells were plated in 0.5 ml culture medium in the organ culture dishes (Falcon No. 3037, Oxnard, CA). This cell system was utilized for determining the effect of 40 ng/ml HCG or 20 $\mu$M forskolin on the change in percentage of 3$\beta$-HSD-positive cells.

*RIA for steroids*

Steroid RIA was employed on the unextracted crude samples. The crude culture media were diluted in 50 mM Tris buffer, pH 8.0. The amount of $P_4$ was measured as described by Bar-Ami *et al.* [3] using tritiated hormone (Amersham Radiochemical Centre, Amersham, Bucks., England) labeled at six places and rabbit anti-$P_4$ (Bio-Makor, Rehovot, Israel). This antiserum has very low cross-reactivity (measured at 50% displacement), which did not exceed 2.5% for a similar steroid such as 5$\alpha$-pregnane-3,20-dione. Assay sensitivity and intra- and interassay coefficients of variation were 50 pg/ml and 2 and 8%. In all instances, data represent the net steroid level obtained after subtraction of the steroid contents of serum-supplemented medium.

*Cell harvest and counting*

At the end of the incubation period, the G-L cells or hCC were detached as described previously [20]. Briefly, following incubation of the cells for 20 min in a buffer consisting of 0.05 M phosphate-buffered saline, pH 7.4, with 1% (w/v) sodium EDTA and 5% (v/v) dimethyl sulfoxide (both from Sigma), most of the cells were detached from the plastic substrate. Detachment of cells was completed by repeating pipetting. The G-L cells from two culture wells were collected into a single Eppendorf test tube. Also, each individual cumulus was collected into a single Eppendorf test tube. The detachment solution was removed by centrifugation at 300 *g* for 15 min at 22°C and decanting of the supernatant. Nuclear staining was observed by adding 50 $\mu$l of crystal violet solution (5 mg/100 ml in 0.15 M trisodium citrate) and incubating at 37°C for 20–30 min. Nuclei were counted in a hemocytometer; this method resulted in reproducible data.

*Determination of 3$\beta$-HSD activity*

3$\beta$-HSD activity was determined according to modifications by Barkey *et al.* (unpublished data) of the method of Levy *et al.* [15]. The reaction solution was prepared as follows: Ten milligrams of nicotinamide-adenine dinucleotide (NAD, Sigma) were dissolved in 3 ml of an already prepared solution consisting of Tris–HCl (110 mM), sodium cyanide (0.224 mM), and $MgCl_2 \cdot 6H_2O$ (11 mM). The NAD-containing solution was mixed with 1.67 ml of a solution consisting of 40 mg nitroblue tetrazolium, 0.5 ml *N*,*N*-dimethylformamide, and 9.5 ml double-distilled water, as well as with 1.33 ml of double-distilled water. This solution was buffered to pH 8 by NaOH. The final reaction solution contained 0.935 ml of the above mixture and either 0.065 ml of *N*,*N*-dimethylformamide (control) or 0.065 ml *N*,*N*-dimethylformamide containing 0.4 mg dehydroepiandrosterone (DHA). Cells of each individual cumulus or G-L cells of a single woman were harvested as indicated above. The cells were divided into two Eppendorf test tubes. The cells were suspended in 100 $\mu$l of the reaction solution in the absence (control) or presence of DHA (experimental). Following incubation for 1 h at 37°C, the cells were visualized under a light microscope in a hemocytometer. Cells containing 3$\beta$-HSD activity were stained blue violet. The degree of 3$\beta$-HSD activity was expressed as percentage of stained cells per total cell count. The addition of the control group was to assure that the measured value of 3$\beta$-HSD-positive cells in the DHA-containing solution was specific.

About 2–3 h after aspiration, in 11 COC two small pieces were cut from each individual cumulus mass. Each piece was placed immediately in a single Eppendorf test tube and incubated for 20 min with a testicular hyaluronidase solution [50 $\mu$l of 20 IU/ml enzyme (Sigma)]. Then the hCC in the hyaluronidase solution were diluted by adding 1450 $\mu$l of culture medium. After mixing and centrifugation at 300 *g* for 15 min, the supernatant was decanted, and the reaction solution for 3$\beta$-HSD determination was added with and without DHA, as detailed above.

To validate the approach for assessing 3$\beta$-HSD activity in our cell system, the optimal pH and length of incubation time were determined. In addition, 3$\beta$-HSD activity was tested in various nonsteroidogenic

cells. In any of these cases, the percentage of 3β-HSD-positive cells was substantially low, such as 0.5% in epithelial cells collected from rat mammary gland.

*Statistical analysis*

Experimental data are presented as the mean ± SEM. The data were subjected to one-way analysis of variance (ANOVA), followed by unpaired Student's *t*-test for individual comparisons between means.

## RESULTS

*Altered rate of $P_4$ secretion and 3β-HSD activity during 7 days' culture*

Daily $P_4$ secretion by G-L cells increased 1.6-fold ($P < 0.001$) and 3.1-fold ($P < 0.001$) during days 3–5 and 5–7 of culture, respectively, compared to steroid secretion during days 0–3 (Fig. 1). In hCC, however, $P_4$ secretion during days 0–3 was 2.5-fold ($P < 0.001$) and 2.9-fold ($P < 0.001$) higher than in hCC during days 3–5 and 5–7, respectively (Fig. 1).

The percentage of G-L cells manifesting 3β-HSD activity increased concomitantly with progress of the culture period, from 39.5 ± 3.1% shortly after aspiration to 70 ± 4.9 after 5–7 days' culture ($P < 0.001$) (Fig. 2). hCC showed a similar pattern; that is, 3β-HSD activity increased with progress of the culture period, from 24.3 ± 3.4 about 2–3 h after aspiration to 58.5 ± 2.1 ($P < 0.001$) after 3–5 days' culture (Fig. 2).

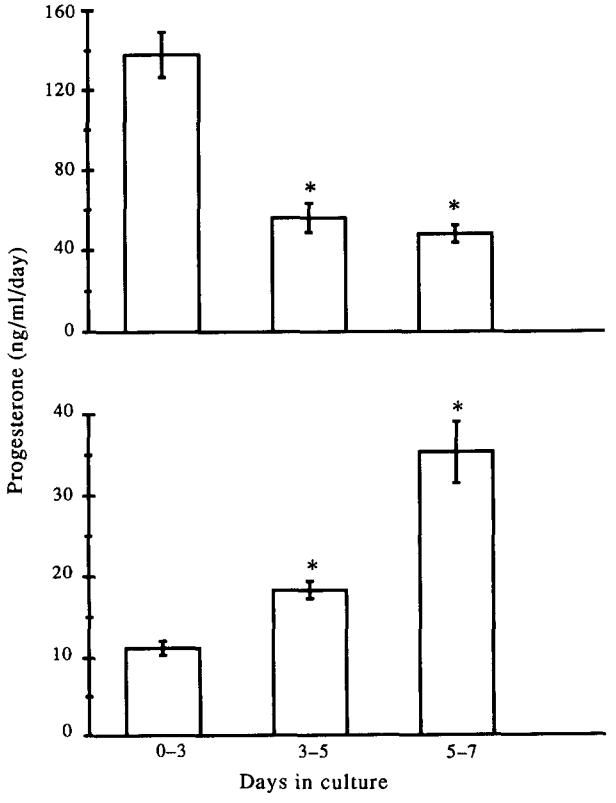


**Fig. 1. *In vitro* accumulation of $P_4$ in hCC (upper panel) and in G-L cells (lower panel), both of which were cultured for 7 days as detailed under Experimental. Data were subjected to one-way ANOVA. The altered $P_4$ secretion with extension of the culture period gave the following statistical values: hCC, $F = 42.3$, $P < 0.001$; G-L cells: $F = 9.6$, $P < 0.001$, *$P < 0.001$ vs the corresponding values on days 0–3 of culture, by Student's *t*-test.**

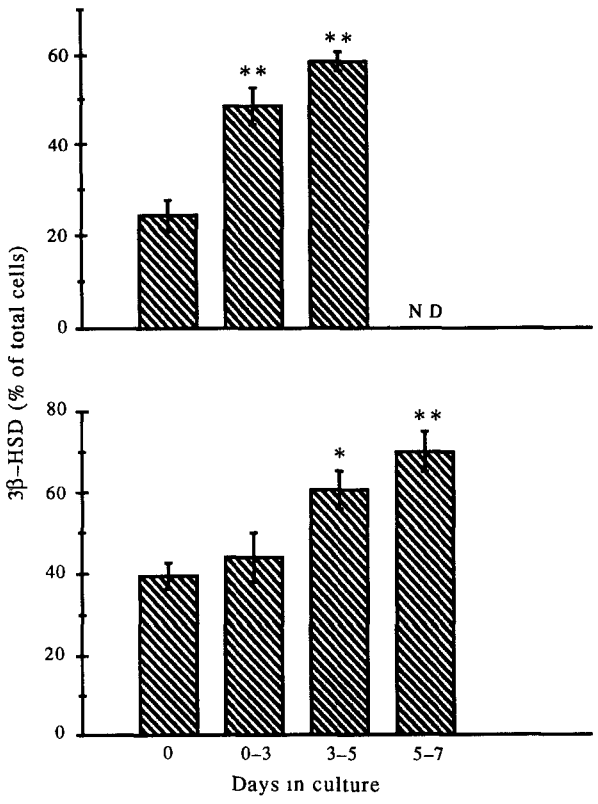


**Fig. 2. Degree of 3β-HSD activity in hCC (upper panel) and in G-L cells (lower panel) during 7 days of culture, where day of aspiration is day 0. Data were subjected to one-way ANOVA for the hCC or G-L cells. Thus, in the presence of DHA the following statistical values were observed: in the hCC, $F = 28.3$, $P < 0.001$; in the G-L cells, $F = 4.1$, $P < 0.001$. Other details are as described in the legend to Fig. 1. *$P < 0.01$, **$P < 0.001$, vs the corresponding values on day 0, by Student's *t*-test. ND, not done.**

When hCC were removed by hyaluronidase shortly after aspiration and cultured for 5 days with HCG or forskolin, the percentage of 3β-HSD-positive cells increased from 46.5 ± 3.0 to 72.4 ± 7.1 ($P < 0.01$) and 79.8 ± 5.8 ($P < 0.001$), respectively. Similarly, in G-L cells cultured for 5 days with HCG or forskolin, the percentage of 3β-HSD-positive cells increased from 66.7 ± 3.4 to 78.5 ± 4.0 ($P < 0.05$) and 81.2 ± 5.0 ($P < 0.05$), respectively.

*Correlation of oocyte fertilizability to $P_4$ and 3β-HSD*

Degree of 3β-HSD activity and rate of $P_4$ secretion were correlated to oocyte fertilizability in G-L cells and hCC. In the fertilized oocytes the corresponding hCC mass secreted 1.9-fold ($P < 0.001$) and 1.6-fold ($P < 0.02$) more $P_4$ during days 0–3 and 3–5 of culture, respectively, than hCC mass of unfertilized oocytes (Fig. 3). The percentage of 3β-HSD-positive cells was

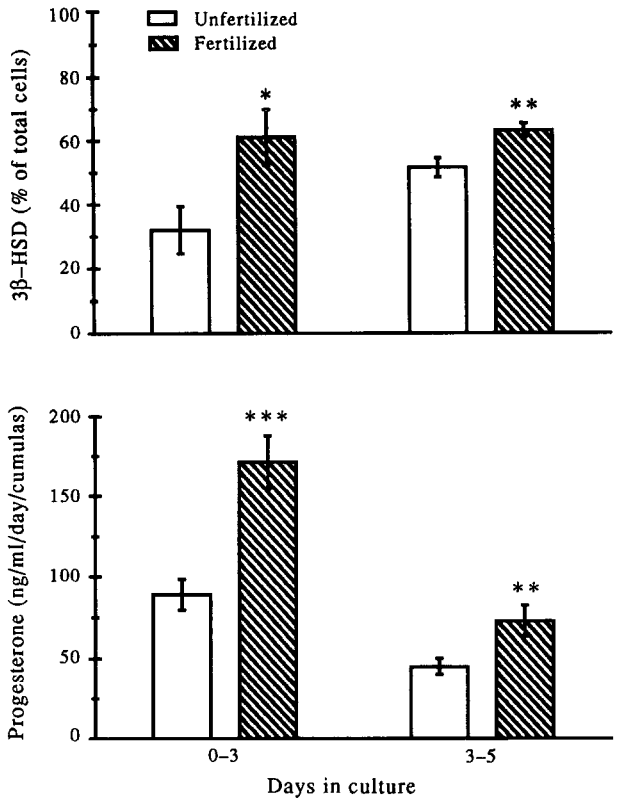


**Fig. 3. Evaluation of degree of 3β-HSD activity and $P_4$ secretion in individual hCC in relation to fertilization of corresponding oocyte. Data were subjected to one-way ANOVA. Thus, the altered percentage of 3β-HSD-positive hCC gave the following statistical values: $F = 5.4$, $P < 0.01$. The change in $P_4$ secretion gave the following statistical values: $F = 14.06$, $P < 0.001$. *$P < 0.05$, **$P < 0.02$, ***$P < 0.001$, vs the corresponding values in hCC of unfertilized oocytes, by Student's $t$-test.**

1.9-fold ($P < 0.05$) and 1.2-fold ($P < 0.01$) higher in hCC mass of oocytes undergoing fertilization, measured after 3 and 5 days, respectively, than the 3β-HSD value in hCC mass of unfertilized oocytes (Fig. 3). Furthermore, when the hCC were examined about 2–3 h after COC aspiration, it appeared that the percentage of 3β-HSD-positive cells was $31.3 \pm 3.4$ ($n = 6$) in hCC corresponding to the fertilized oocytes and $15.9 \pm 3.8$ ($n = 5$) in the hCC of the unfertilized oocytes.

Since the G-L cells were pooled from all follicles yielding mature COC of each individual woman, it was impossible to correlate in a given follicle the competence of the G-L cells for $P_4$ secretion with the fertilizability of the corresponding oocyte. Therefore, the degree of 3β-HSD activity and the rate of $P_4$ secretion of the pooled G-L cells in a given woman were correlated with the average fertilization in the corresponding oocyte population of that woman. Thus, in women yielding an oocyte population manifesting a fertilization rate above 75%, 3β-HSD activity and $P_4$ secretion were 1.4-fold ($P < 0.01$) and 2.0-fold ($P < 0.02$) greater, respectively, during days 3–5 of culture, and 1.3-fold ($P < 0.05$) and 1.7-fold ($P < 0.01$) greater, respectively, during days 5–7 of culture, than in women yielding an oocyte population achieving a fertilization rate of less than 50% in either culture period (Table 1). However, no significant difference was noted in $P_4$ secretion after days 0–3 of culture (Table 1) or in percentage of 3β-HSD-positive cells when examined shortly after aspiration (data not shown) in relation to successful fertilization of the corresponding oocytes.

## DISCUSSION

The data collected in the present study were limited to stimulated follicles yielding mature mucified COC. This study demonstrated that the induction of COC mucification by HCG did not always advance synchronously with the other ovulatory processes such as an increase in hCC and G-L cell $P_4$ secretion. However, a correlation between oocyte fertilization and hCC and G-L cell $P_4$ secretion was observed.

Administration of HCG at IVF/ET causes tremendous changes in the various follicular compartments. Among these changes, the oocyte

*Table 1. Correlation of oocyte fertilizability to G-L cell $P_4$ secretion and 3β-HSD activity**

| Days of culture | Fertilization rate (% of fertilized oocytes) | | | | | |
|---|---|---|---|---|---|---|
| | 0–50 | | 51–75 | | 76–100 | |
| | $P_4$[a] | 3β-HSD[b] | $P_4$ | 3β-HSD | $P_4$ | 3β-HSD |
| 0–3 | $9.2 \pm 1.1$ | ID | $12.1 \pm 0.9$[c] | ID | $12.0 \pm 4.2$ | ID |
| 3–5 | $11.4 \pm 1.1$ | $48.3 \pm 6.1$ | $23.8 \pm 4.6$[d] | $60.0 \pm 5.5$ | $22.2 \pm 1.9$[e] | $68.1 \pm 1.9$[d] |
| 5–7 | $38.7 \pm 3.7$ | $58.6 \pm 5.7$ | $44.9 \pm 6.1$ | $65.1 \pm 6.3$ | $64.5 \pm 5.3$[e] | $75.7 \pm 6.0$[c] |

*Values represent mean ± SEM. Data were analyzed by Student's $t$-test. Comparisons were made between the group with 0–50% fertilization rate and the groups with more than 50% fertilization rate. ID, insufficient data to be presented.

[a]Amount of steroid accumulation during indicated days of culture at ng·ml/day normalized per $10^3$ cells.

[b]Percentage of 3β-HSD-positive cells in the presence of DHA. In the absence of DHA, the percentage of 3β-HSD-positive cells did not exceed 20% of the total cells. This value did not vary significantly among the different groups that were classified by fertilization rate.

[c]$P < 0.05$; [d]$P < 0.02$; [e]$P < 0.01$.

resumes meiosis from the diplotene state to second metaphase [21, 22], and $P_4$, instead of estradiol-17$\beta$ ($E_2$), becomes the major steroid product of the G-L cells [23]. The changes in the steroidogenic pattern apparently occur due to a change in the activity of various enzymes.

The conversion of pregnenolone into $P_4$ is carried out by the microsomal–enzymatic complex exerting the activity of both 3$\beta$-HSD and 3-oxosteroid isomerase [24]. In human, follicles excised at the early follicular stage contain a detectable amount of mRNA encoding $P$-$450_{SCC}$, which probably is localized in the thecal tissue [14]. The quantity of the mRNA for $P$-$450_{SCC}$ and of the enzyme itself increases significantly shortly before ovulation, and $P$-$450_{SCC}$ is present in large quantity in the corpus luteum [12, 14]. On the other hand, mRNA encoding other enzymes essential to steroid biosynthesis, such as cytochrome $P$-450 aromatase and 3$\beta$-HSD, was detected only in the corpora lutea, but not in follicles excised at different stages of growth from the human ovary [14]. Thus, the detection of a significant increase in percentage of 3$\beta$-HSD-positive cells in either hCC or G-L cells collected at the periovulatory state in follicles yielding fertilizable oocytes may suggest that these follicles undergo adequate luteinization compared to follicles yielding oocytes that fail to undergo fertilization at IVF/ET.

In G-L cells, both $P_4$ secretion (Fig. 1) and the percentage of 3$\beta$-HSD-positive cells (Fig. 2) increased with elongation of the culture period. These observations are in agreement with previous studies. Thus, $P_4$ secretion in G-L cells did not decrease in the course of 5 days' culture [20] or longer [25]. The concomitant increase in both processes may suggest that the increase in $P_4$ secretion of G-L cells was a consequence of the increase in activity of 3$\beta$-HSD. In hCC, however, the increase in percentage of 3$\beta$-HSD-positive cells with elongation of the culture period (Fig. 2) was not associated with a parallel increase in $P_4$ secretion (Fig. 1). Furthermore, this pattern of change in hCC-$P_4$ secretion and 3$\beta$-HSD activity was not altered, whether or not the corresponding oocyte had been fertilized at IVF. The reason for the decrease in hCC-$P_4$ secretion and for the difference between hCC and G-L cells is not clear. However, a new series of studies has indicated a significant decrease in hCC-$P_4$ secretion which could be alleviated by supplying the cells with 20$\alpha$-hydroxycholesterol or low-density lipoprotein (C. Khoury and S. Bar-Ami, in preparation). Thus, these data may suggest that in the hCC there is insufficient endogenous cholesterol reservoirs or that cholesterol transport to the mitochondria is less efficient in long-term culture.

COC performance in terms of fertilizability and cleavability has also been studied in correlation with the steroid contents in FF and steroid secretion by G-L cells. There have been contradictory conclusions concerning the correlation between FF steroid contents and the fertilizability and cleavability of the corresponding oocyte. It is generally agreed that oocyte fertilizability is associated with a greater concentration of $E_2$ and/or a higher $E_2/P_4$ ratio [26–28]. However, some studies have indicated that the level of FF-$E_2$ concentration in follicles yielding oocytes that undergo cleavage and give rise to viable embryos is lower than the FF-$E_2$ in follicles yielding oocytes that fail to do so [29]. In other studies no correlation was found between FF-$E_2$ level and functionality of the corresponding oocyte [30]. Contradictory data have also been reported about a correlation between FF-$P_4$ level and functionality of the corresponding oocyte [5, 6, 26, 27, 30].

When oocyte functionality and serum $E_2$ and $P_4$ levels have been compared, a clear correlative trend has not been reported by various researchers. Thus, contradictory data have been demonstrated regarding a correlation between IVF/ET outcomes and the secretory patterns of these steroids [28, 31, 32].

Taken together, it is very hard to derive a strong connection between oocyte functionality and $P_4$ and $E_2$ levels in the FF of the corresponding follicles or in the systemic serum. The difficulty may be associated with the dynamic changes in $E_2$ secretion. Serum $E_2$, which progressively increases with follicular growth and maturation as well as shortly after HCG administration, significantly decreases at the time of oocyte pickup. Thus, when a low level of $E_2$ is measured, it is hard to associate it with the lower potential of the follicles to secrete steroids or with the decrease in $E_2$ level that occurs about 30 h following LH/HCG administration. On the contrary, in G-L cells the levels of $P$-$450_{SCC}$ and 3$\beta$-HSD/3-oxosteroid isomerase progressively increase, with a dramatic rise at the final stages of follicular growth and maturation and following LH/HCG stimulation. Therefore, differences in the degree of activity of these enzymes and cell potential for *in vitro* $P_4$ secretion in hCC and G-L cells should be a better candidate for evaluating oocyte functionality.

In small follicles, or follicles which did not reach their full size and maturity, LH is less effective in inducing granulosa cell luteinization [33], dispersion and mucification of the cumulus mass [33, 34], and oocyte meiotic maturation [33, 35]. In the follicles yielding mature mucified COC there is a correlation between the cumulus culture morphology and oocyte fertilizability and steroidogenic activity of the corresponding hCC and G-L cells [3, 8]. Figure 3 and Table 1 provide supplementary data indicating that the increase in percentage of 3$\beta$-HSD-positive cells in hCC and G-L cells as well as the rate of $P_4$ secretion in the hCC and G-L cells were significantly higher when the corresponding oocytes were fertilized. The increase in 3$\beta$-HSD activity in hCC and G-L cells in the presence of HCG or forskolin further supports our hypothesis that higher 3$\beta$-HSD activity manifests advanced luteinization of these cells. Therefore, as a

result of induction of the ovulatory process by HCG *in vivo*, the higher increase in percentage of 3β-HSD-positive cells should indicate that the follicle is sufficiently ripe to undergo proper luteinization of the follicular cell. Additionally, higher fertilizability of the corresponding oocyte is generally expected in mature follicles in response to HCG stimulation.

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