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ALTERED STEROIDOGENIC PATTERN OF HUMAN GRANULOSA-LUTEIN CELLS IN RELATION TO CUMULUS CELL CULTURE MORPHOLOGY*

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Summary—It has been reported that human cumulus–oocyte complexes (COC) retrieved at a stimulated cycle manifest an asynchrony between oocyte meiotic maturation and cumulus mucification. However, when mature COC were subdivided into subtypes marked by the culture morphology of their cumulus cells following 3 days' culture, successful fertilization and cleavage were approximately 1.5-fold lower in mature COC yielding cumulus cells aggregated into clumps (type A and B COC) than in mature COC yielding homogeneously spread cells (type C–D COC). To determine whether the existing relationship between cumulus culture morphology and oocyte functionality in the various COC types (A–D) could be extended to another follicular compartment—the granulosa-lutein (G-L) cells—basal steroid secretion by the corresponding G-L cells was evaluated within 5 days of culture. Over the first 3 days of culture, secretion of progesterone was 3-fold lower and secretion of testosterone (T) was 2.5-fold higher in cultures of G-L cells from follicles yielding type A COC than in type C–D COC. During days 4 and 5 of culture, G–L cells were incubated with or without 10^{-7} M 3β-hydroxy-5-pregnen-20-one (pregnenolone), dehydroepiandrosterone (DHA), 4-androstene-3,17-dione (androstenedione), or T. The pattern of progesterone level noted over the first 3 days of culture was not altered in the presence of pregnanolone, DHA, androstenedione, or T. Addition of pregnenolone, DHA, androstenedione, or T increased T level 2.5-, 5.6-, 7.3-, and 17.7-fold, respectively, in cultures of G-L cells from follicles yielding type A COC, but did not significantly alter T level in cultures of G-L cells from follicles yielding type C–D COC. In cultures of G-L cells from follicles yielding type A COC, addition of androgens unsaturated at position 4 preferentially increased oestradiol-17β (E_2) level, whereas in cultures of G-L cells of type C–D COC, DHA and androstenedione preferentially increased E_2 level. Taken together, the asynchrony between oocyte and cumulus activity could be diminished when the activity of various follicular cell compartments is evaluated according to cumulus culture morphology rather than cumulus expansion and mucification. The present study suggests that follicles yielding mature COC represent a non-homogeneous population in which G-L cells from follicles yielding type A–B COC manifest a less luteinized state than those from follicles yielding type C–D COC.

INTRODUCTION

Human *in vitro* fertilization/embryo transfer (IVF/ET) treatment involves the selection of mature follicles and oocytes. One of the criteria for maturity is the morphology of cumulus–oocyte complexes (COC). Thus, well-dispersed cumulus cells in a massively mucified cumulus may suggest that the human oocyte is mature and fertilizable [1–3]. Usually, granulosa cells exhibit increasing secretory levels of progesterone and oestradiol-17β (E_2) as the follicle matures, whereas following the endogenous surge of LH or after administration of LH/HCG, the granulosa cells of the Graafian follicle change their main steroidogenic secretory products from oestrogens to progestins [4]. However, in small- or medium-sized follicles, LH is less effective in inducing granulosa cell luteinization [5], dispersion and mucification of the cumulus mass [6], and oocyte meiotic resumption [7, 8].

In unstimulated cycles in women, the state of meiotic maturation and the degree of columus mass expansion and mucification are coupled [1]. However,

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in stimulated cycles an asynchrony between these two processes has been noted in some COC with fully expanded and mucified cumulus [2, 9]. Furthermore, although mature COC may appear similar at the time of aspiration, an inherent variation exists in their competence for fertilization and subsequent cleavage [1]. We recently reported a new approach to the classification of various types of mature COC in terms of competence for fertilization and subsequent embryonal cleavage [3]. Thus, mature COC were subgrouped by indirect means, according to the prospective culture morphology of their cumulus cells following 3 days' culture, as type A–D COC: type A, compact cumulus clumps; type B, partially spread clumps; type C, non-homogeneously spread cells; and type D, homogeneously spread cells. Fertilization and subsequent embryonal cleavage rates of type D COC were approximately 1.5-fold higher compared to type A COC [3]. Furthermore, secretion of progesterone during 3 days of culture was approximately 2-fold higher by cumulus cells of type D compared to type A. It was hypothesized that the type of culture morphology produced by the cumulus cell mass is determined by the degree of maturation and/or atresia of the follicle from which it originated. The present study was carried out in granulosa-lutein (G-L) cells obtained from individual follicles yielding mature COC. We attempted to correlate in a given follicle the basal secretion of progesterone, testosterone (T), and E_2 by G-L cells with the type of culture morphology (A–D) of the follicle's cumulus cells.

EXPERIMENTAL

Subjects

Women admitted to the IVF/ET programme at Rambam Medical Center who had normal menstrual cycles and whose husbands had been proven fertile were the subjects of this study. 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 gonadotrophin (Pergonal; Serano Laboratories, Randolph, Mass, U.S.A.) or 150 IU/day human FSH (Metrodin; Serno Laboratories) and 150 IU/day Pergonal. All women received 10,000 U HCG (Chorigon; Ikapharm) 36 h before aspiration of oocytes.

G-L cell cultures

G-L cells were isolated from the follicular fluid of individual follicles only when the accompanying COC was characterized as mature at the time of aspiration. The COC were removed and cultured under proper conditions for fertilization, and the follicular fluid of each individual follicle was centrifuged at 300 *g* for 10 min to recover G-L cells in the pellet. G-L cells were resuspended in culture medium consisting of Ham's F-10 medium (Gibco, Grand Island, N.Y., U.S.A.) buffered with 25 mM sodium bicarbonate (Gibco) and 10 mM Hepes (Gibco) and enriched with 1 mM glutamine (Gibco), 5% foetal calf serum (Sigma Chemical Co., St Louis, Mo., U.S.A.), and streptomycin (50 IU/ml)/penicillin (50 mg/ml) solution (Gibco). Red blood cells were allowed to sediment in 35-mm Petri dishes for 20 min, and the floating clumps of G-L cells were collected with a Pasteur pipette. This procedure was repeated 2–3 times until the preparation of G-L cells was relatively clear. Ten thousand viable G-L cells/200 μl were plated in 96-well plates (Falcon, Oxnard, Calif., U.S.A.), six wells per treatment group; cell viability was assessed using trypan blue for the dye-exclusion test [10].

In vitro incubation

G-L cells were cultured for 3 days in a humidified 5% CO_2–95% air incubator at 37°C. After 3 days, medium was collected and frozen at −20°C for steroid determination. G-L cell cultures were incubated for another 2 days in the presence of medium alone (control) or of 10^{-7} M of either 3β-hydroxy-5-pregnen-20-one (pregnenolone), dehydroepiandrosterone (DHA), 4-androstene-3,17-dione (androstenedione), or T (all from Sigma). Steroid hormone levels were also assayed in medium collected at the end of the incubation period and frozen at −20°C. Then, after G-L cells were detached following incubation of G-L cells for 20 min in a buffer consisting of 0.05 M phosphate-buffered saline, pH 7.4, with 1% (w/v) EDTA and 5% (v/v) dimethyl sulphoxide (both from Sigma), G-L cells from two wells were collected into a single Epindorph test tube. After centrifugation (600 *g* for 15 min at 22°C) and decanting of the supernatant, 50 μl of crystal violet (50 ng/100 ml in 0.1 M sodium citrate) were added for nuclear staining. Nuclei were counted in a haemocytometer after 10–20 min staining at 37°C; this method was reproducible.

COC culture

Oocyte IVF and embryonal cleavage were performed in Ham's F-10 medium buffered with 25 mM sodium bicarbonate and enriched with 2 mM pyruvate (Gibco), 10% human foetal cord serum, and streptomycin (50 IU/ml)/penicillin (50 mg/ml). Each individual COC was co-cultured with 100,000 motile spermatozoa for about 18 h in a separate culture dish to allow oocyte IVF. The oocyte was denuded of its cumulus-corona cells to view fertilization, which was marked by the appearance of 2 pronuclei in the ooplasm. The denuded oocyte was removed to another dish for the subsequent embryonal cleavage. The remaining cumulus cells were left for another 2 days in the original culture dish (i.e. 3 days' total culture period), and their culture morphology was evaluated as previously described [3] (Fig. 1).

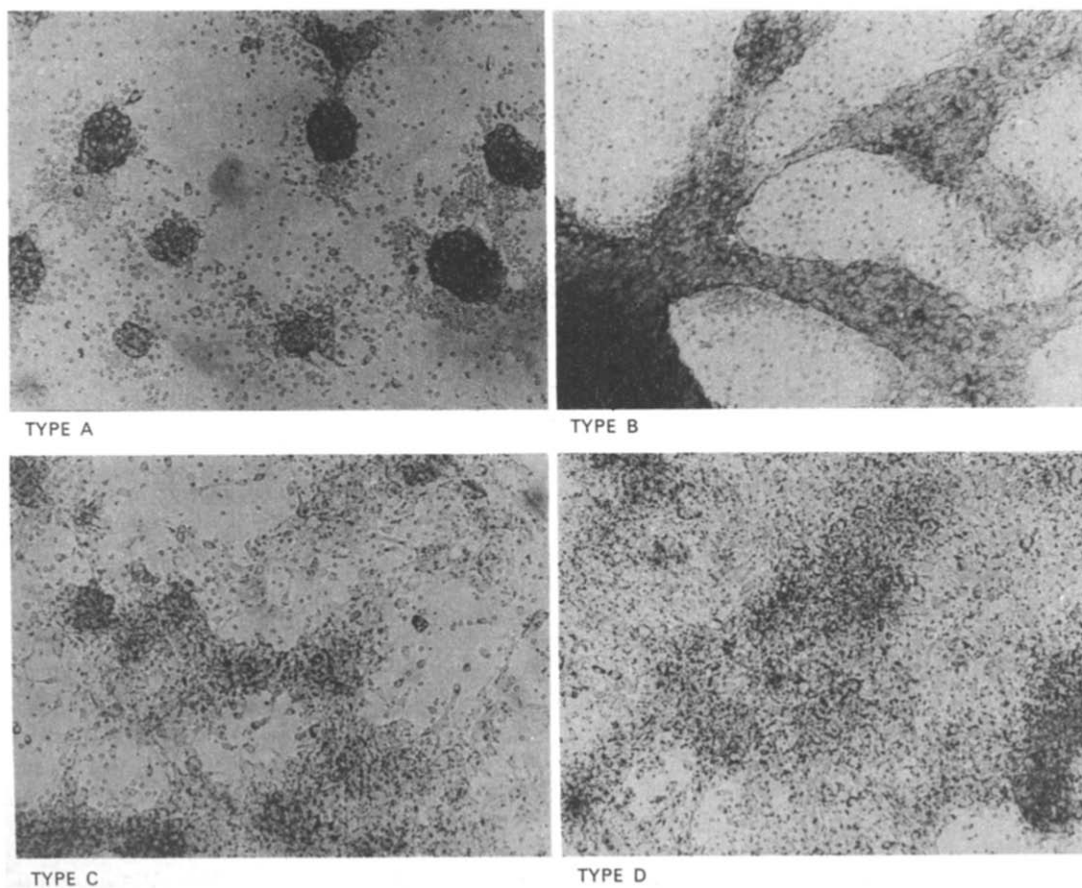

Fig. 1. Light photomicrographs of cumulus culture morphology following 3 days' culture. Each plate represents cumulus cells obtained from an individual mature COC. Each cumulus cell mass yielded a specific type of culture morphology: type A, compact clumps; type B, partially spread clumps; type C, non-homogeneously spread cells; and type D, homogeneously spread cells.

Radioimmunoassays (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. T was measured as described by Barkey *et al.*[11], using T-3-O-CMO-iodohistamine [^{125}I] (Nuclear Research Center-Negev, Beer Sheva, Israel) and rabbit anti-T-7α-bovine serum albumin antiserum (Bio-Yeda, Rehovot, Israel). The amounts of progesterone and E_2 were measured as described by Braw *et al.*[12], using tritiated hormones (Amersham Radiochemical Centre, Amersham, Bucks, England) labelled at six places and rabbit anti-progesterone or anti-E_2 (Bio-Yeda), respectively. These antisera have very low cross-reactivity (measured at 50% displacement), which did not exceed 2.1, 2.5, and 2.5% for the most similar steroid in T, progesterone, and E_2 RIA, respectively. Assay sensitivity and intra- and inter-assay coefficients of variation were as follows: T, 2 pg/ml, 3 and 7%; progesterone, 50 pg/ml, 2 and 8%; and E_2, 20 pg/ml, 4 and 12%, respectively.

Statistical analysis

Experimental data are presented as the mean $\pm$ SEM of measurements from 12–46 different follicles. Data were subjected to individual comparison between means, using unpaired Student's *t*-test. The level of statistical significance was set at $P < 0.05$.

RESULTS

Basal steroid secretion of G-L cells

For the purpose of simplicity, follicles yielding mature type A, B, C, or D COC after 3 days in culture were defined as type A, B, C, or D follicles, respectively. Secretion of progesterone, T and E_2 by G-L cells of a given follicle was evaluated in relation to the type of culture morphology of the follicle's COC (Fig. 2). Steroid secretion during the first 3 days of culture is presented per initial number of G-L cells inoculated, i.e. 10,000 cells per 200-μl well. Progesterone secretion was significantly greater in G-L cell cultures of type C–D than in G-L cell cultures of type A follicles (456 $\pm$ 78 vs 153 $\pm$ 27 ng/well, respectively; $P < 0.005$). T secretion showed an opposite pattern, exhibiting lower levels in G-L cell cultures of type C–D follicles than in type A (7.2 $\pm$ 1.2 vs

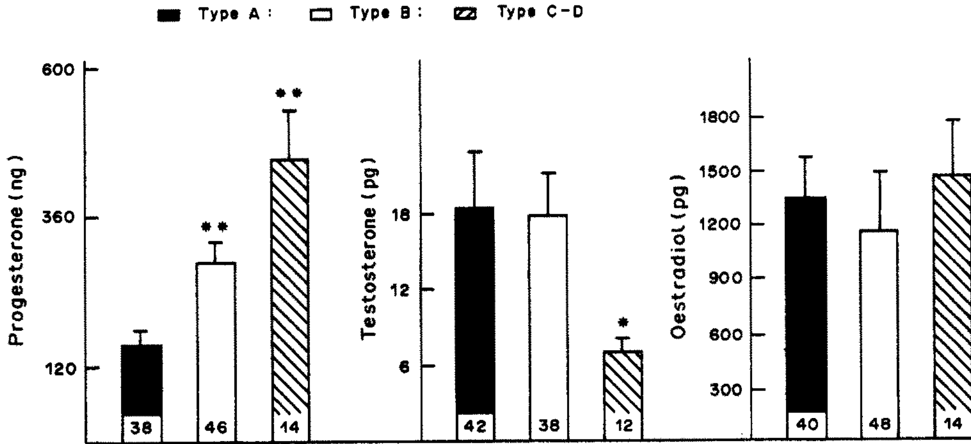

Fig. 2. Basal secretion of various steroids in culture by G-L cells obtained from individual follicles. In each follicle, G-L cells were defined according to the culture morphology of the yielded cumulus cells, as type A (■), type B (□), and type C–D (▨). G-L cells were carefully collected from individual follicles, and 10,000 viable G-L cells were plated per well. Data represent the mean ± SEM of total steroid secreted in each 200-μl well per 10,000 cells during 3 days' culture, with duplicate samples of each follicle (numbers at the base of each bar indicate the number of follicles tested). $^*P < 0.02$; $^{**}P < 0.005$ vs G-L cells from type A follicles.

18.6 ± 4.8 pg/well, respectively; $P < 0.05$). E_2 levels were comparable in all four follicular types. However, E_2/T ratio, which represents aromatase activity, was 203 in type C–D, which is about 3-fold higher than 65 and 72 in type B and A follicles, respectively.

During 5 days in culture, the percentage of G-L cells of type A, B, and C–D follicles increased from 100% (10,000 cells/well) to 119 ± 21, 148 ± 48, and $183 \pm 18\%$, respectively. Therefore, steroid accumulation during days 4 and 5 of the culture period was normalized per 10,000 cells for proper comparison of steroid output of G-L cells of the various follicular types. Rate of basal progesterone and T secretion remained unaltered during days 4 and 5 of culture, compared to the first 3 days of culture (Figs 2–4), whereas basal E_2 secretion was significantly reduced (Figs 2 and 5). Thus, G-L cells had an average E_2 level of 1332 ± 222, 1152 ± 140 and 1458 ± 426 pg/well during the first 3 days of the culture period in type A, B, and C–D follicles, respectively (Fig. 2), whereas E_2 values dropped to 304 ± 66, 252 ± 40, and 440 ± 76 pg/well during the last 2 days of culture (days 4 and 5) in type A, B, and C–D follicles respectively (Fig. 5).

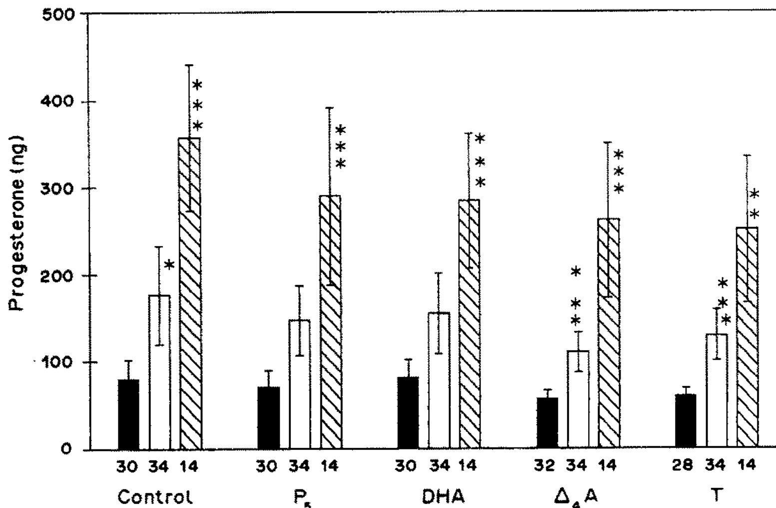

Fig. 3. Progesterone secretion by G-L cells of individual follicles during days 4 and 5 of the culture period in the presence of various steroid substrates: pregnenolone (P_5), dehydroepiandrosterone (DHA), 4-androstene-3,17-dione (Δ_4A), and testosterone (T). Data represent the mean ± SEM of total steroid secreted in each 200-μl well per 10,000 cells during 2 days' culture, with duplicate samples of each follicle (numbers at the base of each bar indicate the number of follicles tested). Other data are as described in the legend to Fig. 2. $^*P < 0.02$; $^{**}P < 0.01$; $^{***}P < 0.005$ vs G-L cells from type A follicles.

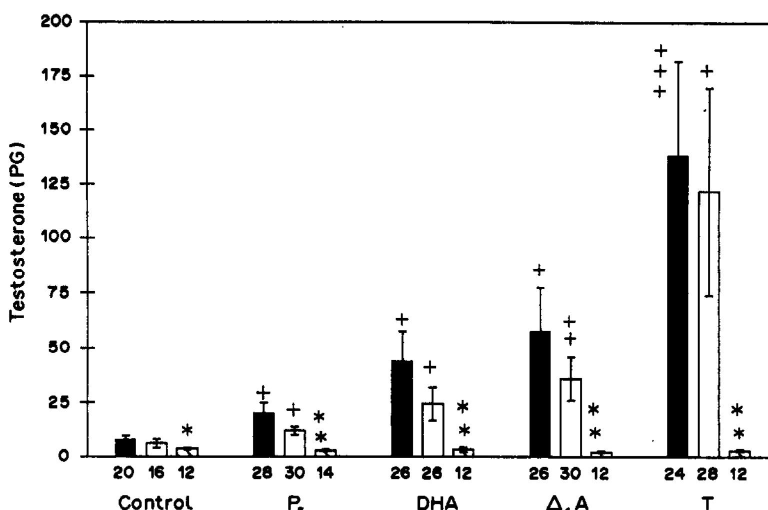

Fig. 4. Testosterone secretion by G-L cells of individual follicles during days 4 and 5 of culture. *$P < 0.05$; **$P < 0.02$ vs G-L cells from type A follicles. †$P < 0.05$; ††$P < 0.01$; ††† < 0.005 vs control G-L cells belonging to the same type of follicle. Other details are as described in the legends to Figs 2 and 3.

Steroid secretion by G-L cells in the presence of various steroid substrates

Levels of progesterone, T, and E_2 during 2 additional days of culture (days 4 and 5) in the presence of various steroid substrates were evaluated in relation to the type of culture morphology of the follicle's COC (Figs 3–5).

Progesterone level (Fig. 3) was significantly higher in cultures of G-L cells from type C–D than from type A follicles. This pattern was repeated in all treatment groups. Upon comparison of the effects of pregnenolone and the different androgen substrates on the control cultures of each morphological type, no significant change was noted in progesterone level by any of the substrates added.

Addition of pregnenolone or androgen substrates did not alter the T levels in the conditioned medium of G-L cells from type C–D follicles compared to control (Fig. 4). However, addition of pregnenolone, DHA, androstenedione, and T increased T levels approximately 2.5- ($P < 0.05$), 5.6- ($P < 0.02$), 7.3- ($P < 0.02$), and 17.7-fold ($P < 0.005$), respectively, in cultures of G-L cells from type A follicles, as well as 2- ($P < 0.02$), 4- ($P < 0.02$), 6- ($P < 0.01$), and 20.3-fold ($P < 0.02$), respectively, in cultures of G-L cells from type B follicles, compared to the corresponding control group (Fig. 4).

Addition of pregnenolone, DHA, androstenedione, and T increased E_2 level approximately 1.9- ($P < 0.05$), 6.3- ($P < 0.001$), 8.1- ($P < 0.001$), and 9.4-fold ($P < 0.001$), respectively, compared to the control group, in the conditioned medium of G-L cells from type A follicles. In cultures of G-L cells

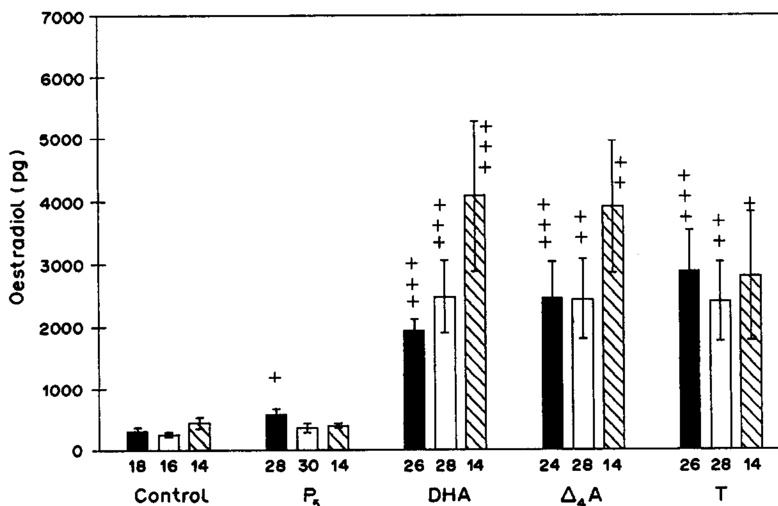

Fig. 5. Oestradiol-17β secretion by G-L cells of individual follicles during days 4 and 5 of culture. †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$ vs control G-L cells belonging to the same type of follicle. Other details are as described in the legends to Figs 2 and 3.

Table 1. Change in oestradiol-17β/testosterone (E_2/T) ratio in granulosa-lutein (G-L) cells in relation to type of cumulus culture morphology

Addition	Type of cumulus culture morphology A	B	C–D
None	51 ± 19	46 ± 17	122 ± 31*
Dehydroepiandrosterone	44 ± 16	101 ± 39	1243 ± 593*
4-Androstene-3,17-dione	43 ± 18	67 ± 25	1774 ± 707†
Testosterone	21 ± 9	18 ± 8	583 ± 336

*$P < 0.05$. †$P < 0.02$. P values refer to differences from E_2/T ratio in G-L cells collected from follicles yielding type A columus.

from type C–D follicles, addition of pregnenolone, DHA, androstenedione, and T increased E_2 level approximately 0.9- (not significant), 9.3- ($P < 0.005$), 8.8- ($P < 0.005$), and 6.4-fold ($P < 0.05$), respectively, compared to the control group (Fig. 5). The E_2/T ratio, calculated from the basal secretions of E_2 and T, was 51 in type A, 46 in type B, and 122 in G-L cell cultures from type C–D follicles. Addition of DHA, androstenedione, and T significantly increased the E_2/T ratio to 1243, 1774, and 583, respectively, in G-L cell cultures from type C–D follicles. However, these androgen substrates barely altered the E_2/T ratio, compared to the corresponding control group, in G-L cell cultures from type A or B follicles (Table 1).

Table 2 shows the absolute amount of steroid measured in culture medium before the amount of steroid was normalized per 10,000 cells. Data in Table 2 suggest that the added androgen substrates at a concentration of 10^{-7} M (approx. 5.8 ng/200-μl well) were entirely metabolized into E_2 in G-L cells of type C–D follicles. However, in G-L cells of type A follicles, between 60% (DHA) and 40% (T) of the added androgen was not utilized for E_2 production (Table 2).

DISCUSSION

The morphological state of the cumulus mass in terms of its expansion and mucification has been used to determine the maturity of the COC in mammals such as rat [13] and human [1, 2]. In mature COC, fertilization and cleavage rates were found to be 2.5- and 3.5-fold higher, respectively, than in immature COC [3]. A more refined morphological survey of cumulus cells revealed different types of morphology of COC which were considered mature at the time of aspiration by the conventional methods [3]. The present study was initiated to explore a possible relationship between the morphological subdivisions of mature COC and the steroidogenic activity of the relevant G-L cells. High amounts of basal progesterone are known to be produced by human G-L cells in culture over an 8-day period [14], in amounts comparable to our study. When comparing the steroid outputs of G-L cells after 3 days in culture, the higher accumulation of progesterone and the diminution of T seen in cultures of G-L cells from type C–D follicles are similar to the mode of steroidogenic pattern of granulosa cells of a mature Graafian follicle following the LH surge or HCG/LH administration [15]. On the other hand, the steroidogenic pattern of G-L cells obtained from type A or B follicles is similar to that of less mature follicles [15]. Thus, granulosa cells of the Graafian follicle respond to HCG administration by an acute decrease in both 17α-hydroxylase and 17,20-lyase, causing a decrease in androgen secretion [16, 17], and an increase in cholesterol side-chain-cleavage cytochrome P450, causing an increase in progestins secretion [18, 19]. A similar change in the steroidogenic pattern might be happening in the G-L cells and cumulus cells from type C–D but not type A–B follicles.

The higher E_2/T ratio in cultures of G-L cells from type C–D vs type A follicles (Table 1) may suggest a higher aromatase activity in G-L cells from type C–D follicles. In accordance with reports by other researchers [15, 20], who consider the degree of aromatase activity to be a criterion of follicular maturity, we may conclude that type C–D follicles are more mature than type A–B follicles. The higher fertilization and cleavage rates in oocytes of type C–D follicles [3] may provide further support for our conclusion. Incubation for 2 additional days (days 4–5 of the culture period) showed no apparent change in progesterone and T secretion by G-L cell cultures of any of the follicular types (A–D) compared to days 0–3. Basal E_2 secretion during days 4 and 5 of culture diminished between 3-fold (type A–B) and 2-fold (type C–D), as compared to basal E_2 secretion during the first 3 days of culture, suggesting a decrease in the degree of, or a reduction in, the supply of androgen-supported aromatase activity. A 2-fold decrease in aromatase activity of human G-L cells during long-term culture was also reported by Bernhisel *et al.* [21].

Addition of pregnenolone after 3 days of culture significantly increased T and E_2 levels 2.6- and 1.9-fold, respectively, in cultures of G-L cells from type A follicles and 2.2- and 1.4-fold, respectively, in G-L cells from type B follicles. However, no apparent changes in T or E_2 levels were noted when pregnenolone was added to G-L cells from type C–D follicles. This difference may indicate that in G-L cells from type A–B follicles, pregnenolone is metabolized into androgens and oestrogens, whereas in G-L cells from type C–D follicles pregnenolone is not metabolized into T and subsequently into E_2, probably due

Table 2. Absolute amount of oestradiol-17β (E_2) in granulosa-lutein (G-L) cell culture media following 2 days' culture with various steroid substrates

Addition	E_2 secretion (pg) by G-L cells of follicles yielding cumulus cells of type A	B	C–D
None	362	360	792
Pregnenolone	685	512	706
Dehydroepiandrosterone	2299	3541	7336
4-Androstene-3,17-dione	2923	3483	7027
Testosterone	3418	3432	5050

to the decrease in 17α-hydroxylase and 17,20-lyase activity. Hence, the difference in pregnenolone metabolism may lend further support to the concept of inadequate luteinization of G-L cells from type A–B follicles.

Addition of various androgen substrates (approximately 5.8 mg/200-μl well) caused a selective and significant increase in T level in G-L cells from type A and B follicles compared to control, but no noticeable change in T level in G-L cells from type C–D follicles. Actually, in cultures of G-L cells from type C–D follicles, the entire amount of the added DHA or androstenedione and almost all the added T were metabolized into E_2, as seen in Table 2. In G-L cells from type A follicles, the added androgen substrates were only partially utilized for E_2 production (DHA, 40% utilization; androstenedione, 50% utilization; T, 60% utilization) (Table 2). The reduced metabolism of the various androgens into E_2 by G-L cells from type A-B follicles was partially associated with the limited increase in cell number (1.3-fold) compared to the greater increase in cell number (1.8-fold) in G-L cells from C–D follicles. However, Fig. 5 indicates that a reduced production of E_2 per 10,000 cells was noticeable in G-L cells from type A–B compared to G-L cells from type C–D follicles. Thus, a lower aromatase activity might be the reason for the decrease in E_2 production in G-L cells from type A follicles, as suggested by the E_2/T ratio presented in Table 1. Since 60% of added T was metabolized into E_2 in the G-L cells from type A follicles, the actual measured level of T was approximately 11 times smaller than the amount of T not utilized for E_2 biosynthesis: approximately 180 vs 2000 pg, respectively. Probably, the T that was not utilized for E_2 production was metabolized into other undefined steroids. The preferential utilization of DHA by G-L cells from type C–D follicles and of T by G-L cells from type A–B follicles, compared to the other androgen substrates, may suggest that in less luteinized G-L cells (type A–B), androgens unsaturated at position 4 may be preferred substrate.

Addition of androgen substrates after 3 days of culture did not alter the pattern of progesterone secretion, which remained similar to that of untreated G-L cells, i.e. increasing amounts of progesterone in G-L cells from type C–D vs type A follicles. The lack of influence of androgens on progesterone secretion has been documented by others [21], although in rodents androgens alone or with FSH have been shown to stimulate progesterone secretion [22, 23]. Progesterone level was not significantly altered in conditioned medium of G-L cells cultured in the presence of pregnenolone, probably due to the minute amount added (10^{-7} M ≅ 5.8 ng/200-μl well).

The greater secretion of progesterone by G-L cells from type C–D follicles may suggest that these follicles could form a corpus luteum with a more appropriate steroidogenic activity than in type A–B follicles. Preliminary studies in our laboratory may have shed more light on this issue. In G-L cell cultures from type C–D follicles, LH and FSH induced 3- and 1.4-fold increases, respectively, in progesterone secretion. However, LH caused almost no effect, and FSH caused a 40% decrease in progesterone secretion in G-L cells from type A follicles (S. Bar-Ami, D. Aminpoor, H. Gitay-Goren and J. M. Brandes, in preparation). This finding may suggest that type A–B follicles will develop into poorly active corpora lutea in relation to their impaired progesterone secretion, which may be inadequate to support embryonal implantation.

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