ANDROLOGY

Effect of Androgen Substrates on the Steroidogenic Pattern of Cumulus Cells: Correlation with Cumulus Culture Morphology

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Background: *In previous studies, higher progesterone secretion was observed in mature versus immature cumulus–oocyte complexes. In mature cumulus mass that become homogeneously spread in culture (type C/D) progesterone secretion was higher than in partially (type B) or totally (type A) aggregated morphology. In sharp contrast, estradiol-17β secretion was significantly higher in type A than type C/D cumulus.*
Purpose: *Our purpose was to assess whether the decreased estradiol-17β level in type C/D cumulus culture is caused by deficiency of substrates.*
Methods: *The different cumulus types were incubated with or without 10^{-7} M dehydroepiandrosterone, 4-androstane-3, 17-dione, or testosterone. The levels of estradiol-17β, testosterone, and progesterone, were measured after 24 hr of culture.*
Results: *The addition of dehydroepiandrosterone or 4-androstane-3,17-dione significantly increased the estradiol-17β levels in all types of cumulus cells, whereas the addition of testosterone was less effective. In all types of cumulus cells the testosterone levels increased significantly on adding these androgen substrates. In the type C/D cumulus, the testosterone increased to lower levels compared to type A cumulus cells. In the presence of these androgens progesterone secretion is significantly reduced in type A cumulus cells. In type C/D cumulus cells, however, progesterone levels were significantly higher than in type A. The estradiol-17β/testosterone and progesterone/estradiol-17β ratios, which partially resemble the degree of aromatase activity and the degree of selectivity for progesterone secretion, respectively, were higher in type C/D than in type A cumulus cells.*
Conclusions: *In type C/D cumulus the significant increase in estradiol-17β secretion in the presence of various androgens suggests that, under basal conditions, androgen is less available for estradiol-17β biosynthesis compared to type A cumulus. Furthermore, the higher progesterone secretion in type C/D cumulus may suggest that the follicles yielding type C/D cumulus cells are more mature than the follicles yielding type A cumulus.*

KEY WORDS: androgen; cumulus cells; estradiol-17β; progesterone.

INTRODUCTION

Following the ovulatory surge of lutenizing hormone (LH) and follicle stimulating hormone (FSH), the cumulus–oocyte complex (COC) of the preovulatory follicle undergoes dramatic changes, including cessation of contact between the cells of the cumulus mass and between the oocyte and the cumulus cells, resumption of meiotic maturation, accumulation of hyaluronic acid (1,2), and an increase in progesterone (P_4) secretion (3). In small or medium-sized follicles, however, LH is less effective in inducing similar changes in the cumulus mass (4) and oocyte (5,6). In addition, when immature or intermediate COC are retrieved, the cor-

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responding cumulus cells secrete less P_4, and their steroidogenic pattern is different from that of cumulus cells of the mature COC (7). Furthermore, in the immature or intermediate COC, the corresponding oocyte has lower fertilization and cleavage rates than mature complexes (8,9). Only occasionally is a viable pregnancy obtained from an immature or intermediate COC (8,10,11).

In previous studies we demonstrated that in a stimulated cycle the mature COC manifest a nonhomogeneous population in terms of in vitro fertilization/embryo transfer (IVF/ET) outcomes. Characterization of the mature COC population according to the culture morphology of the corresponding cumulus cells suggested a subdivision of this COC population and their corresponding follicles (7,12) These subdivided COC yielded cumulus cells that differed in their potential to secrete various steroids. The cumulus masses that became homogeneously spread in culture (type C/D) secreted significantly lower estradiol-17β (E_2) than the cumulus masses producing aggregated morphology (type A) (7). The present study was undertaken to evaluate whether the addition of androgen substrates would overcome the differences between type A and type C/D cumuli in E_2 secretion. For this purpose we have used a model system that was successfully applied previously for human granulosa-lutein (G-L) cells (12). The degree of E_2 secretion was evaluated using three androgens unsaturated at either position 4 or position 5.

MATERIALS AND METHODS

Subjects

Women with normal menstrual cycles who were undergoing IVF/ET due to infertility caused by absent or blocked fallopian tubes participated in this study. Multiple follicular growth was induced with FSH (Metrodin; Teva, Petah Tiqva, Israel), 150 IU/day, plus human menopausal gonadotropin (Pergonal; Teva), 150 IU/day. Ovulation processes were induced with one intramuscular injection of 10,000 U human chorionic gonadotropin (hCG; Chorigon; Teva).

In Vitro Incubation

Human COC were collected 36 hr after hCG administration, usually between 0800 and 1200. The complexes were placed immediately in 1 ml of culture medium (see below), each individual COC in the central well of a separate organ-culture dish (Falcon), and cultured at 37°C in 5% CO_2–95% air. Eighteen to twenty hours after the addition of sperm, the oocytes were mechanically denuded of their corona cells to view fertilization and transferred to a new dish filled with fresh medium for further embryonic development. Each dish containing the cumulus cells originated from individual COC was incubated for 2 additional days (3 days total) to evaluate morphology of cumulus cells. Grading of COC for degree of maturation and examination of cumulus culture morphology were carried out as described previously (7,12).

Following the first 3 days of culture, the cumulus cells were cultured for an additional day in the presence of medium alone (control) or of a 10^{-7} *M* concentrations of one of three androgens: dehydroepiandrosterone (DHA), 4-androstene-3,17-dione (An), or testosterone (T) (all from Sigma Chemical Co., St. Louis, MO, U.S.A.).

Medium Preparation

The culture medium consisted of Ham's F-10 powder (GIBCO, Grand Island, NY, U.S.A), 9.8 g/L, buffered with sodium bicarbonate (GIBCO), 2.1 g/L, achieving a pH of 7.4, and supplemented with antibiotic solution (GIBCO) to achieve final concentrations of 50 IU/ml penicillin and 50 μg/ml streptomycin. During days 0–3 of culture, this medium was also supplemented with 2 m*M* calcium lactate (Calbiochem–Behring, La Jolla, CA, U.S.A) and inactivated human fetal cord serum (10%, v/v). Preparation of human cord serum was as described previously (7). On day 4 the cells were cultured in a nonidentical medium from which lactic acid and human fetal cord serum were omitted but which was supplemented with 2 m*M* glutamine, 10 m*M* Hepes (GIBCO), and inactivated fetal calf serum (GIBCO) (5%, v/v). The ingredients of either medium were dissolved in purified water (BDH Chemicals, Poole, U.K.) to achieve 280–290 mOsm/kg. The media were then sterilized through 0.22-μm filters, 0.5-L size (Nalge Co., Rochester, NY, U.S.A.).

Radioimmunoassay (RIA) for Steroids

The media collected at the end of the incubation period (day 4 of culture) were preserved frozen at −20°C for later determination of steroid hormone levels. Steroid RIA was employed on the unextracted crude samples. The crude culture media were diluted in 50 m*M* Tris buffer, pH 8.0. T was measured as described by Barkey *et al.* (13) 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 P_4 and E_2 were measured as described previously (7).

Cell Harvest and Counting

At the end of the culture period, the culture media were removed for RIA, and the cumulus–corona cells were collected and counted. Detachment of cumulus cells was achieved by incubation for 20 min in a buffer consisting of 0.05 *M* phosphate-buffered saline, pH 7.4, with 1% (w/v) disodium EDTA and 5% (v/v) dimethyl sulfoxide (both from Sigma). The cumulus cells obtained from each individual COC were collected into a single Eppendorf test tube. After centrifugation (600*g* for 15 min at 22°C) and decanting of the supernatant, 50 μl of crystal violet (50 mg/100 ml in 0.1 *M* trisodium citrate) was added for nuclear staining. Nuclei were counted in a hemocytometer after 10–20 min of staining at 37°C; this method was reproducible and produced results similar to those obtained by counting unstained intact granulosa cells from rats, pigs, and humans.

Data Analysis

Statistical analysis was conducted with Student's *t* test for independent means. Results are expressed as means ± SE.

RESULTS

E_2 secretion was four fold lower ($P < 0.05$) in type C/D versus type A cumulus cells. The addition of the various androgens significantly increased the E_2 level in all types of cumulus cells. However, the addition of T was less effective in increasing E_2 secretion than were the other two androgens (Fig. 1). In DHA-, An-, and T-treated cells versus untreated cells, the E_2 level increased by 16-fold ($P < 0.001$), 18-fold ($P < 0.001$), and 3.2-fold ($P < 0.001$), respectively, in type C/D cumulus cells, compared to 2-fold ($P < 0.01$), 3.5-fold ($P < 0.001$), and 1.8-fold ($P > 0.05$), respectively, in type A cumulus cells (Fig. 1).

In the basal condition the T level is 1.8-fold higher ($P < 0.05$) in type C/D compared to type A cumulus cells (Fig. 2). The addition of the various androgens significantly increased the T level in all types of cumulus cells. However, the addition of DHA, An, or T increased T levels to only 2.2-fold ($P < 0.05$), 3.9-fold ($P < 0.05$), and 9.8-fold ($P < 0.01$), respectively, in type C/D cumulus cells, versus 8.9-fold ($P < 0.001$), 10.4-fold ($P < 0.02$), and 36-fold ($P < 0.001$), respectively, in type A cumulus cells (Fig. 2).

In type C/D cumulus P_4 accumulation in the conditioned medium was 1.4-fold ($P > 0.05$) higher than in type A cumulus cultures (Fig. 3). Addition of the various androgens significantly decreased P_4 secretion in type A cumulus cells. However, in type C/D cumulus

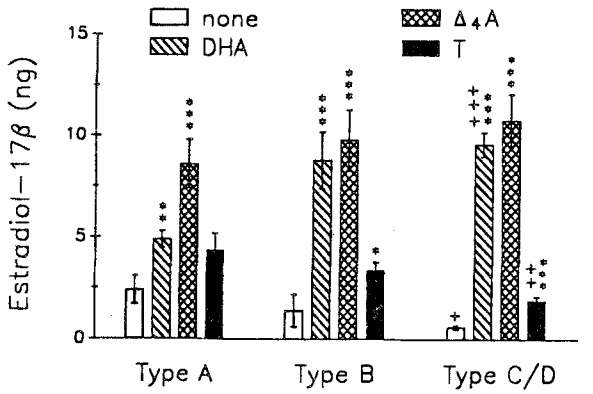

Fig. 1. Accumulation of estradiol-17β in conditioned medium of cumulus cells that were cultured with androgens for 1 day. initially each mature cumulus mass was cultured individually for 3 days in medium alone. At the end of the first 3 days of culture, each cumulus mass was subdivided according to the culture morphology of the cumulus cells as indicated at the bottom of the figure (i.e., type A, type B, and type C/D). Then the culture medium was replaced with fresh medium with or without the various androgen substrates. Data represent the mean ± SE E_2/ml. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to cumulus cells of the same type that were cultured in medium alone. †$P < 0.05$, ††$P < 0.01$, and †††$P < 0.001$ compared to type A cumulus cells that were subjected to a similar in vitro treatment.

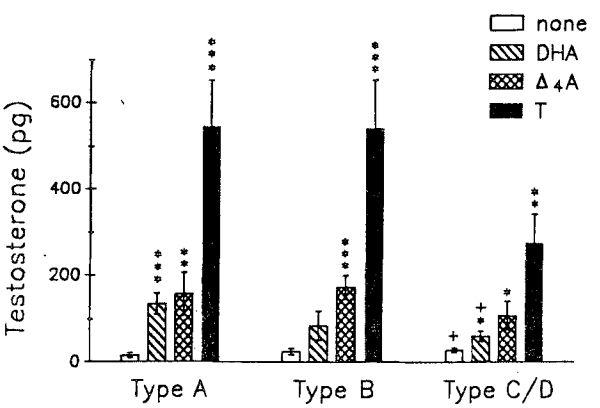

Fig. 2. Levels of testosterone in the conditioned medium of cumulus cells. Other details are as described in the legend to Fig. 1.

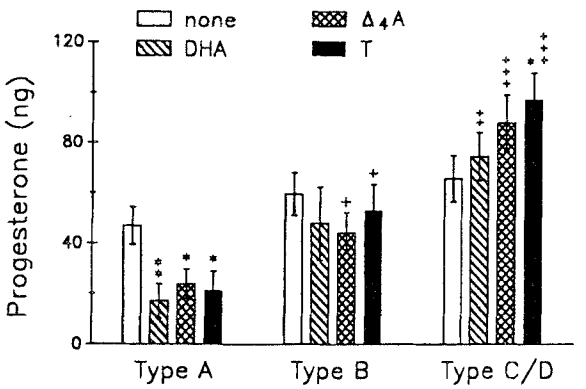

Fig. 3. Levels of progesterone in the conditioned medium of cumulus cells. Other detail are as described in the legend to Fig. 1.

cells, addition of these androgens increased P_4 secretion to levels which were significantly higher ($P < 0.001$) than in type A cumulus cells (Fig. 3). In type C/D cumulus, only in the presence of T did P_4 secretion significantly increased versus cells cultured in medium alone ($P < 0.05$) (Fig. 3).

In type A cumulus cells cultured in medium alone, the P_4/E_2 ratio was 23.7. This value increased by two- and five fold in type B and type C/D, respectively, compared to type A cumulus cells. The addition of androgen substrates significantly reduced the formal values of the P_4/E_2 ratio in all the cumulus types. This reduction in the P_4/E_2 ratios is caused by the increase in E_2 values due to the metabolism of the androgen substrates into E_2. In the presence of the various androgens, the pattern of P_4/E_2 ratios in the various types of cumulus cells was similar to that of cells cultured in medium alone (Table I). The E_2/T ratio was significantly higher in type C/D cumulus cells treated with DHA or An (Table II). However, E_2/T values were

Table I. Change in P_4/E_2 Ratio in Human Cumulus Cells in Relation to Type of Cumulus Culture Morphology and Substrate Added to the Culture Medium[a]

	Type of cumulus culture morphology		
Substrate added	A	B	C/D
DHA	3.5	5.4	7.7
An	2.8	4.5	8.1
T	4.8	15.6	51.1

[a] The individual cumulus masses were initially cultured for 3 days in medium alone. At the end of this culture period the cumulus masses were subdivided according to their culture morphology and cultured for another 24 hr in the absence or presence of different androgen substrates. Following this 24-hr culture period the levels of P_4 and E_2 were measured in the conditioned medium.

Table II. Change in E_4/T Ratio in Human Cumulus Cells in Relation to Type of Cumulus Culture Morphology and Substrate Added to the Culture Medium[a]

	Type of cumulus culture morphology		
Substrate added	A	B	C/D
DHA	36.3	103.5	158.7
An	54.4	56.5	99.8
T	8	6.3	6.9

[a] Levels of E_2 and T were measured in the conditioned medium of the cumulus cells following 24 hr of incubation between day 3 and day 4 of culture. Other details are as described in Table I, footnote *a*.

dramatically reduced in all types of cumulus cells that were treated with T (Table II). No significant difference was noted in the cumulus cell number after exposure to the various androgens (data not shown).

DISCUSSION

In previously reported studies the retrospective subdivision of the mature COC by their 3-day culture morphology correlated with the steroidogenic pattern of the cumulus cells and the developmental competence of the corresponding oocytes (7). The present study demonstrated that steroidogenic patterns observed in the cumulus cells when exposed to the various androgens are also altered in correlation with the type of culture morphology in the cumulus cells. Although in each specific cumulus type the different androgens produced the same type of change in steroid secretion, the various androgens differed in the quantity of the effect. This difference among the various androgens could be related to the significant role or to the availability of the particular androgen in the steroidogenic pattern in the specific cumulus type.

The capacity of human cumulus cells to secrete various steroids has been studied over the last two decades in various culture systems (7,14–16). It has been demonstrated that in the rat the cumulus cell mass acquires the capacity to synthesize P_4 just after germinal vesicle breakdown in the corresponding oocyte (17). In human COC, meiotic resumption of the corresponding oocyte is associated with greater P_4 secretion by the cumulus cells (14,18). In addition, P_4 secretion is increased with progress in morphological maturity from compact cumulus mass to mucified dispersed complex (7,15).

In the present study, type C/D secreted a higher but not a significantly different level of P_4 than type A

cumulus cells. A higher, significant P_4 secretion by type C/D versus type A cumulus cells was shown previously (7). It is possible that the difference in P_4 secretion between type C/D and type A is magnified in a culture period longer than 1 day, as noted in our previous study in which cumulus masses were cultured for 3 days (7). It is also possible that the nonsignificant difference in P_4 secretion that was diagnosed in the present study was due to the decrease in P_4 secretion over a long culture period, as described previously (19). Thus, the initial significant difference in P_4 secretion between type A and type C/D was diminished after 3 days of culture, probably due to lack of further stimulation.

The difference in P_4 secretion between type A and type C/D cumulus cells became highly significant when the cumulus cells were exposed to the various androgens, and more dramatically in the presence of T. Actually, the effect of androgens on P_4 secretion changed from inhibition in type A to stimulation in type C/D cumulus cells. The basis for androgenic action is dependent primarily on the presence of an androgen-responsive system. In humans, androgen receptors have been detected in granulosa cells of the secondary or larger follicles but are missing from primordial or primary follicles (20). The density of androgen receptors is maximal in the newly formed corpora lutea but is reduced thereafter (20).

Blocking of the androgen receptors by cyproterone has been found to reduce significantly the utilization of pregnenolone for P_4 biosynthesis in human ovarian slices within 3 hr of incubation (21). Lack of androgenic action has been noted in human granulosa cells in long-term culture (22). T enhances the FSH-induced P_4 secretion in primate granulosa cells (23). Androgens, via androgen receptors, participate with the FSH-induced degradation of lipoproteins and in utilization of the cholesterol ester core for the biosynthesis of progestin in rat granulosa cells (24). Also in rat granulosa cells (25) and in the luteal cells of pregnant rat (26), androgen enhances P_4 secretion. In the rat luteal cell, androgen enhances cholesterol biosynthesis from acetate and mobilization of cholesterol ester, which leads to more P_4 secretion (26). These various reports of the androgenic stimulation of P_4 biosynthesis are in agreement with the present finding of the enhancement of P_4 secretion in type C/D cumulus cells.

During the first 3 days after COC retrieval in both the type A cumulus cells (7) and the corresponding G-L cells (12), steroidogenic activity manifests a poor response to in vivo hCG stimulation; the opposite has been noted in type C/D cumulus cells (7) and the corresponding G-L cells (12). In this context, there is a resemblance between the cumulus cells and the corresponding G-L cells regarding steroidogenic activity. A similarity in the steroidogenic patterns of the cumulus and G-L cells was also noted when the culture period was elongated for an additional day (present study) or 2 more days (12), respectively. Thus the basic differences in the steroidogenic patterns of the different types of cumulus cells and the corresponding G-L cells were not altered in the presence of androgen substrates.

In polycystic ovary syndrome, follicular development is arrested at 4–7 mm (27,28), which is associated with an increase in androgen levels and a decrease in P_4 and E_2 secretion (29). A similar situation exists in the population of small follicles during the luteal phase of normally cycling women (30). A comparable pattern of steroid levels in follicular fluid has been noted in follicles yielding either immature or failed fertilization oocytes in gonadotropin-stimulated cycles (31). Taken together, the steroidogenic pattern of type A cumulus cells resembles the steroidogenic pattern of growing follicles and, to some extent, small follicles. Similarly, it has been postulated that the COC collected from not fully mature follicles probably yield the type A cumulus cells (7,12).

How androgens inhibit P_4 secretion in type A cumulus cells is not clear. We may suggest an explanation by assuming a similarity between type A cumulus cells and granulosa cells of immature or small follicles. The latter cell type contains a low receptor density for P_4 and androgens and a high receptor density for estrogen (20,32). A high estrogen level is inhibitory of P_4 secretion (33,34). Assuming that type A cumulus cells contain a level of estrogen receptor as high as in granulosa cells of growing follicles, then the estrogen produced from the androgen substrates may inhibit P_4 secretion. In type C/D cumulus cells, the addition of T significantly increased P_4 secretion (Fig. 3) but produced a smaller effect on E_2 secretion (Fig. 1). Assuming that type C/D cumulus cells have the same characteristics as granulosa cells from mature follicles, then the ineffectiveness of E_2 produced when An or DHA was added might be associated with the reduction of estrogen receptor density in mature follicles that were exposed to hCG stimulation (32).

In all types of cumulus cells, An was equally available for E_2 biosynthesis. This finding may suggest that the activity of aromatase is not significantly different in the various cumulus types. Nevertheless, in type A cumulus cells, DHA and T were less available for E_2 biosynthesis as compared to An. Actually, less than 10% of the 10^{-7} *M* T (about 15 ng/dish) was utilized

for the biosynthesis of E_2 in type C/D cumulus cells. On the other hand, about two-thirds of the added An or DHA was utilized for E_2 biosynthesis. This finding may suggest that in type C/D cumulus cells, androgens which are unsaturated at position 4 are less available for E_2 biosynthesis. A similar finding was deduced for human G-L cells which had been collected from follicles yielding type C/D cumulus cells (12). Assuming that progressively mature follicles may yield type C/D cumulus, the preferential utilization of delta 5 androgen may suggest that the maturation or luteinization of the follicle is associated with a shift to the delta 5 pathway.

Human theca interna obtained from preovulatory follicles preferentially metabolized pregnenolone almost entirely into DHA, compared to very little into An (35). This finding may provide other evidence to support our conclusion that E_2 is preferentially biosynthesized either in the cumulus cells (present study) or in human G-L cells (12) from delta 5 androgens. Actually, the steroidogenic pattern in the luteal phase is in favor of the delta 5 pathway compared to the delta 4 pathway in the follicular phase of the human ovary (36). Thus, in type A cumulus cells and the corresponding G-L cells, the lower ability to utilize DHA for E_2 biosynthesis may suggest that the shift into the luteal type of steroidogenesis is not completed.

The importance of cumulus steroidogenesis may be assumed at the time that the COC passes through the fallopian tube and during fertilization. In both processes the effect of steroids has been documented in many studies (37,38). The present study may suggest that lower E_2 secretion in type C/D cumulus cells is due to a lower access to androgens. Furthermore, the initial difference between the various types of cumulus cells with regard to the steroidogenic pattern could not be changed into equal patterns by exogenously supplied androgens.

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