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FOLLICULAR FLUID STEROID LEVELS IN RELATION TO OOCYTE MATURITY AND *IN VITRO* FERTILIZATION

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Summary—Steroid levels in follicular fluid (FF) obtained from stimulated ovaries in patients undergoing *in vitro* fertilization (IVF) were measured by capillary gas chromatography. The correlation between these levels and the maturity of the oocyte, judged from the morphology of the oocyte corona cumulus complex (OCCC) and the fertilizability of the oocytes was analysed. Oocyte maturity was associated with higher FF levels of progesterone, 17-hydroxyprogesterone, 16α-hydroxyprogesterone and 20α-dihydroprogesterone. Follicular fluids containing oocytes that became fertilized had significantly higher levels of 20α-dihydroprogesterone and progesterone and lower levels of androstenedione. Of all the steroids determined, 20α-dihydroprogesterone provides the most significant group differences. Enhanced 20α-dihydrogenation in the presence of decreased 16α- and 17-hydroxylation appears to be an important characteristic of the ultimate ripening stages and early luteinization, at least in stimulated cycles.

INTRODUCTION

Oocyte maturity, as judged from the OCCC morphology, is related to successful fertilization *in vitro*. The OCCC morphology however is a subjective parameter and does not always correlate with the nuclear oocyte maturity. Several authors [1–11] have suggested that follicular fluid steroid levels can be useful for estimating maturity and fertilizability of human oocytes. Those data, however, were derived from radioimmunoassay methods and were mostly limited to evaluations of the concentration of progesterone and estradiol. By using capillary gas chromatography [12] we have measured eight steroids in FF from stimulated ovaries. The aim of this study was to investigate whether a more detailed profile of the follicular fluid steroid content could provide additional data on its relationship with oocyte maturity and fertilization rate *in vitro*.

EXPERIMENTAL

Follicular fluid was obtained by laparoscopic aspiration from 367 follicle aspirates of 75 women whose ovaries had been stimulated with clomiphene citrate, hMG and hCG. The aspiration took place 34–36 h after the hCG injection. Out of 367 oocytes 24 (7%) were classified as immature, 136 (37%) as intermediate and 207 (56%) as mature. Criteria for OCCC morphology were those of Veeck *et al.* [13]. IVF procedures were performed as described by Edwards [14]. Fertilization was defined as the presence of two pronuclei after 18 h.

For the correlation with data on fertilization only those oocytes which were inseminated with normal sperm have been considered. Of these 161 oocytes, 118 (73%) fertilized. The fertilization rate according to the maturity of the oocytes was respectively 82% (mature), 69% (intermediate) and 36% (immature).

Follicular fluid was centrifuged (300 ***g***) within 1 h of aspiration and stored at −20°C until analysis could be done using previously described methods [12].

Nonparametric tests (Mann–Whitney *U*-test and Kruskal–Wallis one-way analysis of variance test) were applied for group comparisons whereas Spearman rank correlation coefficients were calculated to explore relationships between the different steroid levels.

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RESULTS

Follicular steroids and oocyte maturity

Follicular steroid concentrations were grouped according to the oocyte maturity rating (Table 1). A positive correlation was observed between the oocyte maturity and the concentrations of progesterone (Pg), 17α-hydroxyprogesterone (17-Pg), pregnenolone ('Pon), 16α-hydroxyprogesterone (16-Pg) and 20α-dihydroprogesterone (20-DihPg), whereas the reverse applied for androstenedione ('Adione) and estrone (E1). No differences were observed in estradiol (E2) concentrations between the three groups. The most significant association with oocyte maturity was provided by the concentration of 20-DihPg.

To avoid the influence of contamination with blood or flushing fluid (used to improve oocyte recovery) ratios of steroid concentrations with

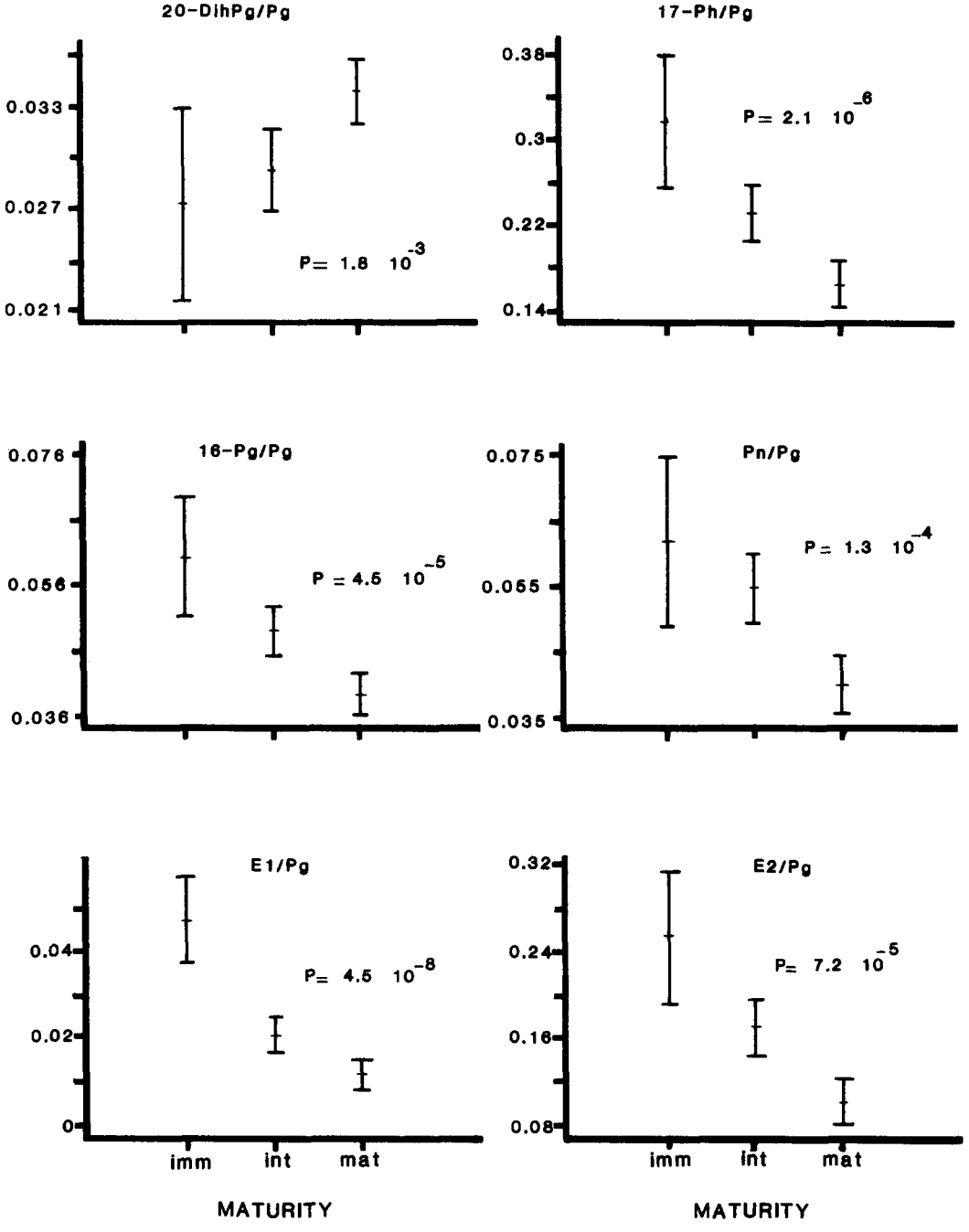

Fig. 1. Mean and 95% confidence intervals of ratios of different steroid concentrations with the progesterone concentration. P = significance of Kruskal–Wallis test.

Table 1. Steroid concentrations (ng/ml) in immature (imm), intermediate (int) and mature (mat) follicles

	Imm ($n = 24$)		Int ($n = 136$)		Mat ($n20$)		
Steroid	Mean	SE	Mean	SE	Mean	SE	P
Progesterone	4376[a]	733	7302[a,b]	1469	9307[b]	312	1.1×10^{-8}
Estradiol	680	80	670[c]	33	744[c]	24	0.027
17-Pg	972[e]	90	1196[d]	69	1395[d,e]	50	9.4×10^{-4}
Pregnenolone	271[f]	48	393[f,g]	26	347[g]	17	3.0×10^{-4}
16-Pg	201[h]	24	294[h,i]	19	333[i]	12	1.3×10^{-4}
20-DihPg	109[j]	32	218[j,k]	17	308[k]	13	6.2×10^{-11}
Estrone	124[l,m]	22	79[l]	7	83[m]	7	0.08
'Adione	112[n]	30	62[o]	10	34[n,o]	4	5.6×10^{-4}
Cortisol	60[p]	10	37[q]	3	51[p,q]	2	2.1×10^{-3}

Values with the same superscripts differ significantly ($P < 0.05$, Mann–Whitney U-test). P: Significance of Kruskal–Wallis test

those of Pg or E2 were calculated. The 20-DihPg/Pg was positively correlated with the degree of oocyte maturity whereas the 17-Pg/Pg, 16-Pg/Pg and Pn/Pg ratios were inversely correlated. A similar trend was observed for the E1/Pg and the E2/Pg ratios (Fig. 1).

Table 2 lists correlation coefficients between the follicular levels of different measured steroids and the progesterone levels. All steroids except androstenedione show a positive relation.

The 17-Pg/Pg, 16-Pg/Pg and 'Pon/Pg ratios correlated significantly ($P < 0.0001$) with progesterone concentrations. The 20-DihPg/Pg ratio however did not correlate significantly ($P = 0.064$) with the progesterone levels.

Follicular steroids and oocyte fertilization

Follicular fluids containing fertilized oocytes had significantly higher 20-DihPg and progesterone levels and lower androstenedione concentration (Table 3). Estradiol levels were comparable in all groups. The most clearcut differences in relation with fertilization were provided by the ratios 17-Pg/E2, 20-DihPg/E2, 16-Pg/E2 and Pg/E2 (ordered in decreasing significance level).

DISCUSSION

The FF steroid levels in our study are comparable with data obtained using RIA methods [1–11] and with our own data on FF from non-stimulated patients [15]. The androstenedione levels are however lower than the reported [1]. This probably is due to cross-reaction of androstenedione antisera with progesterone.

In most studies [1–3], increased follicular progesterone levels and decreased androstenedione levels were found to be associated with progressing oocyte maturity. Some authors, in contrast to our own data, also reported a relation between oocyte maturity and FF estradiol levels [1, 2]. An explanation for this discrepancy may be the type of ovarian stimulation. Indeed the association between oocyte maturity and estradiol levels is confined to cycles stimulated with hMG alone. In clomiphene stimulated cycles, on the contrary, Lobo *et al.* [3] observed an inverse relation between estradiol levels and oocyte maturity and Dlugi *et al.* [20] reported lower follicular estradiol levels in clomiphene versus hMG stimulated cycles.

The increase of progesterone metabolites with advancing oocyte maturity can primarily be ascribed to their strong relationship with the progesterone concentrations. An exception should be made, however, for 20α-dihydroprogesterone: unlike the other metabolites, its relative concentration (i.e. concentration divided by that of Pg) is not related to the Pg

Table 2. Correlation coefficients (r) of different steroids with progesterone (decreasing order of r)

Steroid	r	P
20-DihPg	0.83	<0.00001
16-Pg	0.81	<0.00001
17-Pg	0.78	<0.00001
Pregnenolone	0.78	<0.00001
Cortisol	0.51	<0.00001
Estradiol	0.37	<0.00001
Estrone	0.17	<0.0011
Androstenedione	−0.36	<0.0004

Table 3. Follicular fluid steroid concentrations (ng/ml) and ratios in relation to fertilizability of associated oocytes (mean ± SE)

	Not fertilized ($n = 43$)	Fertilized ($n = 118$)	P
20-DihPg	221 ± 32	300 ± 18	0.009
Progesterone	6878 ± 735	8741 ± 443	0.022
Androstenedione	47 ± 10	29 ± 5	0.026
17-Pg	1142 ± 94	1402 ± 78	0.096
16-Pg	281 ± 27	241 ± 21	0.099
Pregnenolone	337 ± 48	350 ± 23	0.37
Cortisol	46 ± 5	51 ± 4	0.72
Estrone	87 ± 13	96 ± 11	0.84
Estradiol	747 ± 69	722 ± 32	0.95
17-Pg/E2	1.73 ± 0.14	2.1 ± 0.14	0.0013
20-DihPg/E2	0.34 ± 0.05	0.48 ± 0.03	0.0034
16-Pg/E2	0.42 ± 0.04	0.52 ± 0.03	0.0086
Pg/E2	11.0 ± 1.5	13.4 ± 0.7	0.0091
E2/Pg	0.17 ± 0.02	0.12 ± 0.02	0.0095

concentration. The independent increase of 20α-dihydroprogesterone with progressing oocyte maturity (Fig. 1) indicates that the 20α-dihydrogenation activity increases after luteinization. Enzyme studies in rats [16, 17] showed an increased 20α-hydroxysteroid dehydrogenase activity concomitantly with a decrease in the activity of the 17α-hydroxylase and C17,20-lyase enzymes after exposure to ovulatory doses of exogenous gonadotropins. Accordingly, Babalola and Shapiro [18] measured an increase in 20-DihPg and a sharp decrease of all other progestagens in late stage follicles of sows.

In agreement with several other studies [4–7], a correlation between the fertilization rate of oocytes and the FF progesterone was observed. We could however not find a similar correlation for estradiol. Similarly as for the oocyte maturity, the reported association between elevated FF estradiol level and fertilization rate was indeed only observed when hMG without clomiphene had been used to stimulate follicular maturation [1, 2, 6, 8]. Uehara *et al.* [9] did not observe differences for estradiol but noted lower FF testosterone levels in their fertilized group, which agrees with our finding of lower androstenedione levels in this group.

As was found for the relation with oocyte maturity, also concerning oocyte fertilizability, 20-DihPg levels provide a more significant group difference than Pg itself.

There is no general agreement on the relation between FF steroid content and the maturity and fertilizability of the oocyte. Conflicting data may arise from numerous sources: methodology of steroid assays, definition of oocyte maturity and fertilization, exclusion of small follicles, stimulation scheme for follicular maturation and contamination of follicular fluid with blood or flushing medium. The latter problem can be circumvented by expressing steroid levels as ratios. Deichert *et al.* [21] concluded that the FF ratio of E2/Pg was the most sensitive steroid marker for the outcome of fertilization. Similarly we found highly significant differences in the Pg/E2, 17-Pg/E2 and 20-DihPg/E2 ratios between fertilized and unfertilized oocytes.

We conclude from our data that chromatographic analysis of follicular steroids may provide additional criteria to determine the maturity and fertilizability of oocytes in IVF. The selective increase of 20-DihPg during the final stages of follicular development also indicates that an enhanced dihydrogenation of progesterone is a characteristic of the early follicular luteinization.

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