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EVALUATION OF LEYDIG-CELL FUNCTION IN NORMAL PREPUBERTAL AND PUBERTAL BOYS

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SUMMARY

Plasma levels of testosterone (T), estrone (E1) and estradiol (E2) have been studied in 115 normal boys 5–19 years old and related to pubertal stages. The significance of pubertal changes is greater for T than for E1 and E2, and the increase of T begins earlier than the increase of E2.

Response to HCG, 3 × 1500 IU every other day, has been studied in 61 of these subjects. The increase of testosterone from basal to post-stimulatory levels (ΔT) goes up at the beginning of puberty but does not change further. ΔE1 does not increase significantly during puberty. ΔE2 increases at late puberty. All normal prepubertal boys had a definite response of T to HCG, peak level being at least 1·60 ng/ml. The number of injections of HCG and the duration of the stimulation are more important than the doses injected to obtain maximal Leydig-cell stimulation in prepubertal children.

INTRODUCTION

Plasma levels of testosterone and estrogens in prepubertal boys may correlate with both adrenal and testicular activity. Stimulation with human chorionic gonadotropin (HCG), as first shown by Saez and Bertrand[29], allows an evaluation of Leydig-cell secretory activity before puberty. The pubertal development of the Leydig-cells leads to a high spurt of plasma testosterone level and to a lesser increase of plasma estrogens. The aim of this work is to study the pattern of development of Leydig-cell function during childhood and puberty, as evidenced by the level of plasma testosterone and estrogens and their response to HCG stimulation.

PATIENTS AND METHODS

Basal levels of plasma testosterone and estrogens have been evaluated in 115 boys aged 5–19 years. All were normal, either healthy or referred for minor non-endocrine disturbances. HCG stimulation test has been effected in 61 of these subjects: 24 pre-pubertal boys, 30 boys in the course of puberty and 7 pubertal males. The circadian variation of plasma testosterone has been studied in 13 patients of the pre-pubertal group.

The bone age of patients has been evaluated according to the Atlas of Greulich and Pyle[11]. Their testicular volume has been related to the volume of models in a testicular scale[18]. The stages of pubertal development have been evaluated according to Tanner[36].

Blood was collected between 10 and 12 a.m., heparinized and centrifuged, plasma being immediately frozen at −20°C.

Plasma testosterone (T) was radioimmunologically assayed according to Leymarie *et al.*[19]. Rabbit antiserum was obtained using testosterone-3-(0-carboxy) methyloxime-bovine serum albumin as antigen for immunization. Over-estimation of the results by interference of 5α-dihydrotestosterone in the radioimmunoassay was minimal, as shown by comparison of the radioimmunoassay with mass spectrometry [5, 19]. The precision of the method was calculated from duplicate determinations. The intra-assay precision was 10·0% at the 0·06 ng/ml level for a single determination using the same plasma, and 7·1% when the method was run in duplicate. At the level of 2 ng/ml, the coefficients of variation mentioned above were respectively 9 and 6·4%. Concerning the inter-assay precision, the coefficients of variation were for the first level 23·0 and 16·3%, and for the second level 17 and 12·0%. The detection limit was 0·012 ng/ml, when a 2 ml aliquot of plasma was used.

Plasma estrone (E1) and estradiol (E2) were radioimmunologically assayed according to the method of Castanier and Scholler[4] modified by use of microcolumns of Sephadex LH 20 (30 × 40 mm). Rabbit antiserum was obtained using 6-(0-carboxy) methyloxime-BSA as antigens. The inter-assay precision, calculated from the determination of E1 (at a level of 36 pg/ml), was 20% with duplicate and 28% with single evaluation of a 2 ml sample, lower limit of detection being 17 pg/ml. The inter-assay precision for

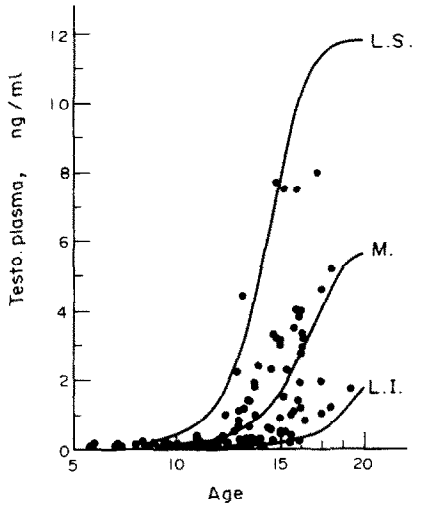

Fig. 1. Individual plasma testosterone levels, mean (M) and range in children and adolescents.

E2 was 9·6% with duplicate, and 14% with single evaluation (at level of 25 pg/ml), the lower limit of detection being 8 pg/ml.

Circadian variation of testosterone levels was studied in 13 pre-pubertal boys, using a venous polythene catheter, and collecting blood at 8 and 11 a.m., 2, 8 and 12 p.m. the 1st day; 4 and 8 a.m. the 2nd day.

HCG stimulation test was effected according to Perheentupa *et al.*[25] with three intramuscular injections of HCG 1500 I.U. every other day, blood being collected at days 0 and 6, i.e. 24 h after the third injection. In six subjects the test was further set up to 9 × 1500 I.U. every other day blood being collected after the third and ninth injection.

Statistical analysis of the data was done using the tests of Wilcoxon[13] and of Fischer and Yates[8] for non-gaussian distributions. Confidence limits for estimating normal ranges were determined using non-parametric methods and Sommerville's tables [35].

RESULTS

Individual testosterone levels, mean value and range values according to age, are recorded in Fig. 1.

Mean basal and post-stimulatory levels of T, E1 and E2 (m ± S.E.M.) and range of individual values have been tabulated according to stages of pubertal development and related to bone age and testicular volume (Table 1).

Basal testosterone levels at each pubertal stage differ significantly from levels at the other stages, the significance being $P < 0{\cdot}01$ from P1 to P2, P2 to P3, P4 to P5 and $P < 0{\cdot}05$ from P3 to P4.

Basal values of E1 differ at a low level of significance ($P < 0{\cdot}05$) between state P1 and the whole of following stages; the difference between stages P2 and P5 is not significant.

Basal values of E2 increase significantly ($P < 0{\cdot}01$) between stages P2 and P3, and this difference is maintained.

Circadian variation of plasma testosterone in 13 prepubertal boys showed an increase in the values at 4 and 8 a.m. (Fig. 2). However the fact that the mean value at 8 a.m. on the second day was higher than on the first day cannot be explained.

Post-stimulatory testosterone levels after 3 × HCG 1500 I.U. are recorded on Table 1 and Fig. 3. At each of the five pubertal stages, post-stimulatory levels differ significantly from basal levels ($P < 0{\cdot}01$). In the prepubertal group (P1) the lower value of plasma testosterone after simulation was 1·60 ng/ml at a confidence level of 90% ($P = 0{\cdot}10$). From the pre-pubertal group P1 to the group in early puberty P2, mean post-stimulatory testosterone levels increased significantly ($P < 0{\cdot}01$). But after the onset of puberty, i.e. from stage P2 to stage P5, the increase of mean post-stimulatory testosterone was small and no longer significant. Similarly, ΔT (the difference from basal to post-stimulatory levels of testosterone) increased significantly from prepubertal to pubertal subjects ($P < 0{\cdot}01$) but remained unchanged from early to late puberty (Fig. 4, Table 2).

In six normal pre-pubertal boys, plasma testosterone was assayed once after three injections of HCG 1500 I.U. and again after nine injections. In five of them, the level after nine injections was much higher than after three injections (Table 3).

Post-stimulatory values of E1 and E2 after 3 ×

Table 1. Basal and post-stimulatory levels

Pubertal stage	Bone age (years)*	Testicular volume* (ml)	Basal plasma levels* T (ng/ml)	E1 (pg/ml)	E2 (pg/ml)	Post-stimulatory plasma levels* T (ng/ml)	E1 (pg/ml)	E2 (pg/ml)
	8·5 ± 0·4	3·5 ± 0·2	0·14 ± 0·02	40 ± 6	17 ± 4	3·37 ± 0·43	63 ± 12	24 ± 10
1			(0·05–0·60)	(17–70)	(8–50)	(1·56–9·50)	(26–143)	(8–112)
	$n = 44$	$n = 44$	$n = 42$	$n = 14$	$n = 14$	$n = 24$	$n = 10$	$n = 10$
	12·6 ± 0·2	5·9 ± 0·4	0·65 ± 0·09	56 ± 8	17 ± 2	7·46 ± 1·06	83 ± 11	40 ± 9
2			(0·19–1·95)	(17–94)	(8–35)	(2·4–18·50)	(49–134)	(14–81)
	$n = 28$	$n = 28$	$n = 28$	$n = 14$	$n = 14$	$n = 17$	$n = 8$	$n = 8$
	13·3 ± 0·2	8·1 ± 0·8	1·91 ± 0·28	57 ± 6	34 ± 5	9·18 ± 1·25	81 ± 9	47 ± 7
3			(0·82–4)	(40–80)	(10–60)	(4·7–17·5)	(66–98)	(30–70)
	$n = 14$	$n = 14$	$n = 14$	$n = 8$	$n = 9$	$n = 9$	$n = 6$	$n = 6$
	14·5 ± 0·3	10·3 ± 0·6	2·92 ± 0·38	48 ± 9	24 ± 5	9·69 ± 2·65	108	46
4			(1·1–5·2)	(17–89)	(11–48)	5·7–17·5)	(83–133)	(18–75)
	$n = 13$	$n = 13$	$n = 11$	$n = 7$	$n = 7$	$n = 4$	$n = 2$	$n = 2$
	15·6 ± 0·3	13·7 ± 0·7	4·66 ± 0·58	77 ± 21	33 ± 0·5	10·86 ± 0·35	104 ± 24	88 ± 14
5			(1·2–8)	44–240)	(10–58)	(9·70–12·00)	(66–220)	(61–145)
	$n = 16$	$n = 16$	$n = 14$	$n = 10$	$n = 10$	$n = 7$	$n = 6$	$n = 7$

* Mean ± S.E.M., (range), n = number of subjects.

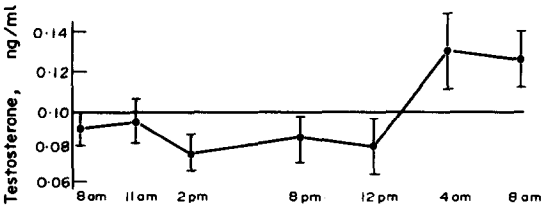

Fig. 2. Circadian variation of plasma testosterone in 11 prepubertal boys (mean ± S.E.M.)

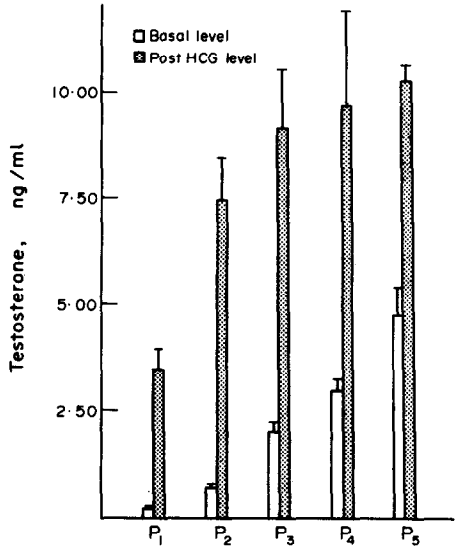

Fig. 3. Basal and post-stimulatory levels of plasma testosterone (mean + S.E.M.) in prepubertal (P1) and pubertal (P2 to P5) boys.

HCG 1500 I.U. did not differ from basal values in prepubertal group P1. After the onset of puberty, response of E1 and E2 to HCG stimulation became significant: $P < 0{\cdot}05$ at stages P2, P3, P4; $P < 0{\cdot}01$ at stage P5. The increase of estrone after stimulation (ΔE1) did not differ significantly from one pubertal stage to the other. The increase of estradiol (ΔE2) did not differ from stage P1 to P2, P3, P4, but rose significantly ($P < 0{\cdot}05$) from middle puberty (P3 and P4) to late puberty (P5) (Table 2).

DISCUSSION

The present study confirms that levels of plasma testosterone in children and adolescent males correlate with pubertal development, testicular volume and

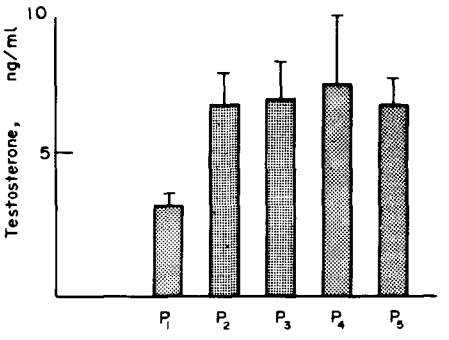

Fig. 4. Increase from basal to post-stimulatory levels of plasma testosterone (mean + S.E.M.) in prepubertal (P1) and pubertal (P2 to P5) boys.

bone age. Mean values in this study are similar to those reported by August *et al.*[1]. There are minimal differences with values reported by Winter *et al.* and by Frasier *et al.*[10, 39, 40] whereas lower values have been found in other studies on prepubertal and pubertal boys[9, 12, 38]. Although sampling of subjects may have been a factor of disparity it is probable that methodological differences were mainly responsible.

Circadian variation of testosterone has been demonstrated in adult males [6, 7, 20, 22, 34]. A recent study [17] confirms that this variation in adults may be significant, with a peak of the mean levels of hourly integrated testosterone concentrations at 6–7 a.m. Present data agree with a similar peak at 4–8 a.m. in prepubertal boys. However an adrenal stress induced by the study should account for the level at 8 a.m. on the second day, higher than on the first day.

Pre-pubertal mean values of plasma estrone and estradiol are somewhat higher in the present study than in other reported studies [14, 30, 39], and this may also be related to technical differences. In the present method water blank value but no plasma blank value was substracted from the experimental value. This probably led to a slight overestimation of the oestrogen concentration during the first stages of pubertal development and thus could lessen a possible gradual increase in plasma levels. Saez and Bertrand[30], using corticotropic suppression with dexamethasone and gonadotropic suppression with fluoxymesterone, have demonstrated that in pre-pubertal children the main source of estrone is the adrenal cortex. In adult males, it has been evidenced by selective venous catheterizations that both adrenals [2] and tes-

Table 2. Mean differences (± S.E.M.) between basal and post-stimulatory levels

Pubertal stage	P1	P2	P3	P4	P5
ΔT	3·17 ± 0·43	6·78 ± 1·03	6·98 ± 1·37	7·42 ± 2·04	6·74 ± 0·93
(ng/ml)	(24)*	(17)	(9)	(4)	(7)
ΔE_1	16 ± 14	30 ± 2	23 ± 10		45 ± 21
(pg/ml)	(9)	(8)	(8)		(6)
ΔE_2	3 ± 2	12 ± 9	14 ± 6		67 ± 14
(pg/ml)	(9)	(8)	(8)		(7)

* Number of cases.

Table 3. Plasma testosterone after 3 × 1500 I.U. and 9 × 1500 I.U. in 6 prepubertal boys

Patients	Plasma testosterone (ng/ml) Basal	After 3 × 1500	After 9 × 1500
J.E.	0·19	9·10	21·00
T.H.	0·15	3·45	9·00
P.P.	0·12	1·60	7·40
M.B.	0·17	4·65	5·05
P.F.	0·12	5·00	10·44
P.V.	0·29	7·34	9·35

tes [3, 15, 23, 32] are sources of estrogens. Present data indicate that puberty in males increases plasma levels of E1 and E2. Thus, mean values at late puberty are about twice pre-pubertal mean levels and the difference is significant.

As suggested by many previous reports [10, 25–29, 33, 39, 41] the present study demonstrates that, in normal pre-pubertal boys, HCG stimulation definitely increases plasma testosterone although the post-stimulatory levels are not the same in these different studies. The number of injections, the doses injected and the duration of the test may be important factors in these discrepancies. Published studies report data obtained after one single injection [28, 33, 37, 41], after three to five daily injections [21, 22, 24, 26–28, 31, 39] or after three to seven injections on alternate days [10, 25, 29]. In these studies, the dose of HCG varied from 800 to 5000 I.U. at each injection or from 1500 to 25,000 I.U. cumulatively, and the time of blood sampling was variable. Such conditions do not allow direct comparison of results.

In prepubertal boys, it appears that post-stimulatory testosterone levels are related to the duration of the test rather than to the doses of HCG injected. Using five injections of 800–5000 I.U., Rivarola *et al.*, observed that the testosterone response in children was not dose-related [27]. A 3-day test with three daily injections of 2000 I.U. in pre-pubertal males [39] increased testosterone less than the 6-day test with three injections of 1500 I.U. on alternate days as in the present study. The preliminary data reported here suggest that nine injections every other day may lead to higher Leydig-cell stimulation in pre-pubertal testes than three injections, but this effect of time may be dose dependent: thus, it is greater with injections of 2000 I.U. (39) than of 1000 I.U. [16].

Finally, it seems that the Leydig-cell response to HCG stimulation in pre-pubertal boys is slower and weaker than in pubertal subjects. Optimal response of prepubertal testes needs more than one injection and probably spaced injections. Maximal response may require more than three injections.

Calculation of the Δ values of testosterone and estrogens at different stages of pubertal development permits an evaluation of Leydig-cell maturation. In the conditions of the present study, ΔT rises at the beginning of puberty but does not increase further after stage P2, whereas ΔE1 does not increase significantly at any stage and ΔE2 increases at late puberty (P5). Thus, estradiol response to HCG seems a better index of complete endocrine maturation of the testes than testosterone response.

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