Effect of human chorionic gonadotrophin on plasma and urine testosterone in boys with delayed puberty

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Rudd, B. T., Rayner, P. H. W., Smith, M. R., Holder, G., Jivani, S. K. M., and Theodoridis, C. G. (1973). *Archives of Disease in Childhood*, 48, 590. **Effect of human chorionic gonadotrophin on plasma and urine testosterone in boys with delayed puberty.** The plasma testosterone and/or urine testosterone levels after 1500 units of human chorionic gonadotrophin (HCG) for four days have been measured in 29 male children (aged 8 years–18 years 7 months) with clinical evidence of delayed puberty. Children with constitutional delayed puberty, and all but 3 children with cryptorchidism, had similar mean incremental rises in testosterone. 3 of 4 children with Prader-Labhart-Willi syndrome had a poor urine testosterone response to HCG. Children with testicular atrophy failed to respond to HCG and were clearly separated from the other three groups. The short-term plasma testosterone response at 4 hours after HCG (1500 units once) in children with constitutional delayed puberty was variable and of limited value in assessing their Leydig cell function. In contrast, 3 children with bilateral cryptorchidism showed incremental rises proportional to age or maturational status.

There was a small but positive correlation between the plasma and urine testosterone incremental changes after four days of HCG for all patients, suggesting that either urine or plasma testosterone measurements are suitable indices of testicular function. Basal levels of urine testosterone were no direct guide to the response to HCG.

Current information favours the view that the hypothalamic-pituitary-gonadal axis in boys and girls is active in the preadolescent as well as the adolescent period of development. A study of 100 normal boys and 149 normal girls in the age range 5 to 18 years by Blizzard *et al.* (1970) has shown that follicle-stimulating hormone (FSH) and luteinizing hormone (LH) production is demonstrable at all ages and correlates well with stages of sexual maturation. However, an overlap exists, and basal levels of these hormones are no direct guide to future pubertal status. The enigma as to whether the hypothalamic-pituitary release of gonadotrophin is regulated by maturational changes at this level, or whether the target organs (the gonads) also undergo enzymatic changes with time, remains unresolved. In the latter context, the recent comprehensive studies of Zachmann (1972) on the response of males to a single injection of HCG support the concept of maturational changes at gonadal level with increasing age. These studies of Zachmann are particularly

pertinent when trying to measure gonadal function in boys of different ages with suspected gonadal insufficiency. In the present study we have attempted to assess the merits or otherwise of measuring urine or, alternatively, plasma levels of testosterone in boys with clinical evidence of delayed puberty, before and after stimulation with HCG. Data are also included on the short-term (4 hours) effect of HCG on plasma testosterone levels when compared to long-term (1–4 days) stimulation.

Materials and methods

Plasma testosterone was measured by a modification of the competitive protein binding procedure of Rosenfield, Eberlein, and Bongiovanni (1969) after two-stage thin layer chromatography. Highly diluted 3rd trimester pregnancy plasma was used as the source of binding protein and dextran-coated charcoal for separation of free from bound label, as previously described (Moshang *et al.*, 1970). 1β - 28 tritiated testosterone (TRK 162 specific activity 55 Ci/mmol) was used as labelled hormone (Radiochemical Centre, Amersham). All data were corrected for 'method blank' run with each batch of

TABLE I
Diagnostic categories of patients studied

Case no.	Age (yr) (mth)	Bone age (yr) (mth)	Sexual development (Tanner score I-IV)*	Diagnosis
1	15 7	12	III	Constitutional delayed puberty
2	14 8	10 6	I	" " "
3	15 7	14	III	" " "
4	13 9	ND	I	" " "
5	13 9	10	II	" " "
6	16	12	I	Male, Turner's syndrome (delayed puberty)
7	13 6	9	I	Constitutional delayed puberty (short stature)
8	15 2	12	I	" " "
9	11 11	11	I	Prepubertal gynecomastia (normal stature)
10	15 2	12	III	Constitutional delayed puberty
11	15 10	13 6	II	Prader-Labhart-Willi syndrome
12	13	12	II	" " "
13	12 9	11 6	I	" " "
14	13	11	II	" " "
15	11 4	11	I	Unilateral cryptorchidism
16	14	11 1	II	" "
17	13 3	ND	II	" "
18	14	12	I	" "
19	12 3	12 6	I	Bilateral cryptorchidism
20	15 7	12	III	" "
21	13 5	13	II	" "
22	15 1	12 6	I	" "
23	16	13	II	" "
24	9 8	ND	ND	" "
25	10 7	10	I	Anorchid
26	8 11	7	I	" "
27	13	12	I	Primary testicular atrophy (1 testis)
28	8 6	8 6	I	" " " (cryptorchid)
29	16 2	14	II	" " "

ND, not known. *Tanner (1962).

samples studied. The value for this blank varied in our hands from not detectable to 0.1 ng.

Urinary testosterone was measured by an improved procedure based on the method of Rudd *et al.* (1969). The modifications were two-stage thin layer chromatography (ligroine-85% methanol—on cellulose, cyclohexane:ethyl acetate (9:1)—on silica gel impregnated glass fibre) after β -glucuronidase hydrolysis and hexane partition. Values omitting the acetylation stage, as previously described, gave identical results. Details of these modifications will be published elsewhere.

Heparinized blood (5–10 ml) was collected by venepuncture at 10 a.m. each day under basal conditions, or after long-term stimulation with HCG (Pregnyl, Organon Ltd.) 1500 units. Plasma was separated within 30 to 60 minutes of collection and aliquots stored at -20°C before analysis.

Urine collections were made every 24 hours without preservative, and creatinine content measured on the Autotechnicon autoanalyser, as an index of adequate collection. The patient's sexual development was classified according to the procedure described by Tanner (1962). Bone age was assessed from Greulich and Pyle (1959).

Patients studied for plasma and urinary testosterone assay.

Group 1. Normal children, and prepubertal children

in hospital. Baseline 24-hour urines were collected from 10 normal, healthy, ambulant children (age range 5 years–11 years 7 months) of British parentage, for baseline testosterone measurements in the prepubertal range. The urine testosterone levels ranged from 0 to $2.9\text{ }\mu\text{g/day}$ (no. = 10). For ethical reasons, only baseline plasma testosterone levels were measured in a group of normal children in hospital (age range 5–13 years). These children had been screened for suspected adrenal insufficiency by stimulation with ACTH. All responded adequately as judged by plasma cortisol measurements. Their basal levels of plasma testosterone varied from 0 (not detectable) to 40 ng/100 ml (no. = 20). All were prepubertal (Tanner Stage 1).

Group 2. Children in hospital with delayed puberty. 29 children (age range 8 years–18 years 7 months) were studied (Table I). There were (a) 10 children with constitutional delayed puberty, of which 1 had concurrent prepubertal gynecomastia and 1 was a male with Turner's syndrome; (b) 4 with Prader-Labhart-Willi syndrome; (c) 10 with cryptorchidism (6 bilateral); and (d) 5 with primary testicular failure (2 of whom were anorchid on surgical exploration).

Blood was taken under basal conditions and then the first injection of 1500 units HCG (Pregnyl) was given (day 1). In the whole of the group 2 patients, injections of HCG (1500 units) were continued for a further 3 days (days 2–4, total 4 injections), and blood collected (at 10

TABLE
Long-term (days 1-4)

Case no.	Plasma testosterone (ng/100 ml)			
	Basal†	Day 3	Day 5	Net mean change
1	137	295	357	+199
2	0	158	273	+215
3	30	227	191	+179
4	17	97	95	+79
5	36	376	236	+270
6	114	325	500	+298
7	16	183	239	+195
8	12	149	184	+152
9	0	75	84	+80
10	0	92	225	+158
11	41	71	148	+15
12	—	—	—	—
13	—	—	—	—
14	—	—	—	—
15	24	66	77	+47
16	34	177	236	+172
17	—	—	—	—
18	—	—	—	—
19	56	187	343	+209
20	136	262	286	+138
21	13	184	163	+160
22	6	23	157	+84
23	—	—	—	—
24	17	55	46	+33
25	0	5	20	+13
26	38	20	47	-4
27	0	16	13	+15
28	0	35	34	+35
29	20	4	22	-7

*Poor urine collections as judged from creatinine content.

†Day of first injection.

Zero level of plasma testosterone not detected.

a.m.) where possible on days 3 and 5 (23 children). A baseline 24-hour urine was also collected, and further 24-hour urine collections were made on days 1-2, 2-3, 3-4, and 4-5 of the HCG injections (20 children).

The plasma testosterone levels obtained after a single injection of HCG (1500 units) at 4 hours will be referred to as 'short-term low dose responses'. The testosterone responses obtained in plasma or urine after multiple (4 days) HCG injection will be referred to as 'long-term high dose responses'.

Results

Basal levels. Baseline urinary testosterone levels for the normal ambulant prepubertal children ranged from 0 to 2.9 $\mu\text{g}/24$ hours, and, on average, showed a slight increase with increasing age (Fig. 1). In contrast, the basal levels from all the children in hospital were much more variable and could not be used as a direct guide to their gonadal status or subsequent response to HCG stimulation. In particular, 11 of the 20 patients who had urine testosterone measured had prepubertal basal levels; but 6 responded adequately after 4 days of HCG stimulation (Cases 1, 2, 3, 4, 12, 18, in Table II). One patient (Case 15), with unilateral cryptorchidism, had urine testosterone in the prepubertal

FIG. 1.—Basal urine testosterone in normal children and patients vs age. ● prepubertal normals, × constitutional delayed puberty, ○ Prader-Labhart-Willi syndrome, ▲ unilateral cryptorchid, □ bilateral cryptorchid, ▽ primary testicular failure. Broken line indicates upper limit of prepubertal range.

II

HCG stimulation test

Urine testosterone ($\mu\text{g}/24 \text{ hr}$)					
Basal	Days 1-2	Days 2-3	Days 3-4	Days 4-5	Net mean change
1.2	6.2	2.4	24.9	*	+10
0.3	2.3	2.3	6.8	16.2	+6.3
1.8	4.1	4.0	12.0	*	+4.9
2.6	5.2	25.0	8.0	9.7	+9.4
—	—	—	—	—	—
3.0	10.2	*	16.4	11.9	+12.8
—	—	—	—	—	—
—	—	—	—	—	—
—	—	—	—	—	—
3.2	3.7	6.8	2.7	7.8	+2.1
0.3	0.7	2.5	6.1	5.6	+3.4
1.4	1.7	3.2	5.2	—	+2.0
5.1	*	3.9	*	6.6	+0.1
6.2	4.9	3.1	2.4	7.1	-1.8
3.5	4.9	8.2	8.0	18.0	+6.3
0.3	0.5	3.3	0.9	0.8	+1.1
0.9	2.0	7.0	8.0	17.0	+9.8
4.4	4.8	9.0	8.9	11.4	+4.1
14.1	25.9	51.6	70.4	69.0	+28.3
10.4	10.7	32.0	23.8	34.8	+14.9
—	—	—	—	—	—
2.6	1.5	10.4	3.4	6.8	+2.9
—	—	—	—	—	—
1.5	0.6	0.8	1.8	2.4	-0.1
3.3	2.9	2.1	1.2	2.0	-1.2
1.8	0.7	1.5	0.4	2.1	-0.6
—	—	—	—	—	—
—	—	—	—	—	—

range and yet did not respond to HCG. Similarly, 2 of the patients with Prader-Labhart-Willi syndrome (Cases 11 and 14) had basal levels in the postpubertal range, but their responses to HCG were poor.

Short-term low dose responses to HCG (children with constitutional delayed puberty and bilateral cryptorchidism). 3 children with constitutional delayed puberty (Cases 1, 2, and 3) had variable incremental rises of plasma testosterone for their age (14 years 8 months–15 years 7 months) at 4 hours after a single injection of HCG (Table III). In contrast, 3 patients with bilateral cryptorchidism (Cases 19, 20, and 21) showed positive increases in plasma testosterone at 4 hours, which rose with increasing age. The data suggested that the short-term low dose effect of HCG on plasma testosterone levels is to increase the production rate of testosterone proportional to age and/or maturational status of the child.

Long-term high dose responses to HCG (constitutional delayed puberty). The long-term high dose responses (4 days' stimulation) in

the children with constitutional delayed puberty were striking. As anticipated, both the plasma and urinary testosterone levels continued to rise with successive doses of HCG. In particular, a rise was noted in urine testosterone within the first 24 hours of the first injection of HCG, but the peak responses were quite variable, and occurred most frequently after 2 or 3 injections of HCG (Table II).

TABLE III

Short-term (4 hr) responses to HCG (children with constitutional delayed puberty or cryptorchidism) in plasma testosterone (ng/100 ml)

Case no.	Age (yr) (mth)	Basal	4 hr	Increment
Constitutional delayed puberty				
1	15 7	137	230	+93
2	14 8	0	28	+28
3	15 7	30	40	+10
Cryptorchidism				
19	12 7	56	122	+66
20	15 7	136	280	+144
21	13 5	13	97	+84

Interpretation of responses to HCG. The preliminary findings in the children with constitutional delayed puberty suggested that because of the wide range of testosterone levels encountered in plasma or urine, which appeared to be related to duration of stimulation and maturational status of the patient, a better means of assessing the significance of the change would be required before patients could be classified as responsive to HCG or not. For this purpose, the following computation was arrived at.

The basal levels of plasma testosterone or urine testosterone were subtracted from the mean incremental rise throughout the course of the test. The figure in each case (positive or negative) was designated as the net mean change in urine or plasma testosterone. A significant change in testosterone was taken as any positive value exceeding the upper limit of the normal prepubertal range as found under basal conditions. That is, 40 ng/100 ml for plasma, or 2.9 μ g/24 hours for urine testosterone.

Fig. 2a shows the mean changes in plasma testosterone for individual patients within the groups of constitutional delayed puberty, Prader-Labhart-Willi syndrome, cryptorchidism, and primary testicular atrophy. Patients with primary testicular atrophy were clearly differentiated from cryptorchidism and constitutional delayed puberty;

whereas patients with cryptorchidism had similar responses to patients with constitutional delayed puberty. Albeit, by the criteria adopted, i.e. a response exceeding the upper prepubertal level (about 40 ng/100 ml), 1 patient with cryptorchidism had a reduced response (Case 24). The 1 patient with Prader-Labhart-Willi syndrome (Case 11) also failed to respond. A similar pattern emerged when the incremental rises in urinary testosterone were plotted (Fig. 2b). All 5 of the 10 patients with constitutional delayed puberty that were studied had urine testosterone values greatly in excess of the upper limit for prepubertal basal testosterone. 5 of 8 children with cryptorchid testes were also responsive. No response was elicited in 3 patients with primary testicular atrophy. Noteworthy is the poor response in 3 of 4 patients with the Prader-Labhart-Willi syndrome; an observation which is in accord with the view that these patients may have a gonadal insufficiency secondary to a lack of gonadotrophin production.

The correlation of the mean incremental change in plasma testosterone *vs* urine testosterone after HCG stimulation was calculated. The correlation coefficient (*r*) for all the patients studied was 0.509, and was just significant at the 5% level ($P < 0.05$). These results suggest that the mean incremental change in either plasma or urine testosterone is a suitable index of a response to HCG when given in the long term (4 days' stimulation).

FIG. 2.—(a) Net mean change in plasma testosterone. (b) Urine testosterone after 4 days of HCG stimulation. Broken line indicates upper limit of prepubertal range.

Discussion

The data presented confirm and extend earlier reports that HCG stimulation in male children is a useful diagnostic aid in the differential diagnosis of hypogonadism.

The basal levels of plasma or urine testosterone in our patients were unrewarding when trying to assess the likely response of the gonads to HCG stimulation. The marked differences in basal levels of testosterone and subsequent response or not of the gonads to HCG indicate that an extra-gonadal source of testosterone contributes to the testosterone pool under basal conditions, probably from the adrenals. It is highly unlikely to be a direct secretion of testosterone from the adrenals, in view of the clear evidence of Zachmann (1972) in males with absent gonads and the studies of Aarskog (1970) in patients receiving dexamethasone which show a minor contribution to urine testosterone excretion from the adrenals. The most likely explanation is that of peripheral conversion of adrenal steroid precursors (Δ^4 androstenedione) to testosterone glucuronide. This has been shown in normals and patients with the adrenogenital syndrome (Horton and Frasier, 1967; Rudd and Rayner, 1970).

In our studies the long-term stimulation (4 days) of the gonads in children with constitutional delayed puberty and in those with cryptorchidism shows, with three exceptions, similar changes whether plasma or urinary testosterone was used as the index of responsiveness. These observations support the findings of Rivarola, Bergadá, and Cullen (1970) and Winter, Taraska, and Faiman (1972) who showed a significant increase in plasma testosterone after HCG (2000–5000 IU for 3–5 days) in two similar groups of children. The present findings, especially when mean incremental rises are studied after long-term stimulation, clearly separate those patients with anorchia or primary testicular atrophy from those with responsive Leydig cells, irrespective of age. Those patients with hypogonadism secondary to lack of pituitary luteinizing hormone may reveal inadequate responses, as shown in children with Prader-Labhart-Willi syndrome. The short-term (4 hours) effect of 1500 units HCG in the small group of children with constitutional delayed puberty or cryptorchidism was illuminating. It was our intention in the first instance to make use of this single injection technique to measure plasma testosterone in our patients. This would have reduced time in hospital. However, the results to date suggest that the short-term testosterone response to this dose of HCG is related to maturational status. That is, the younger or less mature the patient, the poorer the response.

Noteworthy are the findings of Zachmann (1972) who showed that a single injection of 5000 units of HCG was sufficient to elicit responses in children in whom gonadotrophin deficiency was not demonstrable within 3 to 6 days of the injection.

It appears, therefore, that (1) a low dose (1500 units) elicits a response within hours in those subjects only who are fully mature at gonadal level; (2) low dose responses (1500–5000 units) will elicit responses in the sexually immature when blood or urine levels of testosterone are measured at a later stage (24–72 hours) after a single injection.

The question of the 'best dose' of HCG remains an open one (800–5000 units have been employed). Nevertheless, our regimen of 1500 units once for short-term studies, or 1500 units four times for long-term stimulation, has proved entirely satisfactory, a single dose representing approximately 30 times the concentration of equivalent levels of luteinizing hormone circulating in the adult male.

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