CLINICAL STUDY

Evaluation of endocrine testing of Leydig cell function using extractive and recombinant human chorionic gonadotropin and different doses of recombinant human LH in normal men

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Abstract

Background: The functional testing of endocrine testis uses extractive human chorionic gonadotropin (ehCG). Recombinant human hCG (rhCG), avoiding any contamination, should replace ehCG. Moreover, a functional evaluation with recombinant human LH (rhLH) would be closer to physiology than a pharmacological testing with hCG.

Methods: The study was conducted in normal men. We first evaluated the dose–effect of ehCG on plasma testosterone and estradiol levels, before and after injection of either hCG or vehicle. Secondly, the responses to the optimal dose of ehCG were compared with those of rhCG. Thirdly, we investigated the dose–effect of rhLH, on steroid hormone secretion. LH, testosterone, and estradiol plasma levels were measured after the injection of either rhLH or placebo.

Results: ehCG induced dose-dependent increases in plasma estradiol and testosterone levels. They respectively peaked at 24 and 72 h after the injection. The most potent dose of ehCG (5000 IU) induced results similar to those observed with 250 µg (6500 IU) rhCG. By comparison with placebo, rhLH induced a significant and dose-dependent increase in plasma testosterone levels 4 h after the injection. Peak response of testosterone to rhLH and rhCG was significantly correlated. rhLH did not induce significant change in plasma estradiol level.

Conclusions: In normal men, a single i.v. injection of 150 IU rhLH induces a 25% rise in plasma testosterone levels by comparison with placebo. At the moment, the dynamic evaluation using hCG remains the gold standard test to explore the Leydig cell function. The use of 250 µg rhCG avoiding any contamination should be recommended.

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Introduction

In normal adult men, steroid hormone secretion by the Leydig cell is physiologically regulated by luteinizing hormone (LH). The gonadotropin acts through the stimulation of a G-protein-coupled receptor positively linked to adenylyl cyclase and exclusively expressed by the interstitial Leydig cells. In adult man, the activation of this receptor adenylyl cyclase system induces the synthesis and the secretion of testosterone (T) and estradiol (E₂). During puberty, the gonadotrophin-releasing hormone (GnRH)-induced pulsatile release of follicle-stimulating hormone (FSH) is responsible for the increase in testis volume. Simultaneously, LH pulses induce a progressive rise in plasma T and E₂ levels. Human chorionic gonadotropin (hCG) is able to stimulate sex steroid secretion by the testis through the activation of the same receptor of the Leydig cell membrane, rightly named LH/hCG receptor. In contrast to LH, hCG was easily available from the urine of pregnant women. hCG has a

long plasma half life (1, 2) and induces a sustained and prolonged stimulation of Leydig cell steroidogenesis (3, 4). For this reason, hCG was used as a treatment of various testicular diseases including cryptorchid testes (5–7), male hypogonadotropic hypogonadism (8), and in association with human menopausal gonadotropin, male infertility (9, 10). On the other hand, hCG testing of Leydig cell function was of interest for the search of the presence of intra-abdominal testes in case of bilateral ectopia (6, 7), for differentiating delayed puberty from hypogonadotropic hypogonadism (11–13), and for the etiological diagnosis of gynecomastia (14, 15). Various modalities of testing have been proposed in terms of hCG doses, the number of injections, and times of blood drawing (3, 11, 13, 16). The widely used hCG testing of Leydig cell function in adulthood is performed with one injection of 5000 IU hCG and measurements of plasma T and E₂ before and every morning of the 3 days following the injection (12, 17–19). The availability of recombinant human gonadotropins, which allows avoidance of the

potential risk of pathogenic agent transmission, enables elaboration of new modalities of endocrine testicular testing. The aim of the present study was: i) to evaluate the effect of different doses of extractive hCG (ehCG) on the steroidogenic response of the testis, ii) to compare the optimal dose of ehCG with that of rhCG on the same parameters, and iii) with the reference of results of hCG stimulation, to investigate a Leydig cell response to tests with different doses of recombinant human LH (rhLH) injected intravenously in order to obtain a fast steroidogenic response.

Subjects and methods

Subjects studied and protocols

Twenty adult men were included in this randomized single-blind study that was approved by the Institutional Ethic Committee of Basse Normandie, France. The subjects who gave their informed consent to the study protocol were divided into two groups.

Group I The first group included eight normal men (aged 18–30). They were taking no medication and did not have any history of disease or infertility. They were recruited from the list of healthy volunteers of the Clinical Investigation Center (INSERM 0204). Clinical examination including the BMI (20–24 kg/m²) was normal. All hormone parameters (including basal and GnRH-stimulated gonadotropins, basal, and hCG-stimulated testosterone (T) and estradiol (E₂), sex hormone-binding globulin (SHBG), and plasma inhibin B) were normal.

Blood was drawn in order to measure basal morning (0800 h) plasma T and E₂ levels. Then, either vehicle alone or a dose (50, 500, or 5000 IU) of hCG (Organon SA, Puteaux, France) was injected. Its stated potency was 2500 IU/mg in terms of the first International Reference Reagent (IRR) 99/688. hCG doses were injected intramuscularly in a random order. Blood was withdrawn 1, 3, and 5 h later and then every morning for 3 days (24, 48, and 72 h after the injection) to determine T and E₂ in response to the stimulation. A 2-week interval separated each step of the study.

Group II The second group included 12 normal men (aged 18–30) who responded to similar criteria as in group I. They were recruited from the list of healthy volunteers of the Clinical Investigation Center (INSERM 0204) and from the healthy volunteers recruited in Caen University Hospital. Clinical examination of the BMI (20–24 kg/m²) was normal. All hormone parameters (including basal and GnRH-stimulated gonadotropins, basal and hCG-stimulated T and E₂, SHBG, and plasma inhibin B) were normal. The study comprised six steps, each separated by 2 weeks.

In the first step, blood was drawn in order to measure basal morning (0800 h) plasma T and E₂ levels. Then, 5000 IU extractive ehCG, (Organon SA, Puteaux, France) were injected intramuscularly, and the blood was subsequently withdrawn every morning for 3 days to determine T and E₂ levels in response to the stimulation.

During the four following steps, after a basal blood sampling (0800 h) either vehicle or a dose (75, 150, or 225 IU) of recombinant human LH (rhLH, Luveris, Serono-France, Boulogne, France) was injected in which the stated potency was 18 187 IU/mg in terms of the second International RP (IRP) 80/552 (20). rhLH doses were injected intravenously in a random order. Then, the blood was withdrawn at the following times (30, 60, 90, 120, 150, 180, 240, and 300 min) in order to measure LH, T, and E₂ plasma levels.

The last step of the study was performed similarly to the first one but used 6500 IU rhCG, (Ovitrelle, Serono-France, Boulogne, France). Its stated potency was 26 000 IU/mg in terms of the first IRR 99/688.

Hormone assays

LH, FSH, hCG, and SHBG were measured by their respective chemiluminescent assays (Immulate 2500, Siemens Diagnostics, La Garenne Colombe, France). Plasma LH assay was calibrated according to first IRP 68/40 and second IRP 80/552. Plasma T was assayed after organic extraction. Plasma T levels measured by RIA (testosterone RIA, Immunotech, Beckman-Coulter, Villepinte, France). Normal ranges of adult men were T=10.4–34.5 nmol/l. E₂ was measured by RIA (estradiol RIA, Diasorin SA, Antony, France). Normal values in adult men were below 157 pmol/l. Inter-assay imprecision (coefficient of variation) is 8.2 and 4.6% for T (mean value=11.9 nmol/l) and E₂ (mean value=345 pmol/l) respectively.

Statistical analysis

Statistical analysis used an ANOVA with three parameters: subject, treatment, and time of blood drawing. As to whether this first step of analysis evidenced a difference in the evolution with time of these parameters, a new analysis was done either with constant time or constant treatment using Student's *t*-test with the correction of Bonferroni. The relationships between the dose of treatment and the hormone responses observed were studied with Pearson's correlation test. A similar analysis was performed to evaluate the relationships between the peaks of testosterone obtained in the various conditions of testing. Statistical differences were evaluated by applying Student's *t*-test for unpaired and paired data as appropriate. Comparisons were noted statistically significant at an α -level of less than 0.05.

Table 1 Basal and peak values of plasma testosterone and estradiol (mean $\pm$ S.E.M.) in normal men (group I) in response to the injection of vehicle alone or 50, 500, and 5000 IU extractive human chorionic gonadotropin (ehCG).

	Testosterone base (nmol/l)	Testosterone peak (nmol/l)	Estradiol base (pmol/l)	Estradiol peak (pmol/l)
Vehicle	13.9 $\pm$ 1.8	14.4 $\pm$ 1.8	68.7 $\pm$ 7.3	70.9 $\pm$ 10.3
50 IU ehCG	14.0 $\pm$ 1.6	19.2 $\pm$ 2.5	72.7 $\pm$ 4.4	91.8 $\pm$ 16.2
500 IU ehCG	13.6 $\pm$ 2.0	23.4 $\pm$ 3.3	67.2 $\pm$ 7.7	143.2 $\pm$ 14.7
5000 IU ehCG	13.6 $\pm$ 0.9	26.6 $\pm$ 3.5	76.4 $\pm$ 15.8	260.3 $\pm$ 25.3

Results

Group I

Mean basal plasma T level was 13.88 ± 1.84 nmol/l in the testing using vehicle alone. Similar basal plasma T levels were obtained before the injection of the different doses of ehCG (Table 1). Plasma T did not vary after vehicle injection. By contrast, plasma T levels peaked to 19.17 ± 2.50 , 23.44 ± 3.29 , and 26.62 ± 3.47 nmol/l after the injection of 50, 500, and 5000 IU ehCG respectively. Highest plasma T levels were observed earlier (48 h) with 50 and 500 IU than with 5000 IU ehCG injection, where peak was observed at time +72 h (Fig. 1). Mean peak values of plasma T observed after each dose of ehCG injection were significantly higher ($P < 0.01$) than their respective basal values. Plasma T rise after 5000 IU ehCG was significantly

higher ($P < 0.01$) than that observed with either 50 or 500 IU ehCG. Plasma T peak with 500 IU was significantly higher ($P < 0.01$) than that observed with 50 IU ehCG.

Mean basal plasma E₂ level was 68.65 ± 7.34 pmol/l in the testing using vehicle alone. Similar basal plasma E₂ levels were obtained during each stage of the study (Table 1). They did not significantly change after either vehicle or 50 IU ehCG injection. By contrast, plasma E₂ levels increased to 143.17 ± 14.68 and 260.27 ± 25.33 pmol/l after the injection of 500 and 5000 IU ehCG respectively. Highest plasma E₂ levels were observed 24 h after the injection of either 500 or 5000 IU ehCG (Fig. 1). Mean peak values of plasma E₂ observed with either 500 or 5000 IU ehCG were significantly higher ($P < 0.01$) than their respective basal values and plasma E₂ rise after 5000 IU hCG was

Figure 1 Left column: mean $\pm$ S.E.M. of plasma estradiol (upper panel) and testosterone levels (lower panel) in normal men (group I) in response to the injection of vehicle alone ($\diamond$) or 50 (Δ), 500 ($\square$), and 5000 IU ($\blacksquare$) ehCG. Statistical significance: * $P < 0.05$, ** $P < 0.01$. Right column: mean $\pm$ S.E.M. of plasma estradiol (upper panel) and testosterone levels (lower panel) in normal men (group II) in response to the injection of either 5000 IU ehCG ($\square$) or 6500 IU rhCG ($\blacksquare$). Statistical significance: *** $P < 0.001$.

Figure 2 Dose–effect relationship between hCG dose and E₂ (upper panel) and T (lower panel) peak response (mean $\pm$ S.E.M.) to the stimulation.

significantly higher ($P < 0.01$) than that observed with 500 IU hCG.

There were clear dose–effect relationships between the doses of hCG used and the peak responses of both T and E₂ (Fig. 2).

Group II

Mean basal plasma T levels were 16.75 ± 1.49 and 16.23 ± 1.70 nmol/l before the injection of 5000 IU ehCG and 6500 IU rhCG respectively. They rose to 38.10 ± 3.74 and 36.61 ± 3.40 nmol/l 72 h after the

respective injection of 5000 IU ehCG and 6500 IU rhCG. These two peaks did not differ but were significantly ($P < 0.001$) higher than their respective basal values (Fig. 1).

Mean basal plasma E₂ levels were 67.91 ± 6.61 and 80.02 ± 8.07 pmol/l before the injection of 5000 IU ehCG and 6500 IU rhCG respectively. They rose to 297.72 ± 35.61 and 358.29 ± 46.62 pmol/l 24 h after the respective injection of 5000 IU ehCG and 6500 IU rhCG. These two peaks did not differ but were significantly ($P < 0.001$) higher than their respective basal values (Fig. 1).

Mean basal plasma LH level was 3.7 ± 0.4 U/l on the testing using vehicle alone. Similar basal plasma LH levels were obtained before the injection of the different doses of rhLH (Table 2). Plasma LH levels did not vary after vehicle injection. By contrast, they increased to 28.3 ± 6.7 , 53.1 ± 16.7 , and 41.9 ± 8.2 U/l after the injection of 75, 150, and 225 IU rhLH respectively. The peak value was obtained 30 min after rhLH injection. Then, plasma LH levels progressively dropped to reach basal values 6 h later (Fig. 3).

Before testing with rhLH, mean basal plasma T level was 16.88 ± 1.90 nmol/l. Similar basal plasma T levels were obtained before the injection of the different doses of rhLH (Table 2). Plasma T progressively declined to 14.14 ± 1.66 nmol/l 5 h after the injection of placebo. By contrast, they increased to 17.92 ± 1.98 , 18.72 ± 2.18 , and 19.21 ± 1.70 nmol/l during the same time after the i.v. injection of 75, 150, and 225 IU rhLH respectively (Fig. 3). By comparison with the placebo testing, there were statistically significant differences (at least $P < 0.03$) in mean T plasma levels on times 180, 240, and 300 min following rhLH injection. The statistical highest difference ($P < 0.001$) was observed at the time + 300 min after injection of 225 IU rhLH.

There was a clear dose–effect relationship between the doses of rhLH used and the highest plasma T level reached after stimulation (Fig. 4). The highest plasma T levels attained after the injection of either 150 or 225 IU rhLH were significantly ($P < 0.01$ and $P < 0.05$ respectively) correlated with the peak increase in T in response to the injection of 250 μ g rhCG (Fig. 4).

In contrast to T, no significant difference was observed at any time in plasma E₂ levels whatever rhLH dose was used (Fig. 3).

Table 2 Basal and peak values of plasma luteinizing hormone (LH) and testosterone (mean $\pm$ S.E.M.) in normal men (group II) in response to the injection of vehicle alone or 75, 150, and 225 IU recombinant human LH (rhLH).

	LH base (U/l)	LH peak (U/l)	Testosterone base (nmol/l)	Testosterone peak (nmol/l)
Vehicle	3.7 ± 0.4	2.9 ± 0.3	16.9 ± 1.9	14.1 ± 1.7
75 IU rhLH	4.0 ± 0.4	28.0 ± 6.7	16.5 ± 1.9	17.9 ± 2.0
150 IU rhLH	4.0 ± 0.6	53.1 ± 16.7	16.5 ± 1.8	18.7 ± 2.2
225 IU rhLH	3.8 ± 0.5	41.9 ± 8.2	17.4 ± 1.8	19.2 ± 1.7

Figure 3 Left column: plasma LH (mean $\pm$ S.E.M.) in normal men (group II) in response to the injection of vehicle alone (□) or 75, 150, and 225 IU rhLH (■). Middle column: plasma testosterone (mean $\pm$ S.E.M.) in normal men in response to the injection of vehicle alone (□) or 75, 150, and 225 IU rhLH (■). Right column: plasma estradiol (mean $\pm$ S.E.M.) in normal men in response to the injection of vehicle alone (□) or 75, 150, and 225 IU rhLH (■). Statistical significance: * P < 0.05, ** P < 0.01.

Discussion

The effects of an acute injection of ehCG on the yield of T and E_2 are well demonstrated in animal and man (16, 17, 21). In normal adult men, plasma E_2 and T levels reach a peak 24 and 72 h respectively after a single i.m. injection of a pharmacological dose (1500–5000 IU) of ehCG (12, 14, 15, 17–19, 22). The stimulating effect of ehCG, mediated through the activation of the LH/hCG receptor of the Leydig cell, involves the amplification of the enzymatic pathways of sex steroid synthesis. Furthermore, a gonadotropin-induced activation of Leydig cell aromatase leads to a rise in E_2 synthesis (14, 17, 23). Multiple injections of ehCG give similar results as a single one (16, 24, 25). A prolonged exposure to elevated doses of hCG induces a desensitization process in rat and man (22, 26, 27). Such a phenomenon is observed in men with hCG-secreting tumors (28–30). The desensitization occurs as soon as 24 h after ehCG injection and modifies the testicular hormone response. It stems from both a stimulation through the activation of the membrane LH/hCG

receptor as well as a transient downregulation of these receptors (30–32). As a result of these combined events, the testicular response to ehCG shows a biphasic pattern with an initial testosterone rise within 12 h, followed by decline (3, 33, 34). Then, a second testosterone peak appeared 48–72 h after the stimulation (33–35). By contrast, this biphasic response does not occur with LH, which may account for various factors. They include respective plasma half lives of the hormones: short for LH, prolonged for hCG (1, 2); reversibility of the binding to the receptor: possible for LH, not possible for hCG (36, 37); and pattern of plasma levels: pulsatile for LH and sustained for hCG. With the aim of shortening the testing and to limit the drawbacks of the desensitization process, we initially studied the effect of lower doses of ehCG than that actually used. Hormone levels were measured on the early and then usual times of evaluation of the Leydig cell response to the stimulation.

The injection of the lowest dose of ehCG (50 IU) was not followed by any steroid hormone change. By contrast, the injection of 500 IU induced significant increases in plasma E_2 and T levels that peaked

Figure 4 Left part: dose–effect relationship between rhLH dose and T peak response (mean $\pm$ S.E.M.) to the stimulation. Right part: correlations observed between the peak responses of T to 250 μ g (6500 IU) rhCG and the highest plasma T levels reached after injection of either 150 IU (higher panel) or 225 IU rhLH (lower panel).

respectively 24 and 48 h later. Even though peaks reached were significantly higher than with 500 IU ehCG, similar patterns of response of both steroid hormones were observed using 5000 IU ehCG. These results suggest that, without consideration of the ehCG dose used, the most relevant times of testing are +24 h for E_2 and +48 h for T measurements. As evidenced by the dose–effect relationship, 5000 IU ehCG should be

preferred to lower doses. Indeed, the hCG-induced E_2 rise was significantly higher with 5000 IU ehCG. Using this dose would allow limitation of the testing to 48 h. Relevant hormone measurements could be plasma E_2 level at time +24 h and/or T level at time +48 h after ehCG injection, which agree with previously published data (16, 18, 19, 34). Indeed, plasma T levels measured at times +48 h and +72 h did not significantly differ and very likely gave the same information to the clinician.

Available recombinant hCG will logically replace ehCG for treatments and endocrine testing. Thus, we evaluated the effects of recombinant human chorionic gonadotropin (rhCG, packaged as 250 μ g = 6500 IU) in comparison with that of ehCG (5000 IU) in the same healthy volunteers. The amplitude of steroid rises and the patterns of change were similar between the two tests. On the basis of the results of the present study, we can offer as a standard testing the i.m. injection of 250 μ g (6500 IU) rhCG after a basal blood drawing and then the measurement of plasma E_2 levels 24 h later and/or plasma T levels at time +48 h.

The effects of i.v. rhLH, administered in different doses, were studied in the same healthy men. We searched to specify if rhLH could be used as a stimulus as relevant as rhCG to test the Leydig cell function. The objectives were to develop a shorter and more physiological dynamic testing of the endocrine testis. A paradigm using a previous gonadotroph desensitization with GnRH analogs would have led to more clear changes in plasma T levels in response to rhLH but would have been far from physiology. For this reason, we chose to study the responses to rhLH without any modulation of gonadotropin secretion. Obviously, rhLH injection at any dose was followed by a stimulation of T secretion. By comparison with the spontaneous physiological decrease in plasma T during morning, T progressively rose to a highest value obtained 4 h after rhLH i.v. administration. This agrees with the data obtained by several authors who used repeated rhLH pulse infusion in normal men (38–40). The injection of rhLH not only induced a 12% rise in mean plasma T from basal levels but also acted against the physiological decline in plasma T levels that spontaneously decreased by 15% between 0800 and 1200 h. As to whether there was a linear relationship between the highest plasma T value reached and the injected dose of rhLH, statistically similar results were obtained with 150 and 225 IU rhLH. Interestingly, plasma T levels measured 240 min after rhLH injection were significantly correlated with the peak of T obtained 48 h after the stimulation with rhCG. Despite the differences in both bioavailability and time courses of action of rhLH and hCG given by different routes, the ‘response’ to an acute i.v. rhLH injection could hence be a reflection of the responsiveness of testicular Leydig cell to a maximal stimulation with rhCG. In contrast to T, no rhLH-induced change was observed in plasma E_2 levels. This probably results in a lesser potency of the stimulation with LH rather than with hCG, likely

explained by the short plasma half life of rhLH and the reversibility of its binding to the LH/hCG receptor (41). On the basis of the present study, a short testing of endocrine testicular function might be proposed. The design could be the following: a blood drawing for a basal T measurement has to be performed at midday on day 1 of the test. On day 2 at 0800 h, 150 IU rhLH should be injected intravenously. After 4 h, a second blood drawing has to be done to measure plasma T levels. By comparison with day 1, a rise by 25% of plasma T on day 2 is observed in healthy normal men. Although rhLH represents a more physiological tool than hCG to test the endocrine testis, the results obtained with rhLH in the present study need to be confirmed in a larger series of men and compared with that of the patients with delayed puberty or hypogonadism using the same study design. However promising, these results are preliminary and suggest that, at the moment, the use of rhCG should be kept as a gold standard for the evaluation of Leydig cell function. The measurement of either E_2 or T respectively at 24 and 48 h after the i.m. injection of 250 µg (6500 IU) rhCG, appears like a good design as it gives similar results than 5000 IU ehCG.

In conclusion, a single i.v. injection of 150 IU rhLH in normal adult men induces a 25% rise in plasma T levels by comparison with the reference day. The results do not permit the proposition of a very short testing of steroid hormone secretion of the testis as β_{1-24} ACTH test for adrenal gland. However, confirmed in a larger series of men, they would lead to considering the elaboration of a short and cheap testing of Leydig cell function with two blood drawings separated by 24 h and flanking a single i.v. injection of 150 IU rhLH. At the moment, hCG testing remains the gold standard for the dynamic evaluation of the endocrine testis. Recombinant hCG at a dose of 250 µg (6500 IU) gives the same results as 5000 IU extractive hCG. After a basal blood drawing, the measurement of E_2 or T respectively at 24 and 48 h after the injection of rhCG gives pertinent information on the steroid hormone secretion of the adult testis.

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