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CIRCADIAN RHYTHM OF PLASMA TESTOSTERONE, CORTISOL AND GONADOTROPINS IN NORMAL MALE SUBJECTS*

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SUMMARY

Cortisol, gonadotropins FSH–LH and testosterone were estimated in 13 normal men: in 10 subjects blood samples were collected at 8 a.m., 2 p.m. and 10 p.m. and in the others every 2 h.

The results obtained show significant diurnal variations of cortisol and testosterone with the highest values at 8 a.m. and the lowest at 10 p.m.

On the contrary, the daily variations in the concentration of plasma FSH and LH were less constant: the two hormones when recorded on plasma samples collected every 2 h, did not show a gradual decrease during the day, but oscillations reproducing a jagged line. However the mean values of the evening seem to be lower than those of the morning.

These data confirm the existence of a gonadal diurnal cycle for testosterone, which is probably controlled by the hypophysis, although a close correlation between the cyclicity of gonadotropins and testosterone secretion is not clearly demonstrated.

INTRODUCTION

DIURNAL rhythm has already been demonstrated in several human endocrine activities.

This phenomenon is well documented in the adrenal cortex, where studies have been carried out on the rhythm of cortisol secretion. It has been observed that the lowest concentrations of cortisol are found between midnight and 4 a.m., followed by a sharp rise, with the highest levels between 6 and 8 a.m. [1–11].

The circadian rhythm of cortisol is related to that of ACTH, which is controlled by a complex mechanism located in the CNS, involving the hypothalamus, the limbic structures and the reticular formation [12–18].

The reports concerning diurnal variations of gonadotropins and testosterone in normal men are contradictory; some investigators have demonstrated the presence of a circadian rhythm in FSH–LH [19–20] and in testosterone [19–23], whilst according to others there is a non-significant variation in the concentration of these hormones in blood [24–26].

In the light of these differences of opinion, further investigations were carried out on these biological phenomena, by examining the diurnal behaviour of FSH, LH, testosterone and cortisol in normal working volunteers.

MATERIALS AND METHODS

Studies were performed on 13 normal men who were students and physicians

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from our department aged between 25–56 yrs, who maintained normal working activities during the investigation.

Blood samples for the simultaneous assay of cortisol, gonadotropins and testosterone were collected at 8 a.m., 2 p.m. and 10 p.m. in 10 subjects (cases 1–10), while in 4 cases (nos. 8, 11, 12 and 13) blood samples were collected every 2 h from 8 a.m. to 10 p.m. Case no. 8 was included in both groups.

Carlo Erba spectro-grade solvents were used for plasma extraction and chromatography.

Plasma cortisol was determined using the competitive protein binding technique as described by Murphy[27]: 0·1 ml plasma was extracted twice with 1 ml ethanol in a centrifuge tube. The supernatant was transferred to a small test tube and evaporated to dryness. A standard curve was plotted with ng. 1·5, 3, 6, 9, 12, 15, 18, 21 and 24 of cortisol (Vister Co.).

The CBG isotope solution was prepared with 2·5 ml of dog plasma, 4 μCi [^{3}H]-corticosterone (NEN Chemicals Gmbh, Frankfurt, Germany, S.A. 45 Ci/mmol) and distilled water to 100 ml. 1 ml of CBG isotope solution was added to each test tube, warmed at 37°C for 5 min and cooled to 4°C for 10 min. The separation of the bound corticosterone from the unbound was obtained with 1 ml dextran coated charcoal suspension. After centrifugation, 0·5 ml of the supernatant was transferred to a counting vial for the measurement of radioactivity.

Normal values of plasma cortisol at 8 a.m. are 6–18 μg/100 ml.

Plasma FSH and LH were determined according to the procedure of Wide[28] slightly modified by Fraioli[29].

Anti LH serum was obtained by immunizing New Zealand rabbits with Pregnyl (Organon) in complete Freund's adjuvant. Doses of 15,000 IU were injected intraperitoneally every 2 weeks until a total of 120,000 IU was reached. The serum obtained at the end of the third week after the last immunization showed the highest antibody titer which was assessed by means of hemoagglutination inhibition.

Anti-FSH no. 210 antiserum was kindly supplied by Dr. Diczfaluzy*.

For FSH and LH iodination, H.FSH was kindly supplied by Dr. W.R. Butt† and H.LH was kindly supplied by Dr. A. S. Hartree‡. These were used according to the method of Greenwood *et al.* [30] slightly modified by us[29].

Radioimmunological procedure: 0·1 ml of plasma was added in two dilutions to 0·1 ml of solution containing the immunabsorbent (for FSH 0·5 I.U. HCG–17,000 I.U./mg–were added for each ml of immunabsorbent) and 0·1 ml of labelled hormone (for FSH, the latter was added after a 12 h pre-incubation). After 24 h incubation under slow rotation, the tubes were centrifuged and the solid phase was washed four times with normal saline solution containing 0·5 μ tween–20.

The standard curve was then drawn by plotting semilogarithmically increasing concentrations of the 2nd IRP–HMG against the disintegration counts.

In adult men normal values for FSH and LH are mIU 2·5–9·0/ml and 2–12/ml, respectively.

Plasma testosterone levels were measured by the competitive protein binding

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technique, based on the principles of analysis of Fritz and Knobil[31] and Kato and Horton[32] already described in detail[33–34].

In brief the method is as follows: 1 ml plasma samples were extracted with ethyl–ether which was then washed with 1 N NaOH and water. The dry residue was dissolved in a few drops of methylene chloride: methanol (9:1, v/v) chromatographed on T.L.C. and developed in the benzene: ether system (60:40, v/v). Testosterone was eluted with methylene chloride–methanol (9:1, v/v). The recovery for each determination, which was calculated by adding approximately 800 c.p.m. of 1·2 ^{3}H testosterone (NEN Chemicals Gmbh, Frankfurt, Germany, S.A. 40 Ci/mmol) to the plasma samples, was 75–90%.

The competitive protein binding reaction was carried out with a third trimester pregnancy plasma diluted to 2% with 0·9% (w/v) saline solution, and separation of the bound testosterone from the unbound was obtained with a dextran coated charcoal suspension.

After centrifugation, 0·5 ml of supernatant was transferred to a counting vial and 10 ml of Bray's solution was added. Radioactivity was measured in a liquid scintillation counter (Packard Model 3300). With this technique, the blank values, checked systematically, do not differ significantly from the zero values of the standard curve: a maximum of approximately 0·15 ng per 2 ml of water blank was found in each experiment.

In normal subjects the plasma testosterone levels are 620 ± 160 ng/100 ml in males and 62 ± 22 ng/100 ml in females.

RESULTS

The values of plasma cortisol, FSH-LH and testosterone concentrations evaluated in blood specimens collected at 8 a.m., 2 p.m. and 10 p.m. in normal working men are reported in Table 1.

These results reveal a diurnal varation in plasma cortisol; the highest levels are observed at 8 a.m. with mean values of $10·4 \pm 2·9$ μg/100 ml (range 6–16 μg/100 ml) and the lowest in the evening at 10 p.m. with mean values of $2·3 \pm 1·5$ μg/100 ml (range 1–6 μg/100 ml). The decrease of cortisol is significant with $p < 0·001$, only when the values of 8 a.m. and 10 p.m. are compared.

Diurnal fluctuations of plasma gonadotropins, with maximum concentrations early in the morning and minimum in the evening are also registered: in fact FSH levels are $8·06 \pm 2·81$ mIU/ml (range between 4·8 and 14·2 mIU/ml) at 8 a.m., $6·57 \pm 2·51$ mIU/ml (range 4·2–12·2 mIU/ml) at 2 p.m., $4·28 \pm 1·94$ mIU/ml (range 2·4–8·4 mIU/ml) at 10 p.m. The FSH decrease is significant with $p < 0·01$, only when 8 a.m. and 10 p.m. values are compared.

The drop in plasma LH is also statistically significant ($p < 0·001$) only when the 8 a.m. and 10 p.m. values are compared, the concentrations being $10·17 \pm 3·05$ mIU/ml (range between 4·2–16·2 mIU/ml) at 8 a.m., $8·40 \pm 3·26$/ml (range between 4·4 and 14·4 mIU/ml at 2 p.m. and $4·58 \pm 1·66$ mIU/ml (range from 2·4 to 6·6 mIU/ml) at 10 p.m.

Similar diurnal variations have been found in plasma testosterone. As for the other hormones, plasma levels of testosterone are higher at 8 a.m. with mean values of 834 ± 148 ng/100 ml (range from 646 to 1076 ng/100 ml), lower at 2 p.m. with mean values of 689 ± 180 ng/100 ml (range from 416 to 1077 ng/100 ml), reaching the minimum at 10 p.m. with mean values of 516 ± 136 ng/100 ml

Table 1. Diurnal variations of plasma cortisol, gonadotropins FSH–LH and testosterone in normal male subjects (blood collected at 8 a.m., 2 p.m. and 10 p.m.)

Case	Age	Cortisol–μg/100 ml			FSH–LH mIU/ml		FSH–LH mIU/ml		FSH–LH mIU/ml		Testosterone ng/100 ml		
		8 a.m.	2 p.m.	10 p.m.	8 a.m.		2 p.m.		10 p.m.		8 a.m.	2 p.m.	10 p.m.
1-B.V.	24	14	7	2	10·2	12·8	9·6	13·2	4·2	6·4	660	537	468
2-S.E.	58	9	6	2	14·2	16·2	12·2	14·4	8·4	6·6	888	667	415
3-V.A.	34	10	3·3	3	6·4	4·2	5·3	5·4	4·4	3·6	646	416	258
4-A.G.	20	16	14	6	6·8	9·4	5·4	4·4	2·8	2·8	775	652	542
5-C.S.	31	7	4·5	1	8·2	10·2	6·6	9·2	4·4	6·2	825	656	611
6-C.M.	25	10	4	1	8·6	11·8	7·2	6·8	2·4	4·6	1076	1077	719
7-D.G.F	24	13·5	8·3	1·5	9·8	11·8	4·8	6·4	3·2	2·8	758	687	532
8-P.C.	30	6	5·5	1·5	4·8	8·6	4·9	9·2	3·8	4·2	740	603	500
9-A.D.	27	8·5	2	1·5	5·4	8·8	5·6	8·6	6·8	6·2	1030	865	687
10-S.C.	30	10	4·5	4	6·2	7·2	4·2	6·4	2·4	2·4	951	736	432
Mean Values		10·4	5·9	2·3	8·06	10·17	6·57	8·40	4·28	4·58	834	689	516
SD		±2·9	±3·3	±1·5	±2·81	±3·05	±2·51	±3·26	±1·94	±1·66	±148	±180	±136

(range 258–719 ng/100 ml). The decrease is statistically significant, with $p < 0{\cdot}001$ only when the 8 a.m. and 10 p.m. values are compared.

More detailed studies on the daily variations of these hormones have been carried out by collecting blood specimens at more frequent intervals (8, 10, 12 noon, 2, 6, 8, 10 p.m.) (Table 2).

The results obtained reveal that there is a gradual decrease in the cortisol levels which is significant in all the cases studied: mean values drop from 14·2 μg/100 ml at 8 a.m. to 2·8 μg/100 ml at 10 p.m.

On the contrary the behaviour of plasma gonadotropins is less uniform, and FSH in particular does not vary significantly in cases 11 and 12, whereas in 8 and 13 they appear to decrease with oscillations which do not produce a sharp line. LH gave similar results.

However, on examining the mean values of the two pituitary hormones it can be observed that higher levels are obtained in the morning (8–12 a.m.) than in the evening (6–10 p.m.).

Finally the diurnal variations in plasma testosterone are of particular interest, showing a gradual decrease in all the 4 cases, with mean values of 954 ng/100 ml at 8 a.m. to 579 ng/100 ml at 10 p.m.

DISCUSSION

The findings referred to in this report are of particular interest inasmuch as they refer to results obtained in the simultaneous evaluation of cortisol, gonadotropins FSH–LH and testosterone in single plasma samples from normal subjects.

The characteristic diurnal variations of cortisol were observed in all the cases studied, with early morning values higher than those of late evening. This pattern was found not only in the cases in which blood was drawn 3 times daily, but also in those in which cortisol was assayed every 2 h.

On the other hand, this steroid, if assayed in blood collected every 20 minutes, appears to present a mean of 9 (range 7–13) secretory episodes during the 24 h, so that the variations in concentrations do not form a smooth curve but a jagged line with sharp rises and falls (Helman *et al.* [35], Weitzman *et al.* [36]). However these authors report that "when these same data are averaged for 1 h intervals, the resultant curve conforms quite closely to the often described deceptively smooth circadian curve" [36].

In this investigation we also observed daily variations in the plasma gonadotropins: in at least 7 out of 10 cases where blood samples were drawn 3 times daily, the plasma FSH was higher in the morning than in the evening at 10 p.m. (cases 1, 2, 4–7, 10). A similar pattern was observed for the LH where in at least 8 cases the values in the evening were considerably lower than in the morning (1, 2, 4–8, 10).

More detailed studies were performed on 4 normal subjects by evaluating the plasma gonadotropins in blood samples collected every 2 h. Evening levels of FSH were significantly decreased with respect to the morning values in 2 out of 4 cases (cases 8 and 12), although they did not produce a smooth curve. Similarly LH appeared to decrease in all cases, but with oscillations producing a jagged line. However it can be seen from the mean values that the levels of FSH in the morning (from 8 a.m. to 12 noon) are about 30% higher than those

Table 2. Diurnal variations of cortisol, FSH, LH and testosterone in normal men (blood samples collected every 2 h)

Cases	Age	Cortisol μg/100 ml 8 a.m.	10 a.m.	12 noon	2 p.m.	6 p.m.	8 p.m.	10 p.m.	FSH mIU/ml 8 a.m.	10 a.m.	12 noon	2 p.m.	6 p.m.	8p.m.	10 p.m.
8 CP	30	16	12	12	10	7	4	3	13·8	12·4	4·8	5·2	6·4	3·8	5·2
11 FS	30	18	13	11	7	7	4	1·5	12·0	10·3	11·2	8·8	10·2	12·0	11·0
12 CA	27	10	10	7	7	5	4·2	3	9·2	7·2	6·4	5·9	1·4	nd	1·4
13 TE	25	13	11	10·5	6·2	5	4	4	7·2	V-	6·2	10·0	7·2	6·0	6·0
Mean		14·2	11	10·1	7·5	6·0	4·0	2·8	8·4	9·9	7·1	7·4	6·3	5·4	5·9

Cases	Age	LH m IU/ml 8 a.m.	10 a.m.	12 noon	2 p.m.	6 p.m.	8 p.m.	10 p.m.	Testosterone ng/100 ml 8 a.m.	10 a.m.	12 noon	2 p.m.	8 p.m.	8 pm.	10 p.m
8 CP	30	8·6	4·2	5·4	3·6	4·8	2·2	4·6	1150	992	661	533	506	500	530
11 FS	30	6·8	4·2	3·2	2·4	2·2	4·4	4·4	800	722	574	461	553	415	460
12 CA	27	7·0	7·0	10·6	6·0	4·1	2·9	3·0	766	789	772	636	600	519	525
13 TE	25	6	—	7·6	7·0	4·6	4·1	4·0	1100	1023	1000	955	800	—	802
Mean		7·1	5·1	6·7	4·7	3·9	3·4	4·0	954	881	751	646	614	478	579

recorded in the evening (from 6 to 10 p.m.), while those of LH are about 40% higher.

These results are in agreement with those of Burgher *et al.*[19] and Saxena *et al.*[20] who observed a sharp drop in gonadotropin values between 8 a.m. and 12 noon, with the lowest values between 4 p.m. and 12 midnight, and the highest at 8 a.m. with values approximately 5 times those at 8 p.m.

On the contrary other investigators failed to find a diurnal cycle for LH [37–40]; Faiman and Ryan[41] observed the presence of a diurnal variation only for FSH. In studies on normal men Nankin and Troen[42] have recently demonstrated a repetitive elevation of LH determined every 15 min between 6 a.m. and 6 p.m., with abrupt elevations throughout the day; according to Yen *et al.*[43] since the clearance and metabolism of the gonadotropins remain constant during the day[44, 45] these fluctuations represent a periodic secretory activity of the hypophysis, which probably receives intermittent signals from the hypothalamus, that are not related to the sleep-wake cycle[46].

Finally, diurnal variations have been found in plasma testosterone, with higher values at 8 a.m. than 10 p.m. These results obtained by us in 4 normal subjects in whom blood samples were taken at 2 h intervals confirm these findings. In fact, plasma testosterone concentrations decreased in the evening by about 40% with respect to the morning values.

These data are in agreement with those of Resko and Eik-Nes[22], Southren *et al.*[23], Dray *et al.*[21] and Faiman and Winter[47], who registered diurnal variations in the production of testosterone, but not with those of Kirschner *et al.*[24], Hudson *et al.*[25] and Tait and Horton[26] who found no distinct fluctuations.

According to Resko and Eik-Nes[22] the diurnal variations in plasma testosterone may be related to the adrenal function; Faiman and Winter[47] however demonstrated that the diurnal variations of testosterone are independent from cortisol, since the suppression of the adrenal function by dexamethasone had no effect on the levels and cyclicity of plasma testosterone. On the other hand, the amount of testosterone of adrenal origin is so low that the plasma levels of this androgen would not be influenced by this fraction to such an extent as to produce variations between the early morning concentrations and those of the evening which are 40% lower.

It can therefore be assumed that the diurnal variations in plasma testosterone are either of testicular origin or depend on extragonadal factors such as splancnic extraction and peripheral transformation of this androgen into its metabolites.

Considering the first hypothesis, one would expect the circadian rhythm of testosterone to be regulated by the hypophysis; however, it is impossible to establish a strict correlation between diurnal gonadotropin and testosterone patterns. The diurnal variations in testosterone could therefore be merely dependent on an autonomous testicular mechanism involving testosterone release (gradual decrease in enzyme activities or variations of testicular blood flow?).

As far as the extragonadal factors are concerned, it has been demonstrated that the testosterone metabolic clearance rate, when assayed at various times throughout the day, does not vary significantly[48]. However the reduction in plasma testosterone concentrations might be due to a more progressively active transformation of the androgen into its metabolites at the peripheral level.

Another problem is the observation that these findings are in disagreement

with the current concept which maintains that testosterone and LH are linked by a feed-back mechanism. It is likely that testosterone modulates only the magnitude of the periodic release of gonadotropins programmed by the CNS as has been shown for ACTH and cortisol [49].

In this regard it has been seen that the inhibitory action of testosterone on the pituitary function comes into effect 24 h after the beginning of the treatment with fairly high doses (25 mg/ *pro die*) [39], and in castrated rats the dosage necessary to inhibit LH secretion is 100 μg/100 g body weight *pro die* [50]. On the other hand gonadotropins increase when the concentration of testosterone in plasma reaches very low values as in castrated men and in some cases of hypogonadism.

Therefore, the different behaviour between gonadotropins and testosterone observed in our group of patients could be explained by the fact that this androgen, even though it decreases, does not reach concentrations low enough to activate the feed-back mechanism.

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