Melatonin concentration before and during testosterone replacement in primary hypogonadic men

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Abstract

Objective: To study circadian levels of melatonin in primary hypogonadic adult men before and after testosterone treatment.

Design and methods: Circadian serum melatonin profiles were studied in six men with primary hypogonadism before and during testosterone substitution and compared with an age-matched control group (n = 6).

Results: Hypogonadal patients had higher plasma melatonin concentrations than the control group during day time (34 ± 6.8 compared with 5 ± 4.6 ng/l, means ± s.d.; P < 0.005) and night-time (74 ± 34.5 compared with 30.8 ± 3.2 ng/l). A 3 months course of testosterone replacement treatment in the hypogonadal group was followed by a diminution of the amplified melatonin circadian rhythm, with lower mean values both during the day (34 ± 2.8 compared with 12.7 ± 2.4 ng/l, P <0.001) and at night (74 ± 34.5 compared with 41.5 ± 13.5 ng/l, P <0.01), and a decrease in the total area under the curve (958 ± 318 compared with 475.5 ± 222.9, P = 0.046). There was a significant negative correlation between melatonin (r = −0.69) and testosterone concentrations.

Conclusions: These data indicate that diminished testosterone in male primary hypogonadism is associated with enhanced plasma levels of melatonin, and that testosterone substitution treatment induces a deamplification of the circadian rhythm of melatonin values in humans.

Introduction

The role of melatonin and the pineal gland in physiological and pathological conditions remains to be clearly defined and understood in humans. The involvement of the pineal in the mechanisms of internal synchronization has been extensively documented, and there is general agreement in considering this gland as an internal biological clock. Whereas the involvement of the pineal in the regulation of gonadal function in experimental animals (mainly in seasonal breeders) was demonstrated more than 30 years ago, the involvement of the pineal in the reproductive function in humans is still not well known. Conflicting data have appeared in the past decade, either indicating a relationship between pineal and gonadal functions, or negating it (1–3). Some previous studies have demonstrated that the function of the pineal gland is related to circulating levels of sex steroids, as plasma melatonin levels decrease with age (4, 5), and may be increased in hypogonadism (6), reverting to previous levels when hypogonadism resolves (7, 8); it is not known whether this was causative or coincidental. In contrast, other investigators have been unable to find a correlation between melatonin and gonadal status, either under physiological (pubertal development) or pathological conditions (9–12).

In the present study, we tried to clarify whether melatonin levels are increased in particular hypogonadic condition, primary hypogonadism of adult men. In addition, we examined whether melatonin is subject to modification by replacement testosterone treatment.

Subjects and methods

Six men suffering anorchy were studied during spring and summer time (April to July): four had congenital anorchia and the other two suffered secondary testicular atrophy during infancy. The study protocol was approved by the local ethics committee and all participants were included in the study after their informed consent was obtained. They were admitted twice to the Clinical Research Unit for a 24-h period.

These hypogonadal patients had previously been treated with i.m. testosterone enanthate on a personalized dosage.
schedule in order to obtain a good clinical response. For inclusion in the present study, they were asked to stop replacement treatment for a period of washout of 60 days. After the first admission, the same group of patients recommenced replacement treatment with testosterone enanthate (10 mg/kg per month) for a period of 3 months and then were readmitted for a 24-h sampling.

Blood specimens for measurement of plasma melatonin were withdrawn from an antecubital vein via an indwelling catheter every 2 h from 0900 h to 2300 h, and every hour during the period when the lights were turned off (2300 h to 0700 h); the last sample was taken at 0900 h; during the period of dark (lights off), a dim red light was used in order to prevent darkness disruption. Light intensity was measured using a luxometer (Gossen, Germany) and showed values of 500–5000 lux during the day and less than 300 during the nocturnal period. In this regard, the nocturnal period was conventionally defined as ‘when lights were off’.

Blood samples were collected in EDTA-containing plastic tubes and were immediately spun for 15 min at 1500 9g; plasma was then frozen and stored at −20°C until required for analysis.

Melatonin concentration was measured after previous extraction with diethyl ether, by RIA (Eurodiagnostics, Appelborn, Holland) which uses a radioiodinated $^{125}$I-tracer and a polyclonal antibody raised in rabbits by immunization with N-acetylmethoxytryptophan conjugated to bovine thyroglobulin. Cross-reactivity of the primary antibody with the most important indoleamines is as follows: <0.01% for 5-methoxytryptophan, tryptamine and N-acetyltryptophan, 0.02% for N-acetylserotonin, 0.05% for 5-methoxytryptamine and 1.00% for 6-OH-melatonin. The method has an analytical sensitivity of 5 ng/l; the intraserial coefficient of variation is 8.5% at 32 ng/l and 13.3% at 8 ng/l, and the interserial coefficient of variation is 13.9% at 17.5 ng/l and 16% at 89 ng/l. The samples of the study and control participants were all run in the same assay.

Follicle stimulating hormone (FSH) and luteinizing stimulating hormone (LH) were determined by RIA (Pharmacia AB Upplands, Sweden). The limits of detection for the FSH and LH assays were 0.5 IU/l and the intra- and interserial coefficients of variation were <3% and 6% respectively. LH and FSH were determined only at baseline, not after testosterone treatment.

Serum testosterone was measured by a solid-phase RIA (Cis Bio International, Gil-Sur-Yvette, France) with and intra- and interserial coefficients of variation of <6.5% and 8.5% respectively.

Melatonin and testosterone were measured before and after 3 months of testosterone enanthate: post-treatment samples were obtained 5 days after the last injection.

Melatonin concentration profiles before and during treatment were compared with those from six age-matched controls reported previously (13).

Blood specimens of the study and control participants Anorchic patients

<table>
<thead>
<tr>
<th></th>
<th>Control subjects</th>
<th>Anorchic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index (kg/m$^2$)</td>
<td>23.0 ± 4.5</td>
<td>23 ± 3.8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>26.8 ± 1.8</td>
<td>29.2 ± 7.3</td>
</tr>
<tr>
<td>Serum testosterone (nmol/l)</td>
<td>Before treatment</td>
<td>20.0 ± 5.09</td>
</tr>
<tr>
<td></td>
<td>After treatment</td>
<td>—</td>
</tr>
<tr>
<td>Serum FSH (IU/l)</td>
<td>2.7 ± 1.5</td>
<td>41.1 ± 7</td>
</tr>
<tr>
<td>Serum LH (IU/l)</td>
<td>5.7 ± 1.5</td>
<td>39.2 ± 7</td>
</tr>
</tbody>
</table>

Table 1 Characteristics and hormonal level of controls and patients. Values are expressed as mean ± s.d.

Statistical analysis

The area under the curve (AUC) was calculated from melatonin values obtained at each time point by the triangulation method using the formula $AUC = 1/2(a + b + c) \cdot r$, where $r$ is each distance between sampling points, $a$ is the increase above baseline at the first time point, $b$ is the increase above baseline at the second time point and $c$ is the increase above baseline at the third time point.

A Kruskall–Wallis one-way ANOVA test was used to compare AUC between groups. A Wilcoxon test for unpaired data was used to compare total melatonin AUC before and after testosterone treatment. A Mann–Whitney U test was used to compare post-treatment values in patients with those of controls.

An analysis of variance for repeated measures for two factors (period and treatment) was used to evaluate differences between melatonin concentrations during the diurnal and nocturnal periods.

Correlation analysis was used to assess the relationship between values of testosterone and melatonin AUC in patients before and after treatment.

A cosinor (14) analysis was also performed to confirm that a phase-shifting effect between groups was not present.

Significance was defined as $P<0.05$ (two-tailed). Results are expressed as mean ± s.d. and a 95% confidence interval (CI) is given in the text when relevant.

All data were processed by using the statistical package SPSS (Chicago, IL, USA 1995) and GraphPad Prism2 (San Diego, CA, USA 1995).

Results

At baseline, patients with primary hypogonadism showed testosterone concentrations clearly below the lower limit of reference (Table 1). Melatonin concentrations were higher in these hypogonadal patients than in the control
group, during both day and night-time (34.2 ± 8.8 compared with 5.4 ± 0.5 ng/l during the day; $P < 0.005$; 74.8 ± 34.5 compared with 30.8 ± 3.2 ng/l during the night, $P < 0.01$; means ± S.D.). Three months of testosterone treatment in hypogonadal patients were followed by a decrease in plasma melatonin levels during the day time (12.7 ± 2.4 ng/l, $P < 0.001$) and night-time (41.5 ± 13.5 ng/l, $P < 0.01$) (Fig. 1, Table 2).

When analysed together, the effects of treatment and period did not show an interaction; taken with the cosinor analysis, this indicates that testosterone treatment did not modify the melatonin profile and a phase-shift effect was not observed.

Kruskal–Wallis analysis denoted a significant difference in melatonin AUC within groups ($P = 0.005$). Analysis of the melatonin AUC before and after treatment (Wilcoxon test) showed differences between them (958 ± 318 and 475 ± 229, mean difference 483, 95% CI for the difference 127 to 838, $P = 0.046$). Changes in melatonin AUC in each individual before and after treatment with testosterone are shown in Fig. 2. There were no significant differences between melatonin concentrations in anorchic patients after treatment and those of controls.

Correlation between melatonin AUC and testosterone concentrations was demonstrated ($r = -0.69$); an increase of 1 nmol/l testosterone concentration correlated with a decline in melatonin AUC value of between 5.6 and 26.3.

**Discussion**

In the present work, we studied the possible influencing capacity of male gonadal steroids on plasma melatonin concentrations. Melatonin showed an amplification of its circadian rhythm in all hypogonadal patients studied. Testosterone replacement treatment in the usual dosage for 3 months led to a significant decrease in plasma levels of melatonin during both the day and the night, and in the calculated AUC, approaching and almost reaching the values in control subjects. Complete normalization of circulating levels of melatonin in these hypogonadal patients would probably have required much longer treatment with testosterone, but also probably a complete re-establishment of the physiological circulating testosterone profile, which

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**Table 2** Calculated areas under the curve and mean plasma melatonin concentration of patients and controls.

Values are expressed as median absolute numbers ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Anorchic patients without treatment</th>
<th>Anorchic patients during treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC total</td>
<td>321 ± 117.5</td>
<td>958 ± 318†††</td>
<td>475.5 ± 229*</td>
</tr>
<tr>
<td>Mean plasma melatonin (ng/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>During day</td>
<td>5.4 ± 0.5</td>
<td>34.2 ± 8.8†††</td>
<td>12.7 ± 2.7***</td>
</tr>
<tr>
<td>During night</td>
<td>30.8 ± 3.2</td>
<td>74.8 ± 34.5†††</td>
<td>41.5 ± 13.5**</td>
</tr>
</tbody>
</table>

AUC, Area under the curve. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with without treatment; †† $P < 0.01$; ††† $P < 0.005$ compared with controls.
may never be achieved with the current treatment modalities (15); however, our patients received enough testosterone with their treatment to attain basal values within the normal range as checked on the day when sampling for melatonin was performed, and a correlation between the concentrations of both hormones was found in our group.

It might be argued that the decreased levels of melatonin in hypogonadal patients could have been caused by an increase in hepatic metabolism of the indoleamine by testosterone, as it is known to induce liver enzyme activity. Further investigation by measurement of urinary sulphatoxymelatonin, the main metabolite of melatonin, would probably clarify this point. An increased metabolic rate for melatonin would lead to higher levels of urinary sulphatoxymelatonin, whereas no changes would be observed if melatonin production decreases.

Similar data from hypogonadal patients of different origin (suffering delayed puberty of central origin, and primary hypogonadism) have been reported recently (16–18): these studies also showed that melatonin levels were normalized after testosterone treatment. In addition, a clear decreasing trend in patients with hypogonadism, of whatever origin, was observed. Ozata et al. (17) concluded that the incomplete normalization of early morning melatonin was caused by an insufficient testosterone milieu. A study by Okatani et al. (19) that included women with hypothalamic amenorrhoea also showed that sexual conjugated steroids led to a decrease in melatonin. Taken together, all these data support a negative effect of sexual steroids on melatonin synthesis by the human pineal in both sexes.

Experimental data in animal models seem to indicate further that gonadectomy in the rat induces an increase in the number of pineal β-receptors (20); this phenomenon may offer a physiopathological explanation of our observations, as the positive modulation of β-receptor expression in the pineal may be the cause of an enhanced melatonin synthesis. Other regulatory levels may also be involved as, in steroid-sensitive animal models, melatonin binding sites have been reported in the central nervous system (21, 22).

In conclusion, our present observations of altered pineal function in men with primary hypogonadism are in agreement with our own previous data and those of others (6–8, 16, 17, 23–26), implying a modulatory relationship. Low circulating levels of testosterone in untreated primary hypogonadism appear to be associated with enhanced plasma melatonin concentration, and testosterone replacement is followed by its reduction towards levels similar to those observed in control subjects.

The role of the pineal gland in human reproductive function and its disorder is far from being clarified. Although it will continue to be a matter of scientific debate, our investigation has provided evidence suggesting that plasma testosterone levels exert a negative feedback effect on circulating melatonin levels.

Acknowledgements

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