Original Article

Circadian clock mechanisms in adrenal adenomas are not related to osteopenia and osteoporosis

Konstantinos Katsoulis¹, Dimitrios Ioannidis¹, Georgios Karageorgos¹, George I. Lambrou²

¹Department of Endocrinology, Diabetes and Metabolism, "Amalia Fleming" General Hospital, Athens, Greece;

²First Department of Pediatrics, University of Athens, Choremeio Research Laboratory, National and Kapodistrian University of Athens, Greece

Abstract

Background: It is known that humans live under the strong influence of light/dark cycles associated with the day/night changes created by the 24-hour rotation of the earth. Yet, it is possible that the circadian rhythm could be disrupted from the presence of a disease and/or changes in body homeostasis. The aim of the present study was to search for an association in patients with adrenal adenomas between cortisol circadian rhythm and osteopathies. **Materials and Methods:** Twenty eight patients with a history of adenoma, osteopathy or both participated. Patients were tested for routine biochemical factors as well as cortisol levels at 8 a.m., 1 p.m., 5 p.m. and 11 p.m., as well as ACTH and urine cortisol. **Results:** Significant differences were observed in SGOT levels Free-T₄, FSH and K⁺ in subjects with and without any history of osteopenia or osteoporosis manifesting lower levels as compared to subjects with a history of osteopenia or osteoporosis. At the same time cortisol remained similar among all patient groups. **Discussion:** Although there is no conclusive evidence, cortisol levels appear to remain untouched by the disease states and thus it appears that circadian rhythms are regulated through an alternative circuitry.

Keywords: Osteopenia, Osteoporosis, Adenoma, Cortisol, Circadian rhythm

Introduction

Reduced bone mass, either osteoporosis or osteopenia, is considered to be one of the most worrisome health problems nowadays, with serious physical, psychosocial and economic consequences. It is estimated that the number of fractures in Greece in 2010 was 86,000, with a consequent high public health economic burden¹. Among the causes of secondary osteoporosis, glucocorticoids play a very significant role in its pathogenesis as they affect both osteoblasts and osteoclasts via various mechanisms². Under normal conditions, corticosteroids are necessary for bone development and osteoblast differentiation, possibly via the Wnt/ β -catenin pathway. On the other hand, corticosteroid level elevation can enhance osteoblast and osteocyte apoptosis and therefore reduce their life span. Wnt/ β -catenin pathway plays a significant role in their action and seems that excess of glucocorticoids induces Wnt antagonists, such as Dkk1, Sost, and sFRP-1. In addition, excess of glucocorticoids can also reduce OPG expression, increase Rankl expression and so induce bone resorption³.

Other mechanisms of glucocorticoid-induced osteoporosis

is through hypogonadism, reduced intestinal calcium absorption and hypercalciuria⁴. Moreover, glucocorticoids can affect bone health as they cause myopathy and increase the frequency of falls.

Excess cortisol in the body can be a result of either glucocorticoid overtreatment or endogenous glucocorticoid overproduction. We may have the clinical characteristics of hypercortisolism (i.e. rounded red face, weak muscles, reddish stretch marks, easy bruising), which consist the diagnosis

The authors have no conflict of interest. The present study was supported in part by the National and Kapodistrian University of Athens, Medical School.

Corresponding author: George I. Lambrou, First Department of Pediatrics, University of Athens, Thivon & Levadeias, 11527, Athens, Greece

E-mail: glamprou@med.uoa.gr

Edited by: George Lyritis

Accepted 25 May 2017

Figure 1. One-way ANOVA of estimated variables with respect to the history of osteopenia and/or osteoporosis. In particular, significant differences were observed in SGOT levels with subjects without any history of osteopenia or osteoporosis manifesting lower levels as compared to subjects with a history of osteopenia or osteoporosis (A). Similarly, Free-T₄ (B) and FSH (C) manifested similar behavior as SGOT. On the other hand, K⁺ (D) manifested higher levels in subjects with no history of osteopenia or osteoporosis as compared to the subjects with a disease history (all variables are at the $p < 0.05$ significance level. SGOT: Serum glutamic oxaloacetic transaminase, Free-T₄: Thyroxine, FSH: Follicle Stimulating Hormone, K⁺: potassium ions).

of Cushing syndrome or subclinical hypercortisolism may be present. In any case, hypertension, glucose intolerance or overt diabetes, obesity and hyperlipidemia have been associated with hypercortisolism. Pituitary adenomas, adrenal tumors, ectopic Adrenocorticotropic Hormone (ACTH) or Corticotropin-Releasing Hormone (CRH) hypersecretion, adrenal hyperplasia are the causes of endogenous hypercortisolism and its diagnosis is based on the high levels of 24h urinary free cortisol, on the absence of suppression of plasma cortisol during dexamethasone suppression tests, on the disturbance of cortisol's diurnal rhythm and on increased levels of midnight salivary cortisol⁵.

Glucocorticoid treatment as well as Cushing syndrome are regarded as two very important factors of reduced bone mass and increased fracture risk^{6,7}. Other studies have shown this association for subclinical hypercortisolism as well⁸⁻¹⁶. Although endogenous cortisol secretion follows a circadian rhythm and the absence of this rhythm figures the hypercortisolism, literature is quite poor about the association between cortisol rhythm and osteoporosis or osteopenia.

Further on, it is known that humans live under the strong

influence of light/dark cycles associated with the day/night changes created by the 24-hour rotation of the earth^{17,18}. This cycling has as a result the adaptation of the body physiology in the light/dark cycles. Through this system, human beings synchronize their physical activities, such as motivational behaviors, food intake, energy metabolism, sleep, reproductive activity and immune function¹⁷⁻²⁰. The circadian system consists of central and peripheral components, which are located respectively in the suprachiasmatic nuclei (SCN) of the hypothalamus and virtually all remaining organs and tissues, including the extra-SCN brain^{17,18}. The SCN acts as a "master" clock under the strong influence of light/dark input from the eyes, whereas the peripheral system behaves as a "slave" circadian rhythm generator subjugated by the SCN through as yet unclear neural and humoral connections²¹.

Yet, it is possible that the circadian rhythm could be disrupted from the presence of a disease and/or changes in body homeostasis. Therefore, we hypothesized that circadian clock mechanisms of glucocorticoids are related to osteoporosis and osteopenia. The aim of the present study was to search for an association in patients with

Figure 2. One-way ANOVA of estimated variables with respect to the history of adenoma and osteopathy (with osteopathy we mean both the presence of osteoporosis and osteopenia). In particular, significant differences were observed in SGOT levels with subjects without any history of adenoma and osteopathy manifesting lower levels as compared to subjects with a history of adenoma and osteopathy (**A**). Similarly, Free-T₄ (**B**) and FSH (**C**) manifested similar behavior as SGOT. On the other hand, K⁺ (**D**) manifested higher levels in subjects with no history of adenoma and osteopathy as compared to the subjects with a disease history (all variables are at the $p < 0.05$ significance level. SGOT: Serum glutamic oxaloacetic transaminase, Free-T₄: Thyroxine, FSH: Follicle Stimulating Hormone, K⁺: potassium ions).

adrenal adenomas between cortisol circadian rhythm and osteopathies.

Patients and Methods

Patients

A total number of twenty eight Greek Caucasian patients (seven males and twenty one females) with adrenal adenomas were included in the study. Mean age of all patients was 62.1 ± 11.44 years, where males were 62 ± 7.3 years old and females were 62.2 ± 12.5 years old. Patients were recruited from the endocrinology clinics. The diagnosis of adrenal adenomas was based on computed tomography scan. Patients with active thyroid disease, pituitary disease, liver and kidney disease and other causes of secondary osteoporosis were excluded from the study. All experiments were conducted in compliance with the international biomedical studies stipulations, with reference to the Declaration of Helsinki of the World Medical Association. No personal data of patients were kept, while it was impossible to trace back any personal data from the data collected for the present study.

Methods

Blood samples were obtained after overnight fasting for basic biochemical (liver and kidney function, lipids, glucose, calcium, albumin, phosphorus) and hormonal (thyroid, HPA axis, pituitary, gonad) tests. Urine samples were also collected for 24 hours and cortisol as well as catecholamine were measured. The circadian rhythm of cortisol included cortisol samples at 8 p.m., 1 a.m., 5 a.m. and 11 a.m. Weight, height, body mass index (BMI: kg/m^2) and waist circumference were recorded. The diagnosis of reduced bone mass was made upon DXA measurements or personal history.

Data Analysis

The multi-parameter analyses were performed with the MATLAB® simulation environment (The Mathworks, Inc., Natick, MA). The one- and two-way ANOVA tests were used to test the mean differences between groups. Continuous variables are expressed as median $\pm$ standard deviation unless indicated differently. Correlations between variables were calculated using *Pearson's* Correlation coefficient.

Figure 3. Bar charts of ACTH and Cortisol. Urine cortisol (**A**) and ACTH (**B**) were measured in all patients and it was found that there were no significant differences between the different patients groups (Totals: includes all samples irrespectively to the specific disease group they belong, With OPE/OSP: Includes patients *with* Osteopenia and/or Osteoporosis history irrespectively of adenoma presence, Without OPE/OSP: Includes patients *without* Osteopenia and/or Osteoporosis history irrespectively of adenoma presence, Right Adenoma: Includes patients with a diagnosis of *right adenoma*, irrespectively of the presence of an osteopathy, Left Adenoma: Includes patients with a diagnosis of *left adenoma*, irrespectively of the presence of an osteopathy, With Ampho Adenoma: Includes patients with a diagnosis of a *right and left adenoma*, irrespectively of the presence of an osteopathy, Without Ampho Adenoma: Includes patients *without* a diagnosis of *adenoma*, irrespectively of the presence of an osteopathy, With Adenoma & OSP: Includes patients *with* a diagnosis of *adenoma* and the presence of an osteopathy, Without Adenoma & OSP: Includes patients *without* a diagnosis of *adenoma* and *without* the presence of an osteopathy).

Linear regressions were performed using the $y=ax+b$, $y=ax^2+bx+c$ form and curves were estimated using a least-chi-square approach. K-means, clustered scatter plots as well as Hierarchical Clustering algorithms were implemented with the MATLAB® simulation environment (The Mathworks, Inc., Natick, MA). The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Results

ANOVA Analysis of Estimated Variables

In order to find differences among the studied variables in patients with and without osteopenia and/or osteoporosis we have used analysis of variance. Our analysis showed that estimated variables with respect to the history of osteopenia and/or osteoporosis manifested significant differences. In particular, significant differences were observed in SGOT levels with subjects without any history of osteopenia or osteoporosis manifesting lower levels as compared to subjects with a history of osteopenia or osteoporosis (Figure 1A). Similarly, Free- T_4 (Figure 1B) and FSH (Figure 1C) manifested similar behavior as SGOT. On the other hand, K^+ (Figure 1D) manifested significantly higher levels in subjects with no history of osteopenia or osteoporosis as compared to the subjects with a disease history.

Similarly, when examining the effect of the presence of adenoma simultaneously with osteopathy (including both

osteopenia and osteoporosis) we found comparable results to the previous observation with the presence of osteopathies irrespectively of the presence of adenoma. In particular, we have found that significant differences were observed in SGOT levels with subjects without any history of adenoma and osteopathy manifesting lower levels as compared to subjects with a history of adenoma and osteopathy (Figure 2A). Similarly, Free- T_4 (Figure 2B) and FSH (Figure 2C) manifested similar behavior as SGOT. On the other hand, K^+ (Figure 2D) manifested higher levels in subjects with no history of adenoma and osteopathy as compared to the subjects with a disease history. The similar differences observed between the two groups that is those patients with an osteopathy irrespectively of the presence of adenoma or not as compared to the group of patients with a simultaneous presence of adenoma and osteopathy indicates that both diseases contribute equally to the differences of the estimated variables.

The Effect of Adenoma and Osteopathy on ACTH and Cortisol

We have further investigated the effect of adenoma and osteopathies on the levels of ACTH and Cortisol. In particular, we have found that no significant differences were observed between all estimated groups with respect to urine cortisol and ACTH. In particular, it appeared that there were no significant differences between all patient subgroups including those with osteopathies only irrespectively of the presence of adenoma, or patients with adenoma

Figure 4. Bar charts of Cortisol with respect to time. The different patient subgroups are presented diagrammatically. In particular, there was a significant difference between cortisol levels at 8 a.m. and 11 p.m. between all patients irrespectively of any subgroup, patients with adenoma and osteopathy as well as without adenoma and osteopathy (A). No significant difference was observed among the three groups at the respective time point (A). Further on, similar behavior was observed in all patients irrespectively of any subgroups, patients with osteopathy irrespectively of the presence of adenoma and patients without osteopathy irrespectively of the presence of adenoma (B). Finally, comparing the patients with adenoma or not irrespectively of the presence of osteopathy we have also found that there was a significant difference with respect to time from 8 a.m. to 11 p.m. but no significant differences among the patient subgroups (asterisks depict a difference at the $p < 0.05$ level).

irrespectively of the presence of an osteopathy (Figure 3).

Similarly, an expected effect on circadian rhythms would be the disruption of cortisol levels due to the presence of a specific disease or condition. In the present case it appeared that no significant differences were observed in the levels of cortisol with respect to time in all patient subgroups (Figure 4). In particular, we have found that there was a significant difference between cortisol levels at 8 a.m. and 11 p.m. between all patients irrespectively of any subgroup, patients with adenoma and osteopathy as well as without adenoma and osteopathy (Figure 4A). Further on, no significant difference was observed among the three groups at the respective time point (Figure 4A). Further on, similar behavior was observed in all patients irrespectively of any subgroups, patients with osteopathy irrespectively of the presence of adenoma and patients without osteopathy irrespectively of the presence of adenoma (Figure 4B). Finally, comparing the patients with adenoma or not irrespectively of the presence of osteopathy

we have also found that there was a significant difference with respect to time from 8 a.m. to 11 p.m. but no significant differences among the patient subgroups (Figure 4C).

Discussion

In the present work we hypothesized that the presence of osteopathy and adenoma could influence the circadian rhythm of a patient either by the presence of either disease categories or just one of them. Yet, our results showed that cortisol levels were not influenced by either conditions or a combination of the presence of one disease and absence of the other. We also hypothesized that disease-specific daily fluctuation of local glucocorticoid action promoted by concerted regulation of adenoma and osteopathy mechanisms, respectively, on the circulating cortisol levels and on ACTH, could negatively influence the maintenance of proper glucocorticoid action at all tissues

of the human body. This hypothesis was supported by the fact that loss of this relation, as seen during chronic stress or because of excessive exogenous glucocorticoid administration with extended evening effects, lead to development of *Cushing* syndrome or overlapping metabolic syndrome manifestations and subsequent atherosclerosis and cardiovascular complications²². This is further supported by the high risk of cardiovascular manifestations often observed in people who perform day/night-shift work or are involved in frequent trans-time zone travel, possibly mediated by uncoupling of the SCN and peripheral systems, leading to increased time-integrated exposure of target tissues to glucocorticoids²²⁻²⁴.

Up to date, it is still unknown how the circadian mechanism functions and basic knowledge still remains elusive.

Based on previous knowledge, patients with osteopathies may have received glucocorticoids as part of their therapy for comorbidities, a fact that would be adequate to disrupt their circadian rhythm. Yet, we have found that such a disruption may be due only to the exogenous glucocorticoids as none of the studied diseases affects the circadian rhythm at least as far as the cortisol cycling is concerned. Similar result was observed for ACTH and urine Cortisol in all patient subgroups, i.e. no significant differences were observed between the respective ACTH and urine Cortisol levels.

It is also interesting that subjects without any history of adenoma and osteopathy had lower levels of Free-T₄ and FSH as compared to subjects with a history of adenoma and osteopathy, which was an expected finding given the effect of thyroid hormones on bone metabolism and the increase of FSH levels in menopause (a period in the woman's life when these diseases usually appear).

Although there is no conclusive evidence, cortisol levels appear to remain untouched by the disease states and thus it appears that circadian rhythms are regulated through an alternative circuitry²⁵. The present phenomenon is considered to be of extreme complexity and thus, further research is needed to elucidate the detailed role of circadian circuits in common pathological conditions that extend beyond metabolic and cardiovascular diseases to include psychiatric, inflammatory/autoimmune and sleep disorders, all known to have both circadian and stress system components {Krasselt, 2016 #253;Painter, 2006 #254} but in particular, of cortisol, glucocorticoid receptor, the HPA axis/glucocorticoid signaling system at local tissues, adenomas and osteopathies.

Authors' contributions: KK: Collected data, performed clinical evaluation, drafted the manuscript. DI: Provided data. GK: Provided data. GIL: performed data analysis, drafted the manuscript, proof-edited the manuscript and gave final permission for publication.

References

1. Svedbom A, et al. Osteoporosis in the European Union: a compendium of country-specific reports. Archives of osteoporosis 2013;8:137.
2. Manelli F, et al. Glucocorticoid-induced osteoporosis. Trends in endocrinology and metabolism: TEM 2000;11(3):79-85.
3. Komori T. Glucocorticoid Signaling and Bone Biology. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et métabolisme 2016;48(11):755-63.
4. Chiodini I, et al. [Role of cortisol hypersecretion in the pathogenesis of osteoporosis]. Recenti progressi in medicina 2008;99(6):309-13.
5. Nieman LK, et al. The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline. The Journal of clinical endocrinology and metabolism 2008;93(5):1526-40.
6. Krasselt M, et al. [An update on glucocorticoid-induced osteoporosis]. Deutsche medizinische Wochenschrift (1946) 2016;141(5):352-7.
7. Painter SE, et al. Secondary osteoporosis: a review of the recent evidence. Endocrine practice: official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists 2006;12(4):436-45.
8. Hadjidakis D, et al. Does subclinical hypercortisolism adversely affect the bone mineral density of patients with adrenal incidentalomas? Clinical endocrinology 2003;58(1):72-7.
9. Tsagarakis S, et al. Endogenous subclinical hypercortisolism: Diagnostic uncertainties and clinical implications. Journal of endocrinological investigation 2006;29(5):471-82.
10. Arduc A, et al. A rare presentation of subclinical Cushing's syndrome as a pubic fracture. Internal medicine (Tokyo, Japan) 2014;53(16):1779-82.
11. Arduc A, et al. Higher body mass index and larger waist circumference may be predictors of thyroid carcinoma in patients with Hurthle-cell lesion/neoplasm fine-needle aspiration diagnosis. Clinical endocrinology 2015;83(3):405-11.
12. Goddard GM, et al. Adrenal mild hypercortisolism. Endocrinology and metabolism clinics of North America 2015;44(2):371-9.
13. Chiodini I, et al. Mechanisms in Endocrinology: Endogenous subclinical hypercortisolism and bone: a clinical review. European journal of endocrinology 2016;175(6):R265-R82.
14. Chiodini I, et al. Subclinical hypercortisolism among outpatients referred for osteoporosis. Annals of internal medicine 2007;147(8):541-8.
15. Zografos GN, et al. Subclinical Cushing's syndrome: current concepts and trends. Hormones (Athens, Greece) 2014;13(3):323-37.
16. Francucci CM, et al. Adrenal incidentaloma: effects on bone metabolism. Journal of endocrinological investigation 2008;31(7 Suppl):48-52.
17. Beck IM, et al. Crosstalk in inflammation: the interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases. Endocrine reviews 2009;30(7):830-82.
18. Charmandari E, et al. Peripheral CLOCK regulates target-tissue glucocorticoid receptor transcriptional activity in a circadian fashion in man. PloS one 2011;6(9):e25612.
19. Chrousos GP. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. The New England journal of medicine 1995;332(20):1351-62.
20. Chrousos GP. Glucocorticoid therapy. In: Felig P, Frohman LA, editors. Endocrinology & Metabolism. 4th ed. New York: McGraw-Hill; 2001. p. 609-32.
21. Chrousos GP. Stress and disorders of the stress system. Nature reviews 2009;5(7):374-81.
22. Nader N, et al. Interactions of the circadian CLOCK system and

- the HPA axis. Trends in endocrinology and metabolism: TEM 2010;21(5):277-86.
23. Scheer FA, et al. Adverse metabolic and cardiovascular consequences of circadian misalignment. Proceedings of the National Academy of Sciences of the United States of America 2009;106(11):4453-8.
 24. Ekstrand K, et al. Cardiovascular risk factors in commercial flight aircrew officers compared with those in the general population. Angiology 1996;47(11):1089-94.
 25. Nader N, et al. Circadian rhythm transcription factor CLOCK regulates the transcriptional activity of the glucocorticoid receptor by acetylating its hinge region lysine cluster: potential physiological implications. FASEB J 2009;23(5):1572-83.