

Circadian rhythm of serum 25 (OH) vitamin D, calcium and phosphorus levels in the treatment and management of type-2 diabetic patients

Tariq Masood¹, Rajeev S Kushwaha¹, Ranjana Singh², Shivani Sailwal¹, Himanshu Pandey¹, Amit Varma³, Raj K Singh^{1,*}, Germaine Cornelissen⁴

¹Biochemistry Department, Shri Guru Ram Rai Institute of Medical & Health Sciences, Patel Nagar, Dehradun, India;

²Biochemistry Department, King George's Medical University, Lucknow, India;

³Medicine Department, Shri Guru Ram Rai Institute of Medical & Health Sciences, Patel Nagar, Dehradun, India;

⁴Halberg Chronobiology Center, University of Minnesota, MN, USA.

Summary

The circadian time structure of serum 25 (OH) vitamin D (25-OHD), calcium (Ca) and phosphorus (P) may prove to be helpful in prevention, efficacy and management of diabetes mellitus. Ten newly diagnosed patients with type-2 diabetes mellitus (6 men and 4 women), 30-65 years of age, and 10 age-matched clinically healthy volunteers (7 men and 3 women) were synchronized for one week with diurnal activity from about 06:00 to about 22:00 and nocturnal rest. Breakfast was served around 08:00, lunch around 13:30 and dinner around 20:00. Drugs/nutraceuticals known to affect the vitamin D-calcium metabolism and status were not taken. Blood samples were collected at 6-h intervals for 24 h under standardized, 24-h synchronized conditions. Serum 25-OHD, Ca, P, Ca-P product and Ca-P ratio were determined. A marked circadian variation was demonstrated for 25-OHD in healthy volunteers ($p = 0.030$) and of borderline statistical significance in the diabetic patients ($p = 0.083$) by population-mean cosinor analysis. Similarly, healthy volunteers showed borderline significance for serum Ca, P and Ca-P ratio. The circadian acrophase of Ca occurred later in the patients as compared to healthy controls. Mapping the circadian rhythm (an important component of the broader time structure or chronome, which includes *a.o.*, trends with age and extra-circadian components) of vitamin D and calcium is needed for exploring their role as markers in the treatment and management of diabetic patients.

Keywords: Circadian rhythm, diabetes mellitus, serum vitamin D, calcium, phosphorus, chronoprevention, marker rhythm

1. Introduction

Diabetes rates are increasing around the world, mainly driven by increasing levels of obesity (1). Over the past three decades, the number of people with diabetes mellitus (DM) has more than doubled globally, making it one of the most important public health challenges to all nations. Type-2 diabetes mellitus (T2DM) and

prediabetes are increasingly observed among children, adolescents and younger adults. The causes of the epidemic of T2DM are embedded in a very complex group of genetic and epigenetic systems interacting within an equally complex societal framework that determines behavior and environmental influences. Prevention of T2DM is a 'whole-of-life' task and requires an integrated approach operating from the origin of the disease. It affects more than 300 million individuals in the world with significant morbidity and mortality worldwide (2). In parallel to the increase in the prevalence of DM, there has been a resurgence of vitamin D (vit D) deficiency worldwide (3,4). Though the most well-known role of vit D is the regulation of

*Address correspondence to:

Dr. R K Singh, Biochemistry Department, Shri Guru Ram Rai Institute of Medical & Health Sciences, Patel Nagar, Dehradun, India.

E-mail: singhrk23a@hotmail.com

calcium (Ca) absorption and bone metabolism, it is becoming clear that this hormone has pleiotropic effects with possible role in human health including cancer, autoimmune, infectious, respiratory, and cardiovascular disease (5-9).

Hypovitaminosis D has recently emerged as one of the factors contributing to the development of both type-1 and type-2 DM (10-13). Serum 25 (OH) vitamin D (25-OHD) concentrations were reported to be lower in patients with type-2 DM as compared to non-diabetic controls (14). Since then, many cross-sectional and case-control studies have shown an association between 25-OHD concentrations and type-2 DM (10-13). Vit D is a steroid hormone that has a crucial role in the modulation of bone homeostasis. It has been described as a wonder vitamin because of its possible benefits related to diverse health outcomes including bone disease, coronary heart disease, and type-2 diabetes (15,16). 25-OHD is a circulating metabolite used as a clinical indicator of vit D status. Results from prospective epidemiological studies have shown that low circulating 25-OHD concentrations are associated with an increased risk of developing type-2 diabetes (15,17). Whether or not this association is causal is unknown, however (16), as it may be the result of residual confounding, which is plausible in observational studies of incident type-2 diabetes. Measurements of confounders (*e.g.*, physical activity) are susceptible to errors and are not adequately controlled for in epidemiological studies (17). Although results from clinical trials (19,20) have shown no effect of vit D supplementation on the incidence of type-2 diabetes, these findings require cautious interpretation because of issues with doses, combination treatment with calcium, compliance, and suitable conditions for generalization (15).

There is no mention in the available literature to our knowledge regarding the circadian variation (A daily cycle of biological activity based on a 24-h period and influenced by regular variations in the environment, such as the alternation of night and day) of serum 25-OHD concentrations in diabetic patients. The present study was planned to provide reference values for circadian changes of serum vit D, Ca and Phosphorus (P) in clinical health and to assess any deviation from such norms in diabetic patients in an attempt to understand the role of Ca and vit D in the management and treatment of type-2 DM.

2. Materials and Methods

2.1. Study design

This study was carried out in the Department of Biochemistry, Shri Guru Ram Rai Institute of Medical & Health Sciences, Patel Nagar, Dehradun in collaboration with the Department of Medicine,

Shri Guru Ram Rai Institute of Medical & Health Sciences and Shri Mahant Indires Hospital, Patel Nagar, Dehradun, India. Two groups of subjects were investigated: a study group of 10 newly diagnosed patients (6 men; 4 women), 30 to 60 years of age, and a control group of 10 age matched clinically healthy volunteers (7 men and 3 women). This study was approved by Institutional Ethics Committee. The patients were thoroughly examined to ensure the absence of any other disease known to alter the status and rhythm of the variables examined herein. Prior to the collection of blood samples, participants refrained from taking any drug preparation that would affect or alter the Ca-vit D metabolism. All participants were kept (synchronized) for 1 week to a schedule of diurnal activity from about 06:00 to about 22:00 and nocturnal rest. All subjects took their usual (although not identical) meals three times daily: breakfast around 08:30, lunch around 13:30 and dinner around 20:30, without any change in their fluid intake. At 06:00, 12:00, 18:00 and 00:00, 6 mL of blood was collected from each subject in plain and sterile vials. The serum was separated and analyzed for Ca, P and 25-OHD, using VTROS 5.1 FS (Fusion) chemistry autoanalyzer and VITROS EciQ immunoassay analyzer and commercial kits supplied from Ortho Clinical Diagnostics, India - a division of Johnson & Johnson, USA.

2.2. Statistical analysis

Data were evaluated by conventional statistical analyses and by single and population-mean-cosinor procedures (21-23). Accordingly, the MESOR (Midline Estimating Statistic of Rhythm, a rhythm-adjusted mean), the circadian double amplitude (a measure of the extent of predictable change within a day) and the circadian acrophase (a measure of the timing of overall high values recurring each day) were determined. Furthermore, parameter tests were performed to compare each variable between healthy subjects and DM patients.

3. Results and Discussion

A circadian rhythm was demonstrated for serum vit D in healthy volunteers ($p = 0.030$) by population-mean cosinor analysis. Similarly, a circadian rhythm of borderline statistical significance was also demonstrated for vit D in patients ($p = 0.083$), and in healthy subjects for Ca ($p = 0.070$), P ($p = 0.102$), and the Ca-P ratio ($p = 0.091$) (Table 1). Serum 25-OHD concentration was maximum at 12:00 and minimum at 06:00 in diabetic patients as well as in healthy volunteers. Serum 25-OHD concentrations were numerically lower at all sampling times in patients in comparison to healthy subjects. Whereas the MESOR was not statistically

significantly different between the patients and the healthy controls, the circadian double amplitude was smaller in the patients (1.38 vs. 2.72). The circadian acrophase of serum calcium occurred later in the patients (19:12 vs. 11:15). The double circadian amplitude of serum phosphorus was smaller in the patients and the circadian acrophase occurred almost 14 h later in the diabetic patients in comparison to healthy counterparts. The circadian double amplitude of the Ca-P product was numerically larger in diabetic patients and the circadian acrophase occurred later (at 19:12 vs. 06:39) as compared to healthy controls. The MESOR of the Ca-P ratio was numerically higher in the patients, while the circadian double amplitude was numerically smaller. The circadian acrophase of Ca-P ratio occurred statistically significantly later in the diabetic patients.

Parameter tests were performed to compare each variable between healthy subjects and DM patients. The tests were carried out in 3 ways: first, by using the actual M, A, O estimates; second, by expressing the amplitude as a percentage of the MESOR; and third, by equating amplitudes to 1, thus restricting the test to be an acrophase test (Table 2). Using the original rhythm parameters, a difference in the (A, ϕ) pair is found for Ca ($p = 0.029$), the patients having a later phase and a smaller amplitude. A similar difference in (A, ϕ) pair is also of borderline statistical significance for P ($p = 0.084$). Similar results are obtained after expressing the

amplitude as a percentage of the MESOR, with a (A, ϕ) pair difference found for Ca ($p = 0.026$) and for P ($p = 0.083$). In this case, a difference in (A, ϕ) pair is also of borderline statistical significance for the C-P product ($p = 0.072$). Phase tests do not show any statistically significant difference between the two groups, except for the Ca/P ratio ($p = 0.041$).

A marked circadian variation in serum vit D concentration in healthy volunteers ($p = 0.030$) with a borderline statistical significance in patients ($p = 0.083$) was found. The MESOR and the circadian double amplitude were lower in the patients who had a similar circadian acrophase as the healthy subjects. A lower MESOR and smaller circadian amplitude in diabetic patients has not been previously reported to our knowledge. However, hypovitaminosis D has been reported in both type-1 and type-2 diabetic patients (10-13,14). A borderline statistically significant circadian rhythm was also noticed for serum Ca, P and the Ca-P ratio in healthy volunteers. Altered vit D and calcium homeostasis may play a role in the development of type-2 diabetes. Vit D and calcium intakes were inversely associated with development of type-2 diabetes, and the benefits of the two nutrients appear to be additive. For both vit D and calcium, intakes from supplements rather than from diet were significantly associated with a lower risk of type-2 diabetes (24). The

Table 1. Circadian variation of plasma calcium (mg/dL), phosphorus (mg/dL) and vitamin D (ng/mL) in patients with type-2 diabetes mellitus and age-matched healthy controls

Variable	PR	<i>p</i>	Clinical health			PR	<i>p</i>	Diabetes mellitus		
			M ± CI	2A	Ø			M ± CI	2A	Ø
Ca	66	0.07	8.38 ± 0.93	0.96	-169°	68	0.29	8.42 ± 0.53	0.92	-288°
P	73	0.10	4.79 ± 0.51	0.64	-77°	65	0.50	4.42 ± 1.17	0.50	-289°
Vit D	72	0.03	11.49 ± 1.59	2.72	-200°	76	0.08	10.44 ± 2.47	1.38	-199°
CaxP	74	0.16	40.60 ± 7.26	6.14	-100°	66	0.41	38.30 ± 12.75	10.64	-288°
Ca: P	65	0.09	1.82 ± 0.21	0.44	-203°	84	0.74	2.05 ± 0.32	0.90	-332°

Ca: Calcium; P: Phosphorus; Vit D: Vitamin D; CaxP: Ca-P product; Ca/P: Ca-P ratio; PR: percent rhythm, average proportion of variance accounted for by fit of 24-h cosine curve to individual data series; *p*: *p*-value from zero amplitude (no-rhythm) test; M: MESOR, a rhythm adjusted mean value; 2A: double circadian amplitude, measure of extent of predictable change within a day; Ø: acrophase, measure of the timing of overall high recurring each day, expressed in (negative) degrees with 360° = 24 h and 0° = 00:00; CI: 95% Confidence Interval.

Table 2. Comparison of rhythm parameters between healthy volunteers and DM patients

Population No	k	M	A	Ø	<i>p</i>	Test of equality parameters			
						Parameters(S)	DF	F	<i>p</i>
1. Ca:H	10	8.38	0.48	-169°	0.070	A, Ø	2,34	3.95	0.028
Ca:DM	10	8.42	0.46	-288°	0.297				
2. Vit D:H	10	11.49	1.36	-200°	0.030	A, Ø	2,34	0.70	0.49
VitD:DM	10	10.44	0.69	-199°	0.083				
3. P:H	10	4.79	0.32	-77°	0.10	A, Ø	2,34	2.67	0.08
P:DM	10	4.42	0.25	-289°	0.50				
4. CaxP:H	10	40.60	3.07	-100°	0.16	A, Ø	2,34	2.10	0.13
CaxP:DM	10	38.30	5.32	-288°	0.40				
5. Ca%P:H	10	1.82	0.22	-203°	0.091	A, Ø	2,34	2.01	0.14
Ca%P:DM	10	2.05	0.04	-332°	0.74				

K = number of subjects; DF: Degree of freedom; period: 24 h; *p*: *p*-value from the zero-amplitude test (left) and *p*-value from the test of equality of (A, Ø) pairs between the two groups (right).

mechanisms by which vit D may affect the risk of type-2 diabetes are not clear. Both insulin resistance and impaired pancreatic β -cell function have been reported with vit D insufficiency (11,25-28). These observations together with the finding of vit D receptors in β -cells (29) and the finding of impaired insulin secretory capacity in mice lacking a functional vit D receptor (30) indicate an important role for vit D in regulating β -cell function. Short term intervention studies with vit D supplementation in patients with type-2 diabetes have shown conflicting results (25,31). The mechanisms by which calcium intake may alter diabetes risk are speculative. Abnormal regulation of intracellular calcium affecting both insulin sensitivity and insulin release has been suggested as a potential mechanism to account for the putative association between calcium insufficiency and risk of diabetes (32). The active form of vit D, $1\alpha, 25\text{-(OH)}_2\text{D}_3$, has been associated with metabolism control, cell growth, differentiation, antiproliferation, apoptosis, and adaptive/innate immune responses, besides its functions in the integrity of bone and calcium homeostasis. Therefore, insufficient calcium absorption may be the culprit mechanism for the observed associations in our study, either due to vit D insufficiency (from low intake) or low calcium intake. This hypothesis is further supported by data indicating that calcium is essential in normalizing glucose intolerance due to vit D deficiency *in vivo* (33). An important role of $1\alpha, 25\text{-(OH)}_2\text{D}_3$ has recently been reported in the regulation of molecular clock (34). The delayed circadian acrophase of serum Ca and P and the lower MESOR and reduced circadian amplitude of vit D in type-2 diabetic patients, as observed in the present study, may play a role in the development of the disease and become a responsible risk factor deserving further investigation.

References

- Garland CF, Garland FC, Gorham ED, Lipkin M, Newmark H, Mohr SB, Holick MF. The role of vitamin D in cancer prevention. *Am J Public Health*. 2006; 96:252-261.
- Sherwin R, Jastreboff AM. Year in diabetes 2012: The diabetes tsunami. *J Clin Endocrinol Metab*. 2012; 97:4293-4301.
- Lips P. Vitamin D status and nutrition in Europe and Asia. *J Steroid Biochem Molecular Biol*. 2007; 103:620-625.
- Gannage-Yared MH, Chemali R, Yaacoub N, Halaby G. Hypovitaminosis D in a sunny country: Relation to lifestyle and bone markers. *J Bone and Mineral Res*. 2000; 15:1856-1862.
- Mohr SB. A brief history of vitamin D and cancer prevention. *Annals of Epidemiol*. 2009; 19:79-83.
- Janssens W, Lehouck A, Carremans C, Bouillon R, Mathien C, Decramer M. Vitamin D beyond bones in chronic obstructive pulmonary disease; time to act. *Am J Respiratory Critical Care Med*. 2009; 179:630-636.
- Karatekin G, Kaya A, Salihoglu O, H. Balci, Nuhoglu A. Association of subclinical vitamin D deficiency in newborns with acute lower respiratory infection and their mothers. *European J Clin Nut*. 2007; 63:473-477.
- Cantorna MT. Vitamin D and multiple sclerosis: An update. *Nutrition Reviews*. 2008; 66(Suppl 2):S135-S138.
- Adorini L, Penna G. Control of autoimmune diseases by the vitamin D endocrine system. *Nature Clin Pract Rheumatol*. 2008; 4:404-412.
- Isaia G, Giorginoand S, Adami S. High prevalence of hypovitaminosis D in female type 2 diabetic population. *Diabetes Care*. 2001; 24:1496.
- Scragg R, Sowers M, Bell C. Serum 25-hydroxyvitamin D, diabetes, and ethnicity in the Third National Health and Nutrition Examination Survey. *Diabetes Care*. 2004; 27:2813-2818.
- Scragg R. Vitamin D and type 2 diabetes: Are we ready for a prevention trial? *Diabetes*. 2008; 57:2565-2566.
- Mattila C, Knekt P, Mannisto S, Rissanen H, Laaksonen MA, Montonen J, Reunanen A. Serum 25-hydroxyvitamin D concentration and subsequent risk of type 2 diabetes. *Diabetes Care*. 2007; 30:2569-2570.
- Pietschmann P, Schernthaner G, Woloszczuk W. Serum osteocalcin levels in diabetes mellitus: Analysis of the type of diabetes and microvascular complications. *Diabetologia*. 1988; 31:892-895.
- Pilz S, Kienreich K, Rutters F, de Jongh R, van Ballegooijen AJ, Grubler M, Tomaschitz A, Dekker JM. Role of vitamin D in the development of insulin resistance and type 2 diabetes. *Curr Diab Rep*. 2013; 13:261-270.
- Rosen CJ, Adams JS, Bikle DD, Black DM, Demay MB, Manson JE, Murad MH, Kovacs CS. The non skeletal effects of vitamin D: An Endocrine Society scientific statement. *Endocrine Rev*. 2012; 33:456-492.
- Forouhi NG, Ye Z, Rickard AP, Khaw KT, Luben R, Langenberg C, Wareham NJ. Circulating 25-hydroxyvitamin D concentration and the risk of type 2 diabetes: Results from the European Prospective Investigation into Cancer (EPIC)-Norfolk cohort and updated meta-analysis of prospective studies. *Diabetologia*. 2012; 55:2173-2182.
- Wareham N, Rennie K. The assessment of physical activity in individuals and populations: Why try to be more precise about how physical activity is assessed? *Int J Obes*. 1998; 22:S30-S38.
- De Boer IH, Tinker LF, Connelly S, Curb JD, Howard BV, Kestenbaum B, Larson JC, Manson JE, Margolis KL, Suskovic DS, Weiss NS. Calcium plus vitamin D supplementation and the risk of incident diabetes in the Women's Health Initiative. *Diabetes Care*. 2008; 31:701-707.
- Avenell A, Cook JA, MacLennan GS, McPherson GC. For the RECORD trial group. Vitamin D supplementation and type 2 diabetes: A substudy of a randomised placebocontrolled trial in older people (RECORD trial, ISRCTN 51647438). *Age Ageing*. 2009; 38:606-609.
- Halberg F, Johnson EA, Nelson W, Runge W, Sothorn R. Autorhythmometry procedures for physiologic self-measurements and their analysis. *Physiol Teacher*. 1972; 1:1-11.
- Bingham C, Arbogast B, Comelissen Guillaume G, Lee JK, Halberg F. Inferential statistical methods for estimating and comparing cosinor parameters. *Chronobiologia*. 1982; 9:397-439.

23. Cornelissen G, Halberg F. Chronomedicine. In: Encyclopedia of biostatistics (Armitage P, Colton T, eds.). John Wiley & Sons Ltd., Chichester, UK, 1998; 1:642-649.
24. Pittas AG, Dawson-Hughes B, Li T, Van Dam RM, Willett WC, Manson JE, Hu FB. Vitamin D and calcium intake in relation to type 2 diabetes in women. *Diabetes Care*. 2006; 29:650-656.
25. Borissova AM, Tankova T, Kirilov G, Dakovska L, Kovacheva R. The effect of vitamin D3 on insulin secretion and peripheral insulin sensitivity in type 2 diabetic patients. *Int J Clin Pract*. 2003; 57:258-261.
26. Baynes KC, Boucher BJ, Feskens EJ, Kromhout D. Vitamin D, glucose tolerance and insulinaemia in elderly men. *Diabetologia*. 1997; 40:344-347.
27. Chiu KC, Chu A, Go VL, Saad MF. Hypovitaminosis D is associated with insulin resistance and beta cell dysfunction. *Am J Clin Nutr*. 2004; 79:820-825.
28. Norman AW, Frankel JB, Heldt AM, Grodsky GM. Vitamin D deficiency inhibits pancreatic secretion of insulin. *Science*. 1980; 209:823-825.
29. Johnson JA, Grande JP, Roche PC, Kumar R. Immunohistochemical localization of the 1, 25(OH)2D3 receptor and calbindin D28k in human and rat pancreas. *Am J Physiol*. 1994; 267:E356-E360.
30. Zeitz U, Weber K, Soegiarto DW, Wolf E, Balling R, Erben RG. Impaired insulin secretory capacity in mice lacking a functional vitamin D receptor. *FASEB J*. 2003; 17:509-511.
31. Orwoll E, Riddle M, Prince M. Effects of vitamin D on insulin and glucagon secretion in non-insulin-dependent diabetes mellitus. *Am J Clin Nutr*. 1994; 59:1083-1087.
32. Fujita T, Palmieri GM. Calcium paradox disease: Calcium deficiency prompting secondary hyperparathyroidism and cellular calcium overload. *J Bone Miner Metab*. 2000; 18:109-125.
33. Beaulieu C, Kestekian R, Havrankova J, Gascon-Barre M. Calcium is essential in normalizing intolerance to glucose that accompanies vitamin D depletion *in vivo*. *Diabetes*. 1993; 42:35-43.
34. Gutierrez-Monreal MA, Cuevas-Diaz Duran R, Moreno-Cuevas JE, Scott SP. A role for 1 α , 25-dihydroxyvitamin D3 in the expression of circadian genes. *J Biol Rhythms*. 2014; 29:384-388.

(Received January 13, 2015; Revised January 19, 2015; Accepted February 10, 2015)