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RESEARCH PAPER

Presence of impaired intestinal calcium absorption in chronic hypovitaminosis D and its change after cholecalciferol supplementation: assessment by the calcium load test

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Abstract

Background: Hypovitaminosis D is common in Asian Indians and its functional significance is currently under investigation. Previous studies have reported on the effect of low serum 25(OH)D levels (<50 nmol L^{-1}) on bone mineral density and serum parathyroid hormone values. The present study assessed the effect of chronic hypovitaminosis D in Asian Indians on intestinal calcium absorption and its change after cholecalciferol supplementation.
Methods: Subjects included 29 healthy volunteers [mean (SD) age, 28.4 ± 6.4 years] with low serum 25(OH)D levels on screening. Intestinal calcium absorption was assessed by the 'calcium load test' with 1 g of oral elemental calcium. Subjects were put on a calcium restricted diet 1 week prior to the test. The calcium load test was repeated in 26 of them after 8 weeks of supplementation with oral cholecalciferol (60 000 IU $week^{-1}$).
Results: The mean urinary calcium/creatinine ratio of the study subjects was 0.027 ± 0.023 mg mg^{-1} under fasting conditions and increased to 0.035 ± 0.032 mg mg^{-1} after calcium loading (delta change = 29.6%, $P = 0.33$). After 8 weeks of cholecalciferol supplementation, the mean serum 25(OH)D increased from 18.9 ± 11.9 to 84.4 ± 34.9 nmol L^{-1} ($P < 0.0001$). Concomitantly, the mean urinary calcium/creatinine ratio of the study subjects increased from 0.030 ± 0.024 mg mg^{-1} under fasting conditions to 0.059 ± 0.045 mg mg^{-1} after calcium loading (delta change = 96.6%, $P = 0.008$).
Conclusions: The results obtained in the present study show that chronic hypovitaminosis D in Asian Indians has functional relevance in terms of its effect on intestinal calcium absorption, which improves with cholecalciferol supplementation. These findings support the need for improving the vitamin D status of Asian Indians through dietary supplementation and exposure to sunshine.

Introduction

Hypovitaminosis D is common in Asian Indians and is related to their skin pigmentation and inadequate sunshine exposure (Goswami *et al.*, 2000; Harinarayan, 2005, Vupputuri *et al.*, 2006; Marwaha *et al.*, 2005; Sachan *et al.*, 2005). Up to 90% of them are classified as vitamin D deficient with serum 25(OH) D < 50 nmol L^{-1} (Dawson-Hughes *et al.*, 2005; Hollis, 2005; Grant & Holick, 2005). Although several studies have shown a wide prevalence of hypovitaminosis D in India, its functional significance has been assessed in only a few of them

(Goswami *et al.*, 2000; Vupputuri *et al.*, 2006; Arya *et al.*, 2004).

In humans, intestinal calcium absorption is predominantly vitamin D dependent and involves the activation of vitamin D receptors and enhanced transcription of calcium transporter TRVP6 and calbindin-9k genes (Holick, 2007). Only 10–15% of dietary calcium is absorbed in individuals with vitamin D deficiency. As serum 25(OH)D levels increase, dietary calcium absorption increases progressively until a plateau is reached with the establishment of vitamin D sufficiency [25(OH)D levels > 32 ng mL^{-1}] (Heaney *et al.*, 2003; Barger-Lux & Heaney, 2002). In our earlier studies, we found the average dietary calcium intake of Indian women from lower socioeconomic was low, in the range 345–650 mg day^{-1} (Goswami *et al.*, 2000, 2008). The low dietary intake of calcium is a significant determinant for the development of rickets and osteomalacia among Indian, African and Australasian populations (Ray *et al.*, 2009; Prentice, 2008). The present study assessed the effect of chronic hypovitaminosis D in Asian Indians on intestinal calcium absorption and its change after 8 weeks of supplementation with oral cholecalciferol (60 000 IU $week^{-1}$).

Materials and methods

The study subjects included 29 apparently healthy volunteers (17 males and 12 females) including physicians, students and paramedical staff from the All India Institute of Medical Sciences, Delhi, who were detected to have low serum 25(OH)D levels on screening from January to February 2007. All had normal renal and liver test function tests. Inclusion criteria were: (i) age >18 years; (ii) availability for follow-up for 9 weeks; and (iii) consent for undergoing vitamin D supplementation and assessment of intestinal calcium absorption by the 'calcium load test' (Pak *et al.*, 1975; Sakhaee *et al.*, 1979). Subjects on drugs affecting bone mineral metabolism, such as calcium, vitamin D, glucocorticoids, anti-tubercular drugs, anti-epileptics and thyroxine, were excluded. None of the study subjects were on diuretics. The dietary intake was evaluated using a semi quantitative food-frequency questionnaire and the 24-h recall method. Nutrient content, including calories, carbohydrate, protein, fat, calcium and phytin phosphorus (phytin-P), was calculated using standard tables on the nutritive value of Indian foods published by the National Institute of Nutrition, India (Willett *et al.*, 1985; Gopalan *et al.*, 1996). Fourteen of the 29 subjects were nonvegetarian, with frequency of meat consumption ranging from once a week to once a month. To assess physical activity, all subjects were questioned about engagement in any type of strenuous exercise leading to sweating. Based on their response, all were categorised as inactive (Washburn & Montoye, 1986; Haskell *et al.*, 1980). Although meat consumption and activity levels can affect urinary creatinine excretion, such effects are transient and do not extend beyond 24 h (Mayersohn *et al.*, 1983; Calles-Escandon *et al.*, 1984). None of the study subjects changed their pattern of diet or physical activity during the study period.

Basal assessment

In the first stage of the study, a blood sample was drawn without venostasis from all subjects after an overnight fast. Serum was separated in a refrigerated centrifuge at $1500 \times g$ for 5 min and stored at −20 °C in multiple aliquots for estimations of serum total calcium, inorganic phosphorus, alkaline phosphatase, intact parathyroid hormone (iPTH) and 25(OH)D. All subjects were instructed to abstain from dairy products (milk, curd, cottage cheese) for 1 week before the calcium load test. No major physical activity was allowed during the calcium load test, which was performed as detailed below.

An overnight fast was initiated at 21.00 h and all subjects were given 300 mL of commercially procured sterile water to drink at the onset of the fast and once again at midnight. They were again given 600 mL of water to drink on the following morning at 07.00 h. The source of water was same for all the subjects and its calcium content was only 0.03 mg dL^{-1}. Urine was collected between 07.00 h and 09.00 h in sterile containers rinsed with double-distilled water. Baseline calcium and creatinine levels were estimated in this urine.

At 09.00 h, a standard breakfast was provided, which comprised of 330 mL of fruit juice and seven biscuits [1569 kJ (375 kcal), 100 mg of phosphorus, 25 meq sodium and 29 mg of calcium]. An oral load of 1 g of elemental calcium (two tablets, each, containing 500 mg of elemental calcium and 250 IU of D_3; Elder Pharmaceutical, Navi Mumbai, India) was given along with the breakfast.

Urine was collected again from 09.00 h to 13.00 h for determination of the calcium/creatinine ratio. During this period, 300 mL of water was given at 11.00 h to ensure adequate urine output. Intestinal calcium absorption was measured by calculating the urinary calcium/creatinine ratio before and after oral calcium loading and by estimating the delta change.

Reassessment of calcium absorption after supplementation with Cholecalciferol

Eight sachets of cholecalciferol (each containing 60 000 IU of D_3; Cadila Pharmaceutical, Samba, J & K, India) and 120 tablets of calcium carbonate (each containing 500 mg of elemental calcium and 250 IU of D_3) were given to all

the subjects. They were instructed to have one sachet of cholecalciferol every week and two tablets of calcium carbonate daily (one each with breakfast and dinner) for 8 weeks; weekly reminders were given to ensure compliance. At the beginning of the ninth week, blood samples were drawn for biochemical tests under fasting conditions and repeat calcium load tests were performed after 1 week of abstinence from dairy products. One of the subjects was lost to follow up and two who admitted to irregular intake of cholecalciferol were excluded from the study. A final analysis was therefore performed for 26 subjects. Upon direct inquiry, none of the subjects, including those excluded, complained of any side effects (including gastrointestinal disturbances).

Biochemical estimations

Biochemical estimations were carried out using a semi automated analyser (Hitachi Photometer 4020; Boehringer, Mannheim, Germany) and commercial kits (Roche Diagnostics, Mannheim, Germany). The normal ranges for serum total calcium, inorganic phosphorus and alkaline phosphatase were 2.0–2.6 mmol L^{-1}, 0.8–1.45 mmol L^{-1} and 98–79 IU L^{-1}, respectively. Intra-assay and inter-assay coefficients of variation for serum total calcium, phosphorus and alkaline phosphatase assays were 3.6% and 3.8%, 3.5% and 4.8% and 5.2% and 4.1%, respectively. Serum 25(OH)D levels were measured by radioimmunoassay (Diasorin, Stillwater, MN, USA) and serum iPTH levels were measured with an electrochemiluminescent immunoassay (Roche Diagnostics; normal range: 15–65 ng L^{-1}). Urinary calcium and creatinine estimations were carried out by an *O*-cresolphthalein complexone method and the Jaffe reaction respectively (Connerty & Briggs, 1966; Bonses & Taussky, 1951). Linearity of the urinary calcium estimates were valid in the range 0.8–8.0 mg dL^{-1}. The assays for urinary calcium and creatinine estimations were performed in two batches by the same investigator. The intra-assay coefficients of variation for urinary calcium and creatinine assays were 4.1% and 1.1%, respectively.

Subjects were classified as vitamin D deficient, insufficient or sufficient on the basis of serum 25(OH)D concentrations of <50 nmol L^{-1}, 50–80 nmol L^{-1} and >80 nmol L^{-1}, respectively (Dawson-Hughes *et al.*, 2005; Hollis, 2005; Grant & Holick, 2005). The Institutional Ethics Committee of the All India Institute of Medical Sciences approved the study protocol and written informed consent was obtained from all study subjects.

Statistical analysis

Data are presented as the mean ± SD. Statistical analysis was performed using SPSS statistical software, version 11.5 (SPSS Inc., Chicago, IL, USA). Paired *t*-tests were used to assess the significance of differences in mean values of serum total calcium, inorganic phosphorus, alkaline phosphatase, iPTH, 25(OH)D and urinary calcium/creatinine ratio before and after 8 weeks of cholecalciferol supplementation. $P < 0.05$ was considered statitically significant.

Results

25(OH) D status before and after vitamin D supplementation

Table 1 shows the baseline characteristics and biochemical indices related to vitamin D status before and after supplementation with vitamin D. The mean age and body mass index (BMI) of the study group were 28.4 ± 6.4 years and 24.1 ± 3.9 kg m^{-2}, respectively. The mean BMI was comparable between males and females (24.6 ± 3.0 versus 23.4 ± 5.1 kg m^{-2}; $P = 0.12$). However, the mean age of males was significantly higher than that of females (31.2 ± 6.7 versus 24.2 ± 2.3 years; $P = 0.002$). There was no significant difference between the daily calories intake of male and female subjects: 7.10 ± 1.59 MJ (from 268 ± 72 g of carbohydrate, 49 ± 10 g of protein and 47 ± 11 g of fat) with 543 ± 215 mg of calcium and 558 ± 183 mg of phytin-P. The nutrient content of the diet was similar to that observed earlier in healthy Indians (Goswami *et al.*, 2000; Ray *et al.*, 2009). Abstinence from dairy products lead to a decline in the mean daily calcium intake of the study subjects to 456 ± 191 mg.

At baseline, 25 subjects (96.1%) were deficient in vitamin D and 11 of them had serum 25(OH)D values

Table 1 Biochemical indices before and after 8 weeks of cholecalciferol supplementation

Parameter	Baseline	After 8 weeks of cholecalciferol and calcium supplementation	*P*-value
Serum 25 (OH)D (nmol L^{-1})	18.9 ± 11.9	84.4 ± 34.9	<0.0001
Serum iPTH (ng L^{-1})	67.8 ± 29.6	36.1 ± 9.3	<0.0001
Serum total calcium (mmol L^{-1})	2.17 ± 0.17	2.34 ± 0.19	<0.0001
Serum inorganic phosphorus (mmol L^{-1})	1.32 ± 0.09	1.42 ± 0.29	0.29
Serum alkaline phosphatase (IU L^{-1})	219 ± 80	150 ± 50	<0.0001

iPTH, intact parathyroid hormone.

<12.5 nmol L^{-1} (42.3%). Only one subject had a vitamin D level >49.9 nmol L^{-1} (56.1 nmol L^{-1}). Fourteen of the 26 subjects (53.8%) had biochemical hyperparathyroidism with serum iPTH values >65 ng L^{-1}. Serum 25(OH)D levels showed an inverse relationship with iPTH ($r = -0.364$, $P = 0.067$).

After 8 weeks of cholecalciferol supplementation, serum 25(OH)D levels increased in all the subjects and their mean 25(OH)D levels were in the vitamin D sufficient range (Table 1). Concomitantly, the mean serum iPTH value was normalised in all subjects. There was a significant increase in the mean serum total calcium and a significant decrease in serum alkaline phosphatase. The change in serum 25(OH)D levels after cholecalciferol supplementation showed no significant relationship with BMI ($r = -0.284$, $P = 0.159$).

Intestinal calcium absorption: before vitamin D supplementation

The results of the calcium load test before and after 8 weeks of cholecalciferol supplementation are shown in Table 2. The mean urinary calcium/creatinine increased over baseline values by 0.008 mg mg^{-1} after 4 h of 1 g of oral calcium loading. This rise of 29.6% over baseline was not statistically significant (Table 2).

Intestinal calcium absorption: after vitamin D supplementation

After 8 weeks of supplementation, the mean urinary calcium/creatinine ratio increased over baseline by 0.029 mg mg^{-1} after 4 h of 1 g of oral calcium loading and this increase of 96.6% was statistically significant ($P = 0.008$; Fig. 1, Table 2). None of the subjects had a urinary calcium/creatinine ratio in the hypercalciuric range (>0.2 mg mg^{-1}).

Discussion

The present study assessed the effect of 8 weeks of cholecalciferol supplementation on the intestinal calcium absorption in apparently healthy subjects with vitamin D deficiency. The rise in mean serum 25(OH)D to 84.4 ± 34.9 nmol L^{-1} after cholecalciferol supplementation confirms our previous observations of the potential utility of weekly supplementation with 60 000 IU of cholecalciferol for 8 weeks for restoring circulating 25(OH)D levels to the sufficient range in vitamin D deficient subjects (Goswami *et al.*, 2008).

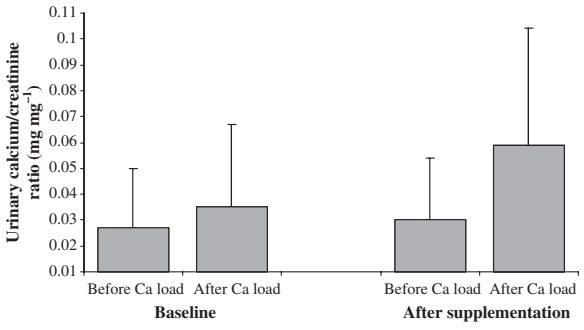

Figure 1 Change in urinary calcium/creatinine ratio in the fasting state and after an oral load of 1 g of elemental calcium before and after 8 weeks of cholecalciferol supplementation.

Absorption of dietary calcium from the intestinal mucosa is critically dependent on vitamin D and its active metabolites. Among the vitamin D deficient populations of India, Africa and Australasia, low dietary calcium compounds the problem, leading to development of rickets and osteomalacia (Ray *et al.*, 2009; Prentice, 2008; Heaney, 2008). The results of the present study show that the rise in mean 25(OH)D levels was associated with a significant improvement in intestinal calcium absorption, as reflected by the calcium load test. These observations provide direct evidence for the compromised intestinal calcium absorption in apparently healthy individuals with vitamin D deficiency. The findings acquire further significance in the context of a recent study showing a lack of bio-adaptation by the intestinal mucosa vitamin D receptor in Indians with chronic hypovitaminosis D (Goswami *et al.*, 2009). The vitamin D receptor mRNA copy numbers in the duodenal mucosa and peripheral blood mononuclear cells showed no significant difference in subjects with 25(OH)D < 25.0 nmol L^{-1} and > 25.0 nmol L^{-1}. Moreover, the vitamin D receptor mRNA levels did not change after cholecalciferol supplementation (Goswami *et al.*, 2008).

Table 2 Calcium load test: before and after 8 weeks of cholecalciferol supplementation

Cholecalciferol supplementation (60 000 IU $week^{-1}$) for 8 weeks	Urinary calcium/creatinine (mg mg^{-1})		*P*-value
	Fasting (after 7 days of calcium free diet)	After 4 h of calcium load (1 g of elemental calcium)	
Before supplementation	0.027 ± 0.023	0.035 ± 0.032	0.33
After supplementation	0.030 ± 0.024	0.059 ± 0.045	0.008

Griffin & Abrams (2005) have reviewed the various methods of assessing intestinal calcium absorption. Currently, the dual isotope tracer test as performed using stable or radioactive isotopes is considered to be ideal for assessing intestinal calcium absorption. In the present study, we used the calcium load test because of the lack of availability of the dual isotope tracer method at our centre. The calcium load test is dependent on the transient elevation of serum calcium and its spillover into the urine after challenge with an oral calcium load. Although the test is simple to perform, there may be wide variation in the results obtained because of individual differences in the renal handling of calcium. In the present study, inter-individual variation in the urinary calcium/creatinine ratio could be excluded because the same subjects were compared before and after cholecalciferol supplementation. However, the dietary calcium intake and frequency of exercise were not re-evaluated after supplementation and therefore potential variability in daily renal calcium excretion caused by changes in dietary salt, calcium intake and exercise during the 8 weeks of supplementation cannot be ruled out. Moreover, the question used to assess the impact of physical activity was validated by Haskell *et al.* (1980) by showing its correlation with serum high-density lipoprotein cholesterol levels, but not with urinary calcium excretion. Previous studies have documented that intense strenuous exercise can (Consolazio *et al.*, 1962) lead to increased calcium excretion in the sweat and increased renal calcium excretion as a result of metabolic acidosis-related increased bone resorption (Ashizawa *et al.*, 1997). Despite these limitations, the calcium load test, as performed in the present study, showed a significant improvement in intestinal calcium absorption after cholecalciferol supplementation.

The significant increase in intestinal calcium absorption observed in the present study is similar to that shown earlier in 34 Caucasian post-menopausal women by Heaney *et al.* (2003). They assessed the area under the curve for serum calcium over 9 h after an oral load of 500 mg of elemental calcium and reported a 65% increase in intestinal calcium absorption at a serum 25(OH)D level of 86.5 nmol L^{-1} compared to 50 nmol L^{-1}. Recently, in a cohort of 319 patients attending an osteoporosis clinic in South Australia, a trend for a rise in intestinal calcium absorption with increasing 25(OH)D levels was reported (Need *et al.*, 2008). Because, in the present study, intestinal calcium absorption and serum 25(OH)D levels were assessed at only two points (i.e. before and after 8 weeks of cholecalciferol supplementation) when serum 25(OH)D levels were in the ideal range, we cannot rule out the possibility of improved intestinal calcium absorption at 25(OH)D levels lower than that achieved in the present study.

Interestingly, in the present study, even after the subjects had attained a vitamin D sufficient state, their mean urinary calcium/creatinine ratios after 7 days on a calcium deficient diet (fasting = 0.030 and post-calcium load = 0.059) were lower than the corresponding ratios observed in Caucasians (fasting = 0.057 and post-calcium load = 0.131). The low urinary calcium/creatinine ratios in our subjects, despite vitamin D supplementation, could be the outcome of a long standing deficiency of vitamin D, with depleted body calcium pools and a reduced spillover into the urine. Besides vitamin D deficiency, the low dietary intake of calcium could also contribute to poor intestinal calcium absorption. Although, in the present study, we did not assess the role of variable dietary calcium intake on intestinal calcium absorption, Sheikh *et al.* (1988) showed increasing intestinal calcium absorption with increasing dietary calcium intake.

The present study, showing the effect of vitamin D supplementation on improved intestinal calcium absorption, taken together with our previous studies showing a relationship between serum 25(OH)D and bone mineral density and serum PTH levels (Vupputuri *et al.*, 2006; Goswami *et al.*, 2000), demonstrates the functional relevance of vitamin D deficiency in Asian Indians. The present study employed an open-label design and therefore there is a need to conduct similar studies using a placebo-controlled, double-blind, randomised design.

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Conflict of interest, source of funding and authorship

The authors declare that they have no conflicts of interest.

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AG conducted calcium load tests and contributed to the writing of the manuscript. NG carried out the radioimmunoassay for serum 25(25(OH)D and PTH and contributed to the writing of the manuscript. NS carried out an assessment of dietary nutrient intake. RG supervised the study, analysed the data and contributed to the writing of the manuscript. All authors contributed to the design of the study. All authors critically reviewed the manuscript and approved the final version submitted for publication.

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