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Vitamin D₃ Repletion in Chronic Kidney Disease Stage 3: Effects on Blood Endotoxin Activity, Inflammatory Cytokines and Intestinal Permeability

Manish P. Ponda, M.D.¹ and Jan L. Breslow, M.D.¹

¹Laboratory of Biochemical Genetics and Metabolism, The Rockefeller University, New York, NY

Abstract

Background—Vitamin D deficiency is common in the general population and even more prevalent in patients with chronic kidney disease (CKD). Low 25-hydroxyvitamin D [25(OH)D] levels have been associated with cardiovascular disease, though a definitive mechanistic link has not been established. Further, it is unclear if repleting vitamin D mitigates the excess risk observed in epidemiologic studies. Because vitamin D may regulate innate immunity and gut epithelial differentiation, we hypothesized that oral cholecalciferol (D₃) would result in decreased blood endotoxin activity, a potential risk factor for cardiovascular disease.

Study Design, Setting & Participants, Intervention—We studied 12 stable outpatients with CKD stage 3 and 25(OH)D deficiency, who received D₃ 30,000 units weekly for 8 weeks. The primary endpoint was the change in blood endotoxin activity.

Results—Baseline endotoxin activity correlated with 25(OH)D levels ($r = -0.60$ $p = .04$). Endotoxin activity decreased by 25% from baseline ($P = 0.03$). Despite the decrease in endotoxin activity, there was no change in intestinal permeability.

Conclusion—The results of this study suggest that vitamin D repletion therapy may have an effect on endotoxin activity in early CKD. Further intervention studies using vitamin D in the CKD population are required.

Keywords

Vitamin D; endotoxin; chronic kidney disease; cardiovascular disease

Introduction

Vitamin D regulates more than mineral metabolism. 25(OH)D can be activated to 1,25-dihydroxy vitamin D 1,25(OH)₂D independent of the kidney by 1- α -hydroxylases expressed in non-renal tissues.¹ These “local” vitamin D systems have been implicated in immune function, cell proliferation and inflammation.^{2–4} While cardiovascular disease has been associated with low 25(OH)D levels, a causal relationship remains elusive.⁵ Such a determination is especially important when deciding whether or not to treat patients with vitamin D. This is particularly relevant for CKD patients, who suffer from a higher prevalence of 25(OH)D deficiency than the general population⁶. Indeed, 25(OH)D repletion is standard clinical practice and is suggested by national guidelines.⁷

Correspondence to MPP, The Rockefeller University, Laboratory of Biochemical Genetics and Metabolism, Box 179, New York, NY 10065, mponda@rockefeller.edu, Phone: 212-327-7631, Fax: 212-327-7165.

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Renal disease is an independent risk factor for cardiovascular disease.⁸ The mechanisms underlying this phenomenon are likely multifactorial and include so-called non-traditional risk factors such as inflammation. Another potential risk factor for patients with advanced kidney disease is chronic, subclinical endotoxemia.^{9–11} Indeed, endotoxemia has been associated with accelerated atherosclerosis, even in apparently healthy volunteers.¹² Subjects with CKD may be predisposed to endotoxemia because of their increased intestinal permeability, which allows for increased bacterial translocation from the gut into systemic circulation.¹³ An increase in blood endotoxin activity as early as CKD stage 3 has also been reported¹⁴.

Vitamin D deficiency impairs the anti-microbial response of human monocytes and macrophages *in vitro* and *ex vivo*.^{2, 15} Further, vitamin D can promote gut epithelial differentiation and enhance tight junction formation.^{16, 17} Therefore, vitamin D repletion in patients may improve clearance of endotoxin by promoting innate immunity and decrease gut translocation by improving gut epithelial integrity. Whether 25(OH)D deficiency is related to blood endotoxin activity (EA), and whether vitamin D repletion can ameliorate blood EA has not been studied. We prospectively tested the hypothesis that vitamin D repletion would lower blood endotoxin activity in subjects with stage 3 CKD. We further hypothesized that vitamin D repletion would result in decreased levels of inflammatory biomarkers and decreased intestinal permeability.

Methods

Subjects

Men and post-menopausal women over the age of 50 were recruited and underwent a screening visit at which kidney function and 25(OH)D levels were measured. Urine was also collected to determine spot total protein/creatinine ratios. Estimated GFR (eGFR) was calculated using the 4-variable MDRD equation.¹⁸ Inclusion criteria were 25(OH)D level < 20 ng/ml and either CKD stage 3 or normal kidney function. Subjects without CKD were invited to participate in our “control arm”, whereas subjects with Stage 3 CKD were invited to participate in our “CKD arm”. We excluded subjects who were taking > 400 IU of ergocalciferol or cholecalciferol, or any dose of activated vitamin D as well as subjects with recent infections or those treated with immunosuppressive agents. For the “effect of meal” arm, subjects were not required to be 25(OH)D deficient.

All study visits took place at The Rockefeller University Hospital. The research protocol was approved by The Rockefeller University’s Institutional Review Board. This study was listed on ClinicalTrials.gov (Identifier NCT00772772).

Study Visits

Control arm—Participants (n=12) returned for a single study visit within 2 weeks of the screening visit at which an overnight fasting blood sample was drawn.

CKD arm—Participants (n=12) returned for a series of 3 study visits, 4 weeks apart. Prior to each visit, subjects were asked to fast for at least 8 hours. Upon arrival to Rockefeller University Hospital, fasting blood samples were drawn and intestinal permeability testing was performed. These subjects were given D₃ 30,000 units orally each week for a total of 8 weeks. Vitamin D₃ was generously provided as Maximum D3 by The BTR Group (Pittsfield, IL). Adherence was determined by pill count after 4 and 8 weeks of therapy.

Effect of meal arm—CKD 3 (n=5) or control subjects (n=4) were asked to fast for at least 8 hours. Upon arrival to Rockefeller University Hospital, fasting blood samples were drawn

to determine baseline EA. Subjects were then given a meal that provided 40% of their estimated daily caloric intake. The meal composition was 40% fat (polyunsaturated:saturated fat ratio = 0.28), 40% carbohydrates and 20% protein. The meal was prepared by The Rockefeller University Hospital's Bionutrition Department. Unconsumed portions were returned to the Bionutrition Department for calorimetric analysis. All subjects consumed >80% of the meal. Blood samples were obtained again at 3 and 5 hours post-prandially.

Endpoints

The primary endpoint was a decrease in EA, as measured by the Endotoxin Activity Assay (Spectral Diagnostics, Toronto, Canada). Secondary endpoints included changes in 25-D levels, inflammatory biomarkers (high sensitivity C-reactive protein [hsCRP], interleukin 6 [IL-6], soluble vascular cell adhesion molecule [sVCAM], soluble intercellular adhesion molecule [sICAM], and tumor necrosis factor alpha [TNF- α]), and intestinal permeability.

Assays

EA was measured per the manufacturer's instructions using whole blood collected in EDTA vacutainer tubes. The assay was performed within 3 hours of sample collection. The EA value is expressed as a fraction of maximum response to endotoxin, therefore individual values range between 0 and 1. 25(OH)D and hsCRP levels were measured in the clinical laboratory of Memorial Sloan-Kettering Hospital. IL-6, sVCAM, sICAM, and TNF- α were measured by ELISA (R&D Systems, Minneapolis, MN). Intestinal permeability was measured by Genova Diagnostics (Asheville, NC). To measure intestinal permeability, subjects consumed an oral mixture of lactulose (5g) and mannitol (1g) (L/M). Subjects were allowed to eat and drink *ad lib* 1 hour later. Urine was collected for 6 hours after L/M administration. Values for intestinal permeability are expressed as the ratio of lactulose/mannitol recovered in a timed 6-hr urine sample. 3 subjects did not have L/M ratios measured because of interfering levels of glucosuria.

Statistical Analysis

Comparisons within the CKD group were made by using one-tailed paired Student's *t*-tests. Comparisons between CKD and control groups were made using unpaired Student's *t*-tests for continuous variables and with the χ^2 test for categorical data. Correlations were assessed by calculating Pearson correlation coefficients. Data are presented as mean values $\pm$ SEM unless otherwise indicated.

Results

Baseline characteristics of subjects with CKD stage 3 and normal kidney function are shown in Table 1. CKD subjects had a mean serum creatinine of 1.47 ± 0.09 mg/dl (eGFR 51 ± 2 ml/min/1.73m²) whereas control subjects had a mean serum creatinine of 1.08 ± 0.03 mg/dl ($P < 0.001$ vs. CKD). Consistent with the comorbid conditions often seen in CKD patients, there was a high incidence of diabetes, hypertension and obesity in the CKD group, in contrast to the non-CKD controls. In CKD subjects, the mean urine total protein to creatine ratio was 0.21 g/g. There was no difference between 25(OH)D levels between CKD and control subjects (15.3 ± 1.2 vs. 15.7 ± 0.9 ng/ml, $P = 0.78$). Consistent with end-organ effects of vitamin D deficiency, CKD subjects had a mean serum PTH level of 75 ± 13 pg/ml, and that of controls was 52 ± 5 pg/ml ($P = 0.11$).

Average 25(OH)D levels were not different between the screening and first study visit in CKD subjects (15.3 ± 1.2 vs. 16.6 ± 1.9 ng/ml, $P = 0.43$; Figure 1). After 4 and 8 weeks of therapy, mean 25(OH)D levels were significantly higher than baseline (29.8 ± 2.3 and

37.4±3.0 ng/ml, respectively; $P < 0.001$ at 4 weeks and $P < 0.0001$ at 8 weeks vs. baseline). The repletion protocol of D₃ 30,000 units weekly was sufficient to correct overt 25(OH)D deficiency for all subjects. Serum calcium and phosphorus levels remained normal for all subjects throughout the study, ranging between 8.5–10 mg/dl and 2.8–4.5 mg/dl, respectively. PTH values decreased by 10±10 pg/ml ($P = 0.34$).

In CKD subjects, baseline EA was negatively correlated with baseline 25(OH)D levels ($r = -0.60$, $P = 0.04$; Figure 2a). There was no correlation between EA and 25(OH)D in non-CKD subjects ($r = -.11$, $P = .73$). After 8 weeks of D₃ repletion, EA decreased 25%, from 0.23±.04 to 0.17±.02 ($P = 0.03$; Figure 2b). The decrease in EA was not correlated with the absolute or relative changes in 25(OH)D levels. Control subjects had a mean EA of 0.20±.04, which was not significantly different from either pre- or post-D₃ repletion values in the CKD group. Indexing EA to levels of lipopolysaccharide binding protein (LBP) yielded similar results (EA per µg/ml LBP).

Given that EA activity values for CKD subjects were not different from control subjects and because previous studies in humans suggest that a high fat meal can elevate levels of blood endotoxin, we hypothesized that fasting EA levels may not reflect the susceptibility to elevated EA in CKD subjects.^{14, 19} Therefore, to examine the potential influence of diet on blood EA, we also determined the effect of a high-fat, high calorie, American-style meal on EA in a subset of both CKD and control participants. However, EA did not change from fasted levels in either CKD subjects or control subjects when measured 3 and 5 hours post-prandially (Figure 3).

Endotoxin is a known inflammatory stimulus with a potential role in atherogenesis.²⁰ Therefore, we sought to measure markers of inflammation that had also been implicated in renal and cardiovascular disease. Baseline EA strongly correlated with serum IL-6 levels in CKD subjects ($r = 0.77$, $p < 0.01$), though not other markers of inflammation. Soluble VCAM was significantly lower after D₃ therapy with mean values decreasing from a mean of 792±66 to 745±59 ng/ml ($P = 0.02$, Figure 4). hsCRP, sICAM, IL-6 and TNF-α were not significantly different before and after D₃ repletion. Markers of inflammation were lower in control compared to CKD subjects, but these differences did not reach statistical significance.

A potential origin of blood endotoxin activity is bacterial gut translocation.^{21, 22} Therefore, we measured changes in gut permeability before and after D₃ therapy by determining the differential absorption and subsequent urinary excretion of defined doses of lactulose and mannitol. Surprisingly, the L/M ratio increased with D₃ therapy from 0.07±.02 to 0.14±.03 ($P = 0.02$, Figure 5), reflecting an increase in small bowel permeability. Values below 0.1 are considered normal.

Discussion

Vitamin D deficiency is highly prevalent in patients with renal disease, and low 25(OH)D levels are associated with worse cardiovascular outcomes. Repletion based on 25(OH)D level is considered standard of care. However, prospective studies of vitamin D therapy in patients with CKD to determine potential mechanisms through which vitamin D repletion could mitigate cardiovascular risk are lacking. We hypothesized that vitamin D deficiency, through impaired innate immunity and impaired gut integrity, would result in elevated blood EA that could be lowered by D₃ therapy. We found that D₃ therapy decreased blood EA, which is consistent with our observation that low baseline 25(OH)D levels are associated with higher blood EA.

Blood endotoxin levels are notoriously difficult to measure accurately using standard methods²³. The traditional limulus amoebocyte lysate method is imprecise and non-specific for blood-based samples²⁴. The EA assay overcomes many limitations by measuring the functional response to endotoxin instead of measuring endotoxin itself, which varies across Gram negative bacterial species. The assay measures the neutrophil respiratory burst in response to opsonized endotoxin. The endpoint is quantitative chemiluminescence generated by through the combination of reactive oxygen species released from blood neutrophils and exogenous luminol. The assay is the only FDA-approved assay for measuring blood endotoxin levels in humans and has been used in validated clinical studies as a predictor of severe sepsis in critically-ill patients²⁵. Therefore, at present, the EA assay may be the most reliable endotoxin assay for human blood samples.

Because ours was a pilot study, the findings must be regarded as hypothesis-generating. The decrease in EA may reflect a favorable change in the balance between appearance of endotoxin in the blood and clearance. With trillions of gut bacteria in the human intestine, translocation is a strong candidate for the source of blood endotoxin. Our measure of intestinal permeability was therefore unexpected – an increased permeability with a decrease in blood EA after vitamin D therapy. This would suggest that despite an increase in translocation, vitamin D therapy had an even greater influence in endotoxin clearance. This is a plausible mechanism as vitamin D can promote activation of blood monocytes. The conclusions we can draw, however, may be limited by the nature of the probes we used. Lactulose (342 Daltons) and mannitol (182 Daltons) are much smaller than endotoxin, and may not reflect changes in gut permeability in the range of molecules the size of endotoxin. Further, lactulose and mannitol, primarily reflect changes in small intestine permeability. The colon, with orders of magnitude greater bacterial load, may be the more relevant source of endotoxin.

CKD patients demonstrated levels of EA which were similar to non-CKD subjects. However, CKD patients are at elevated cardiovascular risk, and therefore a modifiable risk factor is of greater significance to this group. Indeed, small changes in EA may translate into improved outcomes on a population level and chronic endotoxemia need not reach levels seen in acute illness to cause the deleterious effects of chronic inflammation.^{9, 12} The absence of any difference in EA between CKD and control subjects suggests that under the conditions of this study, there is no elevation in blood endotoxin activity attributable to CKD at this range of eGFR. This may also reflect the current debate in the field about whether current GFR estimating equations and staging criteria for kidney disease are too inclusive, especially for older subjects.^{26, 27} Therefore, even though our subjects met current criteria for CKD, with the majority having diabetes and hypertension, their renal disease may not have been advanced enough to manifest elevated EA.

Conclusion

Epidemiologic and observational studies suggest that vitamin D may play a role in health and disease distinct from calcium, phosphorus and PTH homeostasis. However, definitive data from large, prospective clinical trials are lacking. Previous studies of nutritional supplements, including vitamin D, have shown that association is not replicated with intervention.^{28–30} Prior to the availability of outcomes data, an important step is to identify biomarkers as surrogates of cardiovascular risk that respond to vitamin D therapy. This may also help to identify sub-populations that may benefit the most from vitamin D supplementation. We have shown that vitamin D supplementation in patients with CKD stage 3 can lower blood endotoxin activity, a potential accelerant of cardiovascular disease. Larger, prospective studies are required to determine if vitamin D supplementation in CKD is beneficial for cardiovascular outcomes.

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Figure 1.
Serum 25(OH)D levels before and after D₃ therapy.

Figure 2.

(a) Baseline 25(OH)D levels correlated with EA in CKD subjects (b) EA decreased 25% after D3 repletion. There was no difference in baseline EA in CKD subjects compared to controls.

Figure 3. EA was similar before and after a high-fat, high-calorie meal in both CKD and control subjects.

Figure 4.

Effect of D3 Repletion on Circulating Biomarkers of Inflammation. sVCAM levels were reduced from baseline (dark green) with vitamin D therapy (light green), though sICAM, hsCRP, IL-6 and TNF- α levels were not significantly changed. Serum levels in control subjects (gray) were consistently lower than CKD subjects, though these differences were not statistically significant.

Figure 5. Intestinal Permeability. Based on the differential absorption and subsequent excretion of lactulose and mannitol, mean values of small intestinal permeability increased into the abnormal range (normal < 0.1).

Table 1

Baseline Characteristics of Study Participants.

	CKD Subjects (n=12)	Control Subjects (n=12)	P value
Age	61±2	57±1	0.12
% Women	50	50	0.9
Serum Creatinine (mg/dl)	1.47±0.09	1.08±0.03	< 0.001
25(OH)D (ng/ml)	15.3±1.2	15.7±1.2	0.78
BMI (% obese)	33.1±1.7 (58%)	27.3±1.7 (33%)	0.03
% with Diabetes	67%	0%	<0.001
% with Hypertension	67%	17%	0.02
PTH (pg/ml)	75±13	52±5	0.11