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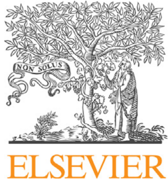

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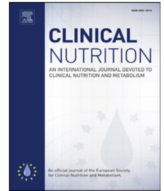

Randomized control trials

High-dose vitamin D_3 reduces circulating hepcidin concentrations: A pilot, randomized, double-blind, placebo-controlled trial in healthy adults

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SUMMARY

Background & aims: *In vitro* studies suggest that vitamin D may reduce hepcidin expression and pro-inflammatory cytokine release from monocytes. However, data assessing the vitamin D-mediated effects on iron recycling in healthy individuals are lacking. We aimed to examine the effect of high-dose vitamin D_3 on plasma hepcidin, inflammatory cytokine, and ferritin concentrations in healthy adults.
Methods: This was a pilot, double-blind, placebo-controlled trial in healthy adults (N = 28) randomized to receive a one-time oral dose of 250,000 IU of vitamin D_3 or placebo. Between- and within-group differences in plasma hepcidin, pro-inflammatory cytokine [interleukin (IL)-1β, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1)], and ferritin concentrations at baseline and 1 week were determined using two-sample and paired *t*-tests, respectively.
Results: At baseline, plasma 25-hydroxyvitamin D [25(OH)D], hepcidin, pro-inflammatory cytokine, and ferritin concentrations did not differ between the two groups, and greater than 70% of subjects in both groups were vitamin D deficient (25(OH)D < 20 ng/mL). After 1 week, plasma hepcidin concentrations decreased by 73% from baseline in those who received vitamin D_3 (geometric mean ratio [GMR] = 0.27 (95% CI: 0.11–0.62); $P = 0.005$); there was no significant change in the placebo group (GMR = 0.73 (95% CI: 0.49–1.09); $P = 0.11$). Plasma cytokine and ferritin concentrations did not change significantly in either group.
Conclusions: High-dose vitamin D_3 significantly reduced plasma hepcidin concentrations in healthy adults 1 week post-dosing, without a change in plasma pro-inflammatory cytokine or ferritin concentrations. These data suggest that vitamin D may have a role in regulating iron recycling by acting independently of changes in pro-inflammatory markers.

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1. Introduction

Vitamin D deficiency and anemia are both prominent nutrition-related public health concerns. In the United States, it has been reported that 32% of adults have vitamin D deficiency as defined by 25-hydroxyvitamin D [25(OH)D] concentrations <20 ng/mL [1]. In 2010, it was estimated that nearly one third of the global population had anemia [2]. Co-existence of vitamin D deficiency and anemia is not uncommon, as poor diets and illness are contributing factors to both conditions [3,4], and chronic diseases, including chronic kidney disease (CKD) and cardiovascular disease, incur high rates of both [5–8]. Recently, vitamin D deficiency was identified as a potential risk factor for anemia, particularly anemia of inflammation, in the general population [9,10].

Anemia of inflammation may develop due to disturbances in iron recycling secondary to pro-inflammatory cytokine-induced

Abbreviations: $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; CKD, chronic kidney disease; GMR, geometric mean ratio; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1.

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increases in the hepatic production of hepcidin, the major iron-regulatory hormone [11]. Elevations in hepcidin promote iron sequestration within cells of the reticuloendothelial system, thus limiting iron availability for erythropoiesis and hemoglobin synthesis [12]. Recent *in vitro* studies suggest a role for vitamin D in down-regulating both pro-inflammatory cytokines and hepcidin [13]. Treatment of cultured human monocytes with vitamin D has been shown to decrease the release of pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-1β (IL-1β) and down-regulate the expression of hepcidin mRNA [14,15]. Furthermore, the hepcidin antimicrobial peptide (HAMP) gene has been found to contain a vitamin D response element, suggesting a mechanism for transcriptional regulation of hepcidin by vitamin D [15].

Despite the strong biological plausibility for the association between vitamin D status and anemia, rigorous randomized controlled trials examining the effect of high-dose vitamin D supplementation on specific biomarkers involved in the pathophysiology of anemia of inflammation, namely pro-inflammatory cytokines and hepcidin, are lacking. It remains unclear whether vitamin D-mediated effects on iron recycling occur due to reductions in inflammation or through direct action on hepcidin expression. Moreover, the down-stream effects of vitamin D on makers of iron status have not yet been elucidated. Therefore, the purpose of this study was to examine the acute effect of high-dose vitamin D_3 supplementation on plasma hepcidin, inflammatory cytokine, and ferritin concentrations in healthy adults to better understand the mechanism by which vitamin D may influence iron recycling. We hypothesized that treatment with vitamin D_3 would reduce circulating hepcidin and plasma inflammatory cytokine concentrations, and increase plasma ferritin concentrations.

2. Materials and methods

2.1. Subjects and protocol

Subjects were participants in a double-blind, randomized, placebo-controlled trial designed to evaluate the impact of a large bolus dose of vitamin D_3 given prior to winter, on 25(OH)D concentrations year round in healthy adults [16]. Briefly, adults between the ages of 18 and 65 who were healthy by self-report were recruited from the Emory University campus in Atlanta, GA between August and December 2012. Participants were excluded if they were currently pregnant or breastfeeding, had granulomatous conditions, a history of kidney or liver disease, diabetes, a history of malignancy, thyrotoxicosis, a history of calcium or bone abnormalities including hyperparathyroidism, osteoporosis, and Paget's disease, an inability to ambulate, an intake of greater than 1000 mg/day of calcium, and/or used medications including antihypertensives, barbituates, anticonvulsants, or steroids. Sex, race, height, weight, time spent outdoors, and vitamin D supplement use were collected via participant self-report. Participants were asked to refrain from taking any additional vitamin D supplementation during the course of the study. A total of 28 participants were randomized to receive a one-time oral bolus dose of 250,000 IU of vitamin D_3 (Biotech Pharmacal, Fayetteville, AR) or matching placebo (Biotech Pharmacal, Fayetteville, AR). This study was approved by the Emory University Institutional Review Board, and is registered at clinicaltrials.gov (NCT01924910). All participants provided written informed consent upon enrollment. The current study used samples drawn from participants at baseline (n = 14 in the vitamin D group; n = 14 in the placebo group) and approximately 1 week (5–10 days) later (n = 13 in the vitamin D group; n = 11 in the placebo group).

2.2. Analytical procedures

Plasma 25(OH)D concentrations were determined using an automated chemiluminescent technique (IDS-iSYS automated machine, Immunodiagnostic Systems, Inc., Fountain Hills, AZ), as previously described [16]. Plasma pro-inflammatory cytokines, interleukin (IL)-1β, IL-6, and IL-8, were measured using a high-sensitivity magnetic bead-based Luminex Performance Assay multiplex kit (R&D Systems, Minneapolis, MN) with a Bioplex analyzer (Bio-Rad, Hercules, CA). Plasma monocyte chemoattractant protein-1 (MCP-1) concentrations were assayed using a bead-based Luminex Performance assay kit (R&D Systems, Minneapolis, MN) on a Bioplex analyzer (Bio-Rad, Hercules, CA). Plasma ferritin concentrations were determined via ELISA (ab108698 – Ferritin Human ELISA kit, Abcam Inc., Cambridge, MA) following manufacturer instructions; the intra-assay CV was 2.48%. A ferritin cut-off value of <12 ng/mL was used to define low iron stores [17].

Plasma hepcidin concentrations were determined using an electrochemiluminescence immunoassay as previously described [14,18,19]. Briefly, streptavidin-coated and blocked 96-well plates were incubated with 25 μL of biotin-labeled capture antibody (4 μg/mL) for 1 h. Plasma samples were diluted 1:50 in assay buffer, added to their respective washed wells, and incubated at room temperature for 1 h. Captured plasma hepcidin was detected with 25 μL of 0.1 μg/mL ruthenium-labeled conjugate hepcidin-specific detection antibody, and hepcidin concentrations were interpolated against a standard curve of reference standard hepcidin (Eli Lilly and Company, Indianapolis, IN, USA) [18,19].

2.3. Statistical analyses

Descriptive statistics were performed for all variables and reported as mean ± SD or geometric mean (95% confidence interval (CI)) for continuous variables, and number (%) for categorical variables. Variables which were not normally distributed were transformed to the natural logarithmic scale; in the case of variables with values of zero (IL-1β and IL-6), a constant of 0.01 was added to all non-missing values prior to log-transformation. Baseline comparisons between the vitamin D and placebo groups were examined using two sample *t*-tests for continuous variables, and χ^2 or Fisher's exact test for categorical variables. Between- and within-group differences in plasma 25(OH)D, cytokine, ferritin, and hepcidin concentrations from baseline to 1 week were evaluated using two sample independent *t*-tests, and paired *t*-tests, respectively. For variables requiring log-transformation, the results were back-transformed so as to be expressed in the original unit of measurement, as geometric means and their corresponding 95% CI. The mean differences between groups and between time points of the log-transformed data were exponentiated (back-transformed) to generate geometric mean ratios (GMR). A GMR of 1 indicates no treatment effect. All analyses were performed in SAS version 9.4 (SAS Institute Inc, Cary, NC) using a two-sided *P*-value of 0.05 to define statistical significance.

3. Results

3.1. Participant characteristics

Baseline demographic and biochemical characteristics for this study population are shown in Table 1. This was a young and predominantly female cohort. The mean body mass index (BMI) was within the normal range for both groups. Most participants were Caucasian and reported spending less than 10 h outdoors per week. Very few participants (n = 5 total) reported regular intake of vitamin D supplements prior to the start of the study. Baseline

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