Efficacy of High-Dose Vitamin D Supplements for Elite Athletes

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ABSTRACT

OWENS, D. J., J. C. Y. TANG, W. J. BRADLEY, A. S. SPARKS, W. D. FRASER, J. P. MORTON, and G. L. CLOSE. Efficacy of High-Dose Vitamin D Supplements for Elite Athletes. *Med. Sci. Sports Exerc.*, Vol. 49, No. 2, pp. 349–356, 2017. **Purpose**: Supplementation with dietary forms of vitamin D is commonplace in clinical medicine, elite athletic cohorts, and the general population, yet the response of all major vitamin D metabolites to high doses of vitamin D is poorly characterized. We aimed to identify the responses of all major vitamin D metabolites to moderate- and high-dose supplemental vitamin D_3. **Methods**: A repeated-measures design was implemented in which 46 elite professional European athletes were block randomized based on their basal 25[OH]D concentration into two treatment groups. Athletes received either 35,000 or 70,000 IU·wk^{-1} vitamin D_3 for 12 wk, and 42 athletes completed the trial. Blood samples were collected for 18 wk to monitor the response to supplementation and withdrawal from supplementation. **Results**: Both doses led to significant increases in serum 25[OH]D, and $1,25[OH]_2D_3$. 70,000 IU·wk^{-1} also resulted in a significant increase of the metabolite $24,25[OH]_2D$ at weeks 6 and 12 that persisted after supplementation withdrawal at week 18, despite a marked decrease in $1,25[OH]_2D_3$. Intact parathyroid hormone was decreased in both groups by week 6 and remained suppressed throughout the trial. **Conclusions**: High-dose vitamin D_3 supplementation (70,000 IU·wk^{-1}) may be detrimental for its intended purposes because of increased $24,25[OH]_2D$ production. Rapid withdrawal from high-dose supplementation may inhibit the bioactivity of $1,25[OH]_2D_3$ as a consequence of sustained increases in $24,25[OH]_2D$ that persist as 25[OH]D and $1,25[OH]_2D$ concentrations decrease. These data imply that lower doses of vitamin D_3 ingested frequently may be most appropriate and gradual withdrawal from supplementation as opposed to rapid withdrawal may be favorable. **Key Words:** 25-HYDROXYVITAMIN D, 24,25-DIHYDROXYVITAMIN D, 1,25-DIHYDROXYVITAMIN D_3, PARATHYROID HORMONE, VITAMIN D

Vitamin D and its metabolites can be described as a group of secosteroid hormones derived primarily from dermal synthesis after ultraviolet B radiation exposure (sunlight) and also from the diet. Cholecalciferol (vitamin D_3), resulting from both skin exposure to ultraviolet B and in limited amounts from dairy products, oily fish, and meat, is considered to be the major contributor to vitamin D concentration (13), whereas ergocalciferol (vitamin D_2) is exclusively derived from the diet of irradiated plants and mushrooms and appears to have less biological significance.

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After the photosynthetic conversion of 7-dehydrocholesterol to pre–vitamin D_3 and subsequently vitamin D_3 (or cholecalciferol, 14) or after dietary intake, vitamin D is transported in the circulation to the liver bound to the vitamin D–binding protein (DBP), where it is hydroxylated at C-25 by the cytochrome P450 enzyme CYP27A1 (25-hydroxylase) to form 25-hydroxyvitamin D (25[OH]D or calcidiol). This metabolite is then carried, again by DBP, to the kidney where at the proximal renal tubule it is hydroxylated by CYP27B1 (1α-hydroxylase) at C-1α to form the biologically active metabolite 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D$ or calcitriol) (1). The active compound $1,25[OH]_2D$ has long been known as a potent modulator of mineral homeostasis via the transactivation of genes related to the maintenance of calcium and phosphate homeostasis. This biological activity is achieved through interaction of $1,25[OH]_2D_3$ with its receptor, the vitamin D receptor (VDR), which heterodimerizes with retinoid X receptor to form a transcriptional complex that can bind to vitamin D response elements in the promoter of vitamin D–regulated genes (11).

In the past decade, the understanding of the biological effects of vitamin D has grown exponentially because of the development of the vitamin D knockout mouse (21) and high throughput gene microarray techniques (2). Vitamin D

is now understood to be not only an important regulator of mineral homeostasis but may be influential in cell proliferation and differentiation (20), innate and acquired immunity (12), and muscle development and repair (25,26) and in the prevention of psychological diseases such as Alzheimer's. This is particularly pertinent given the growing understanding that low vitamin D concentrations (<30 nmol·L^{-1}) are highly prevalent worldwide in general (33) as well as athletic populations (3,23). Despite a rich research base characterizing the biological importance of vitamin D, the regulation of the vitamin D endocrine system is less well understood. Practically, this is an important consideration given that some authors advocate high doses of vitamin D be administered to individuals with osteomalacia/rickets caused by severe vitamin D deficiency (defined < 12.5 nmol·L^{-1}) to ameliorate symptoms (14,18). In the context of professional sport, it is commonplace to supplement entire teams with a blanket approach to vitamin D supplementation, often without basal concentrations being assessed, and with a target concentration of >100 nmol·L^{-1}. Perhaps even more concerning is that the sports teams have access to vitamin D in single capsule at doses up to 50,000 IU (1250 μg), making single dose weekly supplementation with mega doses practically very simple, and without definitive guidelines for supplementation, this could result in more harm than benefit. Such practice is in discord with recommendations set by the European Food Safety Authority, whom advise a safe daily upper limit of 4000 IU·d^{-1} (8), in line with advice portrayed by the U.S. Institute of Medicine (IoM) guidelines for vitamin D intake (32). Notably, the U.S. IoM also state a no adverse effect limit of 10,000 IU·d^{-1}. Evidence does not exist to appropriately define the effect that high-dose blanket supplementation protocols, commonly used in elite sport, have upon the negative regulators of the vitamin D endocrine system, notably 24,25-hydroxylase (CYP24A1 or 24-hydroxylase), which functions to inactivate both 25[OH]D and 1,25$[OH]_2D_3$ (15) by hydroxylation at C-24. It is important to characterize the response high-dose blanket approaches to avoid potentially detrimental effects of too much supplemental vitamin D and contribute toward the establishment of the most safe and effective vitamin D supplementation schemes for elite athletes.

The current study therefore aimed to characterize the serum responses of the major vitamin D metabolites, 25[OH]D, 1,25$[OH]_2D_3$, 24,25$[OH]_2D$, and intact parathyroid hormone (PTH), to high-dose vitamin D supplementation (35,000 and 70,000 IU vitamin D_3 weekly) in an elite professional team sport cohort. It was hypothesized that supplementation would dose dependently increase total serum 25[OH]D and the active metabolite 1,25$[OH]_2D_3$ in a concomitant manner but would also increase the production of 24,25$[OH]_2D$.

METHODS

Participants. Forty-six elite male elite professional team sport athletes volunteered to participate in the current trial (mean ± SD; age = 26 ± 3 yr, height = 1.86 ± 0.6 m, weight = 101.5 ± 11 kg, fat mass = 11.4% ± 3%). Participants underwent a medical screening and provided full informed consent before inclusion into the study. Participants were excluded if they were currently taking vitamin D supplements, using sun beds, or injured at the time of the study. Ethical approval was granted by the ethics committee of Liverpool John Moores University (Ethics code 12/SPS/047). The recruitment for the study began in November 2012, and testing commenced in the same month. The study was concluded in April 2013. The study was conducted at latitude 52°N during the winter months to limit sunlight exposure.

Supplementation. Participants were randomly allocated to either 35,000 or 70,000 IU·wk^{-1} supplemental vitamin D_3 (Maxi Nutrition, UK), herein called moderate and high, respectively. Randomization was achieved with blocking based on baseline serum 25[OH]D and the use of a random number table to allocate participants into balanced groups. The random allocation sequence was allocated by a member of the research team and known by the rest of the research team at the point of supplement administration. The supplemental doses were chosen based on the fact that they represent widely reported supplement strategies (PubMed literature based search), applied experience of the authors in both clinical and elite sporting settings, and also followed the no adverse effect limit set by the U.S. IoM. Supplements were taken orally as a bolus in capsule form on a weekly basis to increase compliance with the protocol, which was 100% as club staff were present during the weekly distribution of supplementation and monitored the ingestion of capsules to track compliance. Supplementation continued for 12 wk at which point supplementation was ceased to monitor the response of vitamin D metabolites to withdrawal. Participants were blinded to the supplement they were receiving. Forty-two players completed the trial, whereas four dropped out or were excluded for the following reasons: one player did not tolerate venipuncture, one player would not comply with the supplementation protocol, and two players used sun beds during the trial. The vitamin D supplements were batch screened by chromatography and mass spectrometry for contaminants and confirmation of vitamin D content stated on the label. Screening was performed in accordance with ISO standard 17025. Sunlight exposure was minimal during the trial because of the latitude at which the players were based. Participants traveled for a 2-d match fixture to a foreign climate at latitude 43°N, in December during a period of significant cloud cover. Thus, players were exposed to minimal amounts of sunlight during the study period, although no direct measurement of UV exposure was taken.

Blood sampling. Blood samples were drawn before supplementation (basal and then at 6, 12, and 18 (withdrawal) wk from the start of supplementation. Blood was obtained from the antecubital vein into two serum separator tubes and two K_2EDTA tubes (Becton; Dickinson and Co., Oxford, UK). Samples were then separated to isolate serum/plasma via centrifugation at 1500 RCF for 15 min at 4°C. Serum/plasma

was extracted and stored at −80°C until required for analysis. All samples were collected in the medical room of a professional sports club.

Analysis of vitamin D metabolites and PTH. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of 25[OH]D_3, 25[OH]D_2, 24,25[OH]$_2D_3$, and 24,25[OH]$_2D_2$ was performed using a Micromass Quattro Ultima Pt mass spectrometer (Waters Corp., Milford, MA). NIST SRM972a traceable 25[OH]D_3 and 25[OH]D_2 calibration standards (Chromsystems, München, Germany) and quality controls (QC; UTAK Laboratories, CA) were purchased commercially, ranging from 0 to 200 nmol·L^{-1}. 24,25[OH]$_2D_3$ and 24,25[OH]$_2D_2$ calibration standards were prepared from certified standards (IsoSciences, King of Prussia, PA) spiked into human vitamin D depleted serum (BBI Solutions, Cardiff, UK), ranging from 0 to 14.8 nmol·L^{-1}. Two hundred microliters of pretreatment solution consist of deuterated 25[OH]D_3-[$^2H_6$] and 24R,25[OH]$_2D_3$-[$^2H_6$] in isopropanol–water 50:50 (v/v) was added to 100 μL of human serum samples, calibration standards, and QC to displace binding proteins. After mixing, the samples were loaded onto supported liquid extraction (SLE+) plates (Biotage, Uppsala, Sweden), which were eluted with 1.5 mL of n-heptane. The extraction procedure was performed by Extrahera positive pressure automation system (Biotage). Eluents were dried under nitrogen, followed by reconstitution with 50 μL 4-phenyl-1,2,4-triazoline-3,5-dione (Sigma-Aldrich, Dorset, UK). After a 30-min incubation period at room temperature, 50 μL of water was added to stop the reaction. Twenty microliters of the derivatized extracts was injected into LC-MS/MS. The separation of vitamin D metabolites was achieved using core-shell C18 2.7 μm 50 × 2.1 mm (Restek, Bellefonte, PA) reversed-phase column. A gradient elution profile was set up using mobile phase (A) LC-MS–grade water and (B) methanol containing 0.2 mM methylamine in 0.1% formic acid. The gradient at the start was 50:50 (v/v) at a column flow rate of 0.4 mL·min^{-1}, which gradually increased to 99% B. 24,25[OH]$_2D_3$/D_2 and 25[OH]D_3/D_2 peaks were eluted at 1.39, 1.42, 1.68, and 1.73 min (see Supplementary Digital Content 1, A chromatogram from an extracted sample containing 86 nmol·L^{-1} of 25[OH]D3/D2 and 5.3 nmol·L^{-1} of 24,25[OH]2D3/D2, http://links.lww.com/MSS/A782). The optimization of MS/MS conditions was conducted by a direct infusion of derivatized standards into the ion source via a T-connector. The precursor to product ion transitions were based on the molecular weight of the methylamine adduct of phenyl-1,2,4-triazoline-3,5-dione-derived products (see Supplementary Digital Content 2, A table highlighting mass spectrometer parameter settings and multiple reaction monitoring (MRM) precursor to product ion transitions for 25[OH]D_3/D_2 and 24,25[OH]$_2D_3$/D_2, http://links.lww.com/MSS/A783).

Measurements of total 1,25[OH]$_2$D were conducted using a commercially available enzyme immunoassay kit (IDS, Boldon, UK). Duplicate samples underwent immunoextraction with a 1,25[OH]$_2$D-specific solid phase monoclonal antibody and incubated overnight with sheep anti-1,25[OH]$_2$D. 1,25[OH]$_2$D-linked biotin was added the next day, followed by horseradish peroxidase labeled avidin to selectively bind to biotin complex. After a wash step, color was developed using a chromogenic substrate (3, 3′, 5, 5′-tetramethylbenzidine). The absorbance of the stopped reaction mixtures was read in a microtiter plate spectrophotometer (Multiskan Go; Thermo Scientific, Finland) at wavelength of 450 nm.

Intact PTH in K_2EDTA plasma samples were determined by electrochemiluminescence immunoassay performed using Roche e601 analyzer (Mannheim, Germany). Two labeled monoclonal antibodies were used to react with the N-terminal fragment (1–37) and the C-terminal fragment (38–84) of PTH, forming a sandwich complex. The antibody complex was magnetically captured and washed to remove unbound substances. A voltage was applied to induce chemiluminescent emission, which was measured by a photomultiplier.

Assay validation. A summary of assay characteristics is described in Supplementary Digital Content 3 (A table highlighting the assay characteristics for each parameter measured, http://links.lww.com/MSS/A784). Linearity of the methods was evaluated by analyzing stock standards made up from reference calibration solutions spiked into human sera. Standard curve was constructed by plotting the analyte response against the concentration of their respective standards. Calibration curves were accepted as linear if the weighted linear regression produced a correlation coefficient (r^2) value of >0.999. The intra- and interassay imprecision of the methods was assessed by running QC materials 10 times within a single run and separately over a 3-month period. Assays were deemed acceptable when the QC results fall within ±2 SD from the mean value. The lower limit of quantification was determined by the lowest concentration quantifiable with a precision CV of 20% for 12 replicates and a minimum peak signal-to-noise ratio of 10:1 (16). Assay recovery was determined by analyzing samples containing a fixed amount of the analyte and by calculating the percentage of the measured value against the sum of endogenous value plus spiking concentration.

Statistical analyses. Comparisons of basal total serum 25[OH]D concentration for the two dose groups were made using an independent *t*-test. The effects of vitamin D dose and time on all repeated-measures variables was determined using linear mixed modeling. Time (basal, weeks 6, 12, and 18) and dose (moderate and high) were modeled as fixed effects and participants as a random effect, with time being modeled as a continuous variable where linear or quadratic responses were observed. The covariance structure that minimized the Hurvich and Tsai's criterion (Corrected Akaike Information Criterion) value was used for the final fitted model for each metabolite. Where significant main or interaction effects were observed, *post hoc* pairwise comparisons were made with Sidak adjusted *P* values. All statistical procedures were conducted using the Statistical Package for the Social Sciences for Windows (version 22; IBM, Armonk, NY), and a two-tailed statistical significance was accepted at the $P < 0.05$ level. Descriptive statistics are displayed as means ± SD. For the

calculation of sample size, Minitab software was used. Pilot work from our laboratories during the winter months suggested that the SD for test–retest serum 25[OH]D concentrations (taken 6 wk apart) in young athletes is ~12 nmol·L^{-1}. To enable the detection of a meaningful 50 nmol·L^{-1} increase in total serum 25[OH]D concentration between presupplementation and postsupplementation with 80% power, six participants per group were required. Thus, the recruitment of an entire squad of 42 players provided a large enough sample size to make valid conclusions from the derived data.

RESULTS

Of the 42 participants that were enrolled onto the trial, 40 were tested for all primary outcome measures. This was due to player commitment to international duty. However, no participants presented with adverse side effects to supplementation during the trial, and thus no participant was withdrawn. Basal (pretreatment) total serum 25[OH]D concentrations were 86 ± 20 and 85 ± 10 nmol·L^{-1} for high and moderate treatment groups, respectively (Fig. 1). These concentrations were not significantly different between groups (t = 0.20, P = 0.84).

Total serum 25[OH]D displayed a significant interaction effect (F = 4.30, P = 0.008) between dose and time. The exploration of this interaction identified that both groups showed significantly elevated 25[OH]D concentrations at weeks 6 (moderate = 108 ± 22 nmol·L^{-1} and high = 122 ± 25 nmol·L^{-1}) and 12 (moderate = 163 ± 47 nmol·L^{-1} and high = 188 ± 66 nmol·L^{-1}). However, upon supplementation withdrawal, the moderate treatment group demonstrated a return to 25[OH]D concentrations comparable with basal by week 18 (P = 0.178) whereas the high treatment did not (P = 0.007; Fig. 2A).

Concentrations of the bioactive metabolite 1α,25[OH]$_2$D$_3$ demonstrated a significant main effect for time (F = 6.13, P = 0.001). In the moderate treatment group, peak concentrations occurred at week 6 (141 ± 58 pmol·L^{-1}). However, a delayed response was observed in the high group with peak 1α,25[OH]$_2$D$_3$ levels occurring at week 12 (112 ± 66 pmol·L^{-1}; Fig. 2B). After supplementation withdrawal, the concentration of 1α,25[OH]$_2$D$_3$ declined significantly in both groups at week 18 (moderate = 107 ± 32 pmol·L^{-1} and high = 104 ± 42 pmol·L^{-1}, P = 0.042) compared with concentrations at week 12 and was comparable with basal by this time point (P = 0.332).

The inactivated metabolite 24,25[OH]D showed comparable values between groups at basal (moderate = 8.3 ± 2.5 nmol·L^{-1} and high = 7.1 ± 1.7 nmol·L^{-1}). Both groups showed significant increases in this metabolite by week 6 (moderate, P = 0.011; high, P = 0.000), which continued to increase between weeks 6 and 12. A significant interaction effect was also detected as the high treatment group displayed markedly higher peak 24,25[OH]D concentrations (17.3 ± 4.5 nmol·L^{-1}) versus moderate treatment (11.8 ± 1.9 nmol·L^{-1}). Interestingly, 1α,25[OH]$_2$D$_3$ declined after supplementation withdrawal, whereas 24,25[OH]D remained significantly elevated at week 18 when compared with basal values in both treatment groups (moderate, 11.4 ± 2.2 and high, 15.7 ± 4.6 nmol·L^{-1}; P = 0.000 for both groups).

Intact PTH was significantly suppressed in both groups by week 6 (moderate = 2.3 ± 0.8 pmol·L^{-1} and high = 1.9 ± 0.4 pmol·L^{-1} vs basal values in moderate = 3.2 ± 2.3 pmol·L^{-1} and high 2.8 ± 1 pmol·L^{-1}) and remained suppressed throughout the trial and after the withdrawal of supplementation (moderate = 2.1 ± 0.8 pmol·L^{-1} and high 2 ± 0.6 pmol·L^{-1}).

Several studies have also examined the ratios of 25[OH]D and 1α,25[OH]$_2$D$_3$ to 24,25[OH]D, and it is evident that additional information can be obtained that is not always obvious when measuring absolute concentrations (17,19,22). In addition, it has been suggested that the ratio of 25[OH]D to 24,25[OH]D is predictive of the 25[OH]D response to supplementation (34), giving important information that surpasses simply measuring the absolute values for these metabolites. Therefore, we also calculated ratio data for the relationships between 25[OH]D and 1α,25[OH]$_2$D$_3$ to 24,25[OH]D. The ratio between 25[OH]D and 1α,25[OH]$_2$D$_3$ showed a significant main effect for time (F = 3.39, P = 0.023) but no group main effect with ratios for both supplemental treatments decreasing over the duration of the study, reaching significance by week 12 (P = 0.039) and increasing toward pretreatment values at week 18 (Fig. 3A). The ratio of 25[OH]D to the inactivated 24,25[OH]D also showed a main effect for time (F = 14.94, P = 0.000) and the absence of a group main effect. Both groups demonstrated a significant lower ratio at week 18 compared with basal (P = 0.000; Fig. 3B). Finally, the ratio of the bioactive 1α,25[OH]$_2$D$_3$ against the inactivated 24,25[OH]D was assessed. A significant interaction effect was observed as the moderate treatment group showed an increased ratio whereas the high treatment group

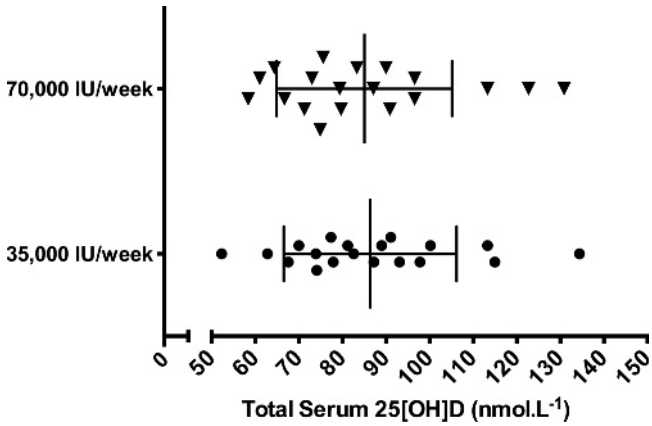

FIGURE 1—Distribution plot of basal serum total 25[OH]D concentrations in participants allocated to the 35,000 IU·wk^{-1} (moderate) and 70,000 IU·wk^{-1} (high) supplemental treatment groups. No significant differences were detected between groups for basal total serum 25[OH]D. Data were normally distributed with no significant difference detected between groups.

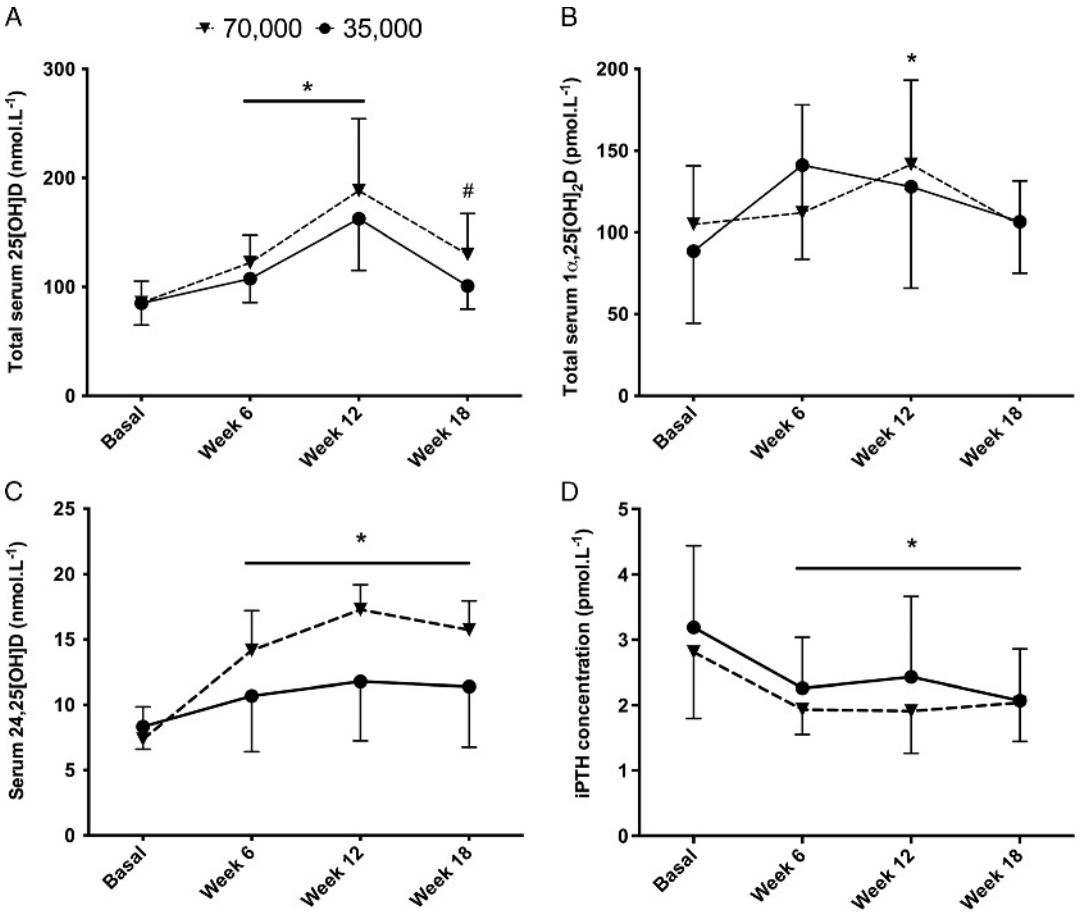

FIGURE 2—Serum responses of the major vitamin D metabolites with treatment of either 35,000 or 70,000 IU·wk^{-1} vitamin D_3 25-hydroxyvitamin D (25[OH]D) (A), 1α,25-dihydroxyvitamin D_3 (1α,25[OH]$_2$D$_3$) (B), 24,25-hydroxyvitamin D (C), and intact PTH (D). Samples were collected before supplementation (basal) and then at weeks 6, 12, and 18 of supplementation. At week 12, supplementation was stopped in both groups. *Significance for both groups compared with basal. #Significance for the 70,000 IU·wk^{-1} compared with basal.

showed an inverse relationship at week 6. However, by week 12, both treatment groups showed a significantly lower ratio of 1α,25[OH]$_2$D$_3$:24,25[OH]D (P = 0.005) that was maintained at week 18 after supplementation withdrawal (P = 0.003; Fig. 3C).

DISCUSSION

The current investigation sought to define the serum responses of the major vitamin D metabolites in a professional athletic cohort to establish the efficacy of a blanket supplementation approach using two commonly used and commercially available doses of vitamin D_3. Our main findings demonstrate that both 35,000 and 70,000 IU·wk^{-1} oral vitamin D_3 supplementation significantly elevated total serum 25[OH]D concentrations. The highest dose led to an initial rapid increase in 1.25[OH]$_2$D but then a decrease in serum 1,25[OH]$_2$D at week 12 when there was a significant increase of 24,25[OH]$_2$D$_3$, which had also been significantly increased at week 6. Resultantly, these responses led to a significantly lower ratio of 1,25[OH]$_2$ D to 24,25[OH]$_2$D from week 6 with the higher, 70,000 IU treatment. After the withdrawal of supplementation, the concentrations of 25[OH]D and 1α,25[OH]$_2$D return to basal values within 6 wk. These data imply that high doses of supplemental vitamin D_3 are sufficient to markedly induce the expression of 24-hydroxylase, leading to the negative control of 1,25[OH]$_2$D activity. Finally, we demonstrate that elevating serum 25[OH]D and 1α,25[OH]$_2$D$_3$ suppresses intact PTH appearance in circulation. This finding is in agreement with previous data published by our group (27) and is underpinned by the understanding that DNA-binding sequences exist in the PTH gene (6), permitting the suppression of the gene when adequate ligand (1,25[OH]$_2$D) is available to induce transcriptional suppression by the VDR (30).

The fact that the concentration of serum 24,25[OH]$_2$D$_3$ did not show a decline along with 1,25[OH]$_2$D after the withdrawal of supplementation has practical implications. The finding suggests that the activity of 24-hydroxylase is sustained after large increases in 1,25[OH]$_2$D and may persist and decrease both the concentration and the subsequent biological activity of 1,25[OH]$_2$D. Evidence is now emerging that the 24,25[OH]$_2$D metabolite may act at the

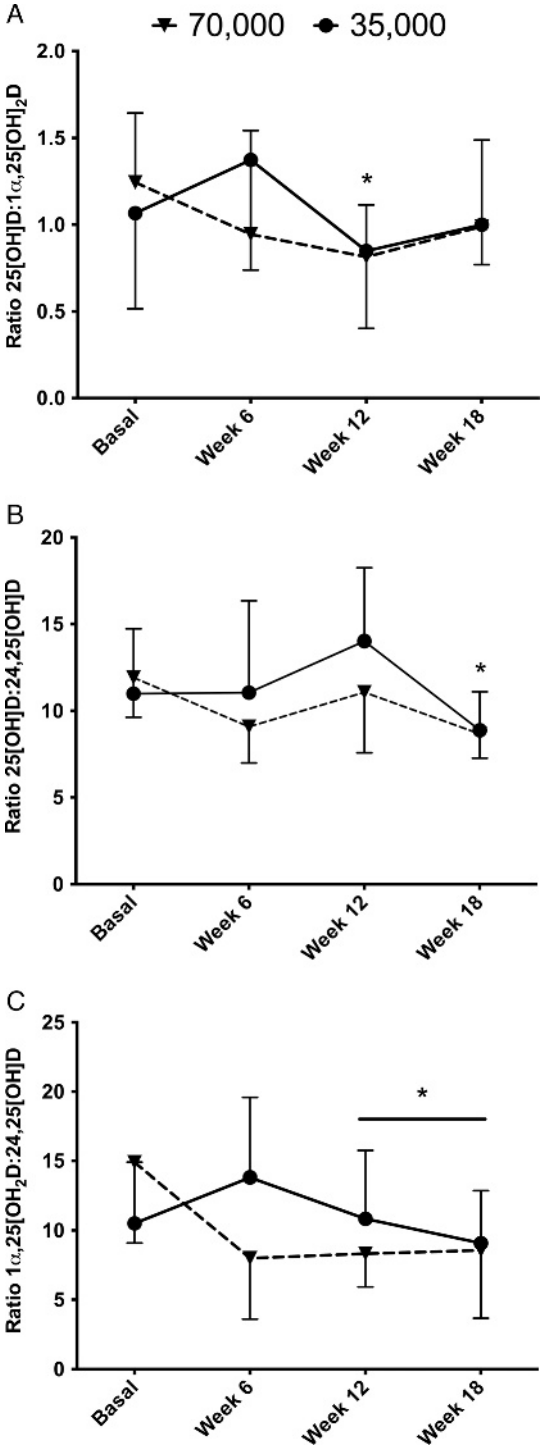

FIGURE 3—Relationships between the major vitamin D metabolites, expressed as ratio at all test time points. A, Ratio of 25-hydroxyvitamin D (25[OH]D) to the biologically active 1α,25-dihydroxyvitamin D_3 (1α,25$[OH]_2D_3$). B, Ratio of 25[OH]D to the inactive metabolite 24,25[OH]D. C, Ratio of 1α,25$[OH]_2D_3$ to 24,25[OH]D. *Significance for both groups compared with basal.

VDR as a "blocking molecule" binding to the VDR decreasing 1,25 $[OH]_2D$ activity (5). Because 24,25$[OH]_2D$ is present in the circulation in nanomole per liter concentration compared with picomole per liter for 1,25$[OH]_2D$, the significantly higher prevailing 24,25$[OH]_2D$ concentrations are liable to contribute to a significant decrease in the activity of the biologically active 1,25$[OH]_2D$. Thus, a dual regulation would appear to be present in subjects receiving high-dose vitamin D supplementation preventing possible toxic effects, namely, 1) the positive stimulation of 24-hydroxylase and 2) the negative control of the VDR activity. This notion is supported by previous mechanistic evidence that has determined the function of 1α,25$[OH]_2D$ in regulating 24-hydroxylase activity *in vitro*. The identification of two VDR in the 5′ region of the CYP24A1 promoter demonstrated that 1α,25$[OH]_2D_3$ could potently transactivate the CYP24A1 gene, inducing a 10- to 100-fold increase in CYP24A1 mRNA to limit the transcription of 1α,25$[OH]_2D_3$ responsive genes (24). Moreover, *in vitro* studies on primary human myoblasts indicate that the induction of 24-hydroxylase is dose dependent (10), which is in agreement with the serum response of 24,25[OH]D seen *in vivo* in the current study.

These findings may explain reported observations of deterioration in skeletal muscle function, increased risk of falls, and increased fracture risk in individuals supplemented with extreme dose vitamin D_3 to correct for severe vitamin D deficiency. As an example, in a large-scale trial (n = 2256), women ≥70 yr old were randomized to either 500,000 IU of vitamin D_3 or placebo. The women randomized to the supplemental vitamin D_3 experienced significantly more falls than the placebo group in the year after dosing of which the falls ratio was greatest in the first month after the one of 500,000 IU dose (29). Furthermore, in a retrospective observational cohort study, very low (<10 nmol·L^{-1}) and high (>140 nmol·L^{-1}) concentrations of 25[OH]D showed an increased risk of all-cause mortality, indicating not only a lower limit but also an upper limit for serum 25[OH]D (7). This hypothesis also lends an explanation for the inconsistency in positive outcomes related to supplemental vitamin D reported by large-scale meta-analyses (4). It is reasonable to suggest that mega dose vitamin D supplements are detrimental to vitamin D target tissues by increasing the production of 24,25$[OH]_2D$, which may act to block the activity of the VDR. It will be necessary to now perform mechanistic studies that clarify the function of 24,25$[OH]_2D$ and to determine whether high-dose supplementation is detrimental to vitamin D signaling through the VDR.

The current trial also had limitations that are important to consider for the design of future work. First, although one of our goals was to use a real-world blanket supplementation approach, we acknowledge that the same protocol used in other athletic cohorts with different body composition, genotype, and lower basal serum 25[OH]D concentrations may yield different results. Determining the response of the vitamin D metabolites to a similar protocol as we have used here across wider athletic cohorts will allow more conclusive recommendations to be made on dosing concentration and frequency. Indeed, we have previously shown that basal 25[OH]D concentrations vary across athletes from different

professional sports (3). This assumption is also true for female cohorts, and as such, we appreciate that our findings cannot be conclusively extended to the female athletic population. Regarding genotype, genotypic variation in the DBP influences the response to exogenous vitamin D (9), and little is known of the variation in genes encoding other vitamin D metabolizing enzymes such as CY24A1 and CYP24B1. We did not perform genotyping, and in light of recent evidence, we fully support genotype–phenotype studies in the context of vitamin D in future. Combining a genotyping approach with vitamin D metabolite ratio data, the latter as we have performed in this study, will offer a great advancement in the understanding of how genotype and supplementation interact and how this can be managed. We also did not measure serum or urinary calcium concentrations, which are markers of vitamin D toxicity and also regulate the PTH response. Measuring Ca^{2+} excretion would add another aspect to our findings; however, we do maintain that the observed increases in 24,25[OH]D are indicative of too much exposure to exogenous vitamin D. Finally, future work should aim to monitor FGF-23, a bone-derived hormone that can function to lower both 25[OH]D and 1α,25$[OH]_2D_3$ by inducing the CYP24 genes (28). FGF-23 may also be a player in regulating the metabolite response to high-dose supplementation, and at present, its role in lowering 25[OH]D by promoting 24-hydroxylase expression is still disputed (31). Extending the current findings to a broader range of vitamin D concentrations coupled with intracellular signaling cascades related to the vitamin D axis will yield the most inferential data, moving toward safer and more effective vitamin D supplementation practices in athletes.

Summary and implications for practice. The data presented here are the first to characterize the response of two major metabolites of vitamin D in response to two high-dose supplementation protocols in healthy professional athletes. The results demonstrate that a blanket approach of high-dose supplementation with 70,000 $IU \cdot wk^{-1}$ not only leads to increased 25$[OH]D_3$ and 1,25$[OH]_2D_3$ concentrations but also stimulates elevated concentrations of the vitamin D metabolite 24,25$[OH]_2D_3$, which has been previously shown to limit the transcriptional activity of 1,25$[OH]_2D_3$. We demonstrated that this negative regulatory effect persists after cessation of vitamin D_3 supplementation even as 1,25$[OH]_2D_3$ concentrations decrease. There are several novel key implications for practice that arise from our current observations. First, we speculate that high-dose bolus supplementation with vitamin D_3 is likely to be detrimental to the intended targeted downstream biological functions because of significant increases in the negative regulatory molecule 24,25$[OH]_2D$. Weekly doses amounting to more than 5000 $IU \cdot d^{-1}$ may need to be reassessed in light of our data. Rapid withdrawal from high-dose supplementation may result in adverse outcomes as the concentration of 24,25$[OH]_2D_3$ remains elevated for several weeks after withdrawal from supplementation despite declines in 1,25$[OH]_2D_3$. If moderate to high doses of vitamin D_3 have been administered, a gradual withdrawal from supplementation is advisable. At present, the optimal approach has not been established. Lower doses administered often (daily) may offer the most potent beneficial biological effects and limit the transactivation of CYP24A1 and the subsequent production of the negative regulatory molecule 24,25$[OH]_2D_3$. Future research must aim to establish the appropriate dose and frequency of administration to achieve a positive increase in both 25$[OH]D_3$ and 1,25$[OH]_2D_3$ while limiting the appearance of increased 24,25$[OH]_2D_3$ concentrations. The generation of 24,25$[OH]_2D$ may be an aspect of the body's defense mechanism to prevent "toxicity" when administered high doses of vitamin D. We postulate that single "super" doses of vitamin D_3 administered on a weekly basis, which is common practice in many professional sporting teams, may result in similar rapid transient increases in 1,25$[OH]_2D_3$ leading to significant increases in the negative regulatory metabolite 24,25$[OH]_2D_3$. Further studies will be required to determine whether the relationship we have observed is seen with higher and lower doses of vitamin D.

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D. J. O. and J. C. Y. T. contributed equally to the manuscript. D. J. O. designed the research, conducted the research, analyzed the data, and wrote the manuscript. J. C. Y. T. designed the research, conducted the research, designed and optimized all analytical techniques, and analyzed the data. W. J. B. conducted the research and provided essential materials for research. A. S. designed the research and conducted the statistical analyses. W. D. F. designed the research, provided essential reagents for the research, analyzed the data, and wrote the article. J. P. M. designed the research, analyzed the data, and wrote the article. G. L. C. designed the research, conducted the research, analyzed the data, performed statistical analysis, wrote the article, and had primary responsibility for the final content.

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