Investigation of the effects of dietary supplementation with 25-hydroxyvitamin D₃ and vitamin D₃ on indicators of vitamin D status in healthy dogs

Robert C. Backus DVM, PhD

Lauren R. Foster DVM

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From the Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211. Dr. Foster's present address is Heart of Texas Veterinary Specialty Center, Round Rock, TX 78664.

Address correspondence to Dr. Backus (backusr@missouri.edu).

OBJECTIVE

To compare the effects of short-term dietary supplementation with vitamin D₃ and 25-hydroxyvitamin D₃ (25[OH]D₃) on indicators of vitamin D status in healthy dogs.

ANIMALS

13 purpose-bred adult dogs.

PROCEDURES

20 extruded commercial dog foods were assayed for 25(OH)D₃ content. Six dogs received a custom diet containing low vitamin D concentrations and consumed a treat with vitamin D₂ (0.33 µg/kg^{0.75}) plus 1 of 3 doses of 25(OH)D₃ (0, 0.23, or 0.46 µg/kg^{0.75}) once daily for 8 weeks followed by the alternate treatments in a crossover-design trial. In another crossover-design trial, 7 dogs received a custom diet supplemented with vitamin D₃ or 25(OH)D₃ (targeted content, 3,250 U/kg [equivalent to 81.3 µg/kg] and 16 µg/kg, respectively, as fed) for 10 weeks followed by the alternate treatment. In washout periods before each trial and between dietary treatments in the second trial, dogs received the trial diet without D-vitamin supplements. Dietary intake was monitored. Serum or plasma concentrations of vitamin D metabolites and biochemical variables were analyzed at predetermined times.

RESULTS

25(OH)D₃ concentrations were low or undetected in evaluated commercial diets. In the first trial, vitamin D₂ intake resulted in quantifiable circulating concentrations of 25-hydroxyvitamin D₂ but not 24R,25-dihydroxyvitamin D₂. Circulating 25(OH)D₃ concentration appeared to increase linearly with 25(OH)D₃ dose. In the second trial, circulating 25(OH)D₃ concentration increased with both D vitamin-supplemented diets and did not differ significantly between treatments. No evidence of vitamin D excess was detected in either trial.

CONCLUSIONS AND CLINICAL RELEVANCE

Potency of the dietary 25(OH)D₃ supplement estimated on the basis of targeted content was 5 times that of vitamin D₃ to increase indicators of vitamin D status in the study sample. No adverse effects attributed to treatment were observed in short-term feeding trials. (*Am J Vet Res* 2021;82:722–736)

Discoveries over the past several decades have revealed that most cells of animal tissues express vitamin D receptors.¹ Many cells additionally have enzymes that convert 25(OH)D, the most abundant circulating metabolite of vitamin D, to the vitamin D receptor ligand and calcitropic hormone

1,25(OH)₂D.^{2,3} Prompted by these findings, many investigations have sought to identify roles that vitamin D may have in illnesses such as cancer, autoimmune disease, diabetes mellitus, and cardiovascular disease.^{4,5} In recent years, some investigators have suggested that most privately owned adult dogs have vitamin D status that is less than optimal for minimizing the risk of neoplastic disease.⁶ Beyond nutritional requirements, beneficial effects of vitamin D administration are currently under investigation. One example of this type of investigation is a report on the use of orally administered vitamin D₃ for treatment of atopic skin disease in dogs.⁷

Prospective studies are needed to determine whether increasing vitamin D status through dietary supplementation in dogs can have a meaningful impact on risks for disease or on clinical outcomes of

ABBREVIATIONS

1,25(OH) ₂ D	1α,25-dihydroxyvitamin D
24,25(OH) ₂ D ₂	24R,25-dihydroxyvitamin D ₂
24,25(OH) ₂ D ₃	24R,25-dihydroxyvitamin D ₃
25(OH)D	25-hydroxyvitamin D
25(OH)D ₂	25-hydroxyvitamin D ₂
25(OH)D ₃	25-hydroxyvitamin D ₃
FGF	Fibroblast growth factor
HPLC	High-performance liquid chromatography
LLOQ	Lower limit of quantitation
PTH	Parathyroid hormone

disease treatment. An impediment to carrying out such studies is the lack of an established safe upper limit for dietary vitamin D in adult dogs. Negative health consequences of hypervitaminosis D in dogs have been described but are mostly reported following consumption of extraordinary amounts of vitamin D (eg, owing to dietary formulation error⁸ or rodenticide ingestion⁹). Another obstacle is that substantial variation in individual responses to ingested vitamin D₃ has been found in studies of people¹⁰ and dogs.^{7,11} Increased dietary vitamin D supplementation may be beneficial for some dogs but may have no effect or even be detrimental in other dogs. The variable response to oral administration of vitamin D₃ imposes a need for repeated evaluation of dogs' vitamin D status as part of the study design.

Diets are conventionally supplemented with one of 2 forms of vitamin D, cholecalciferol (vitamin D₃) or ergocalciferol (vitamin D₂).¹² Of the 2, vitamin D₃ is the most commonly added to dog foods.¹³ We observed that oral administration of a vitamin D₃ supplement affects vitamin D status, as assessed by measurement of serum 25(OH)D₃ concentrations, slowly and unpredictably in dogs.¹¹ Equilibration of vitamin D status occurs only after many weeks of daily ingestion of vitamin D₃. A report¹⁴ published in 1988 indicated that oral administration of 25(OH)D₃ might result in a very rapid increase in the vitamin D status of dogs. Unfortunately, the dosage of 25(OH)D₃ on a body weight basis for achieving this effect was not reported in the publication. Studies of people indicate that the potency of orally administered 25(OH)D₃ is 1.4 to 10 times that of vitamin D₃ to increase circulating concentrations of 25(OH)D₃.^{15,16} A previous study¹⁷ by our group found that the relative oral potency of 25(OH)D₃ to increase serum 25(OH)D₃ concentrations in dogs with low circulating concentrations of vitamin D metabolites is greater than 5 times that of vitamin D₃. Ingested 25(OH)D₃ is expected to have a more acute effect on vitamin D status than is vitamin D₃; the former has greater water solubility than the latter, making mucosal uptake of 25(OH)D₃ more likely and less dependent on dietary fat digestion and absorption.¹⁸ Many processes occur between ingestion of vitamin D₃ and its eventual release into plasma from the liver as 25(OH)D₃. In contrast, 25(OH)D₃ that is absorbed from the alimentary tract directly enters the circulating pool of 25(OH)D₃.¹⁹

Inclusion of 25(OH)D₃ in pet foods has the promise of being an alternative to vitamin D₃ as an effective and rapid means of providing vitamin D to healthy dogs and dogs with disease. Ingested 25(OH)D₃ seems to be a natural source of vitamin D for dogs because they are carnivores.²⁰ Animal tissues used as ingredients in diets for dogs contain variable amounts of 25(OH)D₃ and vitamin D₃.^{21,22} Although concentrations of 25(OH)D in tissues are generally lower than those of vitamin D₃, the 25(OH)D in tissues that are ingested may be nutritionally important, considering the reported high potency of 25(OH)D₃, compared

with vitamin D₃.¹⁵⁻¹⁷ The purpose of the study reported here was to evaluate the potential importance of dietary 25(OH)D₃, versus dietary vitamin D₃, to vitamin D status of dogs. To assess whether consumption of dietary 25(OH)D₃ is common, we additionally sought to determine the concentrations of the vitamin in commercially available dry dog foods. We hypothesized that dogs commonly consume 25(OH)D₃ in commercially available diets and that 25(OH)D₃ added to foods and ingested by dogs would produce circulating 25(OH)D₃ concentrations similar to those observed following oral administration of a vitamin D₃ supplement without causing adverse effects.

Materials and Methods

The prospective, experimental study comprised 3 main parts. First, assays were performed to determine the concentrations of 25(OH)D₃ in a sample of commercially available dry dog foods. Then, a dose determination trial was performed to compare the effects of short-term daily administration of various doses of 25(OH)D₃ (given with 1 dose of vitamin D₂) on vitamin D status in healthy adult dogs fed a diet containing low vitamin D₂ and D₃ concentrations. Last, a short-term feeding trial was performed to compare the effects of a diet supplemented with vitamin D₃ and a diet supplemented with 25(OH)D₃ on indicators of vitamin D status in healthy dogs.

The husbandry and treatment of dogs for the second and third components of the study were reviewed and approved by the University of Missouri Animal Care and Use Committee (Protocols 7956 and 8908) and complied with the *Guide for the Care and Use of Laboratory Animals*²³ and the Animal Welfare Act and regulations.²⁴ The dogs underwent physical examinations twice annually and had annual clinical hematologic and serum biochemical evaluations performed by veterinarians. All dogs enrolled in the study were deemed healthy on the basis of medical history and results of these examinations.

During trials, the dogs were housed in an American Association for Laboratory Animal Science-accredited facility with an environment controlled for light (12-hour light-dark cycle), temperature (21 to 24 °C), and humidity (30% to 70%). The dogs were kenneled in elevated runs with adjustable partitions. This arrangement allowed for daily periods of socialization among dogs and confinement as needed for food presentation and recording of food intake on an individual basis. Additional socialization included regular periods of walking on a leash and release of the dogs together into rooms where runs were located. Water was continuously available. Body weights were determined every 2 to 4 weeks, and the amount of food presented was adjusted as needed for dogs to maintain body condition.

The number of dogs included in each trial was selected on the basis of power analysis for the ability to detect significant treatment effects on the concentration of 25(OH)D₃ in sampled blood. The treatments

of interest were the dose of 25(OH)D₃ in the dose determination trial and D vitamin supplementation type in the dietary supplementation trial. Our previous research indicated that 25(OH)D₃ concentrations from 6 to 7 dogs are normally distributed with sufficiently low variance for detecting a significant ($\alpha = 0.05$) treatment effect with 80% power when the mean difference in 25(OH)D₃ concentrations is 25%.^{11,17}

Assays of 25(OH)D₃ in commercial diets

Commercially produced, extruded, dry-expanded canine diets ($n = 20$) were purchased from local retail stores for assessment of 25(OH)D₃ concentrations. Criteria for diet selection included availability, a high volume of brand sales as learned from manufacturers, or labeled ingredients expected to contain 25(OH)D₃. The analyzed products were over-the-counter diets that were formulated for growth ($n = 1$), maintenance (15), or all life stages (4). The diets were produced by 7 manufacturers, purchased from local retailers, stored in original packaging in a sealed container at 22 to 25 °C, and analyzed before label best-by dates. As inferred from labels, principal sources of animal protein in the diets included chicken ($n = 12$), fish (4), beef (2), lamb (1), and pork (1). The diets that were selected for high sales volume ($n = 12$) were from 2 manufacturers of nationally distributed brands, for which products that were their most popular or most frequently sold were disclosed in response to our inquiry. Diet samples (approx 250 g) from each bag were individually ground and assayed for moisture content at the Experiment Station Chemical Laboratories of the University of Missouri. Concentration of 25(OH)D₃ in ground samples was determined with a modification of the methods described by Jakobsen et al.²⁵ Briefly, 10 g of the sample was mixed with 60 mL of warm (50 °C) water and 500 ng of 25(OH)D₃-[²H₃]^a as internal standard and sonicated for 10 minutes. Tert-butyl methyl ether (40 mL) was added to the resulting slurry, which was mixed and sonicated for an additional 5 minutes. The organic phase of the mixture was separated by centrifugation, dried by centrifugal evaporation, and reconstituted in the mobile phase (2 mL of isopropanol, ethyl acetate, and iso-octane;1:10:89 [vol:vol:vol]) for HPLC. A portion of the reconstituted mixture (0.2 mL) was loaded onto a silica column^b equilibrated with the mobile phase (2 mL/min) at ambient temperature (21 to 24 °C). Eluting fractions at retention times coinciding with 25(OH)D₃ were collected and dried by centrifugal evaporation. The 25(OH)D₃ in residue was derivatized with {4-[2-(6,7-dimethoxy-4-methyl-3,4-dihydroquinoxaliny)ethyl]-1,2,4-triazoline-3,5-dione}^c as described by Kaufmann et al.²⁶ Quantification by liquid chromatography–tandem mass spectrometry of relative abundances of the derivatized dietary and internal standard 25(OH)D₃ was performed at

the Charles W. Gehrke Proteomics Center of the University of Missouri according to the methods of Kaufmann et al.²⁶

25(OH)D₃ dose determination trial

Animals—Six 5.5-year-old sexually intact male ($n = 3$) and female (3) Chinese Crested-Beagle cross-bred dogs were used in this part of the study. The dogs were purpose bred, from the same litter, and considered to be of healthy body weight (range, 6.0 to 10.5 kg). Body condition scores of the dogs were reported during the last month of the trial on a scale of 1 to 9, with a score of 5 considered ideal.²⁷

Diet—A semipurified diet was prepared by the authors to meet or exceed the maintenance requirements of adult dogs¹³ for all nutrients except vitamin D (**Appendices 1 and 2**). However, at the end of the trial, it was discovered that iodine was not included in the mineral mixture. Vitamin D₃ and D₂ concentrations in the diet were 66 and < 20 U/kg, respectively, on a dry-matter basis as determined by commercial laboratory^d analysis. Metabolizable energy density of the diet was estimated to be 5,230 kcal/kg of dry matter, with protein, fat, and carbohydrate contents assumed to contribute 4, 9, and 4 kcal/g of metabolizable energy, respectively. Water was mixed in the diet to form a dough with approximately 50% moisture. The dough was extruded through a grinder plate^e with 0.5-inch-diameter holes to form pellets of a size well accepted by the dogs. The pellets were very lightly top-dressed with a proprietary palatant powder^f before feeding each day; the product was not commercially available, and potential vitamin D content was not provided or measured after application. The prepared food was weighed immediately before it was offered to each dog, and any remaining food was weighed at the time of its removal from the dog's enclosure. The difference in weights represented the amount of food ingested by the dog, which was used to calculate energy intake.

Design—The transition from an extruded, dry-type colony diet⁸ to the semipurified diet was performed over 4 days for all dogs. The semipurified diet was provided without additional supplements for approximately 13 weeks, and during this interval, venous blood samples (3 mL) were collected from the dogs every 2 to 4 weeks. Serum 25(OH)D concentrations were determined to assess vitamin D status. The trial was begun when serum 25(OH)D₃ concentrations were low (≤ 5 ng/mL [12.5 nmol/L])²⁸ and constant. For reference, in using similar analytical methods, our group found a mean serum 25(OH)D₃ concentration of 34 ng/mL in a cohort of healthy adult dogs ($n = 57$) maintained on commercially available diets selected by their owners; the 25(OH)D₃ concentrations ranged from 14 to 60 ng/mL.²⁹ On each day of the trial, prior to being fed, the dogs were offered a portion (3 to 4 g) of a treat^h on which small volumes (6 to 12 μ L) of ethanolic solutions of vitamin D₂ and 25(OH)

D₃ were applied. The solutions were prepared by an author (RCB) by dissolving the vitamers^a in ethanol to concentrations of 0.25 µg/µL. Each dog received all treatments according to a randomized block design with doses calculated on the basis of metabolic body weight. Vitamin D₂ (0.33 µg/kg^{0.75}) was administered with 1 of 3 doses of 25(OH)D₃ (0, 0.23, or 0.46 µg/kg^{0.75}) daily for 8 weeks, with no washout period between the different doses (**Supplementary Figure S1**). When dogs were assigned the 25(OH)D₃ dose of 0 µg/kg^{0.75}, 5 to 7 µL of the ethanol carrier was applied to treats. The described dose of vitamin D₂ was given to all dogs daily to prevent development of signs of vitamin deficiency during periods when no 25(OH)D₃ was administered. All treatments were readily consumed by the dogs.

A sample of jugular or cephalic venous blood (3 mL) was collected by venipuncture every 2 weeks during the trial. The samples were collected approximately 24 hours after food was provided on the previous day (typically between 8:30 and 9:30 AM; all food was typically consumed by the next morning). Serum concentrations of 25(OH)D₃ and 25(OH)D₂ were measured, and 25(OH)D₃ results were monitored to detect patterns indicating plateaued concentrations and the potential for development of vitamin D deficiency or toxicosis. During the last day of each dosing period, an additional blood sample (7 mL) was collected for the measurement of concentrations of other vitamin D metabolites: 24,25(OH)₂D₃ and 24,25(OH)₂D₂ in serum. Concentrations of ionized calcium and PTH in serum and phosphorus and creatinine in plasma were also determined in these samples.

Dietary supplementation trial

Animals—Seven 4-year-old sexually intact male (n = 4) and female (3) Beagles were used in this part of the study. The dogs were purpose bred and from the same litter, with body weights ranging from 8.3 to 10.9 kg. Body condition score was assigned to each dog as previously described²⁷ by a consensus of 4 evaluators prior to onset of the trial.

Diets—Three forms of an extruded, dry-expanded diet that differed only in the source and amount of vitamin D supplementation were manufactured by a vendorⁱ (Appendix 2). One form lacked any vitamin D supplement, the second form was targeted to contain 3,250 U/kg (81 µg/kg) of vitamin D₃ on an as-fed basis, and the third form contained a spray-dried 25(OH)D₃ beadlet product^j that was added to achieve a targeted as-fed dietary 25(OH)D₃ concentration of 16 µg/kg. The beadlet product was a recognized poultry feed additive³⁰ advertised as heat-stable (≤ 90 °C) under pelleting conditions.³¹ The amount of each D vitamin to be added to the diets was determined on the basis of results of a previous study¹⁷ that indicated that 25(OH)D₃ was approximately 5 times as potent as vitamin D₃ for affecting vitamin D status. In that trial, differences in the rates of change of serum 25(OH)D₃ concentration in response to equivalent

25(OH)D₃ and vitamin D₃ dosages (2.3 µg/kg^{0.75}, PO, q 24 h) were used to estimate relative potency of the vitamers. With the exception of the described vitamin D supplementation, the diet was formulated to be nutritionally complete and balanced for canine maintenance.¹³ Ingredients of the diet in decreasing order of relative weight (mass per total mass of food) were as follows: chicken by-product meal, corn, dried egg product, brewers' rice, chicken fat, sorghum, wheat, natural flavor, corn gluten meal, dried plain beet pulp, dicalcium phosphate, potassium chloride, salt, pea fiber, calcium carbonate, vitamin supplement (labeled contents: niacin, thiamin monohydrate, calcium pantothenate, mineral oil, pyridoxine hydrochloride, riboflavin, vitamin B12, folic acid, biotin, and tocopherols), ferrous sulfate, zinc oxide, manganese oxide, copper sulfate, sodium selenite, cobalt carbonate, ethylenediamine dihydroiodide, and choline chloride. Metabolizable energy density of the diet was reported to be 3,860 kcal/kg of dry matter, and the measured moisture content was 4.3% to 5.7%.

Concentrations of proximate, macromineral, methionine, cystine, and vitamin D contents of the diet were determined in ground samples by the Experiment Station Chemical Laboratories of the University of Missouri and by another commercial laboratory^d (Appendix 2). The same methods used for assessment of 25(OH)D₃ in commercial diets were used to determine concentrations of the vitamin in the extruded, dry-expanded diets of the dietary supplementation trial.

Design—All diets were provided daily in amounts determined to maintain the dogs' body weight. Beginning prior to the dietary manipulations and continuing at 2-week intervals throughout the trial, blood samples (3 mL) were collected from all dogs by jugular or cephalic venipuncture for plasma collection and measurement of 25(OH)D₃. The timing of blood sample collection and feeding of the dogs was as described for the dose determination trial. Transition from the dry-type colony diet,⁸ which was supplemented with 4,400 U of vitamin D₃/kg on an as-fed basis, to a trial diet that lacked D-vitamin supplementation was completed over a 5-day period. This diet was provided exclusively for 10 weeks; the dogs were assigned to 2 groups when concentration of 25(OH)D₃ in plasma extracted from the sampled blood declined to a median of 20 to 30 ng/mL.

The groups were balanced by body weight, sex, and number as closely as possible. Initially, 1 group (n = 4) received the experimental diet supplemented with 25(OH)D₃ and the other group (3) received the diet supplemented with vitamin D₃ for 10 weeks (**Supplementary Figure S2**). At the end of the first 10-week period, all dogs were transitioned back to the unsupplemented diet until plasma 25(OH)D₃ concentrations for all dogs again decreased to a median of 20 to 30 ng/mL (9 weeks). The groups were then crossed over and transitioned to the alternate dietary treatment.

During week 10 of each of the 2 vitamin D supplementation periods, an additional blood sample (7 mL) and a pooled urine sample were collected from all dogs. A CBC and biochemical analysis were conducted on the samples. The analyses included determinations of concentrations of PTH and FGF-23 in serum and concentrations of total and ionized calcium, phosphorus, creatinine, and 24,25(OH)₂D₃ and 1,25(OH)₂D₃ in plasma as well as evaluation of variables routinely monitored for health screening. For urine collection, each dog was temporarily kenneled on a cleaned, elevated, plastic-coated steel grate under which previously unused sheets of polyethylene plastic were spread so that falling urine would pool. When a sufficient volume (> 5 mL) of urine was passed, urine was collected and submitted for urinalysis. The time between voiding and urinalysis was variable and ranged from approximately 3 to 14 hours.

Hematologic and urine analysis

Measurement of vitamin D metabolites—Blood samples were added to tubes with no anticoagulant, allowed to clot for 1 hour, and centrifuged at 1,200 X g for collection of serum or added to tubes containing lithium heparin (56 US Pharmacopoeia units) and immediately centrifuged at 1,200 X g for collection of plasma. The serum and plasma extracts were stored frozen (–20 °C) for 2 weeks to 5 months until analyses.

Concentrations of 25(OH)D₃ and 25(OH)D₂ in serum and plasma that were collected for monitoring of vitamin D status were determined with previously described extraction and HPLC methods.^{11,32} For determination of concentrations of 24,25(OH)₂D₃ and 1,25(OH)₂D₃, 1.0 mL of serum or plasma was mixed and incubated at 4 °C overnight (14 to 16 hours) with methanolic solutions (10 µL) of the following internal standards: 50 ng of 3-epi-24R,25-dihydroxyvitamin D₃^a and 120 kBq each of 25-[26,27-³H]-hydroxyvitamin D₃^k and 1α,25-[26,27-³H]-dihydroxyvitamin D₃.^k The vitamin D metabolites were extracted by means of the methods described by Hollis³³ with minor modifications. Briefly, each sample was vortex-mixed for 20 seconds in 1.0 mL of acetonitrile and then centrifuged at 2,000 X g for 20 minutes at 22 °C. The supernatant was collected, vortex-mixed with 1.0 mL of water, and applied to a conditioned solid-phase C18 column.¹ The column was washed with water (5 mL) and a methanol-water solution (70:30 [vol:vol]; 5 mL) and then air-dried for 10 minutes. The vitamin D metabolites were eluted with hexane-methylene chloride solution (90:10 [vol:vol]; 5 mL) and then hexane-2-propanol solution (95:5 [vol:vol]; 5 mL). The eluents were dried by centrifugal evaporation and then reconstituted in HPLC mobile phase, hexane-2-propanol (88:12 [vol:vol]; 250 µL). A portion of the reconstituted product (200 µL) was injected onto an equilibrated (2.0 mL/min) normal-phase column.^b Eluted fractions containing each of the vitamin D metabolites were dried and then further fractionated by reverse-phase HPLC.

The normal-phase fractional residues containing 25(OH)D₃ and 25-[26,27-³H]-hydroxyvitamin D₃ were dissolved in 100 µL of methanol and diluted with 50 µL of water. A portion of the reconstituted product (120 µL) was injected on a heated (50 °C) HPLC column^m equilibrated at 1.2 mL/min with a methanol-water solution (67:33 [vol:vol]). Eluting 25(OH)D₃ was quantified from the area under the curve of peak UV absorbance at 265 nm. Coeluting 25-[26,27-³H]-hydroxyvitamin D₃ was dried, and tritium radioactivity was determined with a liquid scintillation counter.¹¹

The normal-phase fractional residue containing 24,25(OH)₂D₂ and 24,25(OH)₂D₃ was dissolved in 93 µL of methanol and 57 µL of water. The metabolites in the mixture were quantified by reverse-phase HPLC with UV detection as described for quantifying fractions containing 25(OH)D₃, but with modification of the eluting mobile phase to a methanol-water solution (62:38 [vol:vol]).

A portion of the normal-phase fraction containing 1,25(OH)₂D₃ and 1α,25-[26,27-³H]-dihydroxyvitamin D₃ was aliquoted into a liquid scintillation vial, then dried for tritium radioactivity counting. The remaining volume of the fraction was divided equally between 2 borosilicate glass tubes (12 X 75 mm), each containing 10 µL of methanol. Contents of the tubes were dried, and the amount of 1,25(OH)₂D₃ in residue was determined with a commercially available radioimmunoassay kit.^o Manufacturer-provided kit instructions were followed with minor modifications: the standard tubes contained the dried residue of 10-µL aliquots of methanolic dilutions of 1,25(OH)₂D₃ standards^a (0.4-, 1.6-, 6.3-, and 25-ng/mL concentrations) and organic mobile phase (hexane-2-propanol) at a ratio of 88:12 (vol:vol). The volume of mobile phase in the standards was equivalent to that aliquoted to radioimmunoassay tubes containing 1,25(OH)₂D₃ normal-phase fractions (approx 1.5 to 2.0 mL) of plasma extracts. Incubation of sample-containing tubes with the provided radiolabel was extended from 1 to 3 hours before separation of bound from free radiolabels. Following these procedures, 20 pg of 1,25(OH)₂D₃ standard affected a 50% inhibition of binding of the provided radiolabel.

The recoveries of internal standards were determined in 1.0 mL-replicates (n = 6) of pooled canine plasma. Mean ± SD recoveries for 25-[26,27-³H]-hydroxyvitamin D₃, 3-epi-24R,25-dihydroxyvitamin D₃, and 1α,25-[26,27-³H]-dihydroxyvitamin D₃ were 48 ± 18%, 52 ± 7%, and 45 ± 9.0%, respectively.

CBC and plasma biochemical analysis—Samples of whole blood mixed with K₂EDTA (2 mg/mL) and plasma extracted from whole blood mixed with lithium heparin (19 U/mL) were processed and analyzed on the day of collection at the University of Missouri Veterinary Medical Diagnostic Laboratory. Automated analyzers in the laboratory were used for routine clinical cell counting^p and plasma biochemical analysis.^q The laboratory reference intervals were based on

95% CIs for analysis results of samples obtained from 60 to 80 dogs owned by students, staff, and faculty of the University of Missouri College of Veterinary Medicine. These dogs were deemed healthy on the basis of results of physical examination and histories that indicated the absence of disease and no use of medications other than routine preventative treatments.

PTH and FGF-23 analysis—Serum concentrations of PTH in samples collected during the dose determination trial were determined with a direct, 2-site, sandwich-type chemiluminescent immunoassay at a commercial laboratory[†]; the method quantified 1–84 PTH and was validated for use with canine serum by means of dilutional parallelism experiments.⁶ Serum concentrations of PTH in samples collected during the dietary supplementation trial were determined at another commercial laboratory^s with an immunoradiometric assay that used a single polyclonal 1–84 PTH antibody; validity of this assay was supported by previous observation of correlation between PTH concentration and severity of kidney disease in dogs, where severity of kidney disease was assumed proportional to the degree of secondary hyperparathyroidism.³⁴

Concentrations of FGF-23 (intact protein) were determined with a commercially available ELISA kit[†] developed for use with human serum. Validity of use of the assay for canine serum was supported by previous dilutional parallelism observations and findings in dogs with chronic kidney disease in which various degrees of hyperphosphatemia and hyperparathyroidism occur.³⁴

Urinalysis—Urine samples were submitted to the University of Missouri Veterinary Medical Diagnostic Laboratory ≤ 2 hours after collection. The laboratory reported on refractometry, dipstick chemistry, and microscopic sediment findings and sample physical characteristics.

Statistical analysis

In the liquid chromatography–tandem mass spectrometry determinations of 25(OH)D₃ concentration of extruded diet extracts, the lower limit of detection and LLOQ were calculated according to the blank and low-concentration sample formula of Mani et al.³⁵ Variable observations were accepted as normal if means and medians were similar and kurtosis and skew were < 1.0 and > -1.0 . Observations that required square-root transformation for normalization are identified.

A repeated-measures mixed-model ANOVA was used to investigate potential effects of the 25(OH)D₃ doses on normally distributed variables of the dose determination trial. The fixed effects were treatment (dose), block (trial period), and treatment-block interaction, and the random effects were animal (individual dog) and order of treatment. Significance of differences for treatment effects and sampling time were identified with Tukey-adjusted multiple comparisons tests. Friedman 2-way ANOVA was used to investigate

potential effects of the 25(OH)D₃ doses on variables that were not normally distributed.

A repeated-measures, mixed-model ANOVA was used to investigate potential effects of supplementation type (D vitamers dietary treatment) on plasma 25(OH)D₃ concentrations in the dietary supplementation trial. The fixed effects were treatment (vitamer), block (trial period), and treatment-block interaction. The random effects were animal (individual dog) and treatment order. The significance of differences between week 10 variable observations after vitamin D supplementation and those after 25(OH)D₃ supplementation was determined with a paired *t* test when observations were continuous and normally distributed and with a Wilcoxon signed rank tests when observations were ordinal or not normally distributed. Week 10 urinalysis variable observations in the dietary supplementation trial that were descriptive or noncontinuous were assigned to 2 categories that were inclusive of the reported findings, and potential associations between D vitamers supplementation type on the proportion of observations in the categories were determined with McNemar tests.

Central tendency and dispersion of observations throughout are reported as median and range, respectively, even for observations deemed normally distributed, for which mean and median are characteristically similar. Reporting ranges in lieu of SD was considered by the authors as more informative of observational variation given the small sample sizes of the trials ($n = 6$ or 7), which preclude use of formal normality testing. Results summarized in figures are expressed as mean $\pm$ SEM, as the observations were deemed normal and SEM was representative of the degree of certainty for estimation of the mean for a population of dogs. Statistical analyses were conducted with commercial software.^{u,v} For all analyses, values of $P < 0.05$ were considered significant.

Results

25(OH)D₃ in commercial diets

The lower limit of detection of 25(OH)D₃ in the assay for the commercially available diet samples was approximately 0.4 $\mu\text{g/kg}$ of dry matter, and the LLOQ was approximately 3 times this value, 1.1 $\mu\text{g/kg}$ of dry matter. Concentrations of 25(OH)D₃ in 9 of 20 diets were less than the lower limit of detection, and the concentrations in 6 other diets were less than the LLOQ. The coefficient of variation determined from quadruplicate analyses of diet samples with results above the LLOQ ($n = 5$) ranged from 8% to 21%. The median 25(OH)D₃ concentrations in these 5 diets was 1.7 $\mu\text{g/kg}$ (range, 1.1 to 1.7 $\mu\text{g/kg}$) on a dry-matter basis.

25(OH)D₃ dose determination trial

Body condition scores of the dogs reported during the last month of the trial ranged from 4 to 5 of 9. Adverse health effects attributable to D vitamers supplementations were not observed dur-

Table 1—Comparison of median (range) week 8 energy intake, body weight, serum concentrations of vitamin D metabolites, and biochemical indices of vitamin D toxicosis for 6 healthy adult dogs that received a semipurified diet containing low vitamin D₂ and D₃ concentrations and were administered a portion of a treat with vitamin D₂ (0.33 µg/kg^{0.75}) and 1 of 3 doses of 25(OH)D₃ (0, 0.23, or 0.46 µg/kg^{0.75}) applied once daily (approx q 24 h) for 8 weeks in a 25(OH)D₃ dose determination trial.

Variable	25 (OH)D ₃ (µg/kg ^{0.75})			Reference interval	P value
	0	0.23	0.46		
Energy intake (kcal/d)	581 (425–719)	581 (416–719)	581 (378–719)	—	1.0
Body weight (kg)	8.3 (7.0–11.1)	8.2 (7.2–11.7)	8.4 (6.4–11.7)	—	0.997
Serum analytes					
25(OH)D ₂ (ng/mL)*	20.4 (16.2–25.3) ^a	17.5 (12.1–22.3) ^b	17.3 (13.0–24.0) ^b	—	0.032
25(OH)D ₃ (ng/mL)†	3.2 (2.0–5.4) ^a	24.3 (19.2–78.6) ^b	45.1 (27.5–132) ^c	—	0.001
Total 25(OH)D (ng/mL)†	23.1 (19.0–29.5) ^a	42.3 (36.8–97.2) ^b	67.2 (42.4–146) ^c	—	0.001
24,25(OH) ₂ D ₃ (ng/mL)††	13.2 (4.3–24.6)	36.1 (10.6–65.4)	46.6 (42.1–56.3)	—	0.051
Ionized calcium (mmol/L)	1.28 (1.06–1.34)	1.21 (1.01–1.32)	1.23 (1.20–1.37)	1.25–1.45	0.542
PTH (pg/mL)	4.9 (2.4–10.6)	4.9 (2.4–8.5)	3.5 (1.9–6.6)	4.0–38.0	0.855
PTH (pmol/L)§	0.52 (0.25–1.12)	0.52 (0.25–0.90)	0.37 (0.20–0.70)	0.42–4.03	NA
Plasma analytes					
Phosphorus (mg/dL)	3.7 (2.4–4.3)	3.2 (2.1–4.6)	3.9 (2.6–4.7)	2.0–5.0	0.933
Creatinine (mg/dL)	0.4 (0.4–0.6)	0.5 (0.4–0.5)	0.5 (0.4–0.6)	0.6–1.6	0.889

All dogs received the same semipurified diet without supplementary vitamin D for approximately 13 weeks at the start of the dose determination trial and received each of the described treatments immediately prior to daily feeding according to a randomized block design; there was no washout period between dosing periods. Data were not normally distributed except where indicated.

*Data were normally distributed. †Data distribution was normalized by square-root transformation for statistical analysis. ‡Data were available for 5 of the 6 dogs that received the 0.46-µg/kg^{0.75} dose because of sample loss during analysis. §Conversion calculated from the measured value (in pg/mL) on the basis of the molecular weight of intact PTH (9,425 pg/pmol).⁵⁴

— = Not available. NA = Not applicable.

^{a–c}Within a row, results with different superscripted letters are significantly ($P < 0.05$) different.

ing any period of the trial. Although iodine content of the diet was unknown, clinicopathologic signs of hypothyroidism (eg, hypercholesterolemia, nonregenerative anemia, and hypercalcemia) did not develop, and general signs of hypothyroidism were not observed (eg, skin and coat abnormalities, inactivity, weight gain, or diarrhea). Week 8 energy intake, body weight, and concentrations of serum and plasma biochemical analytes other than D vitamers did not differ significantly among 25(OH)D₃ doses (**Table 1**). Circulating concentrations of ionized calcium, phosphorus, and creatinine did not exceed the upper limits of laboratory reference intervals. For most of the 6 dogs, serum concentrations of ionized calcium ($n = 5$) and PTH (4) were slightly below the lower laboratory reference limits.

Week 8 serum 25(OH)D₂ concentrations of dogs were slightly but significantly ($P < 0.05$) less when dogs received 25(OH)D₃ at 0.23 µg/kg^{0.75} or 0.46 µg/kg^{0.75}, compared with the 0-µg/kg^{0.75} dose in addition to vitamin D₂ (**Figure 1**; **Table 1**). When the dogs received 0 µg of 25(OH)D₃/kg^{0.75}, serum 25(OH)D₃ concentrations were significantly ($P < 0.05$) less than coinciding serum 25(OH)D₂ concentrations. During this dosing period, se-

Figure 1—Comparison of mean $\pm$ SEM week 8 plasma concentrations of 25(OH)D₂, 25(OH)D₃, and 25(OH)D for 6 healthy adult dogs that received a semipurified diet containing low vitamin D₂ and D₃ concentrations and were administered a portion of a treat with vitamin D₂ (0.33 µg/kg^{0.75}) and 1 of 3 doses of 25(OH)D₃ (0, 0.23, or 0.46 µg/kg^{0.75}) applied once daily (approx q 24 h) for 8 weeks in a dose determination trial as part of a study to investigate the effects of short-term dietary vitamin D₃ and 25(OH)D₃ supplementation on indicators of vitamin D status in healthy dogs. All dogs received the same semipurified diet without supplemental vitamin D administration for approximately 13 weeks at the start of the trial and received each of the described treatments according to a randomized block design with no washout period between dosing periods. Symbols indicate significant ($P < 0.05$) differences in concentration for a given vitamin D metabolite among the three 25(OH)D₃ doses. *Different from the result for the 0-µg/kg^{0.75} dose. †Different from the results for the 0- and 0.23-µg/kg^{0.75} doses.

Table 2—Targeted and measured vitamin D₃ and 25(OH)D₃ concentrations (reported on an as-fed basis) for 3 forms of a custom-manufactured, extruded, dry-expanded diet that differed only in the type and amount of D vitamer included and were fed to 7 healthy dogs in a dietary supplementation trial.

D vitamer	Concentration			
	Targeted		Measured	
	Vitamin D ₃ (U/kg)	25(OH)D ₃ (μg/kg)	Vitamin D ₃ (U/kg)	25(OH)D ₃ (μg/kg)
None	0	0	< 40	< 1
Vitamin D ₃	3,250	0	2,602	< 1
25(OH)D ₃	0	16	< 40	7

Each D vitamer-supplemented dietary treatment was given for 10 weeks in a crossover-design trial separated by a 9-week washout period. For 10 weeks at the start of the trial and during the washout period, dogs received the same diet with no supplementary D vitamer included. The measured moisture content of the diet was 4.3% to 5.7%. Notice that the units differ between vitamers; 40 U is equivalent to 1.0 μg of vitamin D₃.¹³ Values less than the lower limit of detection were recorded as 0.

rum 25(OH)D₃ contributed approximately 14% of the total serum 25(OH)D concentration.

When 25(OH)D₃ was received at doses of 0.23 μg/kg^{0.75} or 0.46 μg/kg^{0.75}, week 8 serum 25(OH)D₃ concentrations were significantly ($P < 0.05$) greater, with 7.6-fold and 14.1-fold differences in median values, respectively, compared with results for the 0-μg/kg^{0.75} dose (Table 1). The serum 25(OH)D₃ concentrations appeared to increase linearly with respect to the 25(OH)D₃ dose administered (Figure 1). The slopes of individual dog responses to 25(OH)D₃ were positive and indicated that total 25(OH)D concentration rose by a mean of 13 ng for every 0.1 μg/kg^{0.75} of 25(OH)D₃ that was ingested on a daily basis (data not shown).

Week 8 serum samples for the 0-μg/kg^{0.75} 25(OH)D₃ administration period unexpectedly contained 24,25(OH)₂D₃ (Table 1). These results were variable, with a 5.7-fold difference between the highest and lowest 24,25(OH)₂D₃ concentrations for individual dogs. When dogs received 25(OH)D₃ doses of 0.23 or 0.46 μg/kg^{0.75}/d, serum 24,25(OH)₂D₃ concentrations were numerically greater, compared with the concentration for the 0-μg/kg^{0.75} dosing period; however, the differences were nonsignificant ($P = 0.051$). Although 24,25(OH)₂D₃ was quantifiable in serum of the dogs at the end of each dosing period, the vitamin D₂ metabolite 24,25(OH)₂D₂ was not detected in serum samples. This result was not anticipated because a vitamin D₂ supplement was administered orally to all dogs each day, and quantifiable amounts of 25(OH)D₂ were found in serum.

Dietary supplementation trial

The concentration of vitamin D₃ measured in the diet supplemented with vitamin D₃ was numerically lower than the targeted value by 20% (Table 2). The concentration of 25(OH)D₃ measured in the diet supplemented with 25(OH)D₃ was numerically lower than the targeted value by 56%. The concentrations of vitamin D₃ and 25(OH)D₃ in the unsupplemented diet were below the detection limits of analysis methods.

All dogs completed the trial. There were no apparent adverse effects for 6 of the 7 dogs; 1 dog developed an anal gland abscess while receiving the diet supplemented with 25(OH)D₃. The abscess resolved after oral and topical antimicrobial treatment during the supplementation period. Results of week 10 clinical hematologic and biochemical analyses did not differ significantly between the vitamin D₃ and 25(OH)D₃ dosing periods, with 2 exceptions. When dogs received diet supplemented with 25(OH)D₃, plasma glucose concentrations were slightly greater ($P = 0.024$) and reticulocyte counts were slightly lower ($P = 0.031$) than when dogs received the vitamin D₃-supplemented diet (Supplementary Tables S1 and S2). Results for all dogs were within the respective laboratory reference intervals or showed small deviations that were not considered clinically important. Urinalysis results did not differ between the 2 dietary treatments (Supplementary Table S3).

Median body condition score for the 7 dogs at the start of the dietary supplementation trial was 6 of 9 (range, 4 to 7/9). Body weights of the dogs at the end of the trial were not significantly different from those at the start of the trial, and week 10 body weight and daily food intake did not differ significantly between the 2 dietary treatments (data not shown). Median metabolizable energy intake for both dietary treatments was 93 kcal/kg^{0.75} (range, 86 to 136 kcal/kg^{0.75}).

While dogs were receiving the diet not supplemented with a D vitamer, plasma 25(OH)D₃ concentrations among the dogs declined to a median of 22 ng/mL (range, 15 to 35 ng/mL) during the washout period at the start of the trial and to a median of 26 ng/mL (range, 19 to 29 ng/mL) during the washout period between dietary treatments. Plasma 25(OH)D₃ concentrations at the end of these 2 washout periods were not significantly different.

When the dogs received the diet supplemented with 25(OH)D₃, plasma 25(OH)D₃ concentration increased significantly ($P < 0.05$), beginning at the first sampling time of the treatment period (week 2) until concentrations reached an apparent plateau by week 6 (Figure 2). A similar change in plasma 25(OH)D₃ concentration occurred when the dogs received

Figure 2—Comparison of mean $\pm$ SEM plasma concentrations of 25(OH)D₃ for 7 healthy adult dogs that received 3 forms of a custom-manufactured, extruded, dry-expanded diet that differed only in the type and amount of D vitamin included: none, vitamin D₃ (black circles), or 25(OH)D₃ (white circles) in a dietary supplementation trial. Each D vitamin-supplemented dietary treatment was given for 10 weeks separated by a 9-week washout period in a crossover-design trial. For 10 weeks at the start of the trial and during the washout period, dogs received the same diet with no supplementary D vitamin included. Symbols indicate significant ($P < 0.05$) differences between time points for a given dietary treatment. *Value is greater than that for week 0. †Value is greater than those for weeks 0 and 2. ‡Value is greater than those for weeks 0, 2, and 4. See Table 2 for targeted versus measured as-fed concentrations of vitamin D₃ and 25(OH)D₃.

the diet supplemented with vitamin D₃. The plasma 25(OH)D₃ concentrations at each sampling time did not differ significantly between the 2 dietary treatments ($P \geq 0.25$ for all comparisons). However, at the 2-, 4-, 6-, 8-, and 10-week time points, median plasma 25(OH)D₃ concentrations were numerically greater when dogs received the 25(OH)D₃-supplemented diet than when they received the vitamin D₃-supplemented diet. The week 10 plasma concentrations of 24,25(OH)₂D₃, 1,25(OH)₂D₃, total calcium, ionized calcium, and phosphorus, as well as serum concentrations of FGF-23 and PTH, also did not differ significantly between the 2 dietary treatments (Table 3).

Discussion

Some foods that are ingredients of canine diets are known to contain 25(OH)D₃.^{21,22} Quantification of 25(OH)D₃ in commercially available dog foods has not been previously reported to our knowledge. We found detectable amounts of 25(OH)D₃ in many (11/20) of the commercial diets evaluated, but the amounts were low enough to be considered inconsequential. The greatest dietary 25(OH)D₃ concentration was approximately 1.7 $\mu\text{g}/\text{kg}$ on a dry-matter

basis. For a diet with this concentration of 25(OH)D₃ consumed in amounts to support canine maintenance energy requirements (eg, 95 kcal/kg^{0.75}/d),¹³ the daily intake of 25(OH)D₃ would be equivalent to an oral dose of 0.04 $\mu\text{g}/\text{kg}^{0.75}/\text{d}$ and approximately one-sixth of the lowest 25(OH)D₃ dose received by dogs during our 25(OH)D₃ dose determination trial.

We evaluated only extruded dry diets for 25(OH)D₃ content because they are the most common of diet types fed to dogs in the US.³⁶ Wet diets and other diet forms that are high in liver or other meat content and animal fat likely would have contained more 25(OH)D₃ than the extruded diets. Short-duration thermal processing at high temperature with moist heat is unique to extruded diets, and heat can reduce the concentration of 25(OH)D₃ in foods.³⁷ The extent of this reduction depends on the food matrix and the temperature, duration, and method of cooking. The 25(OH)D₃-containing extruded diet that was created for use in the dietary supplementation portion of the present study contained 44% of the targeted amount of 25(OH)D₃ on analysis. It was possible that extrusion reduced 25(OH)D₃ retention, although inefficient extraction of 25(OH)D₃ from the commercial beadlet carrier product during analysis also was possible. The advertised heat stability limit

of that product (90 °C) was lower than temperatures that may be used during extrusion.³⁸ Retention of vitamin D₃ is affected by conditions of extrusion and the vehicle used to provide the supplement.³⁸ Processing loss might similarly have accounted for the finding that measured vitamin D₃ content of the vitamin D₃-supplemented diet used in the dietary supplementation trial was 80% of the targeted value. Quantification of processing effects on 25(OH)D₃ retention in dog foods would be of value to determine in future research considering the high potency of dietary 25(OH)D₃ for affecting vitamin D status and the reputed high bioavailability of ingested 25(OH)D₃, compared with that of vitamin D₃.¹⁸

In administering vitamin D₂ during the 25(OH)D₃ dose determination trial, we assumed similar biopotencies of vitamins D₂ and D₃ after oral administration in dogs. Comparison of vitamin D₂ and vitamin D₃ utilization by dogs was reported not to have been investigated in the 2006 National Research Council publication on the nutrient requirements of dogs.³⁹ The daily dose of vitamin D₂ used in our dose determination trial (0.33 $\mu\text{g}/\text{kg}^{0.75}$) was slightly less than the intake of vitamin D₃ recommended for canine

Table 3—Comparison of median (range) week 10 concentrations of vitamin D metabolites, total and ionized calcium, phosphorus, and hormones involved in calcium and phosphorus homeostasis (PTH and FGF-23) for 7 healthy adult dogs while they were receiving the 2 D-vitamer-supplemented dietary treatments in Table 2.

Analyte	Vitamin D ₃	25(OH)D ₃	Reference interval	P value
Plasma analytes				
25(OH)D ₃ (ng/mL)*	40.4 (27.5–66.6)	63.8 (29.7–95.4)	—	0.335
24,25(OH) ₂ D ₃ (ng/mL)*	24.3 (18.3–37.7)	26.3 (19.0–38.4)	—	0.714
25(OH)D ₃ -to-24,25(OH) ₂ D ₃ ratio	1.32 (1.18–3.02)	1.82 (0.98–3.46)	—	0.390
1,25(OH) ₂ D ₃ (pg/mL)†	64.0 (45.8–81.5)	67.0 (52.9–83.8)	—	0.488
Ionized calcium (mmol/L)*	1.30 (1.25–1.35)	1.30 (1.24–1.35)	1.25–1.45	0.895
Total calcium (mg/dL)	9.9 (9.7–10.5)	9.9 (9.7–10.5)	9.1–10.8	0.750
Phosphorus (mg/dL)	3.5 (2.9–3.6)	3.2 (2.7–3.8)	2.3–5.0	0.448
Serum analytes				
PTH (pmol/L)	0.8 (0.5–1.8)	0.8 (0.5–1.1)	0.50–5.80	0.625
FGF-23 (pg/mL)†	339 (203–953)	297 (97–667)	—	0.572

See Table 1 for remainder of key. See Table 2 for targeted versus measured as-fed concentrations of vitamin D₃ and 25(OH)D₃ in the diets.

maintenance (0.36 µg/kg^{0.75}/d).¹³ Our finding of quantifiable concentrations of 25(OH)D₂ in serum during the final week of treatment (week 8) indicated that the vitamin D₂ is absorbed and 25-hydroxylated after oral administration in dogs. Whether the observed 25(OH)D₂ concentrations were indicative of sufficient vitamin D status was unclear. Serum ionized calcium concentrations of some dogs were below the lower limit of the laboratory reference interval (1.25 mmol/L) when only vitamin D₂ was received in that trial, and the median serum ionized calcium concentration of the dogs was below this limit when the dogs received vitamin D₂ together with 0.23 or 0.46 µg of 25(OH)D₃/kg^{0.75} (median values of 1.21 and 1.23 mmol/L, respectively). The serum PTH measurements obtained at the same time point indicated that the vitamin D₂ dose was likely sufficient. A homeostatic response to hypocalcemia would include increased circulating PTH concentrations, and our results showed that PTH concentrations did not vary significantly according to the amount of 25(OH)D₃ ingested. Furthermore, the observed PTH concentrations for all dogs were much lower than the upper limit of the laboratory reference interval.

The concentration of 25(OH)D₃ in the intestinal mucosa should proportionally reflect the concentration of the metabolite in blood in the absence of ingestion of 25(OH)D₃. In this case, most 25(OH)D₃ is synthesized and released into blood by the liver.⁴⁰ In contrast, ingested 25(OH)D₃ presents a concentration of the metabolite that is many times greater than typical circulating concentrations to the intestinal mucosa.⁴¹ At such high concentrations, 25(OH)D₃ is postulated to have a calcitriol-like effect on the mucosa by acting as a vitamin D receptor ligand.⁴¹ Thus, 25(OH)D₃ ingestion may stimulate absorption of dietary calcium and phosphorus. Given ascribed homeostatic functions of FGF-23 and PTH on circulating phosphorus and calcium concentrations, we hypothesized that dietary 25(OH)D₃ supplementation might result in chronic changes in concentrations of

these hormones that would not be observed with dietary vitamin D₃ supplementation. Nevertheless, 10 weeks of dietary supplementation with 25(OH)D₃ did not result in serum concentrations of FGF-23 or PTH that were significantly different from those when vitamin D₃ was given as a supplement. Therefore, with regard to safety of the amount of 25(OH)D₃ ingested by the dogs of our study, finding a null effect was desired. Chronically high concentrations of FGF-23 are suggested to be associated with damage to the vasculature, heart, and kidneys.⁴²

The concentration of 25(OH)D₂ found in serum of the dogs was only slightly reduced when 25(OH)D₃ was orally administered in our dose determination trial. This observation was consistent with 25-hydroxylation of vitamin D in dogs being insensitive to feedback inhibition by 25(OH)D, a condition reported in other species.³ The oral administration of 25(OH)D₃ caused substantial increases in circulating 25(OH)D₃ concentrations among the dogs in the dose determination trial of the present study, and the changes in serum 25(OH)D₃ concentration appeared linearly proportional to 25(OH)D₃ dose. A similarly effective and linear response to oral administration of 25(OH)D₃ is reported for people.^{43,44} Such a relationship may be valuable for predicting the vitamin D status response of dogs to ingested 25(OH)D₃.

The potency of oral 25(OH)D₃ for affecting vitamin D status in the present study was in contrast to our previous findings on oral administration of vitamin D₃. We reported that serum 25(OH)D₃ concentration in privately-owned dogs changed very little after 9 to 10 weeks of daily ingestion of vitamin D₃ in amounts > 2.3 µg/kg^{0.75}/d.¹¹ A low potency of ingested vitamin D₃ for increasing vitamin D status has been reported in people, but the effectiveness of vitamin D₃ is observed to be conditional. Heaney et al¹⁰ showed that in people with low vitamin D status, a very steep, linear increase in serum 25(OH)D₃ concentration occurs with increasing vitamin D₃ ingestion. However, investigators also showed that when vitamin D status reaches a critical threshold,

an inflection occurs beyond which more vitamin D₃ ingestion causes only a modest rise in serum 25(OH)D₃ concentration, and investigators suggested that the inflection reflects the production of 25(OH)D₃ being no longer principally limited by substrate abundance but dependent on the amount of available 25-hydroxylase, which for many species is believed to be found primarily in the liver.^{3,40} In the present study, we observed that plasma 25(OH)D₃ concentrations effectively doubled by week 6 when the vitamin D₃-supplemented, extruded diet was fed in the dietary supplementation trial. These dogs were fed a diet that reduced plasma 25(OH)D₃ concentrations to values that were considered low (median, 24; range, 15 to 35 ng/mL for both wash-out periods together) prior to receiving the vitamin D₃-supplemented diet. When depleted of vitamin D, 25(OH)D₃ production by dogs may be acutely influenced by an abundance of vitamin D₃ substrate. Under this condition, a so-called near-quantitative conversion of vitamin D₃ to 25(OH)D₃ is suggested to occur in people.¹⁰

In the dietary supplementation trial, the equilibration concentrations—measurements obtained during the periods when continued oral intake of the vitamin affected no further increase in plasma 25(OH)D₃ concentrations—of 25(OH)D₃ and 1,25(OH)₂D₃ in plasma did not differ significantly between the 2 dietary treatments. This finding was serendipitous in that it allowed estimation of potency equivalence of dietary 25(OH)D₃, compared with dietary vitamin D₃ in dogs. In considering the targeted amounts of 25(OH)D₃ and vitamin D₃ in the 2 dietary treatments, 25(OH)D₃ appeared to be approximately 5 times as potent as vitamin D₃ for changing vitamin D status as assessed from plasma concentration of 25(OH)D₃. This potency differential was within the range described for people in recent reports^{16,43,44} indicating ingested 25(OH)D₃ is 1.4 to 5 times as potent as vitamin D₃ for increasing circulating 25(OH)D₃ concentrations.

Our analyses did not detect 24,25(OH)₂D₂ in serum when vitamin D₂ was ingested in amounts sufficient to cause appearance of 25(OH)D₂ in serum during the 25(OH)D₃ dose determination trial. To our knowledge, this was a novel finding that may indicate that dogs poorly convert 25(OH)D₂ to 24,25(OH)₂D₂. The 24-hydroxylation of 25(OH)D is cited as important to skeletal health and protective against vitamin D toxicosis in dogs.⁴⁵ Lacking or diminished 24,25(OH)₂D₂ production might have consequences when vitamin D₂ is used as a sole vitamin D supplement in diets for dogs. Dogs may have a low activity of 24-hydroxylase toward 25(OH)D₂. Species differences in enzymatic activities toward vitamin D₂ metabolites have been reported. Horst et al⁴⁶ suggested that vitamin D₂ is less metabolized by 25-hydroxylase, compared with vitamin D₃ in pigs, whereas 25-hydroxylase appears not to discriminate between vitamin D₂ and vitamin D₃ in chicks. Metabolic discrimination between 25(OH)D₂ and 25(OH)D₃ in people has been suggested, but its

practical relevance to maintenance of vitamin D status is not agreed upon.^{4,12,47}

The median week 8 concentrations of 24,25(OH)₂D₃ that we observed in the 25(OH)D₃ dose determination trial were remarkably high relative to 25(OH)D₃ concentrations, as previously noted for dogs.^{45,48} In previous research, we observed that serum 24,25(OH)₂D₃ concentrations increase following oral administration of 25(OH)D₃, but only after a delay of 3 to 5 weeks.¹⁷ Cause of the delay was unknown, but it might have been from slow induction of 24-hydroxylase activity in dogs. In retrospect, the treatment periods of the dose determination trial reported here should have been extended to allow for equilibration of serum 24,25(OH)₂D₃ concentration. The large interindividual variation that we observed in serum 24,25(OH)₂D₃ concentrations might have reflected blood sampling before the metabolite was equilibrated. Our assumption that 8 weeks would be sufficient time for equilibration of 25(OH)D₃ concentration was made on the basis of a previously calculated plasma half-life during washout of 1.8 weeks for 25(OH)D₃.¹⁷ This assumption appeared to be valid, as plasma 25(OH)D₃ concentrations plateaued by 6 weeks and did not significantly change after this time when dogs received diet supplemented with 25(OH)D₃ in the present study.

A limitation of the present study was uncertainty about the physiological relevance of the observed 25(OH)D concentrations. The concentration of 25(OH)D in circulation is conventionally accepted as an indicator of vitamin D status in many species,⁴⁹ including dogs.^{48,50,51} However, methods for quantifying 25(OH)D concentrations vary, and no gold standard has been established. Chromatography-based methods such as the procedure we used, which had been validated for use on human samples when initially described,³² are considered superior to immunoassays because they are less influenced by sample matrix and allow for simultaneous quantification of vitamin D₃ and D₂ metabolites as well as their C3 epimers, which may circulate in plasma.⁵² The meaning of 25(OH)D₃ concentration thresholds and limits is only tentatively agreed upon for people and, to our knowledge, these thresholds and limits have not been reported for dogs. Circulating concentrations < 12 ng/mL are taken to indicate vitamin D deficiency in human patients, whereas concentrations > 100 ng/mL are cited as indicative of possible toxic consequences of vitamin D excess,^{4,8} and concentrations between 20 and 50 ng/mL are suggested as optimal for skeletal health.⁴ Concentrations of 25(OH)D₃ that are optimal for nonskeletal functions and minimization of disease risk are unknown and are under investigation. The human 25(OH)D₃ concentration thresholds and limits may or may not apply to dogs. Overt signs of hypocalcemia in dogs (eg, muscle twitching, face rubbing, seizure activity, and high circulating PTH concentrations⁵³) were not observed after 8 weeks of the lowest D vitamin dose (when only vitamin D₂ was administered) in our dose deter-

mination trial, when combined serum 25(OH)D₂ and 25(OH)D₃ concentrations ranged from approximately 19 to 29 ng/mL. At the highest D vitamin dose in that trial, the combined serum 25(OH)D₂ and 25(OH)D₃ concentrations ranged from approximately 42 to 146 ng/mL. Under these conditions, signs of vitamin D excess (eg, high ionized calcium and low to undetectable PTH concentrations in serum, decreased food intake, weight loss, and inactivity)⁹ were absent.

Our results indicated that, when included in a custom dry diet, orally administered 25(OH)D₃ was approximately 5 times as potent as vitamin D₃ for increasing the vitamin D status as assessed by circulating concentrations of the 25(OH)D₃ metabolite in adult dogs that had low week 0 concentrations of the metabolite, and the amount of 25(OH)D₃ ingested seemed to increase circulating concentration of 25(OH)D₃ in a linear manner. Although dogs might consume 25(OH)D₃ in commercially available diets, our assay results for several commercial dry foods indicated it was present in amounts that are likely inconsequential for affecting vitamin D status. Short-term 25(OH)D₃ supplementation in the amounts used in this study appeared safe, with no adverse effects deemed attributable to the treatment, in this sample of healthy adult Beagles and Chinese Crested-Beagle crossbred dogs with experimentally induced low initial vitamin D status, and supported median circulating concentrations of 25(OH)D above the minimum and below the maximum values indicative of vitamin D deficiency and excess, respectively, reported for people. These findings provide a basis for evaluation of the use of oral 25(OH)D₃ administration in dogs with disease states and conditions in which vitamin D status is low despite an apparently adequate intake of dietary vitamin D (eg, chronic enteropathies, cholestatic liver disease, atopic skin disease, chronic kidney disease, and lymphoma). However, before generally applying our findings on 25(OH)D₃, additional research is needed. Future studies should be of longer duration and involve greater numbers of dogs with greater genetic diversity than in our investigations. Our findings showed that vitamin D₂ is 25-hydroxylated, as it appeared in plasma as 25(OH)D₂ in substantive concentrations when vitamin D₂ was given at a dosage (0.33 µg/kg^{0.75}/d) close to the National Research Council's vitamin D₃ recommendation for dogs at maintenance (0.36 µg/kg^{0.75}/d).¹³ The importance of our observation that the vitamin D₂ metabolite 24,25(OH)₂D₂ was not detectable in plasma was unknown, and further investigation seems warranted, as vitamin D₂ may be used as the sole source of vitamin D supplementation in dogs.

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The authors declare that there were no conflicts of interest.

Footnotes

- a. Isosciences, Ambler, Pa.
- b. Zorbax Sil (4.6 X 250 mm), Agilent, Santa Clara, Calif.
- c. Key Synthesis, Philadelphia, Pa.
- d. Eurofins Nutritional Analysis Center, Des Moines, Iowa.
- e. Hobart, Troy, Ohio.
- f. Donated by Nestlé Purina, St Louis, Mo.
- g. Laboratory Canine Diet 5006, PMI Nutrition International, St Louis, Mo.
- h. Canine Carry Outs, Beef Flavor, Big Heart Pet Brands, San Francisco, Calif.
- i. DSM Nutritional Products, Parsippany, NJ.
- j. HyD, DSM Nutritional Products, Parsippany, NJ.
- k. PerkinElmer Health Sciences, Shelton, Conn.
- l. Bond Elut, Agilent, Santa Clara, Calif.
- m. Zorbax SB-CN (4.6 X 250 mm), Agilent, Santa Clara, Calif.
- n. Tri-Carb 2000 CA, Packard, Downers Grove, Ill.
- o. AA-56F2, Immunodiagnostic Systems, Gaithersburg, Md.
- p. xT-2000i, Sysmex America, Lincolnshire, Ill.
- q. AU480, Beckman Coulter, Brea, Calif.
- r. VDI Laboratory, Simi Valley, Calif.
- s. Veterinary Diagnostic Laboratory, Michigan State University, Lansing, Mich.
- t. Kainos Laboratories, Tokyo, Japan.
- u. SAS, version 9.4, SAS Institute, Cary, NC.
- v. Excel 2016, Microsoft Corp, Redmond, Wash.
- w. Daily Chef Food Service, Distributed by Sam's West, Bentonville, Ark.
- x. Crisco, JM Smucker Co, Orrville, Ohio.
- y. Now Foods, Bloomington, Ill.
- z. Omega-3 Pet, Nordic Naturals, Watsonville, Calif.
- aa. Dyets, Bethlehem, Pa.
- bb. Nutritionist Pro Nutrition Analysis Software, network version 5.0, Axxya Systems, Stafford, Tex.

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Supplementary Materials

Supplementary materials are available online at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.9.722.

Appendix I

Ingredients (reported on a dry-matter basis) of a semipurified diet low in vitamins D₂ and D₃ that was created for use in a 25(OH)D₃ dose determination trial.

Ingredient	Concentration (g/kg)
Casein (high nitrogen, 30 mesh)	171
Soy protein isolate (92%)	171
Rice (white, long grain) ^w	263
Vegetable shortening ^{*†}	102
Soybean oil ^w	74.6
Whole psyllium husk [†]	60.8
Mineral mixture [†]	51.2
Vitamin mixture [†]	51.2
Potassium chloride	13.2
Sodium propionate	9.0
Magnesium sulfate	8.5
Calcium carbonate	6.8
Tricalcium phosphate	6.4
Marine fish oil ^{†§}	4.9
L-methionine	2.6
L-cystine	2.3
Choline chloride	1.8

Except where indicated, all ingredients were obtained from a single commercial source.^{aa}

^{*}Contained soybean oil, fully hydrogenated palm oil, palm oil, mono- and diglycerides, tert-butylhydroquinone, and citric acid. [†]Contained sucrose (989 g/kg), ferric citrate (8.28 g/kg), zinc carbonate (2.72 g/kg), manganous carbonate (0.245 g/kg), cupric carbonate (0.174 g/kg), and sodium selenite (0.0172 g/kg). [‡]Contained sucrose (998 g/kg), niacin (1.00 g/kg), D-calcium pantothenate (0.400 g/kg), thiamin hydrochloride (0.142 g/kg), riboflavin (0.130 g/kg), pyridoxine hydrochloride (0.065 g/kg), menadione sodium bisulfite (0.050 g/kg), folate (0.012 g/kg), biotin (6.6 × 10⁻³ g/kg), vitamin B12 (8.5 × 10⁻³ g/kg), vitamin E acetate (1,044 U/kg), and vitamin A palmitate (67,500 U/kg). [§]Contained anchovy oil, sardine oil, d-α-tocopherol, eicosapentaenoic acid (170 g/kg), and docosahexaenoic acid (100 g/kg).

Appendix 2 appears on the next page

Appendix 2

Nutrient contents (as fed) per 1,000 kcal of semipurified and extruded diets used in 25(OH)D₃ dose determination and dietary D-vitamin supplementation trials, respectively.

Nutrient	Diet		Recommended allowance
	Semipurified	Extruded	
Proximate composition			
Protein (g)	71	87	25
Fat (g)	43	36	14
Carbohydrate (g)	80	111	—
Fiber (g)	0.2	3.5	—
Ash (g)	4.0	20	—
Protein (kcal)	290	300	—
Fat (kcal)	390	310	—
Carbohydrate (kcal)	320	390	—
Essential amino acids			
Tryptophan (g)	0.5	—	0.4
Threonine (g)	1.4	—	1.0
Isoleucine (g)	1.8	—	0.9
Leucine (g)	3.1	—	1.7
Methionine (g)	1.1	0.8	0.8
Methionine and cystine (g)	2.1	1.3*	1.6
Phenylalanine (g)	1.9	—	1.1
Phenylalanine and tyrosine (g)	4.1	—	1.9
Valine (g)	2.4	—	1.2
Arginine (g)	1.7	—	0.9
Histidine (g)	1.1	—	0.5
Lysine (g)	2.3	—	0.9
Macrominerals			
Calcium (g)	1.3	1.4	1.0
Phosphorus (g)	0.9	1.1	0.8
Potassium (g)	1.7	1.0	1.0
Magnesium (g)	0.2	0.1	0.2
Sodium (g)	0.9	0.6	0.2
Chloride (g)	0.8	1.2	0.3
Trace elements			
Zinc (mg)	23	—	15
Iron (mg)	25	—	7.5
Manganese (mg)	2.0	—	1.3
Copper (mg)	2.0	—	1.5
Selenium (μg)	111	—	88
Iodine (μg)	11†	—	220
Essential fatty acids			
Linoleic acid (g)	10.8	—	2.8
Linolenic acid (g)	1.4	—	0.11
Eicosapentaenoic acid (g)	0.16	—	0.06
Docosahexaenoic acid (g)	0.10	—	0.06
Vitamins			
Choline (mg)‡	425	—	420
Thiamin (mg)	2.1	—	0.6
Riboflavin (mg)	1.6	—	1.3
Niacin (mg)	11	—	4.3
Pantothenic acid (mg)	5.3	—	3.8
Pyridoxine (mg)	0.8	—	0.4
Folate (μg)	355	—	68
Cobalamin (μg)	11	—	8.7
Biotin (μg)	23	—	—
Vitamin K (μg)	605	—	410
Vitamin A (U)	1,553	—	1,263
Vitamin D (U)	83	676§	136
Vitamin E (U)	21	—	7.5

National Research Council recommended daily allowances¹³ for nutrients are provided where applicable. Values for the semipurified diet represent the sum of nutrients in database entries^{bb} for the ingredients, except for chloride, which was determined by laboratory analysis because data were lacking for the ingredients. Values for the extruded diets represent results of laboratory analysis. Three forms of the extruded diet were prepared that differed only in the type and amount of vitamin D supplementation: none, vitamin D₃, or 25(OH)D₃; see Table 2 for targeted vs measured D vitamin content.

*Less than the recommended allowance but equal to the minimum requirement for maintenance of adult dogs.

†Data on iodine content reported only for the casein ingredient; supplemental iodine was inadvertently omitted from the mineral mix in the semipurified diet. ‡Provided as choline chloride in the semipurified diet. §Result shown for the extruded diet supplemented with vitamin D₃; < 11 U in the diets with either no vitamin D supplementation or 25(OH)D₃ supplementation alone.

— = Not available.