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Do Flame Retardants Promote Vitamin D Deficiency?

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Senior Honors Thesis
Biomedical Science – PreMed/PreVet

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ABSTRACT

Vitamin D deficiency in the Unites States has become more prevalent in recent years. Research has shown that environmental chemicals such as flame-retardants induce hepatic enzymes in the cytochrome P450 family such as CYP24 and CYP3A that are important in vitamin D metabolism. To determine if exposure to one class of flame-retardants known as polybrominated diethyl ethers (PBDEs) promotes vitamin D deficiency, 15 rats consumed a diet marginally deficient in Vitamin D - 85 IU Vitamin D/kg diet - for 56 days. On day 28 of the experiment, 7 rats were gavaged daily with 7 mg/kg BW PBDEs and 8 rats were gavaged daily with corn oil, for 28 days. Body weight and food intake were measured three times a week, vitamin D status markers in the urine were measured at weeks 4 and 8 and blood Vitamin D metabolites along with liver weight were measured at euthanization. Liver microsomal vitamin D metabolism, composition and CYP3A enzyme activity were also measured. The final body weight tended to be lower in the treatment animals than in the control but was not significantly different (370.29±40.12 vs. 400.63±31.99, respectively, $p = 0.0636$). Liver from PBDE-treated rats was significantly heavier than liver from control rats (15.67±1.99 vs. 12.71±0.98, respectively) $p < 0.05$. Liver as a percent of body weight was also significantly greater in treatment (4.24±0.2) compared to control (3.18±0.13). There was no significant difference in the lipid composition of the liver or urine metabolites between PBDE-treated and control rats. The inactive metabolites 24, 25-dihydroxy vitamin D_3 and 4β, 25-dihydroxy vitamin D_3 did not show significant difference between control and treatment groups. The active form of 1, 25-vitamin D_3 tended to be lower in the PBDE-treated rats than in the control (0.071±0.027 vs. 0.082±0.018, respectively, $p = 0.187$). When expressed as a ratio to 25(OH)D_3, 4β, 25-dihydroxyD_3 was significantly lower in treatment rats compared to control (0.96±0.18 vs. 1.28±0.38, respectively) and 1, 25-dihydroxyD_3 tended to be lower in treatment compared to control (3.62±0.96 vs. 4.44±0.97, $p = 0.068$). Enzymatic CYP3A levels were significantly higher in PDBE-treated rats than in control (6.047±1.53 vs. 0.103±0.032 nmol/min/mg protein, respectively). The hypothesis that the induction of CYP3A by PBDEs may accelerate vitamin D inactivation, leading to vitamin D deficiency was not supported by the findings, as there was no significant change in serum vitamin D levels in the PBDE-treated rats.

INTRODUCTION

Vitamin D and Health

Vitamin D is an essential nutrient for humans; it is a fat-soluble seco-steroid that can be made by the body. The definition of "vitamin" is a substance that is vital for life but must be consumed in the diet because it cannot be made by the body. Even though we produce vitamin D, it is considered a vitamin because the body cannot make it in adequate amounts. Its function was first discovered during the period between 1910-1930 as the cause of rickets in experiments with sunlight-deprived dogs.[1]

Humans obtain vitamin D from three main sources: sunlight, diet and supplements. In most mammals, when sunlight strikes the epidermis, vitamin D_3 is made from the UV radiation of 7-dehydrocholesterol.[2] In the diet, liver, mushrooms and fortified milk are good sources of vitamin D. Lastly, vitamin D is available as a supplement in pill form, as either vitamin D_3 (animal form) or vitamin D_2 (plant form).

From 2001-2006, two-thirds of the U.S. population over the age of one was considered vitamin D sufficient, but out of the remaining one-third, 25% of the population was considered at risk of inadequacy and 8% were close to deficiency.[3] Suggested reasons for this vitamin D insufficiency are the use of sunscreen, limited outdoor time, or poor diet. The World Health Organization has created the definition of the International Unit as 1.0 IU = 65 pmol of vitamin D. The Institute of Medicine (IOM) suggests that people from ages 1-70 need to consume 600 IU of vitamin D per day.[4] This amount of vitamin D is needed to maintain an adequate circulating serum level of 75 nmol/L required for healthy bone density.[5]

Role of Vitamin D in the Body

Many cells in the body contain the vitamin D receptor, and thus respond to vitamin D. The vitamin D receptor (VDR) is in the nuclear receptor family of transcription factors. When vitamin D binds to the receptor, it becomes a heterodimer with retinoid X receptor and this complex translocates to the nucleus and binds to response elements that effect gene transcription.[6] The many roles of vitamin D are not entirely understood. Active vitamin D (1, 25-dihydroxy vitamin D_3) has been found to stimulate certain cells of the immune system, while the absence of vitamin D stunts growth, impairs bone development, increases insulin production and increases the risk of heart disease and cancer.[7] It is thought that 900 genes respond directly to active vitamin D, primarily the genes for osteocalcin, osteopontin, calbindin, and calcium channels that play key roles in the control of calcium and skeletal homeostasis. The majority of VDR is expressed in the gastrointestinal tract and the kidneys.[8]

The most widely accepted and supported role of vitamin D is its relationship with calcium and bone health. Calcium is essential for maintaining bone strength. Vitamin D facilitates the absorption of calcium in the intestines by up-regulating the calcium binding protein calbinin. In a severe vitamin D deficiency, calcium is not absorbed into the body and thus the parathyroid releases parathyroid hormone (PTH) to stimulate osteoclasts to break down bone and release calcium into the blood stream.[9] Urinary calcium is a good indication of the body's vitamin D status. High levels of urinary calcium may indicate that the body does not have enough or cannot utilize vitamin D to aid in calcium absorption.

Vitamin D Activation

With ultra-violet B radiation, 7-dehydrocholesterol (derivative of cholesterol) undergoes a photoconversion to vitamin D_3 or cholecalciferol in the epidermis. Once in the body, vitamin D circulates in the blood stream and is taken up by adipose tissue or liver for either further metabolism or storage lasting months or even years.[10] The enzymes that are responsible for both the activation and inactivation of vitamin D are part of the cytochrome P450 family of CYP enzymes. The membrane bound CYP enzymes are proteins that use oxygen and NADPH to catalyze reactions for steroid hormones biosynthesis, bile acid biosynthesis and drug metabolism.[8] The family of CYP enzymes used in vitamin D metabolism is located on the internal membranes of the Golgi apparatus and endoplasmic reticulum.

Activation of the vitamin requires two hydroxylations, first at the C-25 site then at the C-1 site. Vitamin D undergoes hydroxylation in the liver, by CYP2R1 at the 25-carbon position forming 25-hydroxy vitamin D_3 or calcidiol (Figure 1). This circulating form of 25-hydroxy vitamin D_3 reenters the blood and the majority travels to the kidneys where it is further metabolized through a second hydroxylation at the 1-carbon position by CYP27B to produce the biologically active form 1, 25-dihydroxy vitamin D_3 also known as calcitriol.[11] The biologically active form of the vitamin is found in very low quantities in the body, usually measured by chromatography, while the circulating 25-hydroxyD_3 is used to gauge vitamin D status.

Vitamin D Inactivation

In order to regulate the hormonal and nutritional functions of active vitamin D, the body has enzymes to inactivate both the circulating 25-hydroxyD_3 and 1, 25-dihydroxy vitamin D_3. High levels of active vitamin D_3 activate CYP24A1, a member of

the cytochrome P450 family of drug metabolizing enzymes that is involved in the biotransformation of more than 50% of drugs.[12] CYP24A1 inactivates 1, 25-dihydroxy vitamin D_3 by hydroxylation at the 23 and/or 24 carbon positions of the molecule to make 1, 24, 25-trihydroxyD_3 and 4β, 25-dihydroxyD_3. CYP24A1 also inactivates the circulating form vitamin D_3, 25-hydroxyD_3, at the 24-carbon position to make inactive 24, 25-dihydroxyD_3 (Figure 1). In addition, another enzyme, CYP3A, can hydroxylate the 25-hydroxyD_3 to form the inactive 24, 25-dihydroxyD_3 in the liver and small intestines.[13] Hydroxylation on the inactive metabolites causes them to become more water soluble for easier excretion through the bile.[14]

Certain drugs such as antiepileptics have been shown to induce the CYP24 and CYP3A enzymes through the gene regulator PXR and ultimately decrease vitamin D_3 levels.[15] The Carey lab is investigating another possible cause of increased CYP3A: synthetic environmental chemicals.

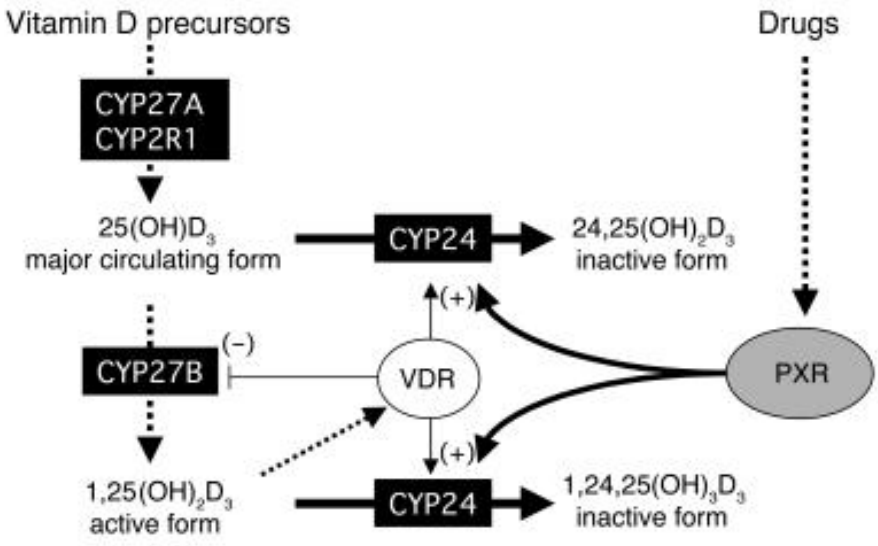

Figure 1. Once ingested in the body, vitamin D_3 is hydroxylated in the liver at the 25-carbon position (major circulating form). 25(OH)D_3 is again hydroxylated in the kidney to the active 1, 25-dihydroxyD_3. Inactivation by further hydroxylation at the 24-carbon position occurs on 1, 25-dihydroxyD_3, which is inactivated to 1, 24, 25-trihydroxyD_3 and 25-hydroxyD_3 can become inactivated to 24, 25-dihydroxyD_3.[15]

Flame Retardants

In the past two decades, studies have revealed that environmental pollutants and widely used chemicals such as DDT and flame-retardants are ingested or inhaled into the body and are stored in adipose tissue.[16] One class of flame-retardants, polybrominated diphenylethers (PBDEs, specifically DE-71) are additives used in chemical mixtures in polyurethane foams as well as many everyday appliances. These consumer products include coating of furniture, carpets, automobiles and airplanes that serve the purpose of inhibiting or delaying the spread of fire.[17] In 2008, the United States, Europe and Asia produced 1.8 million tons of flame-retardants, equivalent to 4.25 billion US dollars.[18] Many classes of PBDE, such as DE-71 have been banned and restricted, as they are believed to absorb into coastal zones and remain in foods like fish and shellfish.

As with drugs, PBDEs induce the CYP3A enzyme, which can metabolize 25-hydroxy vitamin D_3 to inactive forms. *Thus the induction of CYP3A by PBDEs may accelerate vitamin D inactivation, leading to vitamin D deficiency.*

OBJECTIVE

Pilot work from the Carey lab showed that rats fed a normal diet and given PBDEs did not develop vitamin D deficiency compared to rats receiving a corn oil control. However, normal rat diet has an abundance of vitamin D. Therefore, this work examined the effect of PBDEs on the vitamin D status of rats fed a diet that was marginally deficient in vitamin D to reflect the large portion of the U.S. population that is insufficient in vitamin D. It tested the hypothesis that:

Rats fed a marginally deficient vitamin D diet and treated with flame-retardants will develop vitamin D deficiency.

METHODS

This 56 day study was designed based on previous vitamin D work with rats, to measure the physical, biochemical and enzymatic markers of vitamin D status. All rats were fed a diet marginally deficient in vitamin D for the entire duration of the experiment. Physical assessments included food intake, body growth, and liver weight. Biochemical analysis was performed on blood serum, urinary metabolites, liver microsomal metabolic activity and liver lipid composition. Enzymatic measurements included assay of the hepatic CYP3A enzyme.

I. Animals and Diet

Sixteen weaning, male Wistar rats, weighing 75-100 grams were purchased from Charles River Laboratories. Shortly after arrival, one rat died of unknown causes, determined by necropsy, and was not replaced. The remaining 15 rats were fed a diet containing 85 IU/kg diet vitamin D for 56 days, compared to typical diets that contain from 1000 to 3000 IU/kg of vitamin D. The marginal diet of 85 IU was chosen based on the findings of Fleet et al. that serum 25-hydroxy vitamin D_3 levels are highly responsive to vitamin D intake between 50-200 IU/kg diet, which results in $25(OH)D_3$ levels ranging from 25-80 nmol/L (Figure 3).[19] 25-hydroxyD_3 serum would be at the midpoint of the range and sensitive to changes in vitamin D status. Of the 85 IU/kg, the milk protein casein is responsible for 40 IU (J. Fleet, personal communication) and thus 45 IU of vitamin D was fortified in the diet (Figure 2).[19]

Body weight and food intake of each rat was measured three times per week. All rats were housed individually in a controlled room at 70° F with a regular 12-hour light cycle with fluorescent bulbs that had GamTube™ cover slips to block 97% UV radiation below 400 nm. All rats had constant access to food and water.

D01060501 and Preliminary Formula

Rodent Diets With 10 kcal% Fat and Same
With 45 IU Vitamin D added per 4057 kcal

Product #	D01060501		Preliminary Formula	
%	gm	*kcal*	gm	*kcal*
Protein	19.2	*20*	19.2	*20*
Carbohydrate	67.3	70.0	67.3	70.0
Fat	4.3	*10*	4.3	*10*
Total	90.8	*100*	90.8	*100*
kcal/gm	3.85		3.85	
Ingredient	**gm**	***kcal***	**gm**	***kcal***
Casein, Lactic	200	*800*	200	*800*
L-Cystine	3	*12*	3	*12*
Corn Starch	575	*2300*	575	*2300*
Maltodextrin 10	125	*500*	125	*500*
Sucrose	0	*0*	0	*0*
Cellulose, BW200	50	*0*	50	*0*
Soybean Oil	25	*225*	25	*225*
Lard	20	*180*	20	*180*
Mineral Mix S10026	10	*0*	10	*0*
DiCalcium Phosphate	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	0	*0*
Vitamin Mix V13201 (w/o Vit D)	0	*0*	10	*40*
Vitamin D, 100,000 IU/gm	0	*0*	0.00045	*0*
Choline Bitartrate	2	*0*	2	*0*
FD&C Yellow Dye #5	0.025	*0*	0	*0*
FD&C Red Dye #40	0.025	*0*	0	*0*
FD&C Blue Dye #1	0	*0*	0.05	*0*
Total	**1055.05**	***4057***	**1055.05045**	***4057***

Research Diets, Inc. 2010 GaleC01.for

Figure 2. Research Diets, Inc. 85 IU/kg vitamin D diet composition table.

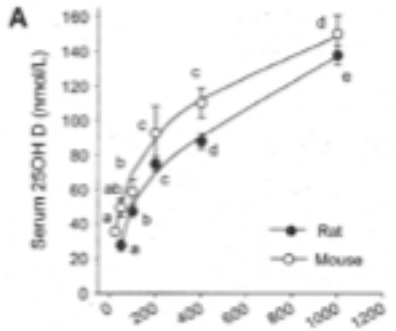

Figure 3. Effect of diets containing 25 to 1000 IU VD3/kg on serum vitamin D metabolite 25-dihydroxyD_3.[19]

II. Chemicals

DE-71 of the penta-PBDE family was donated by Dr. Mike Sanders from NIH and was originally produced by the Great Lakes Chemical Corporation Lot #1550OK07A. An amount of 2 g was dissolved in 2400 µl hexane and combined with 60 mL of corn oil. An additional 51 ml of corn oil was added and the bottle vortexed for 30-60 seconds. Hexane was then evaporated off with compressed nitrogen for 10 hours.[20] The original mixture of 14 mg DE-71/mL was diluted with corn oil to obtain 7 mg DE-71/mL. The same procedure was followed for the control mixture without the addition of PBDE. For the duration of the experiment both bottles were stored at room temperature in a closed box.

III. Animal Treatment

Starting at day 28, fifteen rats were randomly assigned into control and treatment groups. Eight control rats were gavaged with corn oil alone and 7 rats in the treatment group were gavaged with 7 mg PBDE/kg BW per day.[20] Gavaging is the pain-free procedure of scuffing and force-feeding with a ball-tipped metal syringe. The start of gavage was staggered so that four rats, 2 control and 2 treatment, would be euthanized on 4 consecutive days. On day 56 of the experiment, four rats were euthanized by carbon dioxide inhalation after 24 hours of fasting to obtain blood and liver.

IV. Assessing Vitamin D Status

Urine Metabolites

Urine was collected at 2 points during the treatment, following the procedure of Kurien and Schofield at 3 weeks of treatment and at the final 8th week of treatment.[21] Urine was stored at -80°C until being sent to Dr. David Collier, East Carolina University, for calcium and phosphorus measurements (indicators of vitamin D deficiency).

Measurements of creatinine were used to normalize the ratio of calcium or phosphorus between animals.

Blood

About 5.0 mL of blood was removed via cardiac puncture at euthanization using a 5.0 mL heparinized syringe and transferred into BD Vacutainer™ Serum Separation Tubes (Thermo Fisher Scientific). Approximately 2.5 mL of serum was collected after centrifugation at 3000 rpm, 23°C, for 15 minutes. Serum was stored at -80°C until being sent to Dr. Kenneth Thummel at the University of Washington for measurement of 25-hydroxyD_3, 24, 25-dihydroxyD_3, 1, 25-dihydroxyD_3 and 4β, 25-dihydroxyD_3.

V. Liver Microsome Preparation

Livers were removed, rinsed, blotted and weighed before being cut into approximately 2.0 g portions and transported to the Carey Lab on ice or snap frozen with liquid nitrogen at -80°C for later use. One 2.0 g portion for each rat was minced and homogenized in 4 volumes of cold buffer containing 250 mM sucrose, 1 mM DTT, 0.5 mM EDTA, 10% glycerol, 25 mM KCl, and 10 mM HEPES at pH 7.4 and centrifuged for 25 minutes at 20,000-x g. The pellet was discarded and the supernatant containing the internal membranes of the Golgi apparatus and endoplasmic reticulum (microsomes) were collected and buffer was added to the centrifuge tubes before ultracentrifugation at 105,000-x g for one hour, as described by Nash et al.[20] The pellet was resuspended in buffer and spun for a second time at 105,000-x g for one hour. The final pellet was resuspended in 0.1M phosphate buffer (pH 7.5) at 0.8 vol/g tissue. These microsomes were divided into 0.5 mL samples and were stored at -80°C until used to measure CYP3A and vitamin D metabolism. Microsome protein content was measured using a Bio-Rad protein assay kit.

VI. Liver Microsome CYP3A Measurements

BROD Assay

Hepatic microsomal benzyloxyresorufin O-dealkyl (BROD) activity was measured fluorometrically.[20] Reactions were performed in a 3.0 mL cuvette starting with a buffer of 1.25 mL 0.2M HEPES, 250 µL of 50mM $MgSO_4$, and 250 µL of 16 mg/mL BSA. Microsomes (50 µg protein) were added along with 10 µL G-6-PDH and 25 µL of 150 µM benzoxyresorufin. The samples were brought to 2.5 mL with double distilled H_2O and the contents were incubated for 5 minutes in low-light conditions at room temperature. Then the cuvette was placed in the fluorometer in no light at 37°C for 1 minute before addition of 25 µL NADPH. The production of resorufin was measured fluorimetrically to analyze CYP3A activity for 5-10 minutes.

Calculations

The intensity of each sample in duplicate was taken for 10 points around 2 and 4 minutes, averaged and inserted as Y (intensity) into the resorufin standard curve linear regression ($y = 0.2708x + 1.5285$) to solve for X (nM). As the cuvette contains 2.5 mL of solution, nM was converted to nmols by multiplying each number by 0.0025. The point at 2 minutes was subtracted from the point at 4 and divided by 2 to obtain a rate of nmol/min. This number was multiplied by 20 for each sample to achieve the rate at which the enzyme converted 50 µg of protein in units nmol/min/mg protein.

VII. Liver Microsome Vitamin D Metabolism and HPLC

Microsomes (50 µg protein/sample) were incubated with 5.0 or 0.5 mM 25-hydroxyD_3 in 0.1M phosphate buffer, a second buffer (0.1 M KH_2PO_4 and 2 mM EDTA) and 1.0 mM NAPDH for 10, 20 or 30 minutes, following the protocol of Wang et al.[22] The contents of the incubation were extracted thrice with 0.5 mL of chloroform:methanol

(3:1 ratio), which causes the lipid-soluble vitamin D metabolites to become soluble in the organic phase, and transferred to screw-cap test tubes. The tubes were vortexed for 5 seconds and allowed to separate into two layers. From each tube, 1.0 mL of the bottom layer of chloroform was removed and put into a second tube. The organic phase was evaporated to dryness with N_2 gas, wrapped in tinfoil and stored at 4°C.

In Dr. Curran-Celentano's laboratory at UNH, high performance liquid chromatography (HPLC) analysis was performed on an Agilent 1100 series HPLC system equipped with a diode array detector (Agilent Technologies, Santa Clara, CA). The residue from the screw-cap test tubes was solubilized with 200 µl methanol and a 5.0 µl aliquot was injected into a Symmetry C8 (2.1 X 150 mm, 3.5 µm) column (Waters, Milford, MA) at 25°C to separate out the peaks of interest via a linear gradient mobile phase as follows: 0 to 40 minutes, 70-80% methanol; 40.1 to 48 minutes, 90% methanol; and 48.1-63 minutes, 70% methanol. Peaks were monitored and compared to standards at 265 nm in order to measure 25-hydroxyD_3 and the inactive form of 24, 25-dihydroxyD_3 as described by Wang et al.[22]

VIII. Lipid Extraction from Liver

The experiment was run in triplicate using 3, 50 mg samples from each liver following the procedure of Li et al.[23] The liver samples were weighed out and recorded, then homogenized in 0.3 mL of cold phosphate buffer saline at pH 7.4. The homogenate was mixed with 2.0 mL of 3:2 hexane/isopropanol solution, gassed with nitrogen to help prevent oxidation, and sealed with a rubber stopper. The sample was shaken at 23°C for 1 hour, after 0.6 mL of 0.5 M sodium sulfate solution to remove any non-lipids. The sample was vortexed for 1 minute and then shaken at 23°C for 15 minutes. Then, the sample was centrifuged at 1000xg (~2700 rpm) for 10 minutes at room temperature. A

1.0 mL sample of the hexane supernatant (the upper most phase) containing lipids was removed and transferred to a pre-weighed and recorded tube, then dried with nitrogen gas for 20 minutes. Once dried, the tube containing lipids was weighed and recorded immediately and once more 24 hours later.

IX. Data Analysis

A t-test was performed for the main effect of rat PBDE treatment on urinary calcium, urinary phosphorus, blood vitamin D levels, microsomal CYP3A activity and lipid composition of the liver. Statistical significance was set at $p \leq 0.05$.

RESULTS

Animal and Liver Measurements

Final body weight of the PBDE-treated rats was 7.7% lower than controls (Table 1). There was no difference in weight gain between treatment and control rats until day 28 when growth of the PBDE treated rats declined by 6.9% (Figure 4). Liver weight alone was 23% heavier in treated rats than in control and liver weight expressed as a percent of body weight was 33% higher in PBDE-treated rats compared to controls (Table 1). There was no significant difference in the percent lipid composition of the control and treated livers.

	Control	**Treatment**	***P-value***
Final Body Weight (g)	401 ± 32	370 ± 40†	0.0636
Weight Gain After Day 28 (g)	127.38 ± 12.85	118.57 ± 17.04	0.275
Final Liver Weight (g)	12.71 ± 0.98	15.67 ± 1.99*	0.0012
Liver Weight as a % of BW	3.18 ± 0.1	4.24 ± 0.2*	0.0001
% Lipid Composition of Liver	4.55 ± 1.85	5.23 ± 2.28	0.275

Table 1. Physiological findings of 15 rats on a marginally deficient vitamin D diet after 56 days. Values are means ± standard deviation.

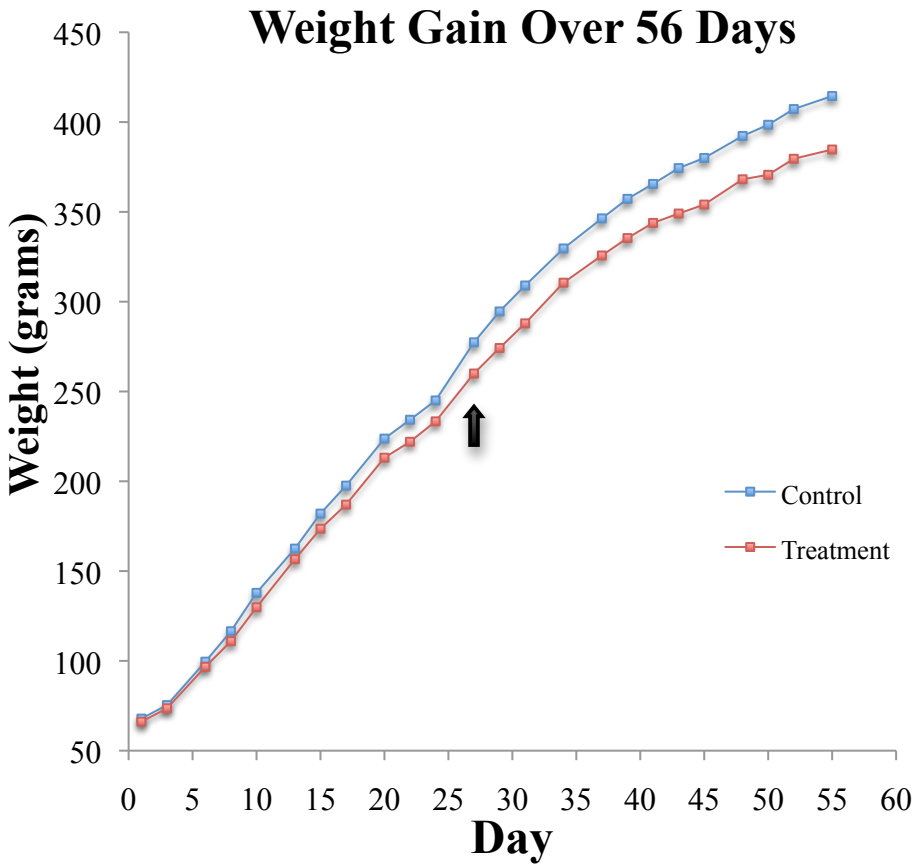

Figure 4. Average weight gain of control and treatment animals over 56 days on 85 IU/kg BW marginal diet of vitamin D. Black arrow indicates start of PBDE chemicals at day 28 of experiment.

Urine Metabolite Measurements

There was no significant alteration in urinary calcium or phosphorus between PBDE-treated and control groups at 4 or 8 weeks (Table 2). The calcium to creatinine ratio in control rats at 4 week tended to be lower than at 8 weeks (p=0.147). There was significant animal-to-animal variation in urinary calcium and phosphorus, as illustrated by standard deviation ranging from 50-143% of the mean.

	Control	Treatment
Week 4		
Calcium/Creatinine	0.154 ± 0.144	0.171 ± 0.145
Phosphorus/Creatinine	0.546 ± 0.696	0.476 ± 0.681
Week 8		
Calcium/Creatinine	0.087 ± 0.032	0.136 ± 0.181
Phosphorus/Creatinine	0.438 ± 0.292	0.451 ± 1.171

Table 2. Urine metabolites during collection at week 3 immediately before PBDE treatment and week 8 at the end of treatment. Values are means ± standard deviation.

Blood Measurements

There was no difference in serum levels of inactive 24, 25-dihydroxyD_3 and 4β, 25-dihydroxyD_3 in PBDE-treated versus control. However, active 1, 25-dihydroxyD_3 level tended to be 13.4% lower in PBDE-treated versus control (Table 3). When each animal's metabolites were expressed as a ratio to storage 25-hydroxyD_3, inactive metabolite 4β, 25-dihydroxyD_3 was 25% lower than control ($p < 0.05$) and active form 1, 25-dihydroxyD_3 was 18.5% lower ($p = 0.068$) in PBDE-treated animals (Table 4).

Vitamin D Metabolites (ng/mL)	Control	Treatment	*P-value*
24, 25 D3	0.794 ± 0.130	0.781 ± 0.332	0.462
4β, 25 D3	0.023 ± 0.003	0.19 ± 0.010	0.373
1, 25 D3	0.082 ± 0.018	0.071 ± 0.027	0.181
25 D3	1.740 ± 0.152	1.896 ± 0.678	0.282

Table 3. Vitamin D metabolites in blood serum

Vitamin D Metabolites/25OH D3 (ng/mL)	Control	Treatment	*P-value*
24, 25 D3	42.77±8.43	38.83±10.33	0.225
4β, 25 D3	1.28±0.38	0.96±0.18*	0.033
1, 25 D3	4.44±0.97	3.62±0.96†	0.068

Table 4. Serum metabolites as a molar ratio to 25-hydroxy vitamin D_3

CYP Enzyme Analysis

There was a significant effect of PBDE treatment on the CYP3A enzyme activity in the liver microsomes prepared from PBDE-treated rats (Figure 5). After 28 days of treatment, the treatment CYP3A activity of 6.047 nmol/min/mg protein was greater by 59 fold ($p < 0.05$) than the control activity of 0.103 nmol/min/mg protein.

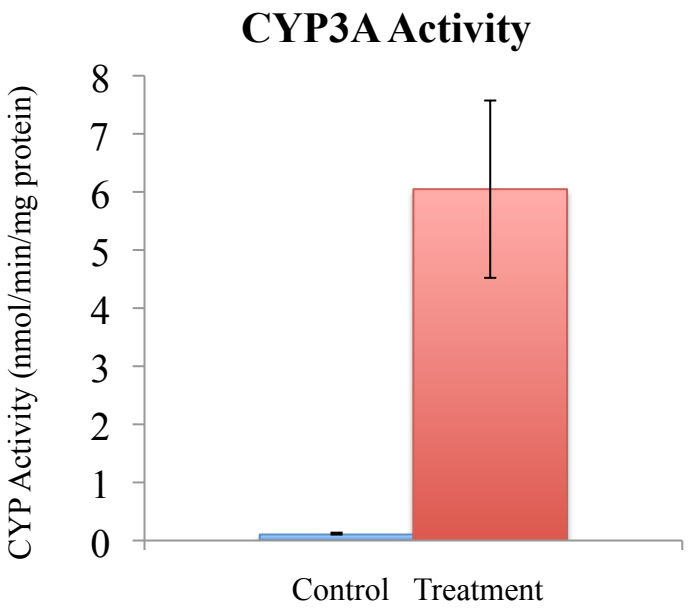

Figure 5. Hepatic CYP3A activity of control and PBDE-treated rats on a diet marginally deficient in vitamin D. Values are means ± standard deviation.

Vitamin D Metabolism

A standard chromatograph of 50 minutes shows three peaks of vitamin D metabolites: 1, 25-dihydroxyD_3 and 24, 25-dihydroxyD_3 both occurring mid-elusion with the larger peak of 25-hydroxyD_3 appearing at 40 minutes (Figure 6). Positive control samples containing no microsomes at 10 min (samples 1, 2) versus no microsomes at 30 min (samples 9, 10) show a 31.6% loss in total 25-hydroxyD_3 in the vial after 30 minutes of incubating. There was no production of the 24, 25-dihydroxyD_3 or 1, 25-dihydroxyD_3 metabolites for control or treatment rats after 10, 20, or 30 minutes of incubation (Table 5).

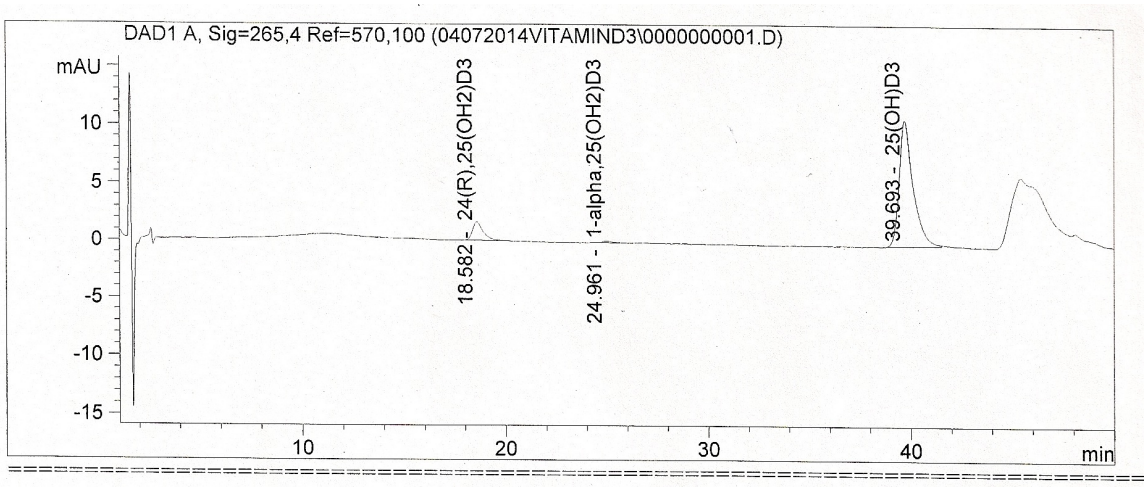

Figure 6. Standard chromatograph of a 50 minute elusion.

Sample ID	Total 25(OH)D3 (nmol)
1 - no microsomes (10 min)	14.49
2 – no microsomes (10 min)	12.14
3 - T4 (10 min)	6.39
4 - T4 (10 min)	13.08
5 - T4 (20 min)	5.59
6 - T4 (20 min)	5.77
7 - T4 (30 min)	8.31
8 - T4 (30 min)	8.29
9 - no microsomes (30 min)	9.77
10 - no microsomes (30 min)	8.45
11- T1 (30 min)	6.82
13 - C1 (30 min)	8.41
14 - C1 (30 min)	6.43
15 - C3 (30 min)	5.29
16 - C3 (30 min)	1.92
17 - T4, no NADPH (30 min)	4.03
18 - T4, no NADPH (30 min)	2.47
19 - C3, no NADPH (30 min)	1.82
20 - C3, no NADPH (30 min)	5.25

Table 5. Total amount of vitamin D 25(OH)D_3 in 2 control and 2 treatment samples analyzed by HPLC out of 16 nmol of 25(OH)D_3. Note: there is no sample 12 due to experimental error.

DISCUSSION

The purpose of this study was to explore the in vivo effects of a commercial PBDE flame retardant mixture of DE-71 on the hepatic CYP3A enzyme responsible for the metabolism of drugs and inactivation pathways in the metabolism of vitamin D. The hypothesis that the induction of CYP3A by PBDEs may accelerate vitamin D inactivation, leading to vitamin D deficiency was not supported by the findings, as there was no significant change in serum vitamin D levels in the PBDE-treated rats.

In contrast to our hypothesis, the measured serum metabolites of vitamin D did not show any difference between the control and treatment groups. A drop in the 4β-dihydroxyD_3 and especially the 24, 25-dihydroxyD_3 was expected in the treatment rats due to the induction and up-regulation of the CYP3A, which causes the formation of these metabolites. Instead, the 1, 25-dihydroxyD_3 active form of vitamin D was slightly decreased by 13.4% in the treatment rats indicating that the active hormone form of the vitamin was being used for unknown purposes in the treatment rats. Taking the active and inactive metabolites for each rat and putting them into a ratio over the storage form of the vitamin 25-dihydroxyD3 explored a possible mechanism for the explanation of serum metabolites findings. The 4β, 25-dihydroyxD_3 inactive metabolite measured in the control rats came from the 25-dihydroxyD_3 more than the rats treated with flame retardant. The active form tended to originate from the 25-hydroxyD_3 more in the control group than treatment. It is possible that the difference between the two groups can be accounted for with another inactive metabolite 1, 24, 25-trihydroxyD_3 that is produced by the enzyme CYP24 in the kidney and was not measured in this study.

In agreement with the findings of Nash et al., the CYP3A enzyme was significantly induced by the presence of the DE-71 flame-retardant. This indicates that there was a greater amount of CYP3A present in the microsome samples of the treatment

group due to transcriptional induction. However, although it is up regulated, no conclusion can be made as to the activity of this enzyme in deactivating vitamin D. The CYP3A enzyme itself has many isomers in humans as well as rats, which implies that the CYP3A enzyme responsible for the inactivation of vitamin D may not be the enzyme analyzed and assayed in this experiment.[24] Thus, multiple enzyme assays on different isomers and the possibly an assay on the CYP24 enzyme shown to inactivate the 1, 25 dihydroxyD_3 into the metabolite not measured in this study (1, 24, 25-trihydroxyD_3) would need to be executed to validate the difference between rat and human enzyme isomers.

Although serum levels of vitamin D did not decrease in PBDE-treated rats, the physiological effects of PBDEs were evident in the enlarged livers and stunted growth of the treatment rats. When comparing the liver as a percentage of the final body weight, the rats treated with PBDEs have livers that make up a significantly larger portion of their body weight. Previously in our laboratory, a summer research project using the same model rat and a higher dosage of flame-retardant (14 mg DE-71/mL) did not show a difference in liver or body weights of the treated animals. This indicates that the combination of PBDE treatment along with an induced marginal deficiency in vitamin D has repercussions on liver size and body weight. An extended study would need to be performed in order to gain any significant validation on the growth and development of marginally deficient rats treated with PBDEs.

The HPLC results indicate that there was no production of the expected inactive 24, 25-dihydroxyD_3 metabolite. Although the peak appears in the standard curve, it is possible that amounts of this metabolite in the samples were under the detection level and thus no peak was seen. Since the positive controls of the samples containing no microsomes degraded over the course of the experiment, the data collected from the

samples is not considered reliable and the procedure will need further modification to adjust for the degradation of total 25(OH)D_3 in the vials.

This experiment is the first to examine low PBDE level exposure in addition to a marginal diet of vitamin D. Extending the findings to fully determine the composition of the enlarged treatment livers and a possible connection to Non-Alcoholic Fatty Liver Disease (NAFLD) could have important implications to low vitamin D levels. Further experimentation is needed to expand our understanding of the in vivo mechanisms of the CYP3A and possibly CYP24 isomers between humans and rats.

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