Effect of feeding 25-hydroxyvitamin D₃ with a negative cation-anion difference diet on calcium and vitamin D status of periparturient cows and their calves

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ABSTRACT

Holstein cows (>1 gestation) were fed 1 of 3 diets during the last 13 d of gestation (ranged from 22 to 7 d). The control diet (16 cows) was formulated to provide 18,000 IU/d of vitamin D₃ and had a dietary cation-anion difference (DCAD) of 165 mEq/kg (DCAD = Na + K – Cl – S). The second diet (DCAD + D) provided the same amount of vitamin D₃ but had a DCAD of –139 mEq/kg (17 cows). The third diet (DCAD + 25D) had no supplemental vitamin D₃ but provided 6 mg/d of 25-(OH) vitamin D₃ [25-(OH)D₃] with a DCAD of –138 mEq/kg (20 cows). Diets were fed until parturition and then all cows were fed a common lactation diet that contained vitamin D₃. Negative DCAD diets reduced urine pH, with the greatest decrease occurring with the DCAD + D treatment. Urinary Ca excretion was greatest for cows fed DCAD + 25D followed by cows fed DCAD + D. Urinary pH was negatively correlated with urinary excretion of Ca for cows fed DCAD + D. No such correlation was observed with the DCAD + 25D treatment because substantial excretion of urinary Ca occurred at moderate urinary pH values for that treatment. Cows fed DCAD + 25D had greater serum concentrations of 25-(OH)D₃ than other treatments from 5 d after supplementation started through 7 d in milk. Concentrations of 1,25-(OH)₂D₃ in serum were greatest in DCAD + 25D cows starting at 2 d before calving and continued through 7 d in milk. Serum Ca concentrations 5 d before calving were greatest for cows fed DCAD + 25D, but at other time points before and after parturition treatment did not affect serum Ca. Incidence of clinical hypocalcemia was not statistically different between treatments, but cows fed DCAD + 25 had the highest incidence rate (12.5, 0, and 20% for control, DCAD + D, and DCAD + 25D). Calves born from cows fed DCAD + 25D had greater concentrations

of 25-(OH)D₃ in serum at birth than calves from other treatments (before colostrum consumption), but concentrations were similar by 3 d of age. Concentrations of 25-(OH)D₃ in colostrum and transition milk were increased by feeding DCAD + 25D, but by 28 d in milk treatment effects no longer existed. Overall, feeding 25-OH vitamin D with a negative DCAD diet increased vitamin D status of the cow and her newborn calf but had minimal effects on calcium status and did not have positive effects on the incidence of hypocalcemia.

Key words: vitamin D, hypocalcemia, dietary cation-anion difference

INTRODUCTION

Peripartum hypocalcemia is common in dairy cows (Reinhardt et al., 2011), is a risk factor for numerous health disorders, and is associated with lower milk yields (Curtis et al., 1985; Gröhn et al., 1990; Massey et al., 1993; Chapinal et al., 2011; Chapinal et al., 2012; Chamberlin et al., 2013; Martinez et al., 2014). Feeding diets with a negative DCAD before partition is an established method of reducing clinical (Lean et al., 2006) and subclinical (Oetzel et al., 1988; Crnkic et al., 2010; Sakha et al., 2014) hypocalcemia.

The biologically active form of vitamin D [1,25-(OH)₂D₃] enhances Ca absorption by the intestine and in concert with parathyroid hormone (PTH) stimulates mobilization of Ca from bone (Horst et al., 1994). Dietary vitamin D₃ must first be hydroxylated to 25-(OH)D₃, which is then converted to 1,25-(OH)₂D₃. Concentrations of 25-(OH)D₃ are used to indicate vitamin D status, but a study with humans suggests that conversion of vitamin D₃ into 25-(OH)D₃ is reduced at high intakes of vitamin D₃ (Heaney et al., 2008). If this occurs in the bovine, higher concentrations of circulating 25-(OH)D₃ may be obtained with direct supplementation of 25-(OH)D₃ compared with supplementing vitamin D₃. Synthesis of 1,25-(OH)₂D₃ from 25-(OH)D₃ is regulated, but direct supplementation of 25-(OH)D₃ compared with vitamin D₃ may increase 1,25-(OH)₂D₃ concentrations to a greater extent. Intramuscular in-

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jections of 4 or 8 mg of 25-(OH)D₃ reduced clinical hypocalcemia in dairy cows, but timing of the injection before parturition was critical (Olson et al., 1973). Dietary supplementation of 25-(OH)D₃ would eliminate the injection timing issue. Supplementing peripartum dairy cows with 25-(OH)D₃ when fed an anionic diet increased plasma Ca concentrations in the peripartum period (Wilkens et al., 2012), but had no effect when supplemented with a diet containing a positive DCAD (Taylor et al., 2008). Anionic diets increase the conversion of 25-(OH)D₃ to 1,25(OH)₂D₃ (Goff et al., 1991); however, in the Wilkens et al. (2012) study, only a mild compensated acidosis was achieved based on urine pH. We hypothesized that supplementing 25-(OH)D₃ to cows fed an anionic diet that induced a substantial metabolic acidosis would enhance Ca status of peripartum cows and reduce the prevalence of clinical and subclinical hypocalcemia. In addition, supplementing 25-OHD₃ should increase concentrations of 25-(OH)D₃ and 1,25(OH)₂D₃ in plasma and colostrum, which may improve vitamin D status of the young calf.

MATERIALS AND METHODS

Cows and Diets

Dietary treatments and all procedures conducted on the cows in the experiment were approved by The Ohio State University Institutional Animal Care and Use Committee. Fifty-four dry Holstein cows (>1 gestation) were blocked into groups of 3 based on gestation number and anticipated calving date. While in the dry cow pen, all cows were fed a common diet that was formulated to meet NRC (2001) requirements, including vitamin D (18,000 IU of supplemental vitamin D₃/d). Cows were moved from the dry cow pen into individual boxstalls 10 d before anticipated calving and fed 1 of 3 prepartum diets (Tables 1 and 2). Treatments were (1) control (supplemental vitamin D₃), (2) supplemental vitamin D₃ with supplemental anions (**DCAD + D**), and (3) 6 mg/d of supplemental 25-(OH)D₃ (Rovimix, Hy-D, 1.25%, DSM Nutritional Products, Basel, Switzerland) plus supplemental anions (**DCAD + 25D**). The DCAD (Na + K - Cl - S) was 165 mEq/kg for control and -138 and -139 mEq/kg for the DCAD + D and DCAD + 25D, respectively. Average duration of the prepartum experimental period was 13 d (Table 3).

During the prepartum phase, all cows were fed 3.5 kg of concentrate DM (contained treatments) and 4.7 kg of a mix of 30:30:40 corn:grass:alfalfa silages DM daily. To ensure consumption of the concentrate mix, the concentrate and silage blend were mixed and offered to the cows at approximately 0800 h. A weighed amount of hay was offered at approximately 1100 h. Additional

hay (measured) was offered at approximately 1600 h based on appetite. Daily feed refusal was measured to calculate daily DMI during the prepartum period. After calving, cows remained in the boxstalls for 3 d and then moved into individual tiestalls and fed a common lactation diet until 28 DIM that contained supplemental vitamin D₃ but no supplemental anions or 25-(OH)D₃. Cows were weighed when moved into the boxstalls and when moved to the tiestalls. Daily milk yields and DMI were measured when cows were in tiestalls. Health treatments were at the discretion of the herd manager and veterinarian. Calcium (i.v., oral gels, or oral bolus) was given when deemed necessary and a blood sample was taken immediately before treatment when practicable (in 2 cases blood was not sampled before Ca administration).

Calves were removed from their dam immediately after birth and not allowed to consume colostrum. The cow was milked and 2 L of fresh colostrum was fed to their calf and another 2 L was fed 12 h later. Calves were fed only colostrum or transition milk from their dams through 6 feedings (2 L/feeding). One calf from a cow fed DCAD + 25D was fed frozen colostrum from another cow fed DCAD + 25D because her dam did not produce sufficient colostrum.

Sampling and Analyses

On average, cows calved 3 d later than anticipated; therefore, the first day of the experiment was designated -13 d. Urine was collected from cows via vulva stimulation on -13 d (before treatment diets were fed), after cows were fed treatment diets for 5 d (averaged 8 d before actual calving), and at 7 DIM. Urine pH was measured immediately using a portable pH meter (Oakton pHTestr 10, Oakton Instruments, Vernon Hills, IL). A subsample of urine was frozen and analyzed later for minerals by inductively coupled plasma spectrometry after microwave digestion and creatinine (Creatinine Urinary Assay Kit No. 500701, Cayman Chemical Co., Ann Arbor, MI). Blood was sampled from the tail vein of cows when moved to the boxstalls, 5 d after diets were initiated and at -2 d before anticipated calving. If cows did not calve on the anticipated date, blood was sampled repeatedly until calving. The blood samples closest to 5 and 2 d before actual calving were used for analysis. Blood was also sampled within 6 h after parturition and at 2 and 7 DIM. Blood was allowed to clot and serum was harvested and frozen in multiple vials until analyzed for Ca (Calcium Liquicolor No. 0150, Stanbio Laboratory, Boerne, TX), P (Phosphorus Liqui-UV No. 0830, Stanbio), Mg (Magnesium Liquicolor No. 0130, Stanbio), BHBA (β-Hydroxybutyrate Liquicolor No. 2440, Stanbio), NEFA [NEFA-HR(2), Wako Chemicals,

Table 1. Composition of prefresh diets and the common lactation diet (% of DM)

Item	Prepartum diet ¹			Lactation
	Control	DCAD + D	DCAD + 25D	
Mature grass hay	37.7	36.7	36.9	—
Midmaturity grass silage	10.4	10.6	10.5	—
Corn silage	10.2	10.6	10.4	30.0
Alfalfa silage	14.4	14.8	14.8	22.0
Corn grain	15.90	16.00	16.10	18.16
Soybean meal	5.62	—	—	8.2
Soybean hulls	3.439	2.379	2.45	5.3
Animal-vegetable fat	0.27	0.27	0.27	0.9
Bio-chlor ²	—	6.35	6.29	—
Magnesium sulfate	0.14	0.14	0.14	—
Calcium chloride	—	0.71	0.67	—
Limestone	0.68	0.24	0.27	1.25
Prepartum supplement ³	1.21	1.21	1.21	—
Lactation supplement ⁴	—	—	—	14.14
Vitamin D premix ⁵	0.051	0.051	—	—
25-(OH)D Premix ⁶	—	—	0.0037	—

¹The control diet contained vitamin D₃ and had a DCAD of 165 mEq/kg. The DCAD + D diet contained vitamin D₃ and DCAD = -139 mEq/kg. The DCAD + 25D diet contained 25-(OH)D₃ and DCAD = -138 mEq/kg.

²Arm & Hammer Animal Nutrition (Church & Dwight Co. Inc., Princeton, NJ).

³Contained 182 g of NaCl, 116 g of MgO, 820 mg of Cu (CuSO₄), 1,520 mg of Zn (ZnSO₄), 30 mg of Se (Na₂SeO₄), 730 kIU of vitamin A, 8,900 IU of vitamin E (Rovimix E-50, DSM Nutritional Products, LLC, Parsippany, NJ), 140 mg of biotin (Rovimix, DSM Inc.), and 1.7 g of monensin (Elanco Animal Health, Inc., Indianapolis IN) per kilogram.

⁴Contained 168 g of soybean meal, 791 g of dried distillers grains, 34 g of NaCl, 148 mg of Cu (CuSO₄), 184 mg of Zn (from Zn-met, Zinpro Inc., Eden Prairie, MN), 50 mg of Se (Na₂SeO₄), 560 kIU of vitamin A, 205 kIU of vitamin D, 3,260 IU of vitamin E, and 125 mg of biotin/kg.

⁵Contained 3,000 IU of vitamin D/g (as vitamin D₃). Formulated to provide 18,000 IU/d.

⁶Rovimix HyD-1.25% (DSM Inc.) containing 12.5 g of 25-(OH)D₃/kg of premix. The diet was formulated to provide 6 mg of 25-(OH)D₃/d.

Richmond, VA], and PTH (Bovine Intact PTH ELISA Kit, Immutopics Inc., San Clemente, CA) using 96-well plates and a Bio-tek Microplate Reader (Bio-tek Instruments, Winooski, VT) with KC4 software. Vitamin

D compounds in serum were assayed using RIA (Hollis et al., 1993; Hollis et al., 1996).

First-milking colostrum, sixth-milking transition milk, and milk on 28 DIM was sampled and frozen until

Table 2. Nutrient composition of diets (DM basis)

Item	Prepartum diet ¹			Lactation
	Control	DCAD + D	DCAD + 25D	
CP, %	14.6	14.5	14.5	17.6
NDF, %	41.8	41.8	41.6	30.0
Ca, %	0.88	1.00	0.90	1.28
P, %	0.30	0.32	0.32	0.36
Mg, %	0.31	0.36	0.37	0.25
K, %	2.00	1.92	1.91	1.42
Na, %	0.13	0.32	0.33	0.43
S, %	0.24	0.36	0.37	0.28
Cl, %	0.89	1.91	1.89	0.51
Cu, mg/kg	15	16	14	16
Mn, mg/kg	64	81	73	52
Zn, mg/kg	60	73	96	65
DCAD, ² mEq/kg	165	-139	-138	230

¹The control diet contained vitamin D₃ and no supplemental anions. The DCAD + D treatment contained vitamin D₃ and supplemental anions. The DCAD + 25D diet contained 25-(OH)D₃ and supplemental anions.

²Calculated as (K + Na) - (S + Cl) expressed as milliequivalents per kilogram of diet DM.

Table 3. Effect of treatment on production measures

Item	Treatment ¹			SEM	P-value ²	
	Control	DCAD + D	DCAD + 25D		DCAD	VD
Prepartum period (cows fed different treatments)						
Days fed treatment	12.4	12.9	13.8	1.03	<0.73	<0.51
Precalving BW, kg	765	743	750	14.0	<0.27	<0.71
Calf birth weight ³ , kg	44.6	44.5	45.2	1.44	<0.97	<0.73
DMI (−10 to −1 d), kg/d	12.2	11.6	11.6	0.43	<0.40	<0.99
Postpartum period (all cows fed a common diet)						
Postcalving BW, kg	697	682	678	13.7	<0.44	<0.82
DMI (3 to 28 d), kg/d	20.4	19.8	19.8	0.84	<0.63	<0.99
Milk (3 to 28 d), kg/d	45.2	41.7	41.7	1.54	<0.11	<0.99

¹During the prepartum period, control diet contained vitamin D₃ and no supplemental anions. The DCAD + D treatment contained vitamin D₃ and supplemental anions. The DCAD + 25D diet contained 25-(OH)D₃ and supplemental anions. Diets were fed starting 10 d before anticipated calving until calving. A common diet was fed after parturition to all cows.

²DCAD = effect of feeding supplemental anions with vitamin D (control vs. DCAD + D); VD = effect of type of supplemental vitamin D (DCAD + D vs. DCAD + 25D).

³Sex of calf affected BW (male > female), but a treatment × sex interaction was not observed ($P > 0.75$).

analyzed for cholecalciferol (vitamin D₃) and 25-(OH)D₃ by Analytical Research Center (DSM Nutritional Products, Ltd.). A blood sample was taken from the jugular vein of all calves immediately after birth (before colostrum was consumed) and again after the calf was fed 6 feedings of colostrum or milk from their dam. Serum was harvested and samples were frozen until assayed for vitamin D metabolites and Ca as described for cow serum.

Feeds were sampled weekly and composited by month. The composited silage samples were lyophilized and ground through a 1-mm screen (Wiley mill, Arthur H. Thomas, Philadelphia, PA). Feed samples were analyzed for DM (100°C for 24 h), ash (600°C muffle oven overnight), NDF (Ankom Fiber Analyzer, Ankom Technology, Fairport, NY) with sodium sulfite and amylase (Ankom #FAA), CP (Kjeldahl N × 6.25), minerals (Ca, P, Mg, K, Na, Cu, Mn, and Zn) by inductively coupled plasma spectrometry after nitric-perchloric acid digestion (concentrates) or oven-ashing (forages), Cl (potentiometric titration, Brinkman Metrohm Titrino 848 Plus, Brinkman Instruments, Westbury, NY), and S (Leco S-144DR Sulfur Combustion Analyzer, Leco Corp., St. Joseph, MI).

Statistical Analyses

The PTH data had an extremely skewed distribution and the transformation that resulted in the most normal distribution was $1/\sqrt{\text{PTH}}$. The data were back-transformed after statistical analyses. Cow and calf serum measures, DMI (standardized to include only the last 10 d of gestation), milk yield, and concentrations of vitamin D metabolites were analyzed using a model (PROC MIXED; SAS Institute, 2011) that included

block (random), day (repeated measure), treatment, and treatment × day interaction. The SLICE option was used when treatment × day interaction was significant ($P < 0.10$). Serum (cow and calf), milk, and urine data were also analyzed within each time point using a model that included the main effect of treatment and the random effect of cow. Two contrasts were used to partition treatment effects within day: effect of DCAD with supplemental vitamin D (control vs. DCAD + D) and effect of feeding 25-(OH)D₃ in a DCAD diet (DCAD + D vs. DCAD + 25D). Almost all the colostrum and milk samples from cows on the DCAD + 25D treatment and all samples of milk at 28 DIM were below the quantification limit (<60 ng/mL) for cholecalciferol. Therefore, vitamin D concentrations in colostrum and transition milk were statistically evaluated using the repeated model, except only control and DCAD + D treatments were used. Relationships among various measures were evaluated using regression (PROC REG of SAS). Incidence of hypocalcemia (subclinical, defined as blood calcium ≤ 8 mg/dL with no clinical signs and clinical defined animals treated with i.v. Ca) was evaluated using PROC FREQ with Fisher's exact test (SAS Institute, 2011). A likelihood-ratio test was used to assess treatment differences in the variation in serum Ca concentrations using the GROUP option of the MIXED procedure.

RESULTS AND DISCUSSION

The number of cows that calved per treatment was 16 for control, 17 for DCAD + D, and 20 for DCAD + 25D. The original protocol called for 18/treatment; however, to ensure adequate numbers of animals for the primary treatment of interest (DCAD + 25D), 3

additional cows were enrolled in the DCAD + 25D treatment. This was because 1 cow fed DCAD + 25D died 2 d after calving and another cow fed DCAD + 25D developed severe hypocalcemia approximately 24 h before calving and Ca therapy had to be administered (no d-0 blood sample was taken). The number of cows that completed the experiment was 15 for control, 16 for DCAD + D, and 19 for DCAD + 25D. One control cow was euthanized on 17 DIM because of a severe injury that occurred when the cow fell. One cow on the DCAD + D treatment was euthanized at 20 DIM after developing an unresponsive abdominal infection following surgery for a displaced abomasum. One cow on DCAD + 25D was found dead in the boxstall at 2 DIM. No health issues were identified and the cow received no veterinary treatments before dying. However, when the d-0 blood sample was analyzed, the cow was classified as subclinical hypocalcemic (6.34 mg/dL). A necropsy was conducted but the cause of death could not be determined.

Treatment did not affect DMI prepartum and calculated average intake of 25-(OH)D₃ was 5.4 mg/d and intakes of supplemental vitamin D for control and DCAD + D treatments were 18,700 and 17,700 IU/d, respectively (Table 3). A meta-analysis (Charbonneau et al., 2006) found a negative relationship between dietary DCAD and DMI; however, DMI of multiparous cows was not affected by negative DCAD diets in several individual studies (Moore et al., 2000; Grünberg et al., 2011; Wilkens et al., 2012). Milk yield and DMI in the first 28 d of lactation were not affected by treatment (Table 3).

Urine Measures

Feeding DCAD diets reduced ($P < 0.01$) urine pH measured 5 d after supplementation began and cows fed DCAD + 25D tended ($P < 0.10$) to have higher urinary pH than cows fed DCAD + D (Table 4). Including 25-(OH)D₃ in diets with a negative DCAD compared with similar diets without 25-(OH)D₃ reduced urine pH in one study (Gibbens, 2012), but not in another (Wilkens et al., 2012). The increased urinary pH for cows fed DCAD + 25D could be caused by the increased Ca concentration in their urine (Constable et al., 2009). The greatly increased urinary Ca excretion when DCAD + D or DCAD + 25D were fed (Table 4) indicates increased Ca flux in those cows. Inducing metabolic acidosis by feeding negative DCAD diets increases bone resorption and reduces bone accretion. Approximately 45% of Ca in the negative DCAD diets was provided by CaCl₂ (no CaCl₂ was included in the control diet), and because the Ca from CaCl₂ is highly available (Goff and Horst, 1993) it may have increased Ca absorption

by cows on those diets. Increasing serum concentrations of 1,25-(OH)₂D also increases Ca absorption from the gut and bone resorption. Excluding cows fed DCAD + 25D, urinary Ca or creatinine was negatively correlated with urinary pH (Figure 1; $r = -0.76$; $P < 0.01$), as reported previously (Grünberg et al., 2011). However, for cows fed DCAD + 25D, urinary pH was not correlated with Ca or creatinine ($P > 0.25$) and urinary excretion of Ca was high even when metabolic acidosis was not extreme. This suggests that for those cows Ca flux was mostly influenced by vitamin D, rather than metabolic acidosis. Assuming a constant urinary excretion of creatinine of 29 mg/kg of BW (Valadares et al., 1999), cows fed DCAD + 25D excreted about 1.7 g more Ca/d via urine than did cows fed DCAD + D (approximately 8 g/d more than control cows). Urine pH or urinary Ca excretion (measured at -5 d) were not correlated ($P > 0.3$) with serum Ca concentrations on -2 d, calving, or 2 DIM, and no apparent relationship between urine measures and prevalence of subclinical or clinical hypocalcemia were observed (discussed herein). By 7 DIM, urinary Ca excretion and urine pH did not differ among treatments (Table 4).

Serum Vitamin D Metabolites and PTH

Because of the high cow-to-cow variation, reaching statistically valid conclusions for the PTH data was difficult. Concentrations of PTH followed the same time profile for all treatments (time $\times$ treatment interaction; $P > 0.6$), with concentrations increasing until calving and then decreasing (Table 5). The time profile was similar to previous studies (Shappell et al., 1987; Wilkens et al., 2012). When all time points were included, cows fed either of the DCAD diets tended ($P < 0.13$) to have lower PTH concentrations than cows fed the control. No differences were observed between the 2 DCAD treatments. Wilkens et al. (2012) reported lower PTH concentrations in cows fed negative DCAD diets compared with those fed positive DCAD, with no effect of feeding 25-(OH)D₃. In Wilkens et al. (2012), treatment mean PTH concentrations were inversely related to plasma Ca concentrations as would be expected, but treatment differences in PTH concentrations were much greater than treatment differences in plasma Ca. We also observed very large numeric differences in PTH between control and cows fed DCAD (with or without 25-OH vitamin D) and only small and usually nonsignificant differences in serum Ca. One function of PTH is to induce activity of renal 1- α -hydroxylase, resulting in increased conversion of 25-(OH)D₃ to 1,25-(OH)₂D₃. Goff et al. (2014) showed that plasma 1,25-(OH)₂D₃ response to PTH depended on whether cows were fed a negative or positive DCAD diet; cows

Figure 1. Relationship between urine pH and excretion of urinary Ca measured 5 d after start of treatments. The regression line includes data from cows fed control and DCAD + vitamin D (DCAD + D) diets ($Y = 1.03 - 0.117X$; $r^2 = 0.58$). The Ca-to-creatinine ratio was not correlated ($P > 0.25$) to urine pH for cows fed the DCAD + 25 (OH) D_3 diet (DCAD + 25D). Color version available online.

fed negative DCAD had much greater increase in plasma 1,25-(OH) $_2D_3$ following PTH administration. Our data generally support the idea that high DCAD reduces tissue sensitivity to PTH in that cows fed control had much higher PTH concentrations but similar serum 1,25-(OH) $_2D_3$ concentrations, ratios of serum 1,25-(OH) $_2D_3$ to 25-(OH) D_3 , and Ca concentrations as cows fed the DCAD + D diet.

Concentrations of 25-(OH) D_3 in serum were affected ($P < 0.001$) by treatment, sampling day, and the day by treatment interaction (Table 5). Time did not affect concentrations of 25-(OH) D_3 of cows fed control or DCAD + D, but for cows fed DCAD + 25D concentrations of 25-(OH) D_3 increased until about -2 DIM and were then maintained through calving and started to decline by 7 DIM. At calving, concentrations of 25-

Table 4. Effect of treatment on urine measures¹

Item	Treatment ²			SEM	P-value ³	
	Control	DCAD + D	DCAD + 25D		DCAD	VD
pH						
-13 d	8.58	8.59	8.58	0.037	<0.91	<0.91
-5 d	8.54	6.23	6.79	0.231	<0.001	<0.09
7 DIM	8.36	8.31	8.14	0.096	<0.76	<0.21
Ca, mg/L						
-13 d	20.7	19.1	32.2	5.3	<0.82	<0.08
-5 d	18.9	342.1	437.9	42.9	<0.001	<0.11
7 DIM	53.3	62.3	105.6	30.3	<0.83	<0.30
Creatinine, mg/L						
-13 d	1162.3	1054.0	1262.4	80.2	<0.35	<0.07
-5 d	1142.3	1073.2	1104.3	89.5	<0.60	<0.80
7 DIM	805.5	796.7	705.2	68.7	<0.93	<0.33
Ca/Creatinine						
-13 d	0.018	0.019	0.026	0.039	<0.94	<0.21
-5 d	0.013	0.319	0.443	0.043	<0.001	<0.04
7 DIM	0.061	0.078	0.127	0.029	<0.70	<0.23

¹At -13 d and 7 DIM cows were fed a common diet within day. The -5-d sample was taken 5 d after treatments started.

²Control diet contained vitamin D_3 and no supplemental anions; DCAD + D diet contained vitamin D_3 and supplemental anions; DCAD + 25D diet contained 25-(OH) D_3 and supplemental anions.

³DCAD = effect of feeding supplemental anions (control vs. DCAD + D); VD = effect of type of supplemental vitamin D (DCAD + D vs. DCAD + 25D).

Table 5. Effect of prepartum diet on serum concentrations of parathyroid hormone (PTH) and vitamin D metabolites in cows¹

Item	Treatment ²			SEM	P-value ³	
	Control	DCAD + D	DCAD + 25D		DCAD	VD
PTH, ⁴ pEq/mL						
–13 DIM	NA	NA	NA	—	—	—
–5 DIM	962	378	625	211	<0.12	<0.32
–2 DIM	NA	NA	NA	—	—	—
Calving	1,568	808	961	441	<0.19	<0.76
2 DIM	1,309	518	883	336	<0.08	<0.22
7 DIM	793	512	635	158	<0.38	<0.62
25-(OH)D ₃ , ng/mL						
–13 DIM	75.5	76.9	72.1	4.3	<0.82	<0.43
–5 DIM	75.7	71.0	173.0	7.1	<0.66	<0.001
–2 DIM	91.1	75.8	270.0	12.4	<0.42	<0.001
Calving	82.1	65.8	274.3	13.1	<0.39	<0.001
2 DIM	74.1	66.9	261.0	10.6	<0.65	<0.001
7 DIM	74.9	59.6	249.5	13.4	<0.43	<0.001
1,25 (OH) ₂ D, pg/mL						
–13 DIM	98.5	94.6	87.9	14.3	<0.85	<0.74
–5 DIM	111.5	104.6	122.9	15.9	<0.77	<0.41
–2 DIM	133.7	98.9	194.1	25.7	<0.37	<0.02
Calving	166.2	151.5	225.3	24.2	<0.67	<0.04
2 DIM	205.2	195.4	320.8	32.6	<0.84	<0.01
7 DIM	125.6	101.2	161.7	17.6	<0.33	<0.02

¹At –13, 2, and 7 DIM all cows were fed a common diet within day. Time and time × treatment effects were significant ($P < 0.01$) for all variables. NA = not analyzed.

²Control diet contained vitamin D₃ and no supplemental anions; DCAD + D treatment contained vitamin D₃ and supplemental anions; DCAD + 25 D diet contained 25-(OH)D₃ and supplemental anions.

³P-values for contrasts: DCAD = effect of feeding supplemental anions (control vs. DCAD + D); VD = effect of type of supplemental vitamin D (DCAD + D vs. DCAD + 25D).

⁴Data were transformed as 1/(SQRT) before statistical analysis and then back transformed.

(OH)D₃ were more than 3 times greater in cows fed DCAD + 25D than for cows fed the other diets. Similar to 25-(OH)D₃, serum concentrations of 1,25-(OH)₂D₃ were affected ($P < 0.001$) by treatment, sampling day, and the day × treatment interaction (Table 5). Time affected concentrations of 1,25-(OH)₂D₃ for all treatments ($P < 0.01$), with peak concentrations at 2 DIM. Feeding DCAD did not affect 1,25-(OH)₂D₃, but starting at –2 DIM through 7 DIM cows fed DCAD + 25D had greater ($P < 0.05$) concentrations than did cows fed DCAD + D. Whereas concentrations of 25-(OH)D₃ were elevated by –5 DIM, 1,25-(OH)₂D₃ was not elevated until –2 DIM, and rather than a 3 to 4 times increase in concentrations as observed with 25-(OH)D₃, concentrations of 1,25-(OH)₂D₃ were about 1.5 times greater for DCAD + 25D than for cows fed DCAD + D.

The number of days individual cows were fed 25-(OH)D₃ ranged from 7 to 24 d and concentrations of 1,25-(OH)₂D₃ at –2 d ($r = 0.84$) and at calving ($r = 0.84$) were positively correlated ($P < 0.001$) with number of days they were fed 25-(OH)D₃ before the blood sample was taken. Serum concentrations of 25-(OH)D₃ were not correlated with duration of feeding ($P > 0.8$).

Although temporal patterns for serum 25-(OH)D₃ were similar to previous studies (Gibbens, 2012; Wilkens et al., 2012), peak concentrations in cows fed 25-(OH)D₃ were greater in our study, likely because 3 mg/d of 25-(OH)D₃ was fed by Wilkens et al. (2012) and Gibbens (2012) whereas cows in our study consumed about 6 mg/d. The temporal pattern for 1,25-(OH)₂D₃ in our study differed from previous studies. In both Wilkens et al. (2012) and Gibbens (2012), feeding 25-(OH)D₃ did not affect concentrations of 1,25-(OH)₂D₃ until after parturition, but in our study concentrations of 1,25-(OH)₂D₃ were higher ($P < 0.01$) for cows fed DCAD + 25D at –2 d and at parturition than for the other treatments. The differences between studies may be a result of the differing supplementation rates (3 vs. 6 mg/d). Peak concentrations of serum 1,25-(OH)₂D₃ occurred at 2 DIM independent of treatment in agreement with previous studies (Barton et al., 1981; Gibbens 2012; Wilkens et al., 2012). Although the amount of 25-(OH)D₃ supplemented was greater in our study, peak concentrations of 1,25-(OH)₂D₃ were similar to those in Wilkens et al. (2012) and Gibbens (2012). The ratio of serum 1,25-(OH)₂D₃ to 25-(OH)D₃ was lower ($P < 0.01$) for cows fed DCAD + 25D

than for cows on other treatments at all time points after supplementation started (data not shown). The lower ratio of 1,25-(OH)₂D₃ to 25-(OH)D₃ for cows fed 25-(OH)D₃ may indicate that elevated concentrations of 1,25 downregulated 1- α -hydroxylase (Engstrom et al., 1987). High concentrations of 1,25-(OH)₂D₃ can also upregulate 24-hydroxylase, which would increase conversion of 25-(OH)D₃ into 1,24,25-(OH)₃ and 24,25(OH)₂D₃ (not measured) which is thought to have limited or no metabolic activity.

Considering the high 25-(OH)D₃ concentrations observed in the treated cows, one might think that 25-(OH)D₃ itself might be directly signaling Ca homeostasis via binding to vitamin D receptor (VDR). However, 25-(OH)D₃ is a poor ligand for VDR, with an approximate 1% cross reactivity with VDR compared with 1,25-(OH)₂D₃ when both are in their free state (Reinhardt et al., 1982). In addition, only about 0.03% of serum 25-(OH)D₃ is free for cell entry (Schwartz et al., 2014), and, based on that assumption, the concentration of free 25-(OH)D₃ in serum, even for cows fed DCAD + 25D, would not be a high enough to act as VDR ligand. Although it cannot be completely ruled out, the high serum 25-(OH)D₃ concentrations observed are unlikely directly activating VDR in this instance.

Serum Minerals and Energy Metabolites

Treatment had little effect on serum Mg, but did influence serum Ca and P concentrations (Table 6). Cows fed diets with negative DCAD prepartum often have greater concentrations of serum Ca than cows fed diets with positive DCAD (Block, 1984; Charbonneau et al., 2006; DeGroot et al., 2010; Grünberg et al., 2011), but no effect was observed in our experiment. Average serum Ca concentrations measured for control cows were in the normal range at all time points, which likely reduced potential responses to supplemental anions. Cows fed DCAD + 25D had greater ($P < 0.01$) concentrations of serum Ca than cows fed DCAD + D at -5 d, but no differences were observed at other time points. Wilkens et al. (2012) and Gibbens (2012) reported that feeding a negative DCAD diet with 3 mg/d of 25-(OH)D₃ increased serum Ca concentrations at parturition and for up to 24 h postpartum, but we observed no effect of treatment on postpartum Ca concentrations. Perhaps the higher daily dose of 25-(OH)D₃ (6 mg/d) used in our study increased concentrations of 1,25-(OH)₂D₃ for a long enough period of time and to a great enough extent to cause a self-induced reduction in tissue responsiveness to the hormone (Reinhardt and

Table 6. Effect of treatment on serum minerals (mg/dL) in cows¹

Item	Treatment ²			SEM	P-value ³	
	Control	DCAD + D	DCAD + 25D		DCAD	VD
Ca						
-13 DIM	9.52	9.68	9.56	0.15	<0.47	<0.56
-5 DIM	9.18	9.27	9.76	0.13	<0.62	<0.01
-2 DIM	9.11	9.30	9.64	0.24	<0.56	<0.35
Calving	8.07	8.24	8.23	0.36	<0.74	<0.98
2 DIM	8.14	8.76	8.50	0.33	<0.22	<0.58
7 DIM	9.49	9.18	9.32	0.19	<0.25	<0.57
P (inorganic)						
-13 DIM	6.13	6.34	6.05	0.12	<0.22	<0.08
-5 DIM	6.78	6.38	7.48	0.20	<0.19	<0.001
-2 DIM	6.40	6.98	8.10	0.28	<0.16	<0.01
Calving	4.60	4.41	5.16	0.30	<0.66	<0.08
2 DIM	5.15	6.12	6.02	0.32	<0.05	<0.82
7 DIM	5.41	5.68	6.06	0.35	<0.60	<0.44
Mg						
-13 DIM	2.49	2.50	2.45	0.044	<0.80	<0.35
-5 DIM	2.46	2.29	2.18	0.049	<0.02	<0.11
-2 DIM	2.33	2.32	2.23	0.042	<0.87	<0.11
Calving	2.62	2.56	2.44	0.064	<0.48	<0.17
2 DIM	2.44	2.56	2.65	0.09	<0.35	<0.42
7 DIM	2.26	2.24	2.13	0.07	<0.81	<0.24

¹At -13, 2, and 7 DIM all cows were fed a common diet within day. Time and time $\times$ treatment effects were significant for Ca and P ($P < 0.01$).

²Control diet contained vitamin D₃ and no supplemental anions; DCAD + D diet contained vitamin D₃ and supplemental anions; DCAD + 25D diet contained 25-(OH)D₃ and supplemental anions.

³DCAD = effect of feeding supplemental anions (control vs. DCAD + D); VD = effect of type of supplemental vitamin D (DCAD + D vs. DCAD + 25D).

Figure 2. Concentrations of serum Ca at calving (<6 h postcalving) and at 2 DIM for cows fed control, DCAD + vitamin D (DCAD + D), and DCAD + 25-(OH) D_3 (DCAD + 25D). The shaded box indicates subclinical hypocalcemia. X = cows that had clinical milk fever and were treated with Ca and no blood sample was taken.

Horst, 1989), thereby eliminating the positive effects observed in previous studies.

Treatment did not affect mean concentrations of serum Ca at calving or at 2 DIM; however, treatment did affect variation among cows in serum Ca concentrations. Serum Ca concentrations were more variable for cows fed DCAD + 25D at 2 DIM than cows fed the other 2 treatments ($P < 0.05$). At calving, serum Ca concentrations were more variable for cows fed DCAD + 25D than cows fed DCAD + D ($P < 0.05$), but variation was similar between cows fed DCAD + 25D and the control diet. Duration of 25-(OH) D_3 supplementation, serum concentrations of 1,25-(OH) $_2D_3$ and 25-(OH) D_3 , urine pH, and lactation number were not significant sources of variation in serum Ca concentrations within the DCAD + 25D treatment. The cause of this greater variation is unknown but may explain why an apparent increase was observed in the prevalence of clinical milk fever but a decrease in subclinical hypocalcemia was noted in cows fed DCAD + 25D compared with cows fed the control diet.

Subclinical hypocalcemia was defined as cows that had serum Ca concentrations ≤ 8 mg/dL without clinical signs (Reinhardt et al., 2011). A case of clinical milk fever occurred when cows required i.v. Ca therapy (therapy was initiated based on clinical observations, not low blood Ca). Treatment did not affect prevalence of subclinical or clinical hypocalcemia ($P > 0.3$). For control, DCAD + D, and DCAD + 25D, 50.0, 41.2,

and 35.0% of cows had subclinical hypocalcemia, and 12.5 (1 case confirmed by low blood Ca and no blood sample was taken from the other case), 0, and 20% of cows (3 cases confirmed by low blood Ca and no blood sample was taken for 1 case) had clinical milk fever, respectively. Subclinical hypocalcemia was identified in 22 (42%) cows sometime during the experiment (Figure 2). These rates are similar to prevalence rates observed in the field (Reinhardt et al., 2011).

Feeding DCAD + D did not affect serum P, except at 2 DIM when concentrations were higher ($P < 0.05$) than control (Table 6). Cows fed DCAD + 25D had elevated ($P < 0.08$) serum P concentrations compared with the DCAD + D treatment, similar to previous studies (Gibbens, 2012; Wilkens et al., 2012). Phosphorus absorption from the intestine is stimulated by 1,25-(OH) $_2D_3$, which could explain the elevated serum P concentrations.

Treatment did not affect concentrations of serum NEFA or BHBA (data not shown). Prepartum NEFA concentrations for all treatments were low (average = 208 μ Eq/L). Postpartum concentrations of NEFA ≥ 0.6 mEq/L are associated with increased disease risk (Ospina et al., 2010). Averages for all treatments and days (excluding the day of calving) were less than this threshold, except for cows fed DCAD + 25D on 2 DIM (0.78 mEq/L). This was likely caused by the greater number of cows with hypocalcemia (Reinhardt et al., 2011). At -2 ($r = -0.66$; $P < 0.001$) and 2 ($r = -0.45$;

Table 7. Effect of prefresh diet of dam on vitamin D metabolites in their milk and in serum from their calves¹

Item	Treatment ²			SEM	P-value ³	
	Control	DCAD + D	DCAD + 25D		DCAD	VD
Milk						
Vitamin D ₃ , pg/mL						
First milking colostrum	471	340	ND	55.1	0.09	—
Sixth milking	324	295	ND	75.4	0.77	—
25-(OH)D ₃ , pg/mL						
First milking colostrum	1,021	891	3690	163.2	0.72	0.001
Sixth milking	458	340	1088	167.7	0.64	0.001
Milk, 28 DIM	358	473	607	168.7	0.37	0.20
Serum of calves						
25-(OH)D ₃ , ng/mL						
Birth	45.7	42.3	80.2	44.4	0.51	0.001
6th feeding	37.1	35.5	57.6	44.3	0.68	0.001
1,25-(OH) ₂ D, pg/mL						
Birth	149.0	136.8	160.1	30.5	0.77	0.59
Sixth feeding	192.3	212.1	257.2	33.6	0.35	0.68
Calcium, mg/dL						
Birth	11.7	11.5	11.3	0.18	0.49	0.26
Sixth feeding	12.0	11.6	11.4	0.19	0.16	0.53

¹Birth samples were taken before consuming colostrum and sixth milking sample was taken within 2 h after calves consumed their sixth feeding of milk. Calves were only fed colostrum or milk from their dams. For milk measures and serum 25-(OH)D₃, time and time × treatment were significant ($P < 0.01$), but only time was significant for 1,25-(OH)₂D₃ ($P < 0.01$). Milk from cows fed DCAD + 25 D did not have measurable concentrations of vitamin D₃ and that treatment was excluded from the statistical analysis.

²Control diet contained vitamin D₃ and no supplemental anions; DCAD + D diet contained vitamin D₃ and supplemental anions; DCAD + 25D diet contained 25-(OH)D₃ and supplemental anions.

³DCAD = effect of feeding supplemental anions (control vs. DCAD + D); VD = effect of type of supplemental vitamin D (DCAD + D vs. DCAD + 25D).

$P < 0.01$) DIM, serum Ca and NEFA were negatively correlated. Subclinical ketosis was defined as BHBA concentrations $>1,200 \mu\text{M}$ (Duffield et al., 1998) and is a risk factor for several health disorders (Ospina et al., 2010). At 7 DIM, incidence of subclinical ketosis was 50, 35, and 20% of cows that were fed control, DCAD + D, and DCAD + 25D (DCAD + 25D $<$ control; $P < 0.06$).

Milk Vitamin D and Vitamin D Status of Calves

No treatment (only control and DCAD + D were included in this analysis), time (colostrum vs. sixth milking), or time × treatment interactions effects were observed for vitamin D concentration in milk (Table 7). The concentrations of 25-(OH)D₃ in colostrum and transition milk (i.e., sixth milking) were greater for cows fed DCAD + 25D ($P < 0.001$), but by 28 DIM concentrations were similar among treatments. Concentrations of 25-(OH)D₃ in colostrum and sixth milking milk were correlated ($P < 0.001$) with serum concentrations of 25-(OH)D₃ (Figure 3). The slope relating concentrations of 25-(OH)D₃ in serum and milk at 2 DIM was essentially the same as reported by Hollis et al. (1986) for humans; the slope relating 25-(OH)D₃ concentrations in serum (sampled at −2 DIM) to colostrum was much greater, indicating different mechanisms regulate concentra-

tions of 25-(OH)D₃ in colostrum compared with milk. For cows fed DCAD + 25D, concentrations of 25-(OH)D₃ in colostrum ($r = 0.60$; $P < 0.01$) and sixth milking milk ($r = 0.57$; $P < 0.01$) were positively correlated with number of days the supplement was fed. Duration of supplementation of 25-(OH)D₃ to the dam was also correlated ($r = 0.46$; $P < 0.05$) with concentration of 25-(OH)D₃ in serum of calves at birth.

Calves from cows fed DCAD + 25D had greater ($P < 0.01$) concentrations of 25-(OH)D₃ in serum at birth (before colostrum was fed) and after their sixth feeding (Table 7). Concentrations of 25-(OH)D₃ decreased ($P < 0.03$) with age for all treatments; whereas concentrations of 1,25-(OH)₂D₃ increased for all treatments (Table 7), but treatment had no effect on concentrations at either age. Rajaraman et al. (1997) reported similar changes with age when calves were fed milk from cows not supplemented with 25-(OH)D₃. Concentrations of serum 25-(OH)D₃ in calves at birth were correlated ($r = 0.79$; $P < 0.001$) with concentrations of 25-(OH)D₃ in the serum of their dams (Figure 4), similar with previous results (Goff et al., 1982). The optimal concentration of 25-(OH)D₃ in serum of calves with respect to immune function have not been determined, but probably range between 30 and 100 ng/mL (Nelson et al., 2012). Elevating serum 25-(OH)D₃ concentrations is generally associated with improved

Figure 3. (A) Relationship between 25-(OH)D₃ in serum of cows 2 d before calving and concentration of 25-(OH)D₃ in colostrum. The regression line includes all treatments ($Y = -0.085 + 0.0145X$; $r^2 = 0.74$). (B) Relationship between 25-(OH)D₃ in serum of cows and concentration of 25-(OH)D₃ in milk both sampled 2 d after calving. The regression line includes all treatments ($Y = 0.16 + 0.0036X$; $r^2 = 0.46$). DCAD + D = DCAD + vitamin D; DCAD + 25D = DCAD + 25-(OH)D₃. Color version available online.

immune function in vitro and in vivo in bovine and human models (Liu et al., 2006; Nelson et al., 2010). The greater concentrations of 25-(OH)D₃ in neonatal calves from cows fed DCAD + 25D may confer some measure of improved immune function.

CONCLUSIONS

Supplementing cows with 25-(OH)D₃ in combination with a negative DCAD diet for the last 13 d of gestation

enhanced vitamin D status of the cows based on serum concentrations of 25-(OH)D₃ and 1,25-(OH)₂D₃. This translated into increased concentrations of 25-(OH)D₃ in colostrum and milk and in the serum of the neonatal calf. A short-lived increase in serum Ca before calving occurred with this treatment, but no effect on serum Ca was observed at calving and in the early postpartum period. Although no statistical differences were observed in the incidence of clinical milk fever, feeding 6 mg/d of 25-(OH)D₃ resulted in the highest incidence

Figure 4. Relationship between concentrations of 25-(OH)D₃ in serum of cows and their calves at birth. Regression includes all treatments. $Y = 11.6 + 0.16X$ ($r^2 = 0.63$). DCAD + D = DCAD + vitamin D; DCAD + 25D = DCAD + 25-(OH)D₃. Color version available online.

rate and it did not reduce subclinical hypocalcemia over DCAD + D diets alone.

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