Zamorano University

Agricultural Science and Production

B.Sc. in Agricultural Sciences

Special Graduation Project

Effect of vitamin D source and amount on serum 25-hydroxyvitamin D levels in pregnant Holstein cows

Student

José Roberto Figueroa Erazo

Advisors

Marielena Moncada, Ph.D.

Corwin Nelson, Ph.D.

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Authorities

KEITH L. ANDREWS

President i.a.

ANA M. MAIER ACOSTA

Vice President and Academic Dean

CELIA O. TREJO RAMOS

Director Department of Agricultural Sciences and Production

JULIO NAVARRO

Secretary General

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Abstract

Vitamin D plays essential roles beyond calcium and bone metabolism, including regulation of immune function, reproduction, and mammary gland physiology. Its two primary forms, cholecalciferol (CHOL) and calcidiol (CAL) differ in origin and bioavailability. While CHOL is synthesized in the skin under ultraviolet B (UVB) exposure and converted to CAL in the liver, modern dairy systems limit sunlight exposure, increasing reliance on dietary supplementation. This study aimed to evaluate the effects of vitamin D source (CHOL vs. CAL) and supplementation amount (0.2 vs. 1.0 mg/day) on serum 25-hydroxyvitamin D 25(OH)D concentrations in pregnant Holstein cows. Twenty-four cows at approximately 63 days of gestation were blocked by parity and performance, assigned to a randomized complete block design with a 2 × 2 factorial arrangement of treatments, and analyzed using repeated measures over time. Treatments included: 0.2 mg CHOL, 1.0 mg CHOL, 0.2 mg CAL, and 1.0 mg CAL. Cows were housed indoors, individually fed a common TMR, and received daily top-dress supplementation for 56 days. Blood samples were collected on days 0, 28, and 56. Serum 25(OH)D concentrations were analyzed using a commercial ELISA kit. Data were analyzed using the MIXED procedure in SAS and least squares means (LSMeans) were used to assess treatment effects. A significant interaction between vitamin D source and amount ($P < 0.001$) was observed. Cows supplemented with 1.0 mg/day of CAL (HCAL) had the highest serum 25(OH)D_3 concentrations (110.31 ± 4.37 ng/mL), while increasing CHOL from 0.2 to 1.0 mg/day did not affect serum concentrations (76.91 ± 5.06 vs. 76.80 ± 4.74 ng/mL). These findings demonstrate that CAL is more effective than CHOL in raising circulating 25(OH)D concentrations.

Keywords: Calcidiol, cholecalciferol, serum, supplementation, vitamin D.

Resumen

La vitamina D desempeña funciones esenciales más allá del metabolismo del calcio y los huesos, incluyendo la regulación de la función inmunológica, la reproducción y la fisiología de la glándula mamaria. Sus dos formas principales, colecalciferol (CHOL) y calcidiol (CAL), difieren en su origen y biodisponibilidad. Mientras que el CHOL se sintetiza en la piel tras la exposición a rayos ultravioleta B (UVB) y se convierte en CAL en el hígado, los sistemas lecheros modernos limitan la exposición solar, lo que incrementa la dependencia de la suplementación dietética. Este estudio tuvo como objetivo evaluar los efectos de la fuente de vitamina D (CHOL vs. CAL) y la cantidad de suplementación (0.2 vs. 1.0 mg/día) sobre las concentraciones séricas de 25-hidroxivitamina D [25(OH)D] en vacas Holstein gestantes. Veinticuatro vacas con aproximadamente 63 días de gestación fueron bloqueadas por paridad y desempeño, asignadas a un diseño en bloques completamente al azar con un arreglo factorial 2 × 2 de tratamientos, y analizadas bajo medidas repetidas en el tiempo. Los tratamientos incluyeron: 0.2 mg CHOL, 1.0 mg CHOL, 0.2 mg CAL y 1.0 mg CAL. Las vacas fueron alojadas en interiores, alimentadas individualmente con una ración total mezclada (TMR) común y recibieron suplementación diaria en forma de top-dress durante 56 días. Se recolectaron muestras de sangre los días 0, 28 y 56. Las concentraciones séricas de 25(OH)D se analizaron mediante un kit ELISA comercial. Los datos se analizaron con el procedimiento MIXED de SAS y se utilizaron medias cuadradas mínimas (LSMeans) para evaluar los efectos del tratamiento. Se observó una interacción significativa entre la fuente y la cantidad de vitamina D ($P < 0.001$). Las vacas suplementadas con 1.0 mg/día de CAL (HCAL) presentaron las concentraciones más altas de 25(OH)D en suero (110.31 ± 4.37 ng/mL), mientras que el aumento de CHOL de 0.2 a 1.0 mg/día no afectó los niveles séricos (76.91 ± 5.06 vs. 76.80 ± 4.74 ng/mL). Estos hallazgos demuestran que el CAL es más eficaz que el CHOL para aumentar las concentraciones circulantes de 25(OH)D.

Palabras clave: Calcidiol, colecalciferol, concentraciones séricas, suplementación, vitamina D.

Introduction

Vitamin D is a fat-soluble vitamin with functions that extend beyond calcium metabolism and bone formation. In cattle, its active hormonal form is also involved in regulating immune responses, reproductive processes, and mammary gland physiology (Nelson and Merriman, 2014).

There are two main forms of vitamin D: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_2, derived from ergosterol in fungi, contributes a small but relevant portion of the total vitamin D intake in cattle (Nelson, 2023). Forages such as alfalfa hay may contain vitamin D_2, though in highly variable concentrations (ranging from 160 to 2,500 IU/kg DM), making them an inconsistent dietary source (Nelson et al., 2016). Moreover, vitamin D_2 is metabolized less efficiently than vitamin D_3, with less than one-third of the potency for increasing serum 25(OH)D concentrations (Norman, 2008; Sommerfeldt et al., 1983). Therefore, supplementation in cattle is typically provided as vitamin D_3. Cholecalciferol (vitamin D_3) is synthesized in the skin following UVB exposure and is subsequently converted in the liver to 25-hydroxyvitamin D_3 (calcidiol) by hepatic 25-hydroxylases (Horst and Reinhardt, 1983). Calcidiol acts as the primary circulating form and a precursor to calcitriol, the biologically active hormone. Calcitriol exerts its effects via widely distributed vitamin D receptors, influencing numerous physiological systems (Norman, 2008).

However, in modern dairy operations, cows are frequently housed indoors, limiting their exposure to sunlight and thereby impairing endogenous synthesis of vitamin D_3. This synthesis is also influenced by environmental variables such as season, latitude, and time of day. As a result, vitamin D status in dairy cattle often relies heavily on dietary supplementation. The National Academies of Sciences, Engineering and Medicine (2021) recommends daily intake of 0.7 mg (28,000 IU) of vitamin D_3. More recently, 25-hydroxyvitamin D_3 (calcidiol) has been introduced as a dietary supplement due to its superior bioavailability, as it bypasses hepatic conversion and induces a more rapid and consistent increase in circulating 25(OH)D concentrations (Poindexter et al., 2023a).

Serum 25-hydroxyvitamin D 25(OH)D concentration is widely accepted as the most reliable biomarker of vitamin D status, reflecting both endogenous synthesis and dietary intake (Nelson et al., 2016). In cattle, normal circulating concentrations typically range from 20 to 50 ng/mL (Horst et al., 1994).

During early lactation, cows experience a significant calcium drain due to milk production. Restoring bone mineral reserves in mid- and late lactation is therefore critical, a process in which vitamin D plays a key role (Hodnik et al., 2020). Therefore, the objective of this study was to evaluate the effects of vitamin D source (cholecalciferol vs. calcidiol) and amount (0.2 vs. 1.0 mg/day) on serum 25-hydroxyvitamin D concentrations in pregnant Holstein cows.

Materials and Methods

Animal Care and different experimental procedures for this study were approved by the University of Florida Institutional Animal Care and Use Committee.

Study Location

The project was conducted at the Dairy Research Unit of the University of Florida in Gainesville, Florida (Figure 1), located at an elevation of 54 meters above sea level. The site receives an average annual precipitation of 1,230 mm and is characterized by a humid subtropical climate (Köppen climate classification Cfa). The study was carried out from February to April, during which average daily temperatures range from 10 °C to 28 °C.

Figure 1

Study Location

Cows and Housing

Twenty-four pregnant Holstein cows were selected from the University of Florida Dairy Research Unit and transferred to the experimental facility at approximately 63 days of gestation. This

early relocation allowed for a period of acclimation to the new environment and training to use individual feeding gates (Calan Broadbent Feeding System, American Calan Inc., Northwood, NH), to which each cow was individually assigned.

The housing facility consisted of two barns equipped with sand-bedded stalls, with bedding replaced twice weekly. Environmental comfort was maintained using two rows of fans positioned above the stalls and a water soaker system with nozzles. Both cooling systems were programmed to activate automatically when ambient temperatures reached or exceeded 20 °C.

Management and Feeding

Cows were fed twice daily at 0700 and 1100 h, using two Calan Data Rangers (computerized feeding systems). The daily diet consisted of a total mixed ration (TMR) composed of forage and concentrate. To minimize exposure to sunlight and control vitamin D intake, all cows were housed indoors throughout the trial. Each cow received its assigned diet individually and had *ad libitum* access to feed.

Table 1

Ingredients of experimental Total Mixed Ration (TMR)

Item	Vit D Experiment Diet
Ingredients, % of diet DM	
Corn Silage	43.10
Oat Silage	4.31
Molasses	3.28
Lactating Grain Mix	49.31
Ingredients, % of Lactating Grain Mix DM	
Ground Corn	36.71
Soyhull	16.43
Amino Plus	10.49
Cottonseed	5.24
Soybean Meal	22.38
Ingredients, % of Lactating Grain Mix DM	
Palmit 80	2.10
No VitD Mineral	6.64

Experimental Design

The study was conducted using a randomized complete block design, with the individual cow serving as the experimental unit. Data was collected from day 0 of treatment application through day 56, with measurements taken on days 0, 28, and 56. Cows were blocked by parity and current lactation performance, and within each block, randomly assigned to one of four treatments arranged in a 2 × 2 factorial design. Treatment allocation was performed using the random number generator function in Excel (Microsoft Corp.).

Treatments

Treatments were administered once daily to each cow as a top-dress to Total Mixed Ration (TMR) for a duration of 56 days. A total of 24 pregnant Holstein cows, at approximately 63 days of gestation, were randomly assigned to one of the following treatment groups:

LCHOL (n = 6): 0.2 mg/day of vitamin D_3 (cholecalciferol)

HCHOL (n = 6): 1.0 mg/day of vitamin D_3

LCAL (n = 5): 0.2 mg/day of 25-hydroxyvitamin D_3 (calcidiol)

HCAL (n = 7): 1.0 mg/day of 25-hydroxyvitamin D_3

Vitamin D sources were prepared from dry powder concentrates containing either 1.25% cholecalciferol (Rovimix D_3 500, DSM Nutritional Products Inc.) or 1.25% calcidiol (Hy-D 1.25%, DSM Nutritional Products). For premix preparation, 56 g of the respective concentrate were mixed into 6,994 g of ground corn. To achieve the desired supplementation levels: the 1.0 mg/day dose was prepared by mixing 2 kg of the vitamin D premix into 8 kg of ground corn, and the 0.2 mg/day dose was prepared by mixing 0.4 kg of the vitamin D premix into 9.6 kg of ground corn.

Each cow received 50 g of the corresponding top-dress premix applied daily to their individual TMR. To ensure full intake, cows were monitored during feeding. The top-dress was thoroughly mixed into the upper layer of the TMR to promote uniform consumption and minimize selective feeding.

Table 2

Composition of Top-Dress Premixes Used for Daily Supplementation

Ingredient	Treatments			
	0.2mg D (Final)	1mg D (Final)	0.2mg HyD (Final)	1mg HyD (Final)
Ground Corn, g	49.984	49.92	49.984	49.92
Rovimix D_3 (500 KIU/g, g	0.016	0.08	0	0
HyD 1.25%, g	0	0	0.016	0.08
Total, g	50	50	50	50
Vitamin D, mg/g	0.004	0.02	0.004	0.02
Final, mg	0.2	1	0.2	1

Note. 0.2 mg D (Final), 1 mg D (Final), 0.2 mg HyD (Final), and 1 mg HyD (Final) refer to the treatments providing a daily supplementation of 0.2 or 1.0 mg of vitamin D, either as cholecalciferol (D_3) or 25-hydroxyvitamin D_3 (HyD/calcidiol).

Table 3

Composition of Bulk Premixes Used to Prepare Daily Top-Dress Treatments

Premix	Treatments	
Ingredient	Premix D_3	Premix HyD
Ground corn, g	6944	6944
Rovmix D3 (500KIU/g), g	56	0
HyD 1.25%, g	0	56
Total, g	7000	7000
Vitamin D, mg/g	0.1	0.1

Measured Variable

The primary response variable in this study was the concentration of serum 25-hydroxyvitamin D 25(OH)D.

Blood Sampling and Processing

Blood samples were collected on days 0, 28, and 56 of the experimental period via coccygeal venipuncture into 10 mL evacuated tubes without anticoagulant, intended for serum separation. After collection, samples were allowed to clot at room temperature and were then placed on ice until processing. Tubes were centrifuged at 3,000 rpm for 30 minutes to separate the serum. Subsequently, serum was aliquoted into three 2 mL cryovials and stored frozen at -20 °C until analysis.

Serum Analysis

Serum 25(OH)D concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (25-OH Vitamin D ELISA Assay Kit, Eagle Biosciences) on samples collected at days 0, 28, and 56.

Spectrophotometer

Optical density was measured using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments), following the manufacturer's instructions for the ELISA procedure.

Statistical Analysis

The analysis was conducted using the MIXED procedure in SAS (SAS Institute Inc., Cary, NC), and least squares means (LSMeans) were used to assess treatment effects. The following linear mixed model was used: $Y = \mu + \alpha + \delta + \tau + (\alpha \cdot \delta) + (\alpha \cdot \tau) + (\delta \cdot \tau) + (\alpha \cdot \delta \cdot \tau) + Cov + b + a + \varepsilon$, where *Y* represents the serum 25-hydroxyvitamin D concentration, μ is the overall mean, α denotes the fixed effect of vitamin D source (SOURCE), δ is the fixed effect of supplementation amount (AMT), and τ is the fixed effect of sampling day (DAY). The terms *(α·δ)*, *(α·τ)*, *(δ·τ)*, and *(α·δ·τ)* represent their respective interaction effects. *Cov* denotes the covariate (initial serum 25(OH)D). The random effects included the block (*b*) and the animal nested within treatment (*a*). Repeated measures over time were modeled using an autoregressive correlation structure of order 1 [AR(1)]. Significance was declared at $P \leq 0.05$.

Results and Discussion

Supplementation with different sources and amounts of vitamin D significantly influenced serum 25-hydroxyvitamin D 25(OH)D concentrations in pregnant Holstein cows. Similarly to (Poindexter et al., 2023b), an interaction ($P < 0.001$) between vitamin D source and amount was observed for serum 25(OH)D concentrations (Table 4).

Feeding HCAL resulted in the highest serum 25(OH)D concentrations (Table 4; Figure 2). In contrast, the amount of CHOL had minimum impact on serum 25(OH)D concentrations. Serum concentrations of 25(OH)D increased with higher CAL supplementation, but not with CHOL, consistent with previous findings (Poindexter et al., 2020).

It has been reported that the liver contains abundant 25-hydroxylases, such as the cytochrome P450 enzymes CYP2J2, CYP2R1, and CYP27A1, which facilitates the efficient conversion of vitamin D_3 to 25(OH)D_3 (Kuhn et al., 2020), however, there is evidence that feedback inhibition can occur during the hepatic conversion of vitamin D_3 to 25(OH)D_3 (Dahlbäck & Wikvall, 1987). This suggests that while CHOL relies on potentially limited or regulated hepatic conversion, direct supplementation with CAL ensures more consistent and effective increases in circulating 25(OH)D.

Table 4

Effect of source and amount of vitamin D fed on serum 25(OH)D concentrations (LSM ± SE)

Item	Treatment[1]				P-Value[2]		
	CHOL		CAL				
	0.2 mg	1 mg	0.2 mg	1 mg	Source (S)	Amt (A)	S × A
Serum 25(OH)D (ng/mL) ± SE	76.91 5.06	76.80 4.74	77.28 5.81	110.31 4.37	<.0001	<.0001	<.0001

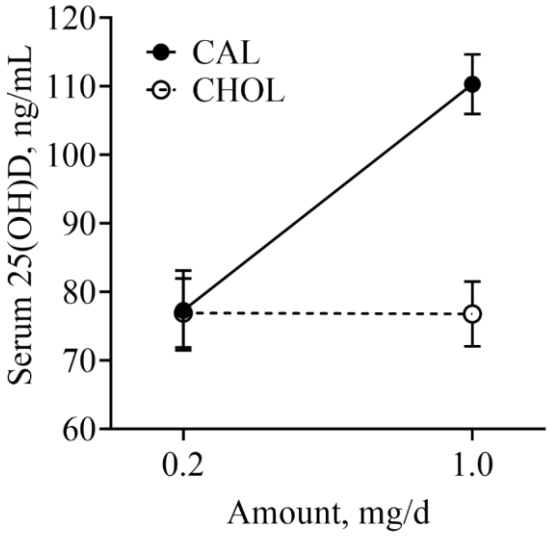
Note. [1]Cows received either 0.2 or 1 mg/d of either cholecalciferol (CHOL) or calcidiol (CAL) for a duration of 56 days. [2]Source = vitamin D source (CHOL vs. CAL); Amt = amount of vitamin D per day (0.2 mg vs. 1 mg); Source × Amt = interaction between source and amount of vitamin D.

As illustrated in Figure 2, serum 25(OH)D concentrations increased over the 56-day supplementation period, particularly in the CAL-supplemented groups. Notably, cows receiving HCAL showed a marked elevation in 25(OH)D compared to other treatments, while CHOL-fed cows exhibited a relatively flat response. A similar response has been observed in previous research, which indicated that increasing dietary vitamin D_3 from 1 to 3 mg/d did not significantly elevate serum 25(OH)D concentrations in mid- to late-lactation dairy cows, suggesting a limited effect of a higher cholecalciferol amount on circulating vitamin D status (Poindexter et al., 2020).

Unlike CAL, increasing the amount of CHOL from 0.2 to 1 mg/day did not substantially improve serum 25(OH)D concentrations, suggesting a saturation of the conversion pathway. Overall, the findings highlight a clear advantage of calcidiol over cholecalciferol in raising serum 25(OH)D concentrations, indicating that the choice of vitamin D source plays a critical role in achieving adequate vitamin D status in pregnant dairy cows.

Figure 2

Effect of treatments on serum 25(OH)D concentrations



Note. Concentrations of serum 25-hydroxyvitamin D 25(OH)D in dairy cows fed diets providing either 0.2 mg/d cholecalciferol (LCHOL, n = 6), 1 mg/d cholecalciferol (HCHOL, n = 6), 0.2 mg/d calcidiol (LCAL, n = 5), or 1 mg/d calcidiol (HCAL, n = 7) during 56 days. Error bars represent the SE.

Conclusion

Feeding calcidiol 25(OH)D_3 to pregnant Holstein cows resulted in a marked increase in serum 25(OH)D concentrations compared with cholecalciferol (vitamin D_3), which had minimal effect regardless of the amount fed. These findings indicate that calcidiol is a more effective source of vitamin D for elevating serum 25(OH)D in cows.

Recommendations

Determine the cost-effectiveness of calcidiol supplementation under commercial dairy conditions.

Evaluate the long-term effects of calcidiol supplementation on cow and calf health outcomes.

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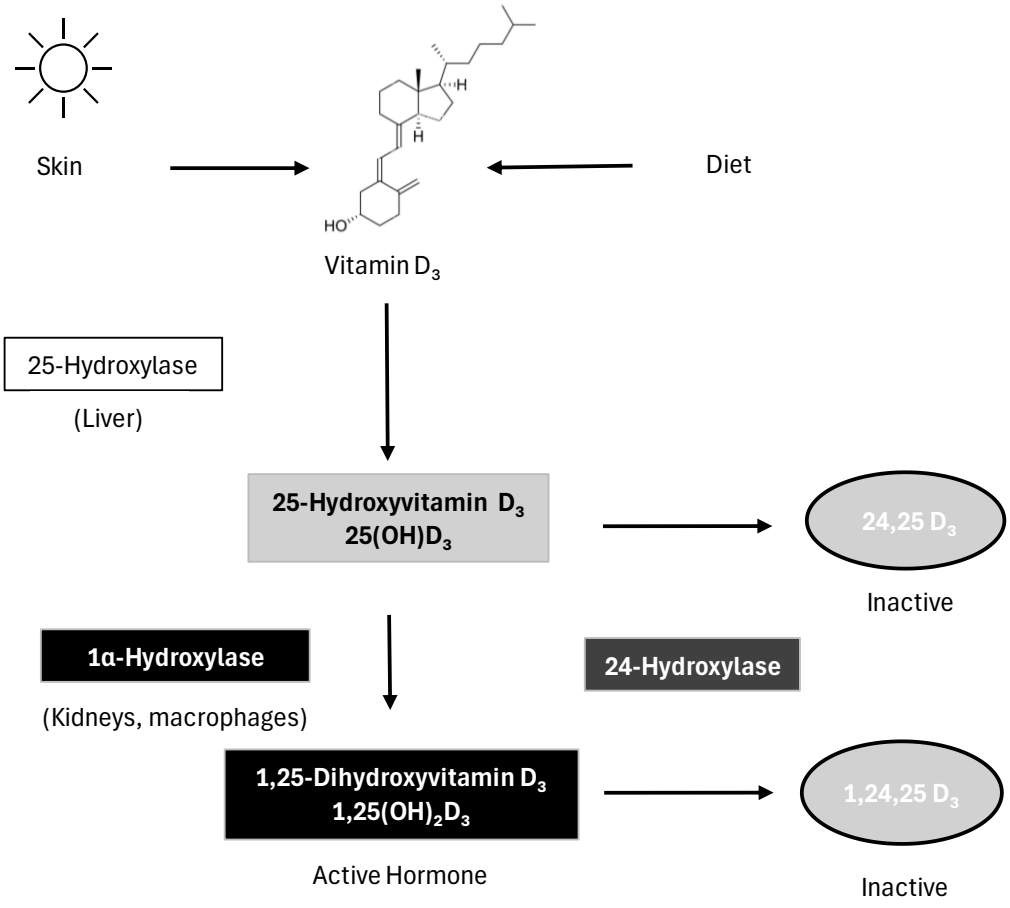
Appendix

Appendix A

Vitamin D Pathway



Note . Adapted from Nelson and Merriman (2014).