Effect of two dosages of prepartum cholecalciferol injection on blood minerals, vitamin D metabolites, and milk production in multiparous dairy cows. A randomized clinical trial.

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ABSTRACT

The objective of the present study was to evaluate the effect of 2 dosages of prepartum cholecalciferol injection on blood minerals, vitamin D metabolites, and milk production. Cows entering their 2nd or greater lactation (n = 158) were randomly assigned to a control group (CON) or one of 2 treatment groups receiving either 6 × 10^6 IU (6VitD) or 12 × 10^6 IU (12VitD) cholecalciferol intramuscularly on d 275 ± 1.2 of gestation. Concentrations of serum total Ca (tCa), phosphate, and Mg were determined on 1, 2, 3, 5, 7, and 10 DIM. For a subsample of 30 cows entering the 3rd lactation (n = 10 per group), these samples were analyzed for cholecalciferol, 25-hydroxycholecalciferol (25-OHD3), and 24,25-dihydroxycholecalciferol (24,25-(OH)2D3). In these cows, we also determined 1,25-dihydroxycholecalciferol (1,25-(OH)2D3), the biologically most active metabolite, on 1, 2, 3, and 5 DIM. Repeated measures ANOVA was performed to evaluate the effect of different dosages of cholecalciferol on blood minerals, vitamin D metabolites, and milk yield over the first 5 test days after calving. Binary outcomes such as retained placenta (RP) and metritis were analyzed using a Chi² test. While the 12VitD treatment increased tCa concentrations on 1, 2, and 3 DIM compared with CON, administration of 6VitD increased tCa concentrations only on 1 DIM. Compared with CON cows and 6VitD cows, 12VitD cows had greater serum phosphate concentration during the first 10 DIM. Furthermore, 6VitD cows had a greater serum phosphate concentrations compared with CON cows. On the contrary, 12VitD cows had lower serum Mg concentrations during the first 10 DIM compared with CON and 6VitD cows. Cholecalciferol was increased by the treatment and decreased quickly until 10 DIM. In respect to 25-OHD3, the 6VitD treatment resulted in a 4.1-fold increase in comparison to the CON group, while a 6.5-fold increase was observed in 12VitD animals. The vitamin D metabolite 24,25-(OH)2D3 increased linearly with 25-OHD3 serum levels, resulting in the highest concentrations in the 12VitD group. An increase of 1,25-(OH)2D3 until 3 DIM was observed in all cows. However, this rise was most pronounced in the CON group. The incidence of RP was 1.9%, 11.5%, and 29.6%, and that of metritis was 11.5%, 15.4%, and 31.5% for CON, 6VitD, and 12VitD cows, respectively. While none of the treated cows exerted clinical signs of hypocalcemia, one cow of the control group incurred clinical hypocalcemia. Cows of the 12VitD group had a lower milk yield over the first 5 DHIA tests compared with the control and 6VitD group (42.2 ± 0.5, 42.0 ± 0.5 and 40.7 ± 0.5kg for control cows, 6VitD cows and 12VitD cows, respectively). Although no negative side-effects were observed in 6VitD cows, we do not recommend the general application of 6 × 10^6 IU cholecalciferol before calving as positive effects on calcium homeostasis were marginal and restricted to the 1st DIM. The present findings confirm that the application of 12 × 10^6 IU cholecalciferol negatively affected milk production on this farm.

Keywords. Vitamin D injection, hypocalcemia

INTRODUCTION

The intramuscular administration of cholecalciferol 5 to 7 DIM before calving is a common strategy to prevent clinical hypocalcemia in dairy cows used in European and Asian countries (Venjakob et al., 2017; Yamagishi et al., 2000). The hydroxylation of cholecalciferol to 25-hydroxycholecalciferol (25-OHD3) in the
liver is not regulated tightly (Jones, 2008). Therefore, a high dose of cholecalciferol results in increased serum concentrations of 25-OH3 and subsequently increased Ca (tCa) concentrations, but informal reports on the efficiency differ (Hodnik et al., 2020; Venjakob et al., 2022). However, randomized controlled studies evaluating the efficacy are scarce.

In an early study on 182 multiparous cows entering ≥3rd lactation, 10 × 106 IU cholecalciferol injected intramuscularly approximately 7 d before expected parturition, reduced the incidence of milk fever, but only in cows that had developed clinical hypocalcemia in previous lactation (Julien et al., 1977). Sadri et al. (2021) administered 8 × 106 IU cholecalciferol intramuscularly to 12 Holstein Friesian cows 2 to 8 d prepartum and reported decreased serum concentrations of ionized Ca, parathyroid hormone (PTH), and bone markers around calving in comparison to the placebo treatment. They concluded that a single cholecalciferol injection did not improve Ca homeostasis around calving (Sadri et al., 2021).

Our group previously evaluated the effect of a greater dose (12 × 106 IU cholecalciferol as an intramuscular injection, 5 d before expected calving) on calcium homeostasis, uterine health, and milk production (Venjakob et al., 2022). Cows that did not calve within 7 d after treatment were re-injected with 10 × 106 IU cholecalciferol. Serum tCa concentrations were greater in treated cows. Gestation length (GL) was affected by the treatment (cows treated once: 278.4 d; cows treated twice: 284.7 d; control 281.3 d). Furthermore, we observed a greater risk for retained placenta (RP; cows treated once: 7.7%; cows treated twice: 4.0%; control: 2.0%) and metritis (cows treated once: 39.3%; cows treated twice: 33.3% control: 21.6%) as well as a reduction in milk yield in treated cows, compared with control cows (control 42.5 kg; cows treated once: 38.8 kg; cows treated twice: 38.7 kg). However, due to the study design (reinjection after 7 d), group allocation (treated once or twice) was not completely independent from GL and our results had to be interpreted carefully.

The present study was conducted (1) to confirm our previous findings on tCa homeostasis, GL, uterine health, and milk yield without injecting the cows for a second time and (2) to gain further insight into the potential mode of action by expanding our analyses of vitamin D metabolites as we had speculated that elevated serum concentrations of cholecalciferol itself or its metabolites might be related to the detrimental effects observed. This knowledge is important in respect to future recommendations on the use of cholecalciferol. Our hypotheses were that a treatment with 6 × 106 IU cholecalciferol would result in improved postpartum Ca homeostasis without exerting negative side-effects on health status and milk production. Based on our former results, we assumed that cows treated with 12 × 106 IU would have a shortened GL and a reduction in milk production in comparison to untreated animals. To verify or reject this hypothesis, the study was conducted without reinjecting cows that had not calved within 7 d in order not to bias the results on GL.

**MATERIALS AND METHODS**

The study was conducted on a commercial dairy farm in northern Germany between July 2020 and December 2020. The farm is located in the federal state of Brandenburg and has approximately 2,600 milking cows, with an average 305-d milk yield of 9,600 kg. All procedures reported herein were approved by the federal authorities (protocol 2347–48–2019). Sample size calculation was performed based on the results of our previous study in which cows entering their ≥2 lactation were treated with one dose of 12 × 106 IU cholecalciferol 5 d before calving had a 3.8 kg reduction in milk yield at first test day compared with untreated cows of the control group (Venjakob et al., 2022). Assuming 80% power, a 95% confidence level, and a SD of 8.0 kg 51 animals per group were needed to detect a similar effect on milk production.

**Transition Cow Management**

Transition cows were managed as recently described (Venjakob et al., 2022). Briefly, cows were dried-off at d 223 ± 10.7 of gestation and moved to the close-up pen at approximately d 255 of gestation. During the close-up period, cows received a negative DCAD diet (DCAD: −31 mEq/kg of dry matter) containing 2,000 IU of cholecalciferol per kg of DM. The TMR was formulated to meet or exceed minimum nutritional requirements (NEL, nutrients, minerals, and vitamins) for dairy cows (NRC, 2001). Ingredients and chemical composition of the close-up diet were described previously (Venjakob et al., 2022). In a subsample of approximately 10 close-up cows per wk urinary pH was assessed. The average urinary pH was 6.7 (±0.99; n = 25), 6.7 (±0.98; n = 25) and 6.8 (±0.99; n = 27) for CON, 6VitD, and 12VitD cows, respectively. At calving, calving ease (eutocia = unassisted calving; dystocia = calving assisted by 1 or more person) were recorded. The calves were separated from the dams immediately after calving and cows received an oral Ca bolus (Bovikalc, Boehringer Ingelheim, Ingelheim am Rhein, Germany) before being moved to the fresh cow pen. Milk records from the federal DHIA equivalent testing system were obtained from the on-farm computer system (HerdeW, version 5.8, dsp-Agrosoft Ltd., Ketzin, Germany).
Treatment Allocation

Before the start of the study, 158 multiparous cows were randomly assigned to one of 3 treatment groups, based on a random list created in Excel (Office 2019, Microsoft Deutschland Ltd., Munich, Germany). Cows were enrolled every Mondays and Thursdays during the study period, based on their gestation length (GL 275 ± 2 d). At enrollment, the body condition score (BCS) was evaluated based on a 5-point scale with 0.25 increments (Ferguson et al., 1994). While cows of the control group were left untreated (CON, n = 52), cows of the 2 treatment groups received a single intramuscular injection of 6 × 10⁶ IU cholecalciferol (6VitD, n = 54; Ursovit D3, Serumwerk Bernburg, Bernburg, Germany), respectively. Cows were injected into one of the 2 hind limbs (i.e., M. semimembranosus, M. semitendinosus). All injections as well as BCS assessment were conducted by the research team. Researchers were not blinded to the treatment.

Disease Diagnosis

Until 10 DIM, daily health checks were performed by the research team including assessment of the general behavior, vaginal discharge, and manure consistency. Detection of clinical mastitis was performed 3 times daily by the farm personnel during regular milking. Clinical mastitis was defined according to Vasquez et al. (2017) as visible signs of inflammation such as redness, swelling, pain, or heat, and alterations such as clots, flakes, discoloration, or abnormal consistency of secretions. When fetal membranes were not expelled within the first 24 h after calving, cows were diagnosed with retained placenta (RP). On 7 DIM, vaginal discharge was assessed (Sheldon et al., 2006). For a subsample of 30 cows entering their 3rd lactation, we quantified the concentrations of 4 vitamin D metabolites. The concentration of 1,25-dihydroxycholecalciferol (1,25-(OH)₂D₃) was determined by a commercial laboratory (Inmmundagnostik AG, Bensheim, Germany) using the ELISA method. Intra- and interassay coefficients of variation were 6.69% and 9.00%, respectively. The lower detection level was 4.80 pg/mL.

Concentrations of calcitriol, 25-OHD₃, and 24,25-dihydroxycholecalciferol (24,25-(OH)₂D₃) were determined at the Institute of Agricultural and Nutritional Sciences (Martin-Luther-University Halle-Wittenberg, Halle/Saale, Germany) by liquid chromatography tandem mass spectroscopy (HPLC-MS/MS) in modification to Bauer et al. (2019). Briefly, 200 µL of serum was mixed with 150 µL potassium hydroxide (10 M), 60 µL ascorbic acid (78.8 mg/ml), 10 µL sodium sulfide (2.125 mg/ml), 50 µL of the internal standard (80 ng/ml d₃-D₃,150 ng/ml d₃-25-OHD₃, and 200 ng/ml d₆-24,25-(OH)₂D₃) and 450 µL ethanol. The prepared samples were overlaid with nitrogen and incubated on a Thermomixer (Thermomixer comfort, Eppendorf, Wesseling, Germany) for 3 h at 37°C under constant shaking (600 rpm). After addition of 200 µL of ultrapure water, the samples were transferred to extraction columns (Extreht NT1, Merck KGaA, Darmstadt, Germany). After incubation for 10 min, samples were extracted with 4 mL n-hexane and 4 mL n-hexane tert-butyl methyl ether. The effluent was collected and mixed with 1 mL water, after 2 min the underlayer was suctioned off. The samples were dried under vacuum (RVC 2–25 CDplus, Christ, Osterode am Harz, Germany). After evaporation, 400 µL acetonitrile and 100 µL 4-phenyl-
1,2,4-triazoline-3,5-dione (0.75 g/l in acetonitrile) were added and the samples were incubated for 1 h at room temperature and then overnight at 4°C. The samples were mixed with 100 µL of ethanol, incubated for 15 min and dried under vacuum afterward. The residues were dissolved in 48 µL methanol and 12 µL 10 mM ammonium format were added. Analysis was performed using a Poroshell column (EC-C18, 50 × 4.6 mm², 2.7 µm) at 40°C in combination with a 1260 HPLC (both Agilent Technologies, Böblingen, Germany) coupled to a QTRAP 5500 mass spectrometer (AB SCIEX, Darmstadt, Germany) and a gradient of 5 mM ammonium format/0.1% formic acid in acetonitrile + water (1 + 1, vol/vol) and acetonitrile (time, min/% acetonitrile/flow, µL/min: 0.0/0/600, 2.1/0/600, 4.0/23.5/600, 6.0/40/600, 8.0/60/600, 10.0/80/600, 11.0/100/600, 16.0/100/600, 18.0/100/1000, 20.0/100/1000, 21.0/0/1000, 24.0/0/800, 25.0/0/600).

Statistical Analyses

Test day data of the first 5 DHIA equivalent test days of each cow and the results from blood analyses were combined using Access (Office 2010, Microsoft Deutschland Ltd., Munich, Germany), exported to Excel spreadsheets, and analyzed using SPSS for Windows (version 25.0, IBM Corp., Ehningen, Germany). Univariable models were calculated to test whether GL at enrollment, GL, interval between enrollment and calving, previous 305-d milk yield, parity, and calving ease was evenly distributed among CON, 6VitD, and 12VitD cows. To analyze the effect of treatment on postpartum blood minerals, vitamin D metabolites, and milk yield, repeated measures ANOVA with first-order autoregressive covariance was performed using GENLINMIXED procedure of SPSS. Eight separate models were calculated to evaluate the effect of the cholecalciferol treatment on serum tCa, Pi, Mg, cholecalciferol, 25-OHD3, 1,25-(OH)2D3 and 24,25-(OH)2D3 concentrations and milk yield. For serum concentrations of tCa, Pi, Mg, cholecalciferol, 25-OHD3, and 24,25-(OH)2D3, repeated measures were conducted on 1, 2, 3, 5, 7, and 10 DIM. For 1,25-(OH)2D3, repeated measures were conducted on 1, 2, 3, and 5 DIM. Milk yield analysis was conducted with repeated measures based on data obtained on test d 1 to 5. According to Dohoo et al. (2009) each explanatory variable was separately analyzed in a univariable model. Exploratory variables tested in the univariable models were treatment (CON vs. 6VitD vs. 12VitD), time (1, 2, 3, 5, 7, 10 DIM for blood minerals and cholecalciferol, 25-OHD3, and 24,25-(OH)2D3 concentration; 1, 2, 3, 5 DIM for 1,25-(OH)2D3 concentration; test d 1, 2, 3, 4, 5 for milk production), parity (parity 2 vs. parity 3 vs. parity ≥ 4), 305-d milk yield in previous lactation (continuous), and BCS at enrollment (normal; BCS of 2.75 to 3.25 vs. fat; BCS ≥ 3.5). If the univariable models resulted in a P-value < 0.1, parameters were included in the final mixed model. In the model of tCa, Pi, Mg, and milk yield, all variables tested were included into the mixed models. Analyzing the effect of treatment on vitamin D metabolites, parity was excluded from the models, as all cows were in 3rd lactation. Furthermore, 305-d milk production of previous lactation (P = 0.89) and BCS at enrollment (P = 0.36) were not included in the mixed model of cholecalciferol, and BCS at enrollment (P = 0.76) was not included in the mixed model for 1,25-(OH)2D3. Selection of the model that best fit the data was performed using a backward stepwise elimination procedure that removed all variables with P > 0.1 from the model. All biologically plausible interactions such as time by treatment, treatment by parity and time by treatment by parity were tested. Whenever the inclusion of time by treatment led to a lower Akaike information criterion, the interaction was forced to remain in the model. In all models, the P-value was adjusted using a Bonferroni correction, to account for multiple comparisons. Variables were declared statistically significant when P < 0.05. As the sample size is not sufficient to analyze binary outcomes such as RP and metritis, disease incidences are described using a Chi² test.

RESULTS

Of the 158 multiparous Holstein Friesian cows, 52, 52, and 54 cows were allocated to the CON, 6VitD and 12VitD group, respectively. Parity (P = 0.47), energy corrected 305 milk yield of previous lactation (P = 0.26), GL at the day of enrollment (P = 0.88), and calving ease (P = 0.48) did not differ between the groups. Treatment with 12 × 10⁶ IU of cholecalciferol resulted in a difference in GL (P < 0.01) and interval between treatment and calving (P < 0.01; Table 1).

Blood Minerals

Serum tCa concentration was affected by time relative to calving (P < 0.01), negatively associated with parity (P < 0.01), body condition score at enrollment (P < 0.01) and 305-d milk production in previous lactation (P < 0.05). Prepartum treatment with cholecalciferol increased serum tCa (P < 0.01; Figure 1). Furthermore, there was an interaction of treatment by time relative to calving (P < 0.05). Cows of the 12VitD group had greater serum tCa concentrations compared with CON cows on 1 (2.03 vs. 1.69 mmol/l; P < 0.01), 2 (1.88 vs. 1.73 mmol/l; P < 0.01), and 3 DIM
(2.06 vs. 1.94 mmol/l; P < 0.05) and greater serum tCa concentrations than 6VitD cows on 1 (2.03 vs. 1.88 mmol/l; P < 0.01) and 3 DIM (2.06 vs. 1.94 mmol/l; P < 0.05; Figure 1). Compared with CON cows, tCa of 6VitD cows was only increased on 1 DIM (1.88 vs. 1.69 mmol/L; P < 0.01).

Serum Pi concentration was affected by time relative to calving (P < 0.01) and increased by prepartum treatment with cholecalciferol (P < 0.01). Compared with control cows, and 6VitD cows, 12VitD cows had greater serum Pi concentrations during the first 10 DIM. Furthermore, 6VitD cows had greater serum Pi concentrations compared with CON cows (1.52, 1.68, and 1.86 mmol/l for CON, 6VitD, and 12VitD, respectively; P < 0.01). We also observed a negative association with parity (P < 0.01) and 305-d milk yield in previous lactation (P < 0.01).

Serum Mg concentration was affected by time relative to calving (P < 0.01), negatively associated with parity (P < 0.01) and BCS at enrollment (P < 0.01) and decreased by prepartum treatment with cholecalciferol (P < 0.01). 12VitD cows had lower serum Mg concentrations during the first 10 DIM compared with CON and 6VitD cows (0.85, 0.81, and 0.77 mmol/l for CON, 6VitD, and 12VitD, respectively; P < 0.01. Furthermore, the interaction of time by treatment (P = 0.08) remained in the model.

Vitamin D Metabolites

Treatment increased the serum concentrations of cholecalciferol (P < 0.05), while concentrations were below the detection limit in the untreated CON group (Table 2). After calving, serum concentrations decreased rather rapidly (Figure 2A). Treatment affected likewise the concentrations of 25-OHD$_3$ (P < 0.01) and 24,25-(OH)$_2$D$_3$ (P < 0.01) throughout the entire observation period (Figure 2B and C; Table 2). Serum concentrations of 1,25-(OH)$_2$D$_3$ showed a more dynamic pattern with the greater increase in the CON group compared with the treatment groups (Figure 2D).

Health Status and Milk Production

Cows were treated on d 275 (±1.2 d) of gestation. In comparison to CON (P < 0.01) and 6VitD animals (P < 0.01), cows of the 12VitD groups calved earlier.
relative to cholecalciferol administration (12VitD: 3.5 ± 0.5 d; 6VitD: 6.1 ± 0.5 d; CON: 6.5 ± 0.5 d). This resulted in a significant difference in GL. Cows in the 12VitD group (GL = 278.5 ± 0.5 d) had a 2.9 d (P < 0.01) and 2.4 d (P < 0.01) shorter GL compared with the CON (GL = 281.4 ± 0.5 d) and the 6VitD group (280.9 ± 0.5 d). In total, 23 out of 158 cows (14.6%) incurred RP and 31 out of 158 (19.6%) were diagnosed with metritis. While there was no difference in the incidence of RP (1.9 [n = 1] vs. 11.5% [n = 6]) and metritis (11.5% [n = 6] vs. 15.4% [n = 8]) between CON and 6 VitD cows, 12VitD cows had a higher incidence of RP (29.6%; n = 16; P < 0.01) and metritis (31.5%; n = 17; P < 0.05) compared with CON cows.

The effect of treatment on milk production was evaluated over the first 5 DHIA equivalent test days. Mean DIM at 1st test were 24.1 (n = 50), 22.8 (n = 46), 26.1 (n = 49) for CON, 6VitD, and 12VitD cows, respectively. Mean DIM at 2nd test were 55.4 (n = 48), 54.6 (n = 46), and 56.8 (n = 48) for CON, 6VitD and 12VitD cows, respectively. Mean DIM at 3rd test were 83.5 (n = 47), 82.5 (n = 45), and 84.8 (n = 48) for CON, 6VitD and 12VitD cows, respectively. Mean DIM at 4th test were 115.6 (n = 46), 113.6 (n = 45), and 117.0 (n = 47) for CON, 6VitD and 12VitD cows, respectively. Mean DIM at 5th test were 146.6 (n = 46), 145.9 (n = 45), and 147.6 (n = 46) for CON, 6VitD and 12VitD cows, respectively. During the first 5 test days, cows of the 12VitD group had a lower milk production than cows of the 6VitD group (40.7 vs. 42.0 kg; P < 0.05) and cows of the control group (40.7 vs. 42.2 kg; P < 0.05; Figure 3). No difference was observed between CON and 6VitD cows (42.2 vs. 42.0 kg; P = 0.76). Furthermore, there was an association between milk production over the first 5 tests and number of test day (P < 0.01), parity (P < 0.01), 305-d milk yield in previous lactation (P < 0.01), and BCS (P < 0.01) at enrollment. The interaction of number of test day by treatment was forced to remain in the model (P = 0.12) as this led to a lower Akaike information criterion.

**DISCUSSION**

As expected, treatment with cholecalciferol increased serum concentrations of cholecalciferol, 25-OHD$_3$ and 24,25-(OH)$_2$D$_3$. 12VitD treatment and to lesser extent also 6VitD treatment increased tCa and Pi concentrations and decreased Mg concentration. In 12VitD cows, GL and milk yield were reduced, while the incidence was RP and metritis were higher in comparison to CON animals.

**Blood Minerals**

In the untreated CON, cholecalciferol concentrations were below our detection limit (5 ng/mL) which is in line with results from Poindexter et al. (2023a). In both treatment groups, administration of cholecalciferol 5 d before expected calving led to an increase not only in cholecalciferol itself but also in serum 25-OHD$_3$ in comparison to the CON group during the entire observation period (Table 2, Figure 2A, Figure 2B). This result was expected as the hydroxylation of cholecalciferol to 25-OHD$_3$ is not regulated tightly (Jones, 2008). Serum concentrations of tCa and Pi were greater in both treatment groups. These results confirm the findings of our former study (Venjakob et al., 2022) and results of an experiment by Poindexter et al. (2023a). This group of authors investigated the effect of increasing prepartum serum concentrations of 25-OHD$_3$ by feeding either 1 or 3 mg 25-OHD$_3$ instead of feeding 1 or 3 mg cholecalciferol. Serum concentrations of 25-

<table>
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<th>Vitamin D Metabolite</th>
<th>6VitD</th>
<th>12VitD</th>
<th>Fixed effects, P-value</th>
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<td>Cholecalciferol (ng/mL)</td>
<td>122.6$^a$</td>
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<td>&lt;0.01</td>
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<td>4.3</td>
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<td>0.05</td>
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<td>1,25-(OH)$_2$D$_3$ (pg/mL)</td>
<td>63.0$^d$</td>
<td>4.3</td>
<td>0.05</td>
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$^a$c<LOQ: Limit of quantification.

$^b$The interaction of time by treatment was forced to remain in the model, as this led to a lower Akaike information criterion.

Table 2. Effect of cholecalciferol treatment 5 d before expected parturition (on d 275 ± 2 of gestation) on postpartum concentrations of cholecalciferol, 25-hydroxycholecalciferol (25-OHD$_3$), 1,25-dihydroxycholecalciferol (1,25-(OH)$_2$D$_3$) and 24,25-dihydroxycholecalciferol (24,25-(OH)$_2$D$_3$) of multiparous dairy cows entering their 3rd lactation (n = 30). Serum samples were analyzed for 1,25-(OH)$_2$D$_3$ on 1, 2, 3 and 5 DIM and on 1, 2, 3, 5, 7, and 10 DIM for all other metabolites. Least squares means (LSM) and standard error of the mean (SEM) are derived from repeated measures ANOVA of cows treated with 12 × 10$^6$ IU cholecalciferol (12VitD, n = 10), cows treated with 6 × 10$^6$ IU cholecalciferol (6VitD, n = 10), and cows of the control group (CON, n = 10). Differences among treatment groups at P < 0.05 are indicated by different superscripts.
OHD₃ increased to 93.8 ng/mL (43 animals), 173.6 ng/mL (46 animals), 58.3 ng/mL (39 animals), and 63.5 ng/mL (45 animals) in cows fed with 1 mg 25-OHD₃, 3 mg 25-OHD₃, 1 mg cholecalciferol, and 3 mg cholecalciferol, respectively. The authors reported increased postpartum tCa with 1 mg or 3 mg oral 25-OHD₃ (2.15 mmol/L and 2.17 mmol/L, respectively) in comparison with cows fed with the same amount of cholecalciferol (2.13 mmol/L and 2.11 mmol/L, respectively). Serum P₄ was affected accordingly (1.75 mmol/L and 1.80 mmol/L vs. 1.72 mmol/L and 1.68 mmol/L). In the present study, higher serum concentrations of 25-OHD₃ in the treated groups increased the tCa and P₄ likewise. The reduction in serum Mg could have been caused by a decrease in renal reabsorption due to lower secretion of parathyroid hormone in the treatment groups associated with greater serum concentrations of tCa (Goff, 2008). From our observations and former results reported in the literature it can be concluded that the effect on serum tCa and P₄ was probably caused by 25-OHD₃.

The biologically most active vitamin D metabolite regulating mineral homeostasis is 1,25(OH)₂D₃. After binding to the vitamin D receptor (VDR) and formation of a heterodimer with the retinoid X receptor, it affects so called vitamin D responsive elements in the promoter regions of target genes which leads to an activation or repression of the transcription of vitamin D dependent genes (Christakos et al., 2016). However, studies using mice lacking the enzyme that converts 25-OHD₃ to 1,25-(OH)₂D₃ showed that 25-OHD₃ can bind to the VDR and induce transcription similar to

![Figure 2. Effect of cholecalciferol treatment 5 d before expected parturition (on d 275 ± 2 of gestation) on serum cholecalciferol (Panel A), 25-hydroxycholecalciferol (Panel B, 25-OHD₃), 24,25-dihydroxycholecalciferol (Panel C, 24,25-(OH)₂D₃), and 1,25-dihydroxycholecalciferol (Panel D, 1,25-(OH)₂D₃) concentrations of multiparous dairy cows entering their 3rd lactation (n = 30) of a single dairy farm. Least squares means (LSM) and standard error of the mean (SEM) were derived from repeated measures ANOVA of cows treated with 12 × 10⁶ IU cholecalciferol (12VitD, n = 10, dark blue), cows treated with 6 × 10⁶ IU cholecalciferol (6VitD, n = 10, light blue), and cows of the control group (CON, n = 10, gray). Serum concentrations of cholecalciferol in CON cows was below the limit of quantification (Panel A). Serum cholecalciferol concentrations of 6VitD and 12VitD cows were associated with time (P < 0.01) and treatment (P = 0.05). Serum concentrations of 24,25-(OH)₂D₃ were associated with time (P = 0.08), treatment (P < 0.01), and body condition score at enrollment. Serum concentrations of 25-OHD₃ were associated with time (P < 0.05) and treatment (P < 0.01). Serum concentrations of 1,25-(OH)₂D₃ were associated with time and (P < 0.01) and treatment (P < 0.05). In all models, time*treatment was forced to remain in the model (P = 0.69, P = 0.39, P = 0.77, and P = 0.13 for the model of cholecalciferol, 25-OHD₃, 24,25-(OH)₂D₃, and 1,25-(OH)₂D₃).](image-url)
1,25-(OH)2D3 (DeLuca et al., 2011). But due to the lower affinity of 25-OHD3 to the VDR it is probably only able to activate the VDR when serum concentrations are above 150 ng/mL (Quesada-Gomez and Bouillon, 2018).

Although a typical increase until 3 DIM was observed in all cows, 6VitD and 12VitD cows had a lower 1,25-(OH)2D3 concentration on 3 and 5 DIM compared with CON cows. From these results we conclude that high serum concentrations of 25-OHD3 had repressed the formation of 1,25-(OH)2D3, either directly as described in the next section, or indirectly via the increase in serum tCa and Pi.

Vitamin D Metabolites

The formation of 1,25-(OH)2D3 out of 25-OHD3 is subject to a strict regulation by several factors like serum Ca, directly and indirectly via parathyroid hormone (PTH), P1, and 1,25-(OH)2D3 itself as a negative feedback (Christakos et al., 2019). The greater concentrations of tCa, especially in the 12VitD group, probably inhibited the activation of 25-OHD3 to 1,25-(OH)2D3 as indicated by the lower concentrations of this metabolite (Table 2, Figure 2D) and induced the inactivation of 1,25-(OH)2D3, either directly as described in the next section, or indirectly via the increase in serum tCa and P1.

Health Status and Milk Production

Although the power of this study is limited (CON: 52 cows, 6VitD: 52 cows, 12VitD: 54 cows), the results on GL, RP and metritis are in line with the observations reported in our previous study (untreated cows: 187, treated once: 135, treated twice: 54) and thus support the hypothesis that 12 × 10^6 IU cholecalciferol 12 can have adverse effects on uterine health. This could be directly linked to the shortened GL, as Vieira-Neto et al. (2017) demonstrated that short GL is a risk factor for RP and metritis.

Compromised uterine health might have decreased DMI and thus milk yield of 12VitD cows. It was demonstrated that postpartum feed intake is compromised in cows with early lactation diseases such as metritis (Huzzey et al., 2007; Pérez-Báez et al., 2019). A direct impact of the treatment on milk production seems unlikely as Poindexter et al. (2023b) showed a positive correlation between postpartum 25-OHD3 and milk yield in cows treated with cholecalciferol or 25-OHD3 orally. In contrast to the present study, health status was not affected in their experiment although serum concentrations of 25-OHD3 were comparable. However, there was also a trend for decreased prepartum DMI.

Future Research Related to Potential Interactions between Steroid and Vitamin D Metabolism

One of the crucial parts in the physiological cascade to induce calving is the induction of placental CYP17A1 that results in an increased placental transformation of progesterone to estrogen (Braun et al., 2012; Kindahl et al., 2002; Schuler et al., 2018). Interestingly, Novola-Mertínex et al. (2017) could show an upregulation of the RNA expression of CYP17A1 and a downregulation of CYP11A1 in cultured human placental cells treated with 1,25-(OH)2D3. CYP11A1 mediates the production of pregnenolone, the precursor of progesterone (Schuler et al., 2018). As described above, 25-OHD3 at high concentrations can bind to the VDR and alter gene expression. In addition, cholecalciferol can be a substrate for CYP11A1, too, which could lead to a reduced formation of progesterone (Slominski et al., 2005). Future studies...
should investigate whether vitamin D treatment could interfere with steroid metabolism, either by altering the RNA expression of CYP450 enzymes or by competing with cholesterol as substrate for CYP11A1. The difference between our study and the experiment done by Poindexter et al. (2023a, 2023b) who supplemented either 1 mg or 3 mg of cholecalciferol or 25-OHD₃ per day orally and did not observe any effect on reproductive traits is the rapid increase in both cholecalciferol and 25-OHD₃ after the injection of cholecalciferol. Furthermore, the treatment administered by Poindexter et al. (2023a, 2023b) did not increase serum concentrations of cholecalciferol as pronounced as the single injection of 12 × 10⁶ IU cholecalciferol which amounts to 300 mg.

Future Research Related to Potential Interactions between Ca Status around Calving and Immune Response

McArt and Neves (2020) differentiated between 3 different types of hypocalcemia evaluating tCa concentrations on 1 and 4 DIM and concluded that a certain degree of transient hypocalcemia around calving is a physiological condition. Other authors showed that there is a relation between endotoxemia and Ca homeostasis. Kvidera et al. (2017) infused cows with LPS, a cell wall component of gram-negative bacteria that elicits a robust and well-characterized immune response. Consequently, ionized Ca concentrations decreased. Horst et al. (2020) quantified the amount of Ca lost during LPS challenge in combination with the eucalemic clamp technique. After LPS infusion, in half of the cows enrolled ionized Ca concentrations were leveled by infusing. The results indicated that intravenous administration of LPS causes a loss of 13.8 g of Ca within 12 h. Compared with cows that were treated with LPS only and showed a transient hypocalcemia, milk yield in cows that were treated and then leveled with Ca infusion was decreased by 15%. Horst et al. (2021) speculated that inflammation-induced hypocalcemia is a protective strategy to remove endotoxins. In the present study, cows of the 12VitD group that had higher serum concentrations of tCa around calving presented with a higher incidence of metritis. Future studies should further investigate whether treatments to prevent hypocalcemia might also have the potential to interfere with endogenous mechanisms regulating serum tCa as a response to the pro-inflammatory effects of calving.

Study Limitation

The present study was based on a former project where cows treated with 12 × 10⁶ IU of cholecalciferol had a 3.8 kg lower milk production at first test day, compared with untreated control cows. Analyzing the association between treatment and milk production, in the present study, the interaction of test day relative to calving by treatment was not significant. We observed however an association with treatment independent from test day between 12VitD and CON and 12VitD and 6VitD cows. As the differences in test day milk yield might be lower between 6VitD and CON cows than between 12VitD and CON cows a more elaborate sample size would be needed to confirm that milk production between 6VitD cows and CON cows is not different. As the study was conducted on a commercial dairy farm and as there had been animal welfare issues after detecting negative downstream outcomes in the treatment group of the previous study, the collaborating institutions decided not to include more cows.

CONCLUSION

The present study confirmed our previous finding that an injection of 12 × 10⁶ IU cholecalciferol led to increased tCa concentrations during the first 3 d after calving but shortened GL and negatively affected milk production, compared with control cows. In contrast, no detrimental effects were observed in cows treated with 6 × 10⁶ IU cholecalciferol. The application of 6 × 10⁶ IU cholecalciferol, however, had only marginal effects at 1 DIM. Therefore, it cannot be recommended for general use to prevent hypocalcemia.

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