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 in multiparous dairy cows. Cows entering their second 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 (SD) of gestation. Concentrations of serum total Ca (tCa), phosphate, and Mg were determined on 1, 2, 3, 5, 7, and 10 d in milk (DIM). For a subsample of 30 cows entering the third lactation (n = 10/group), these samples were analyzed for cholecalciferol, 25-hydroxycholecalciferol (25-OHD₃), and 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 and metritis were analyzed using a chi-squared test. Although none of the treated cows exerted clinical signs of hypocalcemia, one cow in the CON group incurred clinical hypocalcemia. Cows of the 12VitD group had a lower milk yield over the first 5 monthly test days compared with the control and 6VitD group (42.2 ± 0.5, 42.0 ± 0.5 and 40.7 ± 0.5 kg 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 first DIM. The present findings confirm that the application of 12 × 10^6 IU cholecalciferol negatively affected milk production on this farm.

Key words: 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-OHD₃ and subsequently increased serum total 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 third or greater 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 pre-partum 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 at first DHIA equivalent test (control: 42.5 kg; cows treated once: 38.8 kg; cows treated twice: 38.7 kg). However, due to the study design (re-injection 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 second or greater 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 standard deviation 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 (SD) 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 DM) containing 2,000 IU of cholecalciferol/kg of DM. The TMR was formulated to meet or exceed minimum nutritional requirements (NE₄, 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). 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 Monday and Thursday during the study period, based on their GL (275 ± 2 d). At enrollment, the BCS was evaluated based on a 5-point scale with 0.25 increments (Ferguson et al., 1994). Cows of the control group were left untreated (CON, n = 52), and cows of the 2 treatment groups received a single intramuscular injection of 6 × 10^6 IU cholecalciferol (6VitD, n = 52) or 12 × 10^6 IU of cholecalciferol (12VitD, n = 54; Ursovit D3, Serumwerk Bernburg, Bernburg, Germany). Cows were injected into one of the 2 hind limbs. All injections, as well as BCS assessment, were conducted by the research team. Researchers were not blinded to the treatment.

In a subsample of approximately 10 close-up cows per week, 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.

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 metritis (Sheldon et al., 2006), score 4. Cows with score 4 were diagnosed with metritis and mucopurulent (>50% pus present) as score 3, and purulent (≤50% pus present) as score 2, foul smelling pus was categorized as score 1, foul smelling and mucous was categorized as score 0, mucus with flecks of blood, 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 metritis (Sheldon et al., 2006).

Blood Sampling and Analyses of Minerals and Vitamin D Metabolites

At 0800 h, all cows enrolled were restrained in headlocks. Blood samples were drawn on 1 (within 24 h after calving), 2, 3, 5, 7, and 10 DIM. Using a serum blood collection system comprising a sterile needle (Sterican, 1.20 × 40 mm, B. Braun Melsungen AG) and a 9-mL tube without anticoagulants (Sarstedt AG & Co.), all blood samples of cows were drawn from the coccygeal vessels. Blood samples were kept at room temperature (20–25°C) for about 30 min to clot. Thereafter, samples were centrifuged at 2,000 × g and 20°C for 20 min to harvest serum (Heraeus Sepatech Labofuge 200, Heraeus Holding GmbH). Serum was transferred into 5-mL tubes (Sarstedt AG & Co.) and frozen at −20°C. The concentrations of tCa, inorganic phosphate (P_i), and Mg were determined in the laboratory of the Ruminant Clinic, Freie Universität Berlin, Germany using a Cobas 8000c701 (Roche Diagnostics International AG) and test kits LT-Ca 0100, LT-P 0100, and LT-MG 0103, respectively (Labor + Technik Eberhard Lehmann GmbH). The interassay coefficients of variation were 4.15% (Ca = 2.17 mmol/L; n = 30), 3.24% (P_i = 1.74 mmol/L; n = 30), 7.51% (Mg = 0.88 mmol/L; n = 23). The intraassay coefficients of variation were 2.57% (Ca = 2.17 mmol/L; n = 30), 2.10% (P_i = 1.74 mmol/L; n = 30), and 2.85% (Mg = 0.88 mmol/L; n = 23).

For a subsample of 30 cows entering their third lactation, we quantified the concentrations of 4 vitamin D metabolites. The concentrations of 1,25-dihydroxycholecalciferol (1,25-[OH]_2D_3) were determined by a commercial laboratory (Immundiagnostik AG, Bensheim, Germany) using the ELISA method. Intra- and inter-assay coefficients were 6.69% and 9.00%, respectively. The lower detection level was 4.80 pg/mL.

Concentrations of cholecalciferol, 25-OHD_3, and 24,25-dihydroxycholecalciferol (24,25-[OH]_2D_3) were determined at the Institute of Agricultural and Nutritional Sciences (Martin Luther University Halle-Wittenberg, Halle/Saale, Germany) by HPLC-MS/MS in modification to Baur et al. (2019). Briefly, 200 µL of serum was mixed with 150 µL of potassium hydroxide (10 M), 60 µL of ascorbic acid (78.8 mg/mL), 10 µL of sodium sulfide (2.125 mg/mL), 50 µL of the internal standard (80 ng/mL d_5-D_3, 150 ng/mL d_3-25-OHD_3, and 200 ng/mL d_6-24,25-(OH)_2D_3) and 450 µL of 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 of n-hexane and 4 mL of n-hexane/tert-butyl methyl ether. The effluent was collected and mixed with 1 mL of water, after 2 min the underlayer was suctioned off. The samples were dried under vacuum (RVC 2-25 CDplus, Christ,
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, 6 VitD, and 12 VitD 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, 1,25-(OH)2D3 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. Explanatory variables tested in the univariable models were treatment (CON vs. 6 VitD vs. 12 VitD), time (1, 2, 3, 5, 7, and 10 DIM for blood minerals and cholecalciferol, 25-OHD3, and 24,25-(OH)2D3 concentration; 1, 2, 3, and 5 DIM for 1,25-(OH)2D3 concentration; test d 1, 2, 3, 4, and 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 third 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 chi-squared test.

RESULTS

Of the 158 multiparous Holstein Friesian cows, 52, 52, and 54 cows were allocated to the CON, 6 VitD, and 12 VitD group, respectively. Parity (P = 0.47), energy-corrected 305-d 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 × 10^6 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), BCS 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 12 VitD 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; \( P < 0.05 \); 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 P\(_i\) 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 P\(_i\) concentrations during the first 10 DIM. Furthermore, 6VitD cows had greater serum P\(_i\) 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 2C; 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).
Table 2. Effect of cholecalciferol treatment 5 d before expected parturition (on d 275 ± 1.2 [SD] 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 third lactation (n = 30)$^1$

<table>
<thead>
<tr>
<th>Vitamin D metabolite</th>
<th>Fixed effects, $P$-value</th>
<th>CON</th>
<th>6VitD</th>
<th>12VitD</th>
<th>Treatment</th>
<th>Time</th>
<th>Treatment by treatment$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholecalciferol (ng/mL)</td>
<td>&lt;LOQ$^3$</td>
<td>122.6$^a$</td>
<td>25.5</td>
<td>195.5$^b$</td>
<td>27.3</td>
<td>&lt;0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>25-OHD$_3$ (ng/mL)</td>
<td>22.5$^a$</td>
<td>4.6</td>
<td>111.6$^b$</td>
<td>4.3</td>
<td>188.5$^c$</td>
<td>4.1</td>
<td>0.08</td>
</tr>
<tr>
<td>24,25-(OH)$_2$D$_3$ (ng/mL)</td>
<td>1.7$^a$</td>
<td>0.7</td>
<td>10.7$^b$</td>
<td>0.6</td>
<td>18.6$^c$</td>
<td>0.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1,25-(OH)$_2$D$_3$ (pg/mL)</td>
<td>76.8$^a$</td>
<td>4.6</td>
<td>63.0$^b$</td>
<td>4.3</td>
<td>59.4$^c$</td>
<td>4.5</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

$^a$–$^c$Different letters indicate differences among treatment groups at $P < 0.05$.

$^1$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. LSM and 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).

$^2$The interaction of time by treatment was forced to remain in the model, as this led to a lower Akaike information criterion.

$^3$LOQ = limit of quantification.

Figure 2. Effect of cholecalciferol treatment 5 d before expected parturition (on d 275 ± 1.2 [SD] of gestation) on serum cholecalciferol (A), 25-hydroxycholecalciferol (B; 25-OHD$_3$), 24,25-dihydroxycholecalciferol (C; 24,25-[OH]$_2$D$_3$), and 1,25-dihydroxycholecalciferol (D; 1,25-[OH]$_2$D$_3$) concentrations of multiparous dairy cows entering their third lactation (n = 30) of a single dairy farm. Least squares means and SEM were derived from repeated measures ANOVA of cows treated with 12 × 10$^6$ IU cholecalciferol (12VitD, n = 10, dark blue), cows treated with 6 × 10$^6$ 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 (A). Serum cholecalciferol concentrations of 6VitD and 12 VitD cows were associated with time ($P < 0.01$) and treatment ($P = 0.05$). Serum concentrations of 25-OHD$_3$ were associated with time ($P = 0.08$), treatment ($P < 0.01$), and BCS at enrollment. Serum concentrations of 24,25-(OH)$_2$D$_3$ were associated with time ($P < 0.05$) and treatment ($P < 0.01$). Serum concentrations of 1,25-(OH)$_2$D$_3$ were associated with time ($P < 0.01$) and treatment ($P < 0.05$). In all models, time × treatment was forced to remain in the model, as this led to a lower Akaike information criterion. $P = 0.69$, $P = 0.39$, $P = 0.77$, and $P = 0.13$ for the models of cholecalciferol, 25-OHD$_3$, 24,25-(OH)$_2$D$_3$, and 1,25-(OH)$_2$D$_3$, respectively.)
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. Although 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 6VitD cows, 12VitD cows had a higher incidence of RP (29.6%; n = 16; P < 0.01) and metritis (31.5%; n = 17; P < 0.01) compared with CON cows.

The effect of treatment on milk production was evaluated over the first 5 DHIA equivalent test days. Mean DIM at first test were 24.1 (n = 50), 22.8 (n = 46), and 26.1 (n = 49) for CON, 6VitD, and 12VitD cows, respectively. Mean DIM at second 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 third 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 fourth 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 fifth 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 CON group (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$_3$. The 12VitD treatment and to lesser extent also 6VitD treatment, increased tCa and P$_i$ concentrations and decreased Mg concentration. In 12VitD cows, GL and milk yield were reduced, whereas the incidence of 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 and 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 P$_i$ 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-OHD$_3$ 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 of 25-OHD$_3$, 3 mg of 25-OHD$_3$, 1 mg of cholecalciferol, and 3 mg of cholecalciferol, respectively. The authors reported increased postpartum tCa with 1 mg or 3 mg of oral 25-OHD$_3$ (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 P₁ 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 P₁ likewise. The reduction in serum Mg could have been caused by a decrease in renal reabsorption due to lower secretion of PTH 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 P₁ 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 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 the 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 P₁.

**Vitamin D Metabolites**

The formation of 1,25-(OH)₂D₃ out of 25-OHD₃ is subject to a strict regulation by several factors such as serum Ca, directly and indirectly via PTH, P₃, 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 and 25-OHD₃.

Inactivation of 1,25-(OH)₂D₃ and 25-OHD₃ is initiated by a further hydroxylation at position 24 (Jones et al., 2012). The enzyme crucial for this transformation, cytochrome P450 isoenzyme 24A1 (CYP24A1), is activated by Ca, P₃, 1,25-(OH)₂D₃, and inhibited by PTH (Dusso et al., 2005). In sheep, we could show that increased plasma concentrations of 25-OHD₃ induced by an oral administration of 25-OHD₃ resulted in a 22-fold upregulation of renal RNA expression of CYP24A1 (Wilkens et al., 2016). In the present study, serum concentrations of 24,25-(OH)₂D₃ were elevated by the treatment (P < 0.01) during the entire observation period (Figure 2D) indicating a stimulated inactivation of vitamin D metabolites.

**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 × 10⁶ 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 (Poindexter et al., 2023b).

**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, Noyola-Martínez 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 con-
centrations 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 CYP3A50 enzymes or by competing with cholesterol as substrate for CYP11A1. The difference between our study and the experiment done by Poindexter et al. (2023a,b) 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,b) 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 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 eucalcemic 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 Limitations**

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 larger 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.

**CONCLUSIONS**

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 it 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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**REFERENCES**


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