ABSTRACT

Large parenteral doses of vitamin D3 (15 to 17.5 x 10^6 IU vitamin D3) were associated with prolonged hypercalcemia, hyperphosphatemia, and large increases of vitamin D3 and its metabolites in the blood plasma of nonlactating nonpregnant and pregnant Jersey cows. Calcium concentrations 1 day postpartum were higher in cows treated with vitamin D3 about 32 days prepartum (8.8 mg/100 ml) than in control cows (5.5 mg/100 ml). None of the cows treated with vitamin D3 showed signs of milk fever during the peripartal period; however, 22% of the control cows developed clinical signs of milk fever during this period. Signs of vitamin D3 toxicity were not observed in nonlactating nonpregnant cows; however, pregnant cows commonly developed severe signs of vitamin D3 toxicity and 10 of 17 cows died. There was widespread metastatic calcification in the cows that died. Because of the extreme toxicity of vitamin D3 in pregnant Jersey cows and the low margin of safety between doses of vitamin D3 that prevent milk fever and doses that induce milk fever, we concluded that vitamin D3 cannot be used practically to prevent milk fever when injected several weeks prepartum.

INTRODUCTION

Vitamin D has been used to prevent parturient hypocalcemia (PH) or milk fever in dairy cows for many years (4, 14, 15, 16). A variety of routes of administration and concentrations of the vitamin has been tried. Also, a variety of times and durations of administration of vitamin D2 and D3 during the preparturn period has been tried. For instance, feeding 20 to 30 x 10^6 IU of vitamin D2 daily for a maximum of 7 days starting 3 to 8 days prepartum or 3 x 10^6 IU of vitamin D2 daily throughout the year effectively reduced the incidence of PH (13, 14, 15). However, feeding 30 x 10^6 IU of vitamin D2 daily from 20 days before to 1 day after parturition induced extreme toxicity in a Jersey cow (6). Parenteral administration of 10 x 10^6 IU D3 2 to 3 days before expected date of parturition reduced the incidence of PH in cows with a history of PH (20, 21). However, parenteral administration of 10 x 10^6 IU of vitamin D3 daily during the last 10 days of gestation resulted in clinical and pathological toxicity (5, 21, 31). Likewise, parenteral administration of 15 to 20 x 10^6 IU of vitamin D3 in divided doses 30 days prepartum was toxic and produced high mortality (22). Recent findings indicated parenteral administration of 15 x 10^6 IU of vitamin D3 to dry nonpregnant cows resulted in large increases in concentrations of 1α,25-dihydroxyvitamin D [1,25-(OH)2D] in blood plasma beginning about 2 wk after administration (19). This increase in 1,25-(OH)2D persisted for 1 to 3 wk, depending on the dose. This observation prompted us to evaluate the effectiveness of parenteral vitamin D3 given 3 to 6 wk prepartum as a method for prevention of PH. This report describes the response of nonlactating pregnant and nonpregnant cows to massive doses of vitamin D3 administered intramuscularly.

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1 Mention of a trade name, proprietary product, or vendor does not constitute a guarantee or warranty by the US Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may be suitable.

2 Lack of a 2 or 3 subscript implies both the D2 and D3 forms of vitamin D.
MATERIALS AND METHODS

Animals

Blood samples (50 ml) were collected from external jugular veins of 42 Jersey cows by venipuncture with heparinized syringes (20,000 IU/ml). Samples were centrifuged; plasma was collected and stored at -15°C within 2 h after collection.

Analyses

Concentrations of calcium (Ca) and magnesium (Mg) in plasma were measured by atomic absorption spectroscopy (24). Concentrations of vitamin D2 and D3; 25-OH-vitamin D (25-OHDD); 24,25-dihydroxyvitamin D3 [24,25-(OH)2D3]; 25,26-dihydroxyvitamin D3 [25,26-(OH)2D3]; 1α,25-dihydroxyvitamin D [1,25-(OH)2D]; and 25-hydroxyvitamin D3-26,23 lactone (25-OHD3-26,23 lactone) were determined (17, 18, 20). Hydroxyproline in plasma (2) and total inorganic phosphate (Pi) (8) were measured. Concentrations of parathyroid hormone in plasma were determined (1).

EXPERIMENTAL PROCEDURE

Experiment 1a – Nonlactating Nonpregnant Cows

Six mature nonlactating nonpregnant Jersey cows were injected im with 15 x 10⁶ IU D₃ in 5 ml of ethanol. A second injection was given 7 days later, and each cow received one of the following doses: 0, 1 x 10⁵, 1 x 10⁶, 2 x 10⁶, 5 x 10⁶, or 10 x 10⁶ IU vitamin D₃ in 5 ml of ethanol. One additional cow was a control and received a placebo of 5 ml of ethanol for both injections. Cows were housed under confinement conditions throughout the experiment, fed good-quality alfalfa hay and 2 g of a commercial grain mix, and given access to a mineralized salt block. It was calculated that the cows were consuming 80 to 90 g of Ca and 30 to 40 g of P daily. Blood samples were taken at the intervals indicated in Figures 1 to 8. Six months after their first dose of vitamin D₃, cows were necropsied and their tissues were examined grossly for ectopic calcification.

Experiment 1b – Nonlactating Pregnant Cow from the Experimental Herd

A nonlactating pregnant Jersey cow with four lactations from the herd maintained at the National Animal Disease Center was injected im with 5 ml of ethanol:propylene glycol (30:70, vol/vol) containing 15 x 10⁶ IU of vitamin D₃ at 32 days before expected date of parturition. Seven days later, an additional 5 x 10⁶ IU vitamin D₃ was injected similarly. Blood samples were taken at the times indicated in Figure 9.

The cow was fed good-quality alfalfa hay cubes free choice and 2 kg of a commercial dairy concentrate daily until parturition and alfalfa hay cubes free choice and 5 kg of concentrate daily after parturition. Cows were consuming 80 to 90 g of Ca and 30 to 40 g of P daily prior to parturition.

Experiment 2 – Nonlactating Pregnant Cows from Five Commercial Herds

A total of 35 Jersey cows with an average age of 6.5 yr were selected from five herds (Table 1). All cows had a minimum of two lactations. The history of milk fever of cows with three lactations or more was about 50% with a range of 25 to 80%. Six of 18 cows assigned to the control group and 9 of 17 cows in the D₃-treated group had a history of milk fever (Table 1).

Cows from each herd were divided into two major treatment groups (control and vitamin D₃ groups) by alternating treatment groups according to scheduled date of calving. Eighteen control cows were injected im with 5 ml of a sterile ethanol:propylene glycol mixture (30:70, vol/vol) 32 days before expected date of calving. One week later, an additional 2 ml of this same mixture was given.

Seventeen vitamin D₃-treated cows were given injections similar to those given to the control cows, except that the first injection contained 15 x 10⁶ IU vitamin D₃ and the second injection contained 2.5 x 10⁶ IU vitamin D₃. The last four of the vitamin D₃-treated cows scheduled to calve received a placebo injection as the second injection.

Dry cows in all five herds were fed a similar diet. Roughage was corn silage (~7 kg/day per cow) and alfalfa hay (~5 kg/day per cow). Two to 4 kg of concentrate was fed daily to each cow.


RESULTS

Experiment 1a —
Nonlactating Nonpregnant Cows

Magnitude and duration of changes in plasma Ca, Pi, Mg, and hydroxyproline were similar for cows in this experiment regardless of the dose of the second injection. Therefore, data in Figures 1, 2, 3, and 4 represent the mean ± SE of concentrations of Ca, Mg, Pi, and hydroxyproline in blood plasma of 6 cows injected with vitamin D₃. Shaded areas on these figures represent the overall average standard error of the mean of the combined data from all the cows treated with vitamin D₃ during the control sampling period (days —3, —2, and 0). Means for Ca, Mg, Pi, and hydroxyproline of the single uninjected cow that was sampled throughout the experiment fell within the shaded areas in Figures 1, 2, 3, and 4.

Concentrations of Ca in plasma remained fairly constant for several days following the initial injection of vitamin D₃, as in Figures 1 and 2. Hypercalcemia (~20% at lowest point) and hyperphosphatemia (~60% at peak) were observed from day 10 until about 90 days after initial injection of vitamin D₃. The marked, prolonged hypomagnesemia (~40% at lowest point) evident in the same cows (Figure 3) was most pronounced about 30 days after initial injection of vitamin D₃. After an initial decline, mean concentrations of hydroxyproline remained low (~10% decrease) during the first 10 days following the injection of vitamin D₃.

Figure 1. Mean ± standard error of the mean (bars) of plasma calcium concentrations of 6 nonlactating Jersey cows in Experiment 1a injected intramuscularly with 1.5 × 10⁶ IU vitamin D₃ on day 0 and one of the following: 0, 1 × 10⁵, 1 × 10⁶, 2 × 10⁶, 5 × 10⁶, 10 × 10⁶ IU vitamin D₃ on day 7.
days after vitamin D₃ injections (Figure 4) but then rose steadily thereafter to peak at about 40 to 50 days and remained above (~80% at peak) the initial concentration for the remainder of the sampling period (to 90 days).

In Figures 5 to 9, individual concentrations of vitamin D and its metabolites are plotted, and the shaded area represents the mean ± SE of the six cows injected with vitamin D₃. Vitamin D₃ concentrations peaked (150 to 300-fold increase) at about 10 to 12 days (Figure 5) and did not return to normal until at least 90 days after the initial injection. Plasma 25-OHD₃ and 24,25-(OH)₂D₃ concentrations were high (300 to 900% increase) in most cows between 30 and 60 days (Figures 6 and 7). By 90 days, 25-OHD₃ concentrations had returned to their initial concentrations. The 24,25-(OH)₂D₃ concentration remained elevated (300 to 400% increase) for the duration of the experiment. Plasma 25-OHD₂ concentrations decreased drastically after vitamin D₃ injections (Figure 8) and did not return to initial concentrations during the sampling period.

Concentrations of 1,25-(OH)₂D in plasma showed an increase after 10 days, and this lasted until 90 days after the initial injection of vitamin D₃ with the mean peak about 9-fold the initial concentrations at 28 days (Figure 9). Plasma 1,25-(OH)₂D₃ concentration appeared unaffected by the second injection of vitamin D₃.

The cow receiving 10 × 10⁶ IU vitamin D₃ as a second injection showed signs of toxicity. She had about a 50% reduction in feed consumption that persisted for several days. Thereafter, she appeared normal. At necropsy, calcification was in kidney and aorta. Neither clinical signs of toxicity nor soft tissue calcification was in the other 5 cows.

Experiment 1b — Nonlactating Pregnant Cow from the Experimental Herd

Responses of a pregnant Jersey cow given 15 × 10⁶ IU vitamin D₃ 32 days before the
VITAMIN D₃ TOXICITY IN DAIRY COWS

Figure 5. Plasma vitamin D₃ concentrations of 6 nonlactating Jersey cows in Experiment 1a injected intramuscularly with 15 × 10⁶ IU vitamin D₃ on day 0 and one of the doses of vitamin D₃ noted in the figure on day 7. The control cow (A- - -A) received only placebo injections (5 ml ethanol) on day 0 and day 7.

expected date of parturition and a second injection (5 × 10⁶ IU D₃) 1 wk after the first injection are in Figures 10 to 14.

As in Figure 10, Ca concentrations of plasma were increased (~25%) between days 10 and 23, decreased as the udder began to fill preparum, and remained between 9.5 and 10.0 mg/100 ml throughout the peripartal and postpartum periods. Changes in concentration of plasma P₁ (Figure 10) were similar to changes in concentration of plasma Ca. Plasma PTH (Figure 10) concentrations decreased as plasma Ca increased with only slight, if any, increase at parturition, and concentrations were low during the postpartum period.

Plasma vitamin D₃ (Figure 11), 25-OHD₃ (Figure 11), 24,25-(OH)₂D₃ (Figure 12), 1,25-(OH)₂D₃ (Figure 13), and hydroxyproline (Figure 13) were elevated within a few days after injection of vitamin D₃. Plasma 25-OHD₃-26,23 lactone concentrations (Figure 14) increased about 2 wk after the initial injection of vitamin D₃, peaked at about 20 times initial concentrations a few days after parturition, and remained at about 15 times initial concentrations at 48 days. The increase in these blood constituents was similar in time and duration to those of nonlactating, nonpregnant cows of Experiment 1a.

No clinical signs of toxicity were observed throughout lactation.

Experiment 2 - Nonlactating Pregnant Cows from Five Commercial Herds

Nonlactating pregnant cows from five commercial herds were treated with vitamin D₃ similarly to the cow in Experiment 1b to evaluate the effect of superimposing time of maximal stimulation of bone and gut on time of initiation of lactation.

Average duration from first vitamin D₃ injection to calving was 32 ± 1.1 days (Table

Figure 6. Plasma 25-OH-vitamin D₃ (25-OHD₃) concentrations of 6 nonlactating cows in Experiment 1a injected intramuscularly with 15 × 10⁶ IU vitamin D₃ on day 0 and one of the doses of vitamin D₃ noted in the figure on day 7. The control cow (A- - -A) received only placebo injections (5 ml ethanol) on day 0 and day 7.

Figure 7. Plasma 24,25-dihydroxyvitamin D₃ [24,25-(OH)₂D₃] concentrations of 6 nonlactating cows in Experiment 1a injected intramuscularly with 15 × 10⁶ IU vitamin D₃ on day 0 and one of the doses of vitamin D₃ noted in the figure on day 7. The control cow (A- - -A) received only placebo injections (5 ml ethanol) on day 0 and day 7.

None of the vitamin D3-treated cows developed PH even though 53% of them had a history of that disease whereas 22% of control cows developed PH. Thirty-three percent of the control cows had a history of PH.

Changes in plasma Ca, Mg, vitamin D3, 25-OHD3, 24,25-(OH)2D3, 25-OHD3-26,23-lactone, and 1,25-(OH)2D of the two groups of cows about 24 h after calving are in Table 2. Mean Ca concentrations were much higher in cows treated with vitamin D3 (8.8 ± .6 mg/100 ml) than in control cows (5.5 mg/100 ml). This is consistent with the occurrence of PH in control cows. Plasma Mg concentrations were higher in control cows (2.90 ± .15) than in vitamin D3-treated cows (1.90 ± .12), which is consistent with the hypermagnesemia that commonly accompanies PH. Plasma vitamin D3, 25-OHD3, and 24,25-(OH)2D3 concentrations in vitamin D3-treated cows were, respectively, 8, 4, and 10 times those of control cows. Plasma 25-OHD3-26,23 lactone concentrations were undetectable in the control cows; however, treated cows had mean concentrations of 13.3 ± 2.6 ng/ml of this metabolite. Mean 1,25-(OH)2D concentrations in the vitamin D3-treated cows were slightly lower than in the control cows. The severe hypocalcemia of the
control cows would be expected to result in elevated plasma 1,25-(OH)₂D₃, and vitamin D₃ injections in the treated groups, even in the absence of hypocalcemia, would be expected to increase (2×) 1,25-(OH)₂D (15).

Ten of 17 cows injected with vitamin D₃ died (Table 2). Death occurred on the average of 16 days after parturition, which was 48 days following the first injection of vitamin D₃ (Table 1). Eight of the 10 cows that died showed clinical signs of vitamin D₃ toxicity for several weeks prior to death. The other two cows died suddenly during the prepartum period without showing clinical signs of vitamin D₃ toxicity. None of the control cows died.

Clinical Manifestations of Toxicity

The first sign of vitamin D toxicity was inappetence between wk 2 and 3 after initial injection of vitamin D₃. Severe anorexia often persisted for several weeks before death, and the anorexia resulted in severe weight loss. These cows developed a pasty discharge about the eyes and flaccid udders. Some respiratory distress was evident at this time, which included polypnea with labored expiration. Near the time of death, polypnea, rapid pounding pulse, and a ketotic breath were observed. The cows were weak, recumbent, and often showed torticollis and a febrile response (≥107°F).

Animals less severely affected had delayed shedding of their winter hair coat, which had a rough, dry appearance. Inappetence and reduced milk production were observed during the postpartum period. During the latter part of the experimental period, the cows rose with difficulty because of apparent muscle and joint stiffness. Consequently, many of the cows were recumbent much of the time. Excessive water consumption was a common sign. About 50% of the cows that lived for several weeks after the injections developed an accumulation of air under the skin of the neck and backs. Crepitation of the skin in these areas could be detected.

DISCUSSION

Results of Experiment 1a show effects of
large intramuscular doses of vitamin D₃ given to nonlactating nonpregnant Jersey cows. The peak plasma concentration of vitamin D₃ occurred 20 to 30 days before the peak concentration of 25-OHD₃ (Figures 5 and 6). This relationship suggests that the liver initially may be concentrating vitamin D₃ followed by its release upon 25-hydroxylation as suggested (25); or the accumulation of 25-OHD₃ in the plasma initially may be inhibiting liver 25-hydroxylase activity (3); or accumulation may be from a change in 25-hydroxylase affinity for vitamin D₃ (10). There may have been competition between vitamin D₂ and vitamin D₃ for 25-hydroxylation as evidenced by the declining plasma 25-OHD₂ following vitamin D₃ injections (Figure 8).

No clinical signs of vitamin D₃ toxicity were evident in any of the cows in Experiments 1a or 1b despite hypercalcemia and hyperphosphatemia that persisted for several months. Persistent hypercalcemia and hyperphosphatemia were associated with prolonged increases of vitamin D₃ and its metabolites. In addition to the well-documented hypercalcemic and hyperphosphatemic actions of 1,25-(OH)₂D₃, high concentrations of vitamin D₃, 25-OHD₃, and 24,25-(OH)₂D₃ are reported to have biologic activity and binding affinities for intestinal cytosol receptor protein (7). Therefore, the biologic effects of high concentrations of vitamin D₃, 25-OHD₃, and 24,25-(OH)₂D₃ may have contributed in part to the hypercalcemia and hyperphosphatemia. Also, we have isolated two other vitamin D₃ metabolites [1,24,25-(OH)₃D₃ (27) and 1,25,26-(OH)₃D₃ (28, 29)] from the plasma of cows given large parenteral doses of vitamin D₃. These trihydroxylated vitamin D₃ metabolites possess biological activity in cows and, thus, also could contribute to the hypercalcemia and hyperphosphatemia (K. Hove et al., unpublished data).

Plasma 25-OHD₃-26,23 lactone was increased in cows treated with vitamin D₃ in Experiments 1a and 2 (Figure 14; Table 2). This metabolite is normally undetectable in dairy cows (R. L. Horst et al., unpublished) but is present following vitamin D₃ injections in cows (17). No function has yet been proven for this metabolite.

Prolonged increases in plasma 1,25-(OH)₂D₃ concentrations in cows following vitamin D₃ administration reported by us (15) were con-
firmed in this experiment (Figure 9). This response appears unique to the cow, and we sought to capitalize on this response to develop an effective preventative treatment for milk fever. Since plasma 1,25-(OH)₂D peaked about 1 mo after initial injection of vitamin D₃, and the hypercalcemic response (Figure 1) as well as the hydroxyproline response (as indicator of bone resorption) were also near maximum at this time, we sought to optimize the effect of injection of vitamin D₃ by giving it 32 days before parturition (Figure 4). This is several weeks sooner than the currently recommended treatment schedules (13, 15, 21, 23). The use of our treatment schedule, therefore, should result in optimal stimulation of both gut absorption of Ca and bone resorption of Ca and should protect against hypocalcemia even in inappetent cows. When this scheme was tried in a preliminary experiment with a pregnant cow, discussed in Experiment 1b (Figures 10 to 14), no ill effects were observed and the cow appeared well protected against parturient hypocalcemia, as indicated by maintenance of relatively high Ca, Pi, hydroxyproline, and 1,25-(OH)₂D concentrations during the immediate postpartum period.

However, in further experiments with pregnant Jersey cows managed under field conditions (Experiment 2), administration of similar amounts of vitamin D₃ about 32 days preparum resulted frequently in extreme toxic manifestations and death in 10 of 17 cows. The prolonged time between injection and parturition appeared to maximize both the biologic response and the toxic effects of vitamin D₃. Usual recommended procedures are to administer 10 × 10⁶ IU of vitamin D₃ about 1 wk before the expected date of parturition and to repeat the injections, if necessary, at 8-day intervals until three injections have been given (21). The effectiveness of this latter procedure apparently relies on the transient increase in plasma 1,25-(OH)₂D that follows the vitamin D₃ injection (26). The lack of reported toxicity with this method may be partly from lack of large changes in vitamin D₃ metabolites for 10 to 14 days following injections of vitamin D₃ (10) and the large drain of calcium and phosphate induced by lactation that may provide some protection against development of hypercalcemia and hyperphosphatemia and, thus, metastatic calcification. The possibility exists that vitamin D₂ and vitamin D₃ exhibit different toxicity potential in cows due to differences in metabolic clearances or metabolism. If this is so, it might explain the apparent lack of toxicity in some of the studies utilizing vitamin D₂ (14, 15, 16). Also, inasmuch as there is a large discrepancy between the amount of vitamin D required to induce toxicity orally compared to that required when given parenterally, extensive degradation of vitamin D in the gastrointestinal tract of the cows must be present. We recently have demonstrated that extensive degradation of vitamin D₃ occurs in the rumen contents of cows (J. L. Sommerfeldt, unpublished).

In this study, pregnant cows were more prone to vitamin D₃ toxicity than nonlactating nonpregnant cows. This phenomenon may be a result of differences in vitamin D₃ metabolite concentrations between the two groups of cows. Pregnant cows had higher mean 25-OHD₃ and 24,25-(OH)₂D₃ concentrations than nonpregnant cows after vitamin D₃ treatment (compare Table 2 with Figures 6 and 7). Differences in susceptibility to vitamin D₃ toxicity, however, are more likely a result of different sensitivities of target organs to 1,25-(OH)₂D₃, the active form of vitamin D₃. Target organs (bone and intestine) may be more sensitive to 1,25-(OH)₂D₃ in pregnant than nonpregnant cows. The combination of more sensitive target organs and high plasma concentrations of 1,25-(OH)₂D₃ as a result of vitamin D₃ injection would result in more extensive calcification. One other possible explanation of this difference between pregnant and nonpregnant cows would be the placental production of 1,25-(OH)₂D₃ in pregnant cows as described in the rat (11, 32).

Increases in plasma hydroxyproline were indicative of increased bone resorption following administration of vitamin D₃. The prolonged hypercalcemia, in spite of inappetence in many of the cows, supports this assumption. This assumption is consistent with the development of "hypervitaminosis rickets" described in rats and rabbits (9, 12, 30) but conflicts with the report that cows with hypervitaminosis D showed no evidence of increased bone resorption and had exceedingly well mineralized bone (5).

We conclude that administration of large amounts of vitamin D₃ in cows results in a
delayed and prolonged production of most known metabolites of vitamin D, including 1,25-(OH)2D. This treatment is associated initially with a mild hypocalcemia and hypophosphatemia followed by a prolonged period (several months) of hypercalcemia, hyperphosphatemia, and hypomagnesemia. Pregnant cows seem especially susceptible to development of clinical signs of vitamin D3 toxicity when this treatment is administered about 1 mo before parturition. Both the biologic and toxic effects are maximized when the treatment with vitamin D3 precedes parturition by several weeks. We conclude it is unlikely that treatment with vitamin D3 prepartum in this manner can be utilized practically or safely to prevent milk fever because of the resulting toxicity. In subsequent studies (E. T. Littledike and R. L. Horst, published data), substantially lower doses of vitamin D3 given several weeks prepartum avoided toxic manifestations of vitamin D3; however, 6 of 6 cows treated in this manner developed severe hypocalcemia (3 to 4 mg Ca/100 ml) and clinical signs of milk fever postpartum following this treatment. Because of the depressed plasma 1,25-(OH)2D3 concentration associated with the severe hypocalcemia, it is probable that a long-lasting block of 1-hydroxylase was present. We conclude that the margin of safety between toxic and ineffective doses of parenteral vitamin D3 is too narrow to allow use of vitamin D3 in the manner reported for prevention of milk fever in dairy cows.

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