Influence of the combination of 25-hydroxyvitamin D₃ and a diet negative in cation-anion difference on peripartal calcium homeostasis of dairy cows

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ABSTRACT

Around parturition, many dairy cows experience varying degrees of hypocalcemia, which increases the incidence of several diseases in early lactation. In the current study, an established concept of feeding a diet negative in cation-anion difference (DCAD) was combined with oral supplementation of 25-hydroxyvitamin D₃ (25-OHD₃) from d 270 of gestation until parturition. Fifty-six dairy cows were divided into 2 feeding groups (low DCAD and control). Fourteen animals of each group received a daily dosage of 3 mg of 25-OHD₃. From the beginning of the treatment to d 10 after parturition, plasma samples for analysis of 25-OHD₃, 1,25-dihydroxyvitamin D₃, parathyroid hormone (PTH), Ca²⁺, phosphate, the bone resorption marker CrossLaps, and osteocalcin were collected every other day, at calving, and at 6, 12, and 24 h after calving. Urine samples for determination of macrominerals and measures of acid-base status were collected on d 6 of treatment and on d 6 after calving. The induction of a compensated metabolic acidosis by the animals on the DCAD diet could be demonstrated by decreased urinary pH. A linear correlation between treatment duration and the plasma concentration of 25-OHD₃ indicated effective absorption of 25-OHD₃ in supplemented animals. The mean plasma concentrations of Ca²⁺ from d –4 prepartum to d 4 postpartum were significantly higher in animals treated with the combination of the low DCAD diet and 25-OHD₃ supplementation (1.24 ± 0.02 mmol/mL) compared with the 3 other groups (low DCAD: 1.17 ± 0.02 mmol/mL; control diet plus 25-OHD₃: 1.16 ± 0.02 mmol/mL; control diet: 1.18 ± 0.02 mmol/mL). We postulate that the increased tissue responsiveness to parathyroid hormone induced by the low DCAD is crucial for the observed positive effects of the 25-OHD₃ treatment.

Key words: hypocalcemia, 25-hydroxyvitamin D, prevention, dairy cow

INTRODUCTION

Parturient paresis, also known as milk fever, is one of the most frequently occurring diseases in periparturient dairy cows. A meta-analysis of field studies from 1977 to 2009 found that incidence was 3.45 and 3.50% in North American and Australasian studies, respectively, and 6.17% in European studies (DeGaris and Lean, 2008). Milk fever increases the risk of other diseases, such as dystocia, placental retention, mastitis, displacement of the abomasum, and ketosis (Curtis et al., 1983).

Subclinical hypocalcemia (defined as serum Ca concentrations <2 mmol/L), which has a reported incidence of between 25 and 54%, depending on the lactation number (Reinhardt et al., 2011), also has effects on various physiological functions as indicated by reduced abomasal and ruminal motility (Daniel, 1983), impaired smooth muscle function (Al-Eknaib and Noakes, 1989), and increased blood concentrations of NEFA (Reinhardt et al., 2011), all without clinical signs of milk fever.

A concept that aims at the activation of regulatory mechanisms of Ca homeostasis during the last days of gestation is the negative DCAD theory of milk fever prevention. Feeding a diet high in strong anions, widely known as “feeding anionic salts,” has been used to prevent hypocalcemia for the last 40 yr (Ender et al., 1971). The exact mechanisms have not been identified. One possible explanation is that the metabolic acidosis induced by such diets leads to increased tissue responsiveness to parathyroid hormone (PTH; Goff and Horst, 2003). Parathyroid hormone stimulates the mobilization of Ca from the skeleton and decreases renal Ca excretion in monogastric animals (Ramasamy, 2006). Although an effect of acidifying salts on bone resorption was demonstrated in cows (Abu Damir et al., 1994), the renal excretion of Ca was increased in balance studies, which was interpreted as excretion of excess Ca (Oetzel et al., 1991). Furthermore, PTH enhances the conversion of 25-hydroxyvitamin D₃ (25-OHD₃) to 1,25-dihydroxyvitamin-D₃ [1,25-(OH)₂D₃], the biologically active metabolite of vitamin D that stimulates the gastrointestinal absorption of Ca (Ra-

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Table 1. Distribution of cows by lactation numbers¹

Lactation number	Control diet		Low DCAD diet	
	No treatment	Hy-D	No treatment	Hy-D
2	6	8	4	7
3	7	4	5	1
4 to 8	2	2	4	6

¹Cows on a control ration (control), untreated cows on a diet negative in cation-anion difference (low DCAD), cows on a control ration and treated with 3 mg of 25-hydroxyvitamin D₃ (25-OHD₃) daily from d 270 of gestation until parturition (control Hy-D), and cows on low DCAD diet combined with the 25-OHD₃ treatment (low DCAD Hy-D).

masamy, 2006). Increased plasma concentrations of 1,25-(OH)₂D₃ after feeding a diet negative in DCAD were reported (Abu Damir et al., 1994).

The direct application of 1,25-(OH)₂D₃ effectively increased plasma Ca concentrations (Gast et al., 1979). However, because 1,25-(OH)₂D₃ has a relatively short half-life, it is necessary to predict the calving date rather precisely to ensure beneficial effects on Ca balance.

Other studies that investigated the effect of administered 25-OHD₃, the metabolite with a much longer half-life (Jones, 2008), provide conflicting data. The incidence of parturient paresis was decreased in pregnant cows after parenteral administration of 25-OHD₃ (Jorgensen et al., 1978), whereas a hypercalcemic effect could not be demonstrated after oral administration (Taylor et al., 2008).

A concerted action of the regulatory hormones and their respective target organs might improve Ca homeostatic mechanisms of the dairy cow. Provided that a diet negative in DCAD can enhance tissue responsiveness to PTH, it could not only accelerate the mobilization of calcium from the bone, but also the conversion of 25-OHD₃ to 1,25-(OH)₂D₃. Thus, the efficiency of supplementation with vitamin D might be supported by feeding an acidifying diet concurrently.

To test this hypothesis, studies were carried out to investigate the effects of a low DCAD diet alone, an oral supplementation with 25-OHD₃, and the respective combination on periparturient macromineral homeostasis and bone metabolism of a population of late-pregnant dairy cows reaching at least the second lactation.

MATERIALS AND METHODS

Cows, Diets, and Treatments

The study was carried out on a commercial dairy farm with approximately 2,100 lactating cows located in Saxony, Germany, from June to August 2009. The protocol of the animal treatment was approved by the University of Veterinary Medicine, Foundation, Hannover, Germany, and its conduct supervised according to the German Animal Welfare Law.

Ten days before the expected calving (d 270 of gestation), 60 multiparous Holstein cows that had been dried off 8 wk prepartum were randomly divided into 2 feeding groups (low DCAD and control) and housed in a tie-stall barn. The distribution of lactation number within the groups is in Table 1. The BCS (Edmonson et al., 1989) ranged between 2.75 and 4.25, with an average of 3.35 ± 0.1 and no differences between the experimental groups.

While the control group was kept on a TMR supplemented with 150 g of a mineral supplement, the low DCAD group received 450 g per cow per day of a special mineral mixture based on MgCl₂ to acidify the diet (Table 2). Results of the analysis of the TMR fed in the dry period and postpartum are in Table 3. Mineral contents as well as DCAD values were calculated from the analysis of the TMR and the mineral premix fed to the respective group (Table 4). Vitamin A was added to a final daily intake of 75,000 IU per animal and vitamin E was supplemented for a daily intake of 1,000 mg. Refusals were quantified once daily to estimate daily DMI and to adapt the amount of TMR provided.

Fifteen cows of each feeding group received an oral dose of 3 mg of 25-OHD₃ (240 mg Rovimix, **Hy-D**, 1.25%, DSM Nutritional Products, Basel, Switzerland) dissolved in 10 mL of rapeseed oil once daily until parturition.

Table 2. Ingredients of the mineral supplements used in this study¹

Ingredient	Control diet	Low DCAD
Ca, g/kg	107	53
Na, g/kg	79	39
Mg, g/kg	0	128
Cl, g/kg	121	433
Mn, mg/kg	4,000	2,000
Zn, mg/kg	6,000	3,000
Cu, mg/kg	1,000	500
Se, mg/kg	30	15
I, mg/kg	100	50
Co, mg/kg	20	10

¹Animals on the control and low DCAD diets were supplemented with 150 and 450 g per animal and day, respectively.

Table 3. Analyses of the TMR fed in the early dry period (4 to 8 wk prepartum), the late dry period, and postpartum (before addition of the mineral supplement)

Ingredient, % of DM	Early dry period	Late dry period	Postpartum
CP	11.4	14.6	15.7
Crude fat	3.4	3.9	3.3
Starch	9.4	11.0	14.3
Water-soluble carbohydrates	0.5	2.2	4.2
NDF	46.4	47.9	41.4
ADF	29.5	26.1	23.1
ME, MJ/kg of DM	9.7	10.6	11.0
NE _L , MJ/kg of DM	5.7	6.2	6.4

After calving in straw-bedded areas and a colostrum period of 5 d, the animals were housed in freestalls, allotted to different performance groups according to their milk yield, and all fed the same diet for fresh cows (Table 4).

Sample Collection

From d 270 of gestation to d 10 after calving, 2 blood samples (lithium heparin and EDTA served as anti-coagulants) were taken from the coccygeal vein every other day. After parturition, additional samples were taken immediately after calving and at 6, 12, and 24 h postpartum.

As the prepartum samples had been taken every other day, they were classified according to the true day before parturition after calving. Samples taken on d 2 and 1 before calving were classified as sample -2 d, samples taken on d 4 and 3 prepartum as sample -4 d. Urine samples were collected on d 275 of gestation, which was consistent with d 6 of treatment, and on d 6 after calving.

Sample Analysis

After measuring pH and ionized Ca (Ca²⁺) in whole blood using a blood gas analyzer (Chiron Diagnostics,

RapidLab 348, Bayer, Fenwald, Germany), samples were centrifuged (2,000 × *g*, 15 min), and plasma was aliquoted and frozen at -18°C.

Total Ca (Ca_t) and inorganic P (P_i) concentrations were determined by standard diagnostic methods (photometric assays based on methylthymol blue and ammonium molybdate, respectively) in the clinical laboratory of the Clinic for Cattle of the University of Veterinary Medicine, Foundation (Hanover, Germany). Analysis for 25-OHD₃ was carried out using HPLC by the Analytical Research Centre of DSM Nutritional Products (Kaiseraugst, Switzerland). The analysis for 1,25-(OH)₂D₃ was done by Idexx Diavet Labor AG (Bäch, Switzerland). Determination of PTH, the bone formation marker osteocalcin (OC), and the bone resorption marker CrossLaps (CL) was done using commercial ELISA kits according to the manufacturer's instructions (Bovine Intact PTH ELISA Kit, Immutopics Inc., San Clemente, CA; MicroVue Osteocalcin EIA Kit, Quidel Corp., San Diego, CA; Serum CrossLaps, IDS GmbH, Frankfurt am Main, Germany). The detection limits of the test kits were 10 pg/mL (PTH), 0.45 ng/mL (OC), and 0.02 ng/mL (CL). The intraassay CV were 6.0% (PTH), 7.6% (OC), and 2.8% (CL), and the interassay CV were 12.2% (PTH), 7.8% (OC), and 4.5% (CL).

Table 4. Minerals in the rations fed in the early dry period, in the control ration (control) and the diet negative in cation-anion difference (low DCAD) fed during the observation period (late dry period), and in the rations fed postpartum as calculated from the analyses of the TMR and the mineral premix fed to the respective group

Mineral, % of DM	Early dry period	Late dry period		Postpartum
		Control	Low DCAD	
K	ND ¹	1.54	1.49	1.63
Ca	0.72	0.78	0.88	1.00
P	0.37	0.41	0.40	0.50
Na	0.19	0.25	0.23	0.27
Mg	0.23	0.28	0.62	0.36
Cl	ND	0.72	1.77	0.58
S	ND	0.25	0.24	0.27
DCAD, ² mEq/kg of DM	ND	144	-168	206

¹ND = not determined.

²Calculated as (Na⁺ + K⁺) - (Cl⁻ + SO₄²⁻).

Urine samples were analyzed for pH, base acid quotient (**BAQ**: ratio of Na^+ , K^+ , Ca^{2+} , Mg^{2+} , and HCO_3^- to Cl^- , SO_4^{2-} , HPO_4^{2-} , organic acids, and NH_4^+ after titration with HCl and NaOH, respectively), net acid-base excretion (**NABE**: difference between the measures mentioned above), Ca, P, Mg, and creatinine (**Crea**). Calcium, P, Mg, BAQ, and NABE were determined after inductively coupled plasma optical emission spectrometry (Landwirtschaftliche Kommunikations- und Servicegesellschaft mbH, Lichtenwalde, Germany). Creatinine concentrations were determined by a standard diagnostic method (photometric assay based on alkaline picrate) in the clinical laboratory of the Clinic for Cattle of the University of Veterinary Medicine.

Data Presentation and Statistical Analysis

Four animals were excluded from the study for the following reasons: chronic diarrhea (1 cow, DCAD), muscle rupture (1 cow, control Hy-D), contrary behavior (1 cow, DCAD Hy-D), and calving on the first day of treatment (1 cow, DCAD). Three animals (1 cow, control Hy-D, fourth lactation; 2 cows, DCAD Hy-D, fourth and seventh lactation) were treated for hypocalcemia. The cow from the control Hy-D group was treated twice (6 h and 24 h postpartum) with approximately 10 g of Ca s.c. (C-B-Gluconat 38% plus 6%, Belapharm GmbH, Vechta, Germany) immediately after sampling. Due to secondary lesions of the musculoskeletal system, this animal had to be euthanized on d 4 postpartum. The cow from the DCAD Hy-D group entering the fourth lactation was treated once with approximately 10 g of Ca s.c. 3 h after parturition. Although plasma Ca concentrations could be stabilized, the cow developed ketosis (verified by analysis of plasma samples for hepatic enzymes) and died on d 2 postpartum. The cow from the DCAD Hy-D group entering the seventh lactation was treated once with approximately 10 g of Ca s.c. immediately after the sampling 6 h postpartum and recovered completely. Except for 1 sample, all blood samples were taken before the treatments. The data from these animals were not excluded from the statistical analysis.

Thus, for determination of 25-OHD₃, 1,25-(OH)₂D₃, Ca_t, Ca²⁺, and P_i, 14 animals were left in the low DCAD Hy-D group, 13 animals in the low DCAD group, 14 animals in the control Hy-D group, and 15 animals in the control group. Analysis of plasma samples of selected sampling times (d 270 of gestation, 2 d before the actual calving, 12 and 24 h postpartum, and d 2 postpartum) for PTH and OC was carried out for 7 animals randomly chosen from each group, whereas

plasma concentrations of CL were determined for 10 cows per group.

Values are expressed as mean $\pm$ standard error of the mean. All statistical analyses were performed using SPSS 15.0 (SPSS, Chicago, IL). As the data describing the urinary measures and the distribution of lactation number did not all pass the normality test according to Kolmogorov-Smirnov, the Kruskal-Wallis nonparametric test was used for the respective comparisons. If significant differences between the 4 experimental groups were found, the Mann-Whitney test was carried out to reveal effects of the diet or the treatment within the 2 diets.

Comparison of basal (first day of experiment) and peripartal Ca²⁺, Ca_t, P_i, PTH, CL, and OC plasma concentrations was carried out by 3-way ANOVA for repeated measurements with a saturated model of the fixed factors diet, treatment, and lactation number. The latter was clustered into 2 levels (second and third to eighth lactation). As sphericity verified using Mauchly's test could not be assumed for all the data, a Huynh Feldt correction was invented to reveal within-subject effects (time and its interactions). Type III sums of squares were used for detection of between-subject effects (lactation number, diet, treatment). The Fisher Least Significant Difference test was applied for multiple comparisons of the estimated marginal means of plasma concentrations of Ca²⁺, Ca_t, and P_i of the 4 groups at each time point for all animals, cows entering the second lactation, and cows of third or higher lactations separately. For comparisons of estimated marginal means of 25-OHD₃, PTH, 1,25-(OH)₂D₃, OC, and CL it was not distinguished between the lactation number groups. In all cases, *P*-values <0.05 were considered statistically significant.

RESULTS

Feed Intake

The voluntary feed intake was not affected by diets and the Hy-D treatment during the observation period. From d 270 of gestation until calving, the estimated DMI per animal and day was 7.7 ± 0.22 kg in the control and 7.8 ± 0.19 kg in the low DCAD group.

Urine Samples

Results of analyses of urine samples taken prepartum are given in Figure 1. As no differences due to diet or treatment were revealed in samples taken postpartum, the respective data are not shown.

Measures of Acid-Base Status. Six days (d 275 of lactation) after the start of the feeding regimen and

Figure 1. Means $\pm$ SEM of the acid base status [pH; base acid quotient (BAQ); net acid base excretion (NABE)] and macromineral excretion determined in urinary samples taken from cows kept either on a control ration (control) or on a diet negative in cation-anion difference (low DCAD) on d 6 of the experiment. White columns represent untreated animals, and gray columns represent animals treated daily with 3 mg of 25-hydroxyvitamin D₃. Significant effects between the groups revealed by Kruskal-Wallis-test, comparisons of diet and treatment within the feeding groups carried out using Mann-Whitney-test; ** $P < 0.01$; *** $P < 0.001$. Crea = creatinine.

treatment with Hy-D, urinary pH, BAQ, and NABE of animals kept on the low DCAD diet were significantly decreased irrespective of Hy-D treatment, whereas the pH determined in whole blood remained within the physiological range of 7.36 to 7.44.

Urinary Excretion of Macrominerals. Calcium concentrations normalized to Crea were significantly increased by feeding the low DCAD diet. In addition, the Hy-D treatment induced significant increases in both feeding groups. A significant increase in urinary P concentrations due to the Hy-D treatment was only revealed in animals fed the low DCAD diet, whereas neither the low DCAD diet alone nor the Hy-D treatment combined with the control diet had an effect. Furthermore, animals kept on the low DCAD diet showed higher urinary Mg concentrations than those fed the control diet, irrespective of the treatment.

Plasma Concentrations of 25-OHD₃, PTH, and 1,25-(OH)₂D₃

Figure 2 shows plasma concentrations of 25-OHD₃ as a function of treatment duration before parturition. Irrespective of the feeding regimen, the 25-OHD₃ concentrations of untreated animals remained stable within a range from 18 to 48 ng/mL until calving, whereas

oral supplementation resulted in a linear increase up to concentrations of between 160 and 180 ng/mL after 10 d of treatment.

Figure 3 shows the plasma concentrations of 25-OHD₃, PTH, and 1,25-(OH)₂D₃ around parturition as affected by supplementation with Hy-D before calving. The 25-OHD₃ concentrations significantly increased in the Hy-D treated groups until 24 to 48 h after calving and then decreased in all groups within another 24 to 48 h. On d 2 postpartum, plasma concentrations of the low DCAD Hy-D group (144 ± 20 ng/mL) were significantly lower than those of the control Hy-D group (198 ± 15 ng/mL). By d 4 postpartum, 25-OHD₃ plasma concentrations of both Hy-D treated groups were similar. Subsequently, plasma 25-OHD₃ slightly increased and remained relatively stable until the end of the observation period (data not shown).

In all groups, PTH concentrations increased from the beginning of the observation period to d 1 postpartum. An increase could not be detected in the low DCAD Hy-D group (32 ± 12 pg/mL), whereas the greatest changes were in animals treated with Hy-D on the control diet (250 ± 21 pg/mL).

Plasma concentrations of 1,25-(OH)₂D₃ began to increase near parturition and reached peak levels on d 2 after calving. During the first 24 h, as well as on

Figure 2. Plasma concentrations of 25-hydroxyvitamin D₃ (25-OHD₃) of dairy cows as a function of treatment duration. Untreated cows on a control ration (control), untreated cows on a diet negative in cation-anion difference from d 270 of gestation until parturition (low DCAD), cows on a control ration and treated with 3 mg of 25-OHD₃ daily from d 270 of gestation until parturition (control Hy-D), and cows on low DCAD diet combined with the 25-OHD₃ treatment (low DCAD Hy-D); means $\pm$ SEM.

d 4 after parturition, both Hy-D treated groups had significantly higher 1,25-(OH)₂D₃ concentrations than the untreated groups (pooled means over the first 24 h after parturition of Hy-D treated compared with untreated groups: 158 ± 15 vs. 120 ± 15 pg/mL; d 4 postpartum: 127 ± 9 vs. 82 ± 10 pg/mL).

Plasma Concentrations of Ca²⁺, Ca_t, and P_i

Regarding the complete observation period, Ca²⁺ concentrations were influenced by lactation number, diet, an interaction of diet and treatment, and an interaction of lactation number and treatment (Figure 4). For plasma concentrations of Ca_t, an effect of lactation number and the interaction of lactation number and treatment could be demonstrated, whereas the effects of the diet only became apparent at certain time points (Figure 5). Likewise, the effect of diet and treatment on plasma P_i concentrations could not be seen during the entire observation period. However, during the first 24 h postpartum, a diet by lactation number interaction became significant (Figure 6).

On d -4 and -2 prepartum, plasma concentrations of Ca²⁺, Ca_t, and P_i of the Hy-D treated groups were significantly increased compared with untreated animals. At parturition, plasma Ca²⁺, Ca_t, and P_i concentrations declined in all animals. Besides a significant effect of the lactation number on Ca²⁺ and Ca_t concentrations, positive effects of the low DCAD diet could be demonstrated.

In the 24 h following parturition, animals kept on the low DCAD diet and treated with Hy-D maintained

higher plasma Ca²⁺ and Ca_t concentrations. Interestingly, older animals on the control diet and treated with Hy-D had the lowest plasma concentrations of Ca²⁺ and Ca_t. Effects of the HyD treatment combined with the control diet on P_i concentrations after parturition were reversed for second compared with third and greater lactations.

Plasma Concentrations of CL and OC

Plasma concentrations of the bone resorption marker CL and the bone formation marker OC around parturition are shown in Figure 7. Generally, OC concentrations were significantly higher in younger animals than in older cows (overall means of animals of the second lactation vs. animals of the third or higher lactations: 16.9 ± 1.6 vs. 11.5 ± 1.7 ng/mL). Near parturition, CL concentrations increased and OC concentrations decreased in all groups. Significant interactions of time and lactation number were observed for both measures. Younger animals had higher basal values of plasma CL than cows in their third or later lactations (0.649 ± 0.084 vs. 0.460 ± 0.076 ng/mL). The decrease of plasma OC within the first 24 h postpartum was more pronounced in cows of the second lactation (reduced by 14.5 ± 2.9 ng/mL) than older animals (5.5 ± 3.1 ng/mL).

DISCUSSION

Effect of the Feeding Regimen on DMI, Acid Base Status, and Renal Excretion of Macrominerals

In the present study, the DMI during the last 10 d before parturition supported the literature (Marquardt et al., 1977) and was not affected by the diet. Reduced urinary pH, BAQ, and NABE (Figure 1) accompanied by an unaffected pH in whole blood (data not shown) indicate the induction of a compensated metabolic acidosis by the low DCAD diet, which supports other studies (Vagnoni and Oetzel, 1998; Moore et al., 2000).

The increased urinary Ca:crea ratios (Figure 1) indicate higher renal Ca excretion in animals fed a diet low in DCAD, which supports other studies (Vagnoni and Oetzel, 1998). The pH decrease in tubular fluid is suggested to decrease renal reabsorption of Ca (Sutton et al., 1979). The higher Mg excretion (Figure 1) in cows fed the low DCAD diet was probably caused by the higher Mg intake in these animals.

Plasma Concentrations of 25-OHD₃, 1,25-(OH)₂D₃, and PTH

We demonstrated that the concentrations of 25-OHD₃ changed as a function of treatment duration (Figure 2).

Figure 3. Time courses of plasma concentrations of 25-hydroxyvitamin D₃ (25-OHD₃), parathyroid hormone (PTH), and 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] around parturition. Untreated cows on a control ration (control), untreated cows on a diet negative in cation-anion difference from d 270 of gestation until parturition (low DCAD), cows on a control ration and treated daily with 3 mg of 25-OHD₃ from d 270 of gestation until parturition (control Hy-D), cows on low DCAD diet combined with the 25-OHD₃ treatment (low DCAD Hy-D). If no time point is denoted, values reflect pretreatment samples. Data are means $\pm$ SEM. Three-way ANOVA for repeated measurements with the fixed factors diet, treatment, and lactation number revealed effects of time ($P < 0.001$) for all 3 measures. Additionally, an effect of treatment ($P < 0.001$) and an interaction of time $\times$ treatment were found for 25-OHD₃ ($P < 0.001$). For PTH, the statistical analysis revealed effects of diet ($P = 0.031$), time $\times$ diet ($P = 0.018$), and time by diet $\times$ treatment ($P = 0.017$) interactions. For 1,25-(OH)₂D₃, effects of treatment ($P = 0.024$), lactation number ($P = 0.005$), treatment by lactation number ($P = 0.006$), and time by treatment ($P = 0.041$), and time $\times$ lactation number $\times$ diet $\times$ treatment ($P = 0.017$) interactions were found. Results of the comparison of estimated marginal means for all animals irrespective of lactation number are given within the figures.

Figure 4. Plasma concentrations of ionized calcium (Ca^{2+}) for untreated cows on a control ration (control), untreated cows on a diet negative in cation-anion difference from d 270 of gestation until parturition (low DCAD), cows on a control ration and treated with 3 mg of 25-hydroxyvitamin D_3 (25-OHD $_3$) daily from d 270 of gestation until parturition (control Hy-D), and cows on low DCAD diet combined with the 25-OHD $_3$ treatment (low DCAD Hy-D) around parturition; data are given as means $\pm$ SEM. Three-way ANOVA for repeated measurements with the fixed factors diet, treatment, and lactation number revealed effects of time ($P < 0.001$), lactation number ($P = 0.012$), diet ($P = 0.040$), diet by treatment ($P = 0.020$), lactation number by treatment ($P = 0.002$), time by treatment ($P = 0.048$), and time by lactation number by treatment ($P = 0.045$) interactions. Results of the comparison of estimated marginal means are given within the figures.

Figure 5. Plasma concentrations of total calcium (Ca_t) of untreated cows on a control ration (control), untreated cows on a diet negative in cation-anion difference from d 270 of gestation until parturition (low DCAD), cows on a control ration and treated with 3 mg of 25-hydroxyvitamin D₃ (25-OHD₃) daily from d 270 of gestation until parturition (control Hy-D), and cows on low DCAD diet combined with the 25-OHD₃ treatment (low DCAD Hy-D) around parturition; data are means $\pm$ SEM. Three-way ANOVA for repeated measurements with the fixed factors diet, treatment, and lactation number revealed effects of time ($P < 0.001$), lactation number ($P = 0.032$), lactation number by treatment ($P = 0.027$), time by diet ($P = 0.035$), and time by lactation number by treatment ($P = 0.015$) interactions. Results of the comparison of estimated marginal means are given within the figures.

Figure 6. Plasma concentrations of inorganic phosphorus (P_i) of untreated cows on a control ration (control), untreated cows on a diet negative in cation-anion difference from d 270 of gestation until parturition (low DCAD), cows on a control ration and treated with 3 mg of 25-hydroxyvitamin D_3 (25-OHD $_3$) daily from d 270 of gestation until parturition (control Hy-D), and cows on low DCAD diet combined with the 25-OHD $_3$ treatment (low DCAD Hy-D) around parturition; data means $\pm$ SEM. Three-way ANOVA for repeated measurements with the fixed factors diet, treatment, and lactation number revealed effects of time ($P < 0.001$), time by lactation number ($P = 0.030$), time by treatment ($P = 0.025$), and time by lactation number by diet by treatment ($P = 0.003$) interactions. Results of the comparison of estimated marginal means are given within the figures.

Figure 7. Time courses of plasma concentrations of CrossLaps and osteocalcin around parturition. Untreated cows on a control ration (control), untreated cows on a diet negative in cation-anion difference from d 270 of gestation until parturition (low DCAD), cows on a control ration and treated daily with 3 mg of 25-OHD₃ from d 270 of gestation until parturition (control Hy-D), and cows on low DCAD diet combined with the 25-OHD₃ treatment (low DCAD Hy-D). If no time point is denoted, values reflect pretreatment samples. Data are means $\pm$ SEM. Three-way ANOVA for repeated measurements with the fixed factors diet, treatment, and lactation number revealed effects of time ($P < 0.001$) and of time by lactation number (CrossLaps: $P = 0.049$; osteocalcin: $P = 0.014$) for both measures. A tendency was observed for an effect of treatment on plasma concentrations of CrossLaps ($P = 0.065$) and an effect of lactation number on osteocalcin ($P = 0.025$). Results of the comparison of estimated marginal means for all animals irrespective of lactation number are given within the figures.

The daily oral supplementation with Hy-D increased the plasma concentrations of 25-OHD₃ linearly from values within the physiological range (Horst et al., 1981) to peak levels of approximately 200 ng/mL on d 1 and 2 postpartum. Thus, the duration of treatment should be limited to prevent potential toxic effects of 25-OHD₃, which occur if plasma concentrations are elevated above 300 ng/mL (Jones, 2008).

After reaching its maximum concentration, plasma 25-OHD₃ decreased over the next 2 d by about 30%, and then remained relatively stable at supraphysiological levels (Horst et al., 1981) to the end of the observation period (Figure 3). These data reflect the long half-life of 25-OHD₃ of 15 d (Jones, 2008). As the decline of 25-OHD₃ concentrations occurred with the increase of 1,25-(OH)₂D₃, the decrease might have been

caused by an enhanced transformation of 25-OHD₃ to 24,25-(OH)₂D₃ mediated by the 24-hydroxylase, an enzyme upregulated by 1,25-(OH)₂D₃ (Kutuzova and Deluca, 2004).

The plasma PTH concentrations reached peak levels on d 1 postpartum (Figure 3). The significant effect of the diet on PTH concentrations not only reflects the lower stimulation of PTH secretion due to higher periparturient Ca²⁺ concentrations in the low DCAD groups, but also supports the hypothesis of diet-induced ameliorated tissue responsiveness to PTH (Goff and Horst, 2003).

The plasma concentrations of 1,25-(OH)₂D₃ (Figure 3) remained unaffected until parturition. The sudden increase was probably a result of the regulation of 1 α -hydroxylase and the induced transformation of

25-OHD₃ to 1,25-(OH)₂D₃. Expression and activity of 1 α -hydroxylase were stimulated by PTH (Fraser and Kodicek, 1970), which is secreted from the parathyroid gland in response to the transient hypocalcemia at calving (Brown and MacLeod, 2001). Twelve and 24 h postpartum, interactions with time became apparent for 1,25-(OH)₂D₃. Despite the difference in PTH concentrations, the low DCAD Hy-D group had similar 1,25-(OH)₂D₃ plasma concentrations compared with the control Hy-D group. These observations indicated enhanced tissue responsiveness to PTH mediated by the compensated metabolic acidosis in animals fed the low DCAD diet.

Plasma Concentrations of Ca_t and P_i Prepartum

Significantly increased plasma P_i and Ca_t concentrations (Figures 4, 5, and 6) as well as enhanced renal excretion of Ca and P prepartum in animals treated with Hy-D (Figure 1) in combination with slightly decreased bone resorption marker concentrations (Figure 7) prepartum indicate an increased gastrointestinal absorption of Ca and P. But, as the plasma concentrations of 1,25-(OH)₂D₃ were not elevated prepartum, the stimulation of gastrointestinal Ca absorption might have been mediated by the high levels of 25-OHD₃. To verify this hypothesis, direct measurements of gastrointestinal Ca and P absorption would be necessary.

Effect of the Treatment on Macromineral Homeostasis After Parturition

Low DCAD Diet. Cows fed the low DCAD diet prepartum, especially those that entered third or later lactations, had a significantly faster recovery from the peripartal hypocalcemia (Figures 4 and 5). This observation supports Oetzel et al. (1988). Although the exact mechanisms are not yet known, it has been suggested that tissue responsiveness to PTH is enhanced by the low DCAD diet (Goff and Horst, 2003). In the present study, the correlation between plasma Ca²⁺ and PTH 12 h postpartum depends on the feeding regimen prepartum (Figure 8). This corroborates the hypothesis of a more effective regulation of bone mobilization and Ca homeostatic mechanisms, which depend on the transformation of 25-OHD₃ to 1,25-(OH)₂D₃ mediated by PTH in cows kept on the low DCAD feeding regimen.

Combination of the Low DCAD Diet and Hy-D Treatment. The beneficial effect of the low DCAD diet prepartum on peripartal Ca homeostasis was even more pronounced in cows that were additionally supplemented with Hy-D (Figures 4 and 5). Interestingly, these cows had the lowest concentrations of PTH and CL at

Figure 8. Correlations of plasma concentrations of parathyroid hormone (PTH) and ionized Ca (Ca²⁺) in whole blood 12 h after calving in animals kept either on a control ration (control) or on a diet negative in cation-anion difference (low DCAD). Because the slopes of the 2 lines are not different ($P = 0.938$), one slope can be calculated for all the data (-0.284 ± 0.171 ; $R^2 = 0.0865$). The intercepts are different ($P = 0.018$; 1.25 ± 0.04 and 1.14 ± 0.09 , respectively).

the same time (Figures 3 and 7). In contrast, cows in third and later lactations that received the combination of the control diet and Hy-D prepartum had the slowest recovery from the peripartal hypocalcemia (Figures 4 and 5). Thus, the enhanced gastrointestinal absorption of Ca prepartum that has been suggested was probably not the reason for the positive effects of the combined low DCAD and Hy-D treatment. Nevertheless, we can speculate that the 25-OHD₃-mediated increased gastrointestinal absorption of Ca and P prepartum in cows treated with Hy-D leads to a better mineralization of the skeleton, as indicated by unaffected OC but slightly decreased CL concentrations 2 d before calving, which reached statistical significance in the low DCAD Hy-D group (Figure 7). Because bone metabolism is characterized by continuous resorption and formation, a transient decrease in resorption might result in a higher mineral content. In humans, oral Ca loads of 0.2 or 1.0 g, respectively, result in dose-dependent increments of Ca²⁺ between 2 and 8% compared with baseline values and a concurrent decline of PTH (by 20 to 60%) and CL concentrations (by 20 to 50%) within several hours (Zikán et al., 2001). More detailed and direct measurements on bone mineral density would be necessary to confirm the assumption of a higher Ca content of the skeleton because of the Hy-D treatment. For sheep, treatment with 1 α -OHD₃ resulted in increased gastrointestinal absorption and thereby enhanced storage of Ca in bone within 10 d (Braithwaite, 1978). In addition, a recent study provided evidence for direct effects of 25-OHD₃ on bone resorption (Kogawa et

al., 2010), suggesting that the Hy-D treatment in the current study enhanced the Ca content of the bone. Future investigations should take into account that an increased Ca content of the bone alone does not necessarily result in greater mobilization of minerals. The skeletal microstructure, which plays a pivotal role in liberation of Ca and P, might be negatively affected by long-term imbalances of the bone formation and resorption processes.

Combination of the Control Diet and Hy-D Treatment. As shown in Taylor et al. (2008), the Hy-D treatment alone did not have significant beneficial effects on peripartal Ca homeostasis; the older animals in the control Hy-D group recovered more slowly from peripartal hypocalcemia than those without supplementation (Figures 4 and 5). As both Hy-D treated groups had plasma concentrations of 1,25-(OH)₂D₃ 24 h postpartum equal to or greater than those of untreated animals (Figure 3), the differences in Ca homeostasis cannot be explained by insufficient transformation of 25-OHD₃ to 1,25-(OH)₂D₃.

Plasma PTH concentrations after calving depend on the alimentary Ca supply prepartum (Shappell et al., 1987). The Hy-D cows had higher plasma Ca concentrations than the untreated animals (Figures 4 and 5). Additionally, the production of PTH in the parathyroid gland might have been reduced in animals supplemented with Hy-D because of the increased 25-OHD₃ concentrations (Ouseph et al., 1996). Cows kept on the control diet and supplemented with Hy-D had greater plasma PTH concentrations after parturition than those supplemented with Hy-D and fed the low DCAD diet (Figure 3). But when the lower tissue responsiveness to PTH is taken into account, the plasma PTH might not have been high enough to counterbalance the Ca losses with the milk in animals that had received only the Hy-D supplementation and therefore were adapted to circumstances in which the availability of Ca from the gastrointestinal tract allowed increased renal excretion (Figure 1). This assumption might be supported by the PTH concentrations measured by Shappell et al. (1987) that were higher (approximately 700 pg/mL) than the maximum concentrations determined here (about 420 pg/mL). That cows entering the third or later lactations were negatively affected by the combination of the control diet and the Hy-D treatment (Figures 4 and 5) might be explained by a reduced capacity to mobilize calcium from the skeleton, because the number of osteoclasts and the resorptive bone surface are reduced in older animals (Reinhardt et al., 1988). In the current study, the effect of age on bone metabolism was reflected by a smaller relative decrease of OC at parturition in older cows. The decreased postpartum

plasma P_i concentrations in cows of the third or higher lactation kept on the control diet and the Hy-D treatment (Figure 6) might indicate insufficient liberation of minerals from the bone.

CONCLUSIONS

Although the transient hypocalcemia at parturition could not be avoided completely, beneficial effects of oral supplementation with Hy-D in combination with feeding a diet low in DCAD prepartum could be demonstrated. Because the Hy-D treatment alone might have a negative influence on postpartum plasma Ca concentrations, especially in older animals, combination with the low DCAD diet seems to be crucial. At parturition, cows treated with Hy-D might enter the lactation period with a higher skeletal Ca content. Concurrently, the tissue responsiveness to PTH is improved by the low DCAD diet, which facilitated the mobilization of Ca from the bone at calving.

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