Supplementation with difructose anhydride III promotes passive calcium absorption in the small intestine immediately after calving in dairy cows

M. Teramura,*†[1] S. Wynn,* M. Reshalaitihan,‡ W. Kyuno,† T. Sato,† M. Ohtani,† C. Kawashima,‡ and M. Hanada‡
*United Graduate School of Agricultural Sciences, Iwate University, Morioka, Iwate, 020-8550, Japan
†Nippon Beet Sugar Manufacturing Co. Ltd., Obihiro, Hokkaido, 080-0831, Japan
‡Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, 080-8555, Japan

ABSTRACT

The incidence of hypocalcemia increases in high-parity dairy cows because resorption of bone Ca is delayed in these animals, and they appear to have a reduced ability to absorb Ca from the intestine during the early postpartum period. Difructose anhydride (DFA) III has been shown to promote the absorption of intestinal Ca via a paracellular pathway. However, past studies have not reported this effect in peripartum dairy cows. Therefore, we investigated the effect of DFA III supplementation on Ca metabolism during the peripartum period to determine whether DFA III promotes intestinal Ca absorption via this route. Seventy-four multiparous Holstein cows were separated into DFA and control groups based on their parity and body weight. The feed of the DFA group was supplemented with 40 g/d of DFA III from −14 to 6 d relative to calving. The control group did not receive DFA III. At calving (0 h relative to calving), serum Ca declined below 9 mg/dL in both groups. However, serum Ca concentrations were greater in the DFA group than in the control group at 6, 12, 24, and 48 h relative to calving, and the time required for serum Ca to recover to 9 mg/dL during the postpartum period was shorter in the high-parity cows in the DFA group than in those in the control group. Parathyroid hormone concentrations increased immediately after calving in both groups and were greater in the control group than in the DFA group at 12 and 24 h relative to calving. Serum 1,25-dihydroxyvitamin D concentrations increased at 0 and 12 h relative to calving in both groups and were higher in the control group than in the DFA group at 72 h relative to calving. Serum concentrations of the bone-resorption marker cross-linked N-telopeptide of type I collagen (NTX) were not different between the groups during peripartum period, and serum NTX in all cows was lower at 0, 6, 12, 24, 48, and 72 h relative to calving than at −21, 4, and 5 d relative to calving. Thus, DFA treatment induced faster recovery of serum Ca, although bone resorption was restrained. In conclusion, DFA III promotes intestinal passive Ca absorption via the paracellular pathway during the early postpartum period; this absorption is unaffected by aging.
Key words: hypocalcemia, calcium, difructose anhydride (DFA) III, paracellular pathway, dairy cow

INTRODUCTION

Hypocalcemia is a condition in which serum Ca declines due to the disruption of Ca homeostasis by the large change in demand for Ca that occurs at the onset of lactation (DeGaris and Lean, 2008). Hypocalcemia is considered a gateway disease that greatly reduces the chance for full productivity in the ensuing lactational period. Hypocalcemia renders cows more susceptible to other disorders, such as mastitis, displaced abomasum, retained placenta, and ketosis (Curtis et al., 1983). Methods to prevent the occurrence of hypocalcemia have been proposed that involve limiting the Ca supply before calving (Goings et al., 1974), feeding or injecting exogenous vitamin D at 10 to 14 d before calving (Hibbs and Pounden, 1955), and adjusting the DCAD by supplying anions (Block, 1984). However, it is almost impossible to formulate an effective diet containing a very low level of Ca (NRC, 2001), and the effective dose of 1,25-dihydroxyvitamin D [**1,25-(OH)$_2$D**] before calving is close to the toxic dose (Goff and Horst, 1990). Furthermore, it is difficult to adjust the DCAD because anionic salts are of low palatability (Charbonneau et al., 2006), and a large effort is required to collect samples from each cow to determine the pH of the urine. Subclinical hypocalcemia, defined as a plasma Ca concentration of <8 mg/dL, affects approximately 50% of all multiparous cows (Reinhardt et al., 2011); hence, various methods have been proposed to prevent the occurrence of hypocalcemia.

Three routes exist by which to recover reduced serum Ca levels: (1) resorption of Ca stored in bones (bone resorption), (2) dietary provision of Ca, and (3) reabsorption by the renal tubules (NRC, 2001). However, in parturient cows, the total amount of Ca that can be recovered and the extent of urinary Ca excretion are relatively small (Goff, 2006), and Ca homeostasis has been reported to depend on intestinal Ca absorption immediately after calving because bone resorption is delayed for 1 wk or more following calving (Ramberg et al., 1984) unless cows are fed a low-DCAD diet (Kurosaki et al., 2007).

Transepithelial Ca transport in the intestine occurs via 2 routes: a transcellular pathway and a paracellular pathway (Bronner, 1998). The previously mentioned methods of preventing hypocalcemia by limiting Ca supply and injecting exogenous vitamin D before calving promote Ca absorption via the transcellular pathway by stimulating the production of 1,25-$(OH)_2$D. However, because the number of vitamin D receptors (**VDR**) within the intestinal epithelial cells declines with aging (Horst et al., 1990), the ability to absorb Ca through the transcellular pathway of the intestinal epithelial cells is reduced in older cows (Horst et al., 1997). In contrast, Ca absorption via the paracellular pathway occurs via passive transport through tight junctions, which is affected by the Ca concentration gradient (Bronner, 1987) that occurs when cows are administered oral Ca drenches (Goff and Horst, 1993). Tight junctions, which are located at the apical cell–cell interfaces of adjacent epithelial cells, form a continuous intercellular barrier that allows selective ion permeation between these cells (Tang and Goodenough, 2003). Paracellular transport via tight junctions is physiologically regulated by various intracellular signaling pathways (Karczewski and Groot, 2000). Extracellular stimuli, including food factors, drugs, and chemicals, reportedly affect paracellular transport through signal transduction (Nusrat et al., 2000). Medium-chain fatty acids have been shown to increase paracellular transport through the contraction of actin filaments following the phosphorylation of myosin light chains by Ca^{2+}/calmodulin-activated myosin light-chain kinase (MLCK) in Caco-2 cells (Lindmark et al., 1998). Lysophosphatidic acid increases the permeability of tight junctions by causing actin contraction via a Rho-kinase-dependent pathway (Hirase et al., 2001).

Difructose anhydride (**DFA**) III is an indigestible oligosaccharide that is enzymatically synthesized from inulin (Kikuchi et al., 2004) and cannot be degraded by typical strains of intestinal microbes such as *Bifidobacterium*, *Lactobacillus*, and *Bacteroides* spp. (Saito and Tomita, 2000) because the DFA III hydrolytic enzyme is produced only by *Arthrobacter* sp. H65–7 (Sakurai et al., 1997). In vitro experiments conducted using the small intestines of rats (Suzuki et al., 1998; Mineo et al., 2002) and duodenum of cows (Teramura et al., 2015) have shown that Ca absorption via the paracellular pathway can be accelerated by agents that act upon tight junctions. Difructose anhydride III promotes paracellular transport by reduced transepithelial electrical resistance (**TEER**) and enhances the transport of paracellular markers (Suzuki and Hara, 2004, 2006) with alterations to caludin-1, a component protein of tight junctions and actin filament in Caco-2 cells (Suzuki and Hara, 2006). However, the DFA III-induced reduction in TEER is not attenuated by any inhibitors of several kinases mentioned above; thus, the cellular mechanisms underlying the enhancement of paracellular transport by DFA III are distinct from other factors (Suzuki and Hara, 2006). The ruminal microorganisms of dairy cows have difficulty digesting DFA III (Sato et al., 2006). Therefore, intact DFA III reaches the duodenum 1 h after oral ingestion (Nakai et al., 2007), and approximately 70% of DFA III reaches the duodenum intact in dairy cows (Teramura et al., 2015). Furthermore, DFA III promotes in vitro intestinal Ca absorption via the paracellular pathway in cattle and, by supplementing the diet of dairy cows with DFA III during the close-up period (i.e., 3 wk before parturition), postcalving hypocalcemia can be prevented (Sato et al., 2007). In addition, other effects of DFA III have been identified, including an effect on the absorption of IgG from colostrum in newborn calves (Sato et al., 2012) and a positive effect on the overall health of calves (Matsumoto et al., 2009; Takagi et al., 2011).

Feeding DFA III to peripartum dairy cows promotes intestinal Ca absorption via the paracellular pathway, which is unaffected by aging, whereas the cow's ability to absorb Ca via the transcellular pathway of intestinal epithelial cells, which is dependent on the number of VDR within the intestinal epithelial cells, declines with age. However, previous studies did not indicate that feeding DFA III promotes passive intestinal Ca absorption via the paracellular pathway in peripartum dairy cows. Therefore, the objective of this study was to clarify the effect of feeding DFA III on Ca metabolism during the parturition period.

MATERIALS AND METHODS

The study was conducted between October 2012 and April 2014 at the Field Center of Animal Science and Agriculture of Obihiro University of Agriculture and Veterinary Medicine, Japan. The experimental pro-

tocols complied with the regulations of the Guide for the Care and Use of Agriculture Animals of Obihiro University, Japan.

Animals

Twenty-one days before the expected calving day (d 259 of gestation), 74 multiparous Holstein cows were divided into 2 groups of 37 animals (control and DFA groups) consisting of cows of similar parity and BW. The mean values for the control and DFA groups, respectively (means ± SE), were as follows: parity after calving = 3.7 ± 0.3 and 3.7 ± 0.3; BW at 21 d before the expected calving day = 771 ± 12 and 768 ± 10 kg; BCS (Edmonson et al., 1989) at 21 d before expected calving = 3.51 ± 0.02 and 3.54 ± 0.02; and 305-d milk yield during the previous lactational period = 9,465 ± 228 and 9,690 ± 270 kg. The 2 groups were housed in a testing facility containing stalls and Calan doors (Tsuchiya Dairy Equipment Mfg. Co. Ltd., Obihiro, Japan) located in the Center, and they had free access to water and salt mineral blocks (KNZ salt licks, Hengelo, the Netherlands). The cows were fed 10.9 ± 0.1 kg (DM) of a TMR per day when they were dry (Table 1) and were fed grass hay ad libitum from 21 d prepar-tum until calving. After calving, the cows were fed a TMR for lactating cows that was formulated to supply the nutrients required to sustain milk production of 35 kg/d (NRC 2001; Table 1) and grass hay ad libitum. From 14 d before calving until 6 d postpartum, the diet of the DFA group was supplemented with 200 g/d of DFA pellets containing 20% DFA III (Nippon Beet Sugar Manufacturing Co. Ltd., Tokyo, Japan; Table 1). The diet of the control group was supplemented with 160 g/d of control pellets that did not contain DFA III (Table 1). Therefore, the DFA diet was supplemented with 40 g/d of DFA III. To supplement dietary Ca, 200 g of dibasic calcium phosphate (CaHPO$_4$·2H$_2$O; Obanaya Cementex Co. Ltd., Aichi, Japan; containing 46 g of Ca) was administered to all cows at calving and 12 h after calving as a bolus dose in water, and 100 g/d of CaHPO$_4$·2H$_2$O (containing 23 g of Ca) was administered to all of the cows for 6 d postpartum as top-dressing on TMR.

Data and Sample Collection

The amount of feed offered and refused was recorded daily from 14 d before calving until 6 d postpartum. Feed samples were collected once monthly. Samples of

Table 1. Ingredients of the pellets and TMR fed to dry and lactating cows

Ingredient, % of fresh matter	Pellets		TMR	
	Control	DFA[1]	Dry[2]	Lact[3]
Corn silage	—	—	50.0	40.5
Grass silage	—	—	39.0	35.7
Concentrate mix	—	—	8.4[4]	9.6[5]
Grass hay	—	—	—	2.9
Ear corn silage	—	—	—	3.6
Beet pulp	—	—	—	2.3
Rice bran	24.0	20.0	—	1.9
Soybean meal	—	—	2.4	1.8
Rolled barley	—	—	—	1.4
Ground corn	38.0	30.0	—	—
Wheat bran	38.0	30.0	—	—
Vitamin mineral mix[6]	—	—	0.07	0.04
Vitamin E	—	—	0.07	—
MgO	—	—	0.07	0.03
CaCO$_3$	—	—	—	0.10
DFA III[6]	—	20.0	—	—

[1]Difructose anhydride III.

[2]Prepartum period (average from −14 to −1 d relative to calving).

[3]Postpartum period (average from 1 to 6 d relative to calving).

[4]Dry Base 17 (nutrient composition on a DM basis: 20.5% CP, 27.9% NDF, 0.28% Ca, 0.70% P, 0.35% Mg, 1.25% K, 0.04% Na, 0.15% S, and 0.38% Cl; Hokuren Kumiai Shiryo K. K., Hokkaido, Japan).

[5]Mo-Dairy 18 (nutrient composition on a DM basis: 21.4% CP, 24.5% NDF, 1.58% Ca, 0.60% P, 0.31% Mg, 1.28% K, 0.23% Na, 0.38% S, and 0.41% Cl; Nippon Formula Feed Manufacturing Co. Ltd., Kanagawa, Japan).

[6]Contents per gram: 5,000 IU of vitamin A, 1,000 IU of vitamin D, 2 mg of vitamin E, 0.2 mg of vitamin K$_3$, 0.5 mg of vitamin B$_1$, 1 mg of vitamin B$_2$, 0.1 mg of vitamin B$_6$, 1 ng of vitamin B$_{12}$, 6 mg of nicotonic acid, 2 mg of choline chloride, 10 mg of pantothenic acid, 156 µg of Mn (MnSO$_4$), 0.7 mg of Zn (ZnCO$_3$), 50 µg of Fe (FeSO$_4$), 139 µg of Cu (CuSO$_4$), 325 µg of I Ca(IO$_3$)$_2$, 38 µg of Co (CoSO$_4$), 1 mg of methionine, and 0.5 mg of lysine hydrochloride.

Table 2. Chemical composition (% of DM, unless otherwise noted) of pellets, TMR, and hay that were fed to dry and lactating cows

Item	Pellet		Dry[1]		Lactating[2]	
	Control	DFA[3]	TMR	Hay	TMR	Hay
DM, % as fed	88.7	89.5	35.0	86.6	39.9	85.5
CP	14.4	11.5	15.0	14.9	15.4	14.8
NDF	27.5	19.3	41.6	60.3	41.3	62.4
ADF	8.6	6.8	24.6	36.9	24.3	37.3
Neutral detergent insoluble CP	1.7	1.4	2.5	4.0	2.8	5.5
Acid detergent insoluble CP	0.5	0.4	1.0	1.4	1.1	1.3
ADL	2.2	1.7	3.5	4.9	3.5	4.5
Ether extract	5.7	4.7	4.0	3.1	3.8	2.9
Ash	4.4	3.7	7.8	12.0	8.1	11.5
NE_L,[4] Mcal/kg of DM	1.81	1.84	1.52	1.16	1.51	1.19
Ca	0.07	0.07	0.40	0.42	0.65	0.45
P	0.92	0.76	0.42	0.40	0.44	0.41
Mg	0.40	0.35	0.24	0.24	0.27	0.24
K	1.02	0.78	2.36	3.40	2.21	3.28
Na	0.02	0.02	0.05	0.05	0.14	0.04
S	0.16	0.13	0.21	0.29	0.23	0.32
Cl	0.10	0.08	0.48	0.80	0.48	0.56
DCAD,[5] mEq/100 g of DM	14.1	10.4	36.2	44.7	34.8	50.0

[1]Prepartum period (average from −14 to −1 d relative to calving).

[2]Postpartum period (average from 1 to 6 d relative to calving).

[3]Supplemented with difructose anhydride III.

[4]Calculated according to NRC (2001).

[5]Calculated as $(Na^+ + K^+) - (Cl^- + S^{2-})$ (Ender et al., 1971).

the refused feed were collected daily, and their composition was determined weekly (Table 2). The samples were dried in a forced-air oven at 60°C for 24 h and then ground using a Wiley mill (1-mm screen, SM2000; Retsch, Haan, Germany). Subsequently, the contents of DM, CP, NDF, ADF, neutral detergent-insoluble CP, acid detergent-insoluble CP, ADL, ether extract, ash, Ca, P, Mg, K, Na, S, and Cl were determined (Table 2). The N content was quantified using the Kjeldahl method, and the value was multiplied by 6.25 to calculate the CP content (AOAC International, 2003; method 984.13). The NDF content was determined according to the procedure of Van Soest et al. (1991) using α-amylase and sodium sulfate. The ADF content was measured according to AOAC International (2003; method 973.18). The neutral detergent-insoluble and acid detergent-insoluble CP contents were determined according to the procedure of Coblentz et al. (2010). The ADL content was quantified according to AOAC International (2003; method 973.18) and ether extract content according to AOAC International (2003; method 920.39). The ash content was determined after placing the samples in a muffle furnace for 2 h at 600°C (AOAC International, 2003; method 942.05). The concentrations of Ca, P, Mg, K, and Na were obtained according to AOAC International (2003; method 985.01). Briefly, the samples were ashed in a muffle furnace for 2 h at 600°C, and the ash was dissolved in hydrochloric acid, followed by

analysis using inductively coupled plasma spectroscopy (ICPE-9000; Shimadzu Corporation, Kyoto, Japan). The contents of S and Cl were determined using an energy-dispersive fluorescent X-ray spectrometer (JSX-3100RII; Jeol Ltd., Tokyo, Japan). The DCAD content (mEq/100 g of DM) was calculated as $(Na^+ + K^+) - (Cl^- + S^{2-})$ (Ender et al., 1971). The NE_L content was calculated in accordance with NRC (2001), discounting energy yield on the assumption that a dairy cow ingests 3 times the amount required for normal maintenance. The feed mineral intake was calculated by multiplying the amount of DM intake (difference between the amount of DM offered and the amount of DM refused) and the content of each mineral.

Blood samples were collected from the caudal vein using blood collection tubes (Venoject II VP-P100K; Terumo Corporation, Tokyo, Japan) before feeding at −21, −14, −7, −3, and −1 d and 0 h (immediately upon calving), 6, 12, 24, 48, 72 h, and 4, 5, and 7 d relative to calving. The blood samples were centrifuged (2,000 × g, 10 min) and the resultant serum was stored at −30°C until analysis. The serum Ca, P, and Mg concentrations were determined using an automatic biochemical analyzer (TBA120FR; Toshiba Medical Systems Co., Tochigi, Japan). The concentrations of serum parathyroid hormone (**PTH**) at −21, −7, and −1 d, 0, 12, 24, and 72 h, and 7 d relative to calving were determined using a commercial kit (Access Intact PTH; Beckman

Coulter Inc., Brea, CA). The serum concentrations of $1,25\text{-}(OH)_2D$ at -1 d and at 0, 12, 24, and 72 h relative to calving were determined using a commercial RIA kit (Immunodiagnostic Systems Ltd., Boldon, UK). The serum concentrations of the cross-linked N-telopeptide of type I collagen (**NTX**), a bone-resorption marker, were determined at -21, -7, and -1 d, at 0, 6, 12, 24, 48, and 72 h, and at 4, 5, and 7 d relative to calving using a commercial ELISA kit (Osteomark NTX Serum; Alere Medical Co. Ltd., Tokyo, Japan). The serum pH at -21, -14, and -7 d was determined using a blood gas and electrolyte analyzer (Cobas B 221; Roche Diagnostics Japan, Tokyo, Japan).

The cows were milked twice daily, and the milk yield from each milking was measured and sampled during 6 d after calving. The Ca concentration of the milk was determined using inductively coupled plasma spectroscopy (ICPE-9000), after 1 mL of the milk sample was mixed with 0.5 g of cellulose (Wako Pure Chemical Industries Ltd., Osaka, Japan), incinerated in a muffle furnace for 2 h at 600°C and digested using hydrochloric acid (AOAC International, 2003; method 985.01).

Statistical Analysis

The data on serum Ca, P, Mg, PTH, $1,25\text{-}(OH)_2D$, and NTX concentrations and serum pH, feed mineral intake, milk yield, and Ca concentration of the milk were analyzed using repeated-measures ANOVA (JMP 8.0.2; SAS Institute Inc., Cary, NC); only the feed mineral intake data were separated according to pre- and postpartum periods. When an interaction between groups and time was detected, these data were analyzed using Student's t-test for each sampling period. The transition in the concentration of serum NTX in all of the cows (n = 74) was analyzed using paired t-tests. The time required for serum Ca concentrations to recover to a normal range (9 mg/dL; NRC, 2001) during the postpartum period was calculated by fitting the serum Ca transition cubic function curve for each group and parity.

RESULTS

The average number of days that DFA III was administered to the DFA group during the prepartum period was 16.6 ± 1.5 d, with a maximum of 26 d and a minimum of 7 d. Three cows in the DFA group and 1 cow in the control group carried twins. At calving, 1 cow from the control group required assistance due to dystocia.

Blood Components

The serum concentrations of Ca during the prepartum period did not differ according to the treatment and declined in both groups at 0 h relative to calving. However, the serum Ca was higher in the DFA group than in the control group at 6 h ($P < 0.05$), 12 h ($P < 0.01$), 24 h ($P < 0.01$), and 48 h ($P < 0.01$) relative to calving (Figure 1a). During the postpartum period, the time required for the serum Ca to recover to 9 mg/dL was shorter in the DFA group than in the control group at each parity (Table 3).

The serum concentrations of P during the prepartum period did not differ according to the treatment and declined in both groups at 0 h relative to calving. However, the serum P was significantly higher in the DFA group than in the control group at 6 h ($P < 0.05$), 12 h ($P < 0.01$), 24 h ($P < 0.01$), and 48 h ($P < 0.01$) relative to calving (Figure 1b).

The serum concentrations of Mg during the prepartum period did not differ according to treatment and increased in both groups at 0 h relative to calving. However, the concentrations of serum Mg were significantly higher in the DFA group than in the control group at 72 h ($P < 0.05$) and 4 d ($P < 0.05$) relative to calving (Figure 1c).

Serum PTH concentrations did not differ between groups during the prepartum period. During the postpartum period, serum PTH concentrations increased at 0 h relative to calving in both groups and were significantly lower in the DFA group than in the control group at 12 h ($P < 0.05$) and 24 h ($P < 0.01$) relative to calving (Figure 2a).

No significant difference was observed in serum $1,25\text{-}(OH)_2D$ concentrations between the groups during the prepartum period (d -1). During the postpartum period, serum $1,25\text{-}(OH)_2D$ concentrations increased at 0 and 12 h relative to calving in both groups and were significantly lower in the DFA group than in the control group at 72 h relative to calving ($P < 0.01$, Figure 2b).

Serum NTX concentrations did not differ significantly in the 2 groups during the peripartum period (Figure 3a). Serum NTX concentrations in all cows were significantly lower at 0, 6, 12, 24, 48, and 72 h relative to calving than at -21, 4, and 5 d relative to calving ($P < 0.05$, Figure 3b).

Serum pH did not differ according to treatment during the prepartum period (Table 4).

Feed Mineral Intake

We detected no significant difference in the prepartum Ca and K intakes of the 2 groups (Table 4).

(a)

(b)

(c)
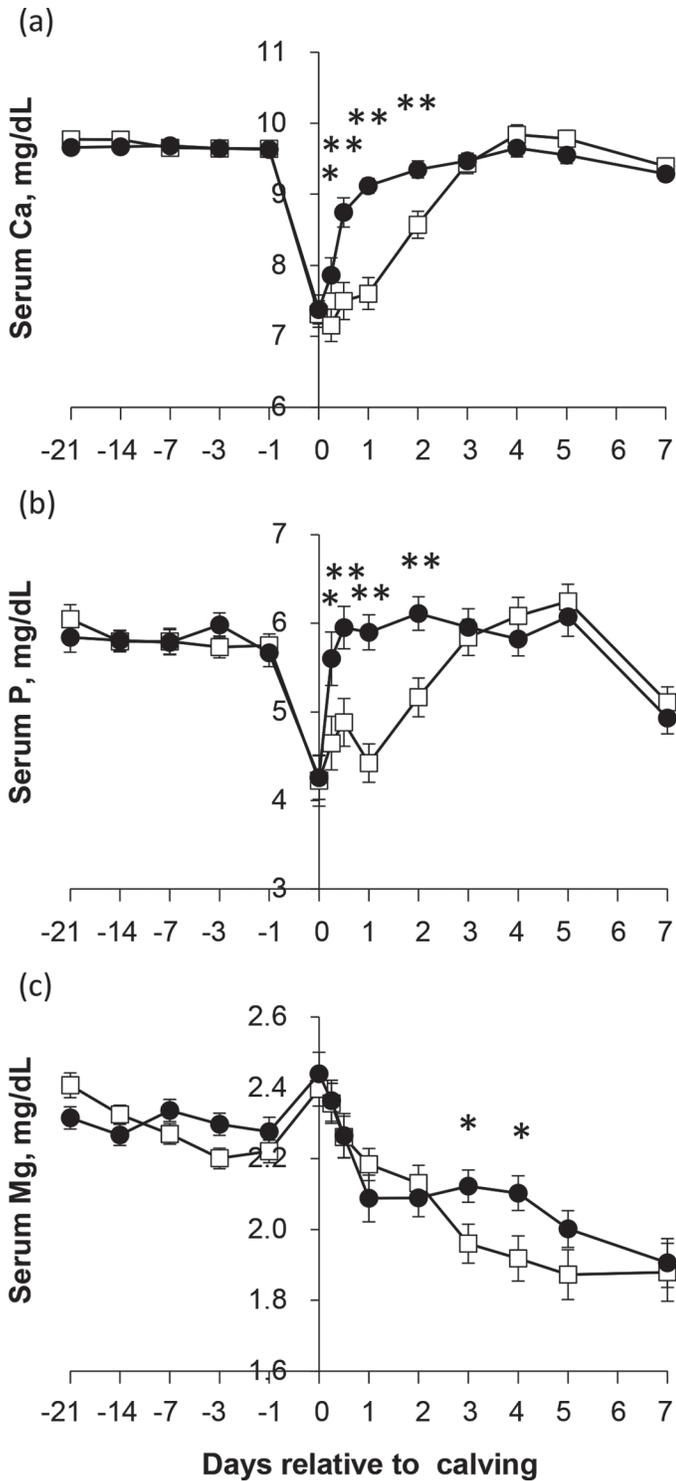
Figure 1. Serum calcium (a), phosphate (b), and magnesium (c) during the peripartum period (−21 to 7 d relative to calving; means ± SEM) in the control group (□; n = 37) and the group supplemented with difructose anhydride III (DFA, ●; n = 37). * and ** indicate significant differences between the groups at each time point (**$P <$ 0.01; *$P <$ 0.05).

(a)

(b)

Days relative to calving
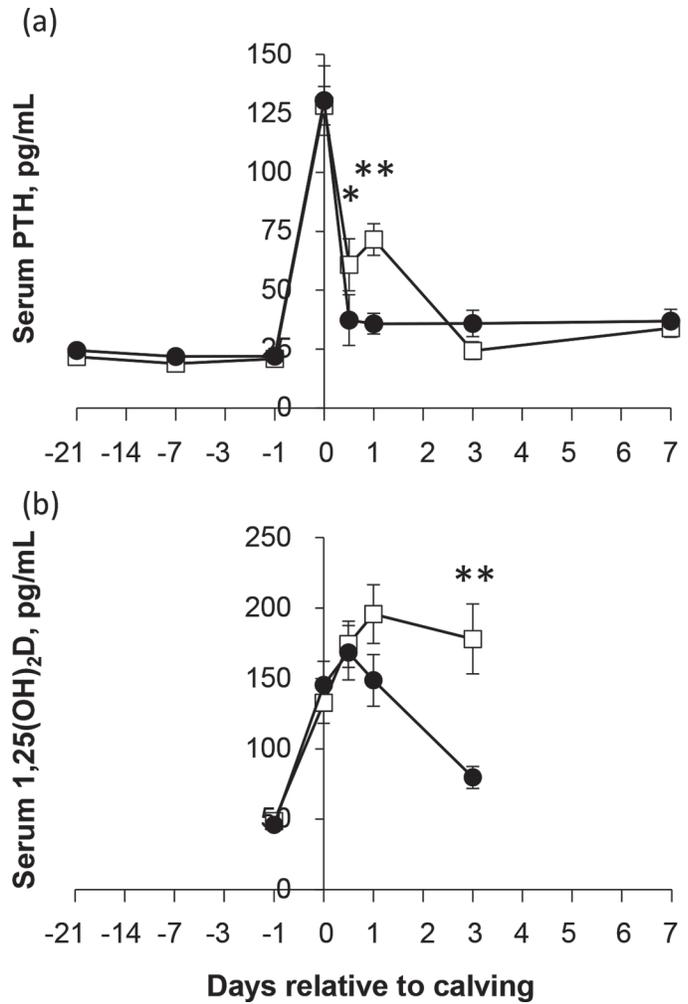
Figure 2. Serum parathyroid hormone (PTH; a) and 1,25-dihydroxyvitamin D (b) during the peripartum period (−21 to 7 d or −1 d to 72 h relative to calving; means ± SEM) in the control group (□; n = 37) and the group supplemented with difructose anhydride III (DFA, ●; n = 37). * and ** indicate significant differences between the groups at each time point (**$P <$ 0.01; *$P <$ 0.05).

Table 3. The time required for the recovery of the serum calcium content to 9 mg/dL during the postpartum period[1]

	Serum Ca recovery time (= 9 mg/dL)			
Parity	Control		DFA[2]	
≥6	74 h	(n = 6)	50 h	(n = 5)
5	59 h	(n = 7)	23 h	(n = 8)
4	40 h	(n = 4)	26 h	(n = 4)
3	60 h	(n = 9)	19 h	(n = 8)
2	63 h	(n = 11)	28 h	(n = 12)

[1]The postpartum serum Ca values for each parity level were fitted to a cubic function curve to calculate the time for the serum Ca concentration to recover to 9 mg/dL.

[2]Supplemented with difructose anhydride III.

However, compared between the groups, postpartum Ca intake was significantly higher ($P < 0.05$, Table 4).

Ca Outflow in Milk

We found no significant difference in Ca concentrations in the milk of the 2 groups (Table 4). Milk yield was significantly higher in the DFA group than in the control group ($P < 0.01$, Table 4). Therefore, the amount of Ca outflow into the milk was significantly higher in the DFA group ($P < 0.01$, Table 4).

DISCUSSION

The previously mentioned risk factors for hypocalcemia include age, breed, contents of dietary Ca and K, DCAD status, and hypomagnesemia (van de Braak et al., 1987; Oetzel, 1991; Goff and Horst, 1997; NRC, 2001). Furthermore, metabolic alkalosis is the most important determinant of the risk of milk fever (Ender et al., 1971). In the present study, parity, breed, offered feed, Ca and K intakes, serum pH during the prepartum period, and serum Mg during the early postpartum period were similar between groups. Therefore, the only difference between the 2 groups was dietary supplementation with DFA III. When the concentration of serum Ca declines, it is compensated for by bone resorption, intestinal Ca absorption, and reabsorption by the renal tubules (NRC, 2001). Among these Ca supply routes, reabsorption via renal tubules was found to be crucially important when anionic salt was added (Schonewille et al., 1999). However, in the present study, anionic salt was not added to the feed, and the cows were therefore offered a high-DCAD feed ($+36.2$ mEq/100 g). Although we could not determine the amount of Ca that was resorbed by the renal tubules, the total amount of Ca that can be recovered by reducing the extent of urinary Ca excretion is relatively small in parturient cows (Goff, 2006), and no difference in this value was found in the 2 groups. Hence, there were 2 main Ca supply routes during the early postpartum period: bone resorption and intestinal Ca absorption.

Bone resorption is inactive during the early postpartum period (Liesegang et al., 2000, 2007; Moreira et al., 2009; Taylor et al., 2009). In the present study, the transition in the concentration of serum NTX, which was used as a bone-resorption marker, suggested that bone resorption was restrained from 0 to 72 h relative to calving. Nevertheless, we observed a higher serum Ca concentration in the DFA group than in the control group from 6 to 48 h relative to calving. These results indicated that DFA III promoted Ca absorption by the intestine immediately after calving.
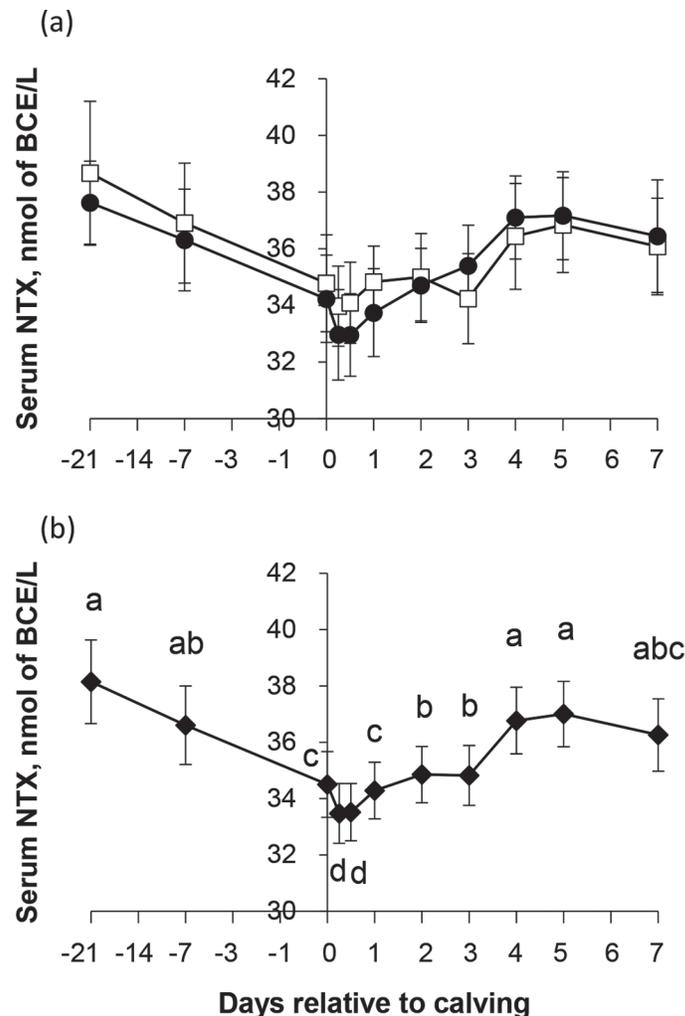
Figure 3. Serum cross-linked N-telopeptide of type I collagen (NTX; means ± SEM) during the peripartum period (−21 to 7 d relative to calving) in the control group (□; n = 37) and the group supplemented with difructose anhydride III (DFA, ●; n = 37) (a) and all cows (♦; n = 74) (b). BCE = bone collagen equivalents. Letters a, b, c, and d indicate differences at $P < 0.05$ at each time point among all cows.

Serum PTH increased at 0 h relative to calving in both of the groups. When serum Ca decreases, the parathyroid glands release PTH, which stimulates bone resorption and increases $1,25\text{-}(OH)_2D$ synthesis to enhance the rate of active intestinal Ca transport via the transcellular pathway and Ca reabsorption through the renal tubules (NRC, 2001). Metabolic alkalosis induces conformational changes in the PTH receptor, which disturbs the tight binding of PTH to its receptor and impairs the physiological activity of PTH, preventing upregulation of bone resorption and the production of $1,25\text{-}(OH)_2D$ (Goff et al., 1991, 2014; Phillippo et al., 1994). In the present study, the average serum pH in both groups was higher than the standard value

for alkalemia (>7.45; Constable et al., 1991) because the cows of both groups were offered a high-DCAD feed. Serum Ca concentration was significantly lower in the control group than in the DFA group during the postpartum period, even though the control group exhibited a high concentration of serum PTH. The reasons for this finding could be the malfunctioning of either the PTH receptor within the bone and kidney or the VDR within the intestinal epithelial cells, or both. Bone resorption was restrained in the early postpartum period, which might have been affected by metabolic alkalosis (Bushinsky, 1996). However, cows in which $1,25\text{-}(OH)_2D$ production is delayed or insufficient account for fewer than 10% of milk fever-affected cows (Goff et al., 1989). Furthermore, many studies have reported that serum $1,25\text{-}(OH)_2D$ increases to a level equal to or greater than that of normal cows in the early postpartum period, although these cows were fed high-DCAD feed (Goff and Horst, 1997; Penner et al., 2008), which affected hypocalcemia (Horst et al., 1978; Goff et al., 2014). Similarly, in the present study, serum $1,25\text{-}(OH)_2D$ increased in both groups at 0 and 12 h relative to calving. Therefore, these results suggested that the most significant factor delaying serum Ca recovery in the control group was the lack of reaction to increasing serum $1,25\text{-}(OH)_2D$ in the intestine, rather than PTH in the kidney in the early postpartum period because of the decrease in the number of VDR within the intestinal epithelial cells that occurs with aging (Horst et al., 1990). In contrast, even though the DFA group also experienced metabolic alkalosis and a decreasing number of VDR within the intestinal epithelial cells, serum Ca was significantly higher and serum PTH was significantly lower than in the control group during the early postpartum period. Thus, the improvement of serum Ca status of the DFA group occurred due to DFA III promoting intestinal Ca absorption, which is independent of PTH and $1,25\text{-}(OH)_2D$ during the early postpartum period. Furthermore, the time required to recover a normal range of serum Ca (≥ 9 mg/dL) following parturition was shorter in the DFA group than in the control group, even in high-parity cows. Moreover, DFA III promoted Ca absorption via the paracellular pathway of tight junctions in the intestine, a process that is unaffected by aging.

Calcium intake increases significantly during the early postpartum period, providing an increasing amount of Ca that could be absorbed in the intestine (Goff and Horst, 1997). Therefore, these conditions could contribute to the rapid recovery of serum Ca during the early postpartum period in the DFA group, as intestinal Ca absorption via the paracellular pathway depends on the concentration gradient (Bronner, 1987).

Hypocalcemia generally occurs when the amount of Ca lost to lactation exceeds that of the supplied Ca (Goff, 2008). In the present study, Ca outflow into the milk was significantly higher in the DFA group than in the control group, even though the time for the recovery of serum Ca during the early postpartum period was shorter in the DFA group. These results suggest that the Ca supply in the DFA group during the early postpartum period exceeded the amount of Ca outflow due to lactation, resulting from the DFA III-induced enhancement of Ca absorption by the intestine.

Table 4. Feed mineral intake, milk yield, and Ca outflow into the milk in the control and DFA (supplemented with difructose anhydride III) groups during the pre- and postpartum periods

| Feed mineral intake, g/d (unless otherwise noted) | Cows | Group | | | | P-value | | |
		Control	DFA	SEM	Groups	Time	G × T [1]
Ca	Dry[2]	46.7	47.8	1.0	0.71	0.12	0.55
	Lactating[3]	130.5	150.9	4.4	0.02	<0.01	0.74
P	Dry	55.3	52.3	1.0	0.79	0.14	0.65
	Lactating	93.4	114.2	3.4	<0.01	<0.01	0.48
Mg	Dry	30.1	28.7	0.5	0.33	0.06	0.48
	Lactating	42.0	51.4	1.5	<0.01	<0.01	0.23
K	Dry	292.7	290.3	6.7	0.90	0.11	0.74
	Lactating	325.0	404.8	12.6	<0.01	<0.01	0.30
Na	Dry	6.8	6.6	0.3	0.37	0.67	0.64
	Lactating	26.7	23.8	1.0	0.65	<0.01	0.17
Serum pH	Dry	7.46	7.46	0.00	0.71	0.93	0.41
Milk yield,[4] kg/d		27.2	31.6	0.8	<0.01	<0.01	0.38
Ca content,[4] %		0.16	0.15	0.00	0.22	<0.01	0.70
Ca outflow,[4] g/d		41.5	47.4	1.2	0.02	<0.01	0.16

[1]Interaction between the groups and time.

[2]Prepartum period (average from −14 to −1 d relative to calving).

[3]Postpartum period (average from 1 to 6 d relative to calving).

[4]Mean values at −21, −14, and −7 d relative to calving.

In the current study, we observed that serum Ca recovered rapidly during the postpartum period in the DFA group; however, we cannot confirm that DFA treatment affected the decline in the serum Ca that occurred immediately upon calving. In contrast, Sato et al. (2007) observed that the decline in the serum Ca immediately upon calving was prevented by supplementing the diet with DFA III during the close-up period. The difference between the results of the 2 studies may involve the management of the prepartum period. Therefore, further investigation of the conditions under which DFA III shows effects is necessary.

Treatment with DFA III promotes Ca absorption via the paracellular pathway, which occurs through passive diffusion driven by the concentration gradient and is independent of the content of VDR within intestinal epithelial cells. Therefore, DFA III dietary supplementation holds promise by potentially showing a universal effect that could protect older cows from hypocalcemia, as these cows are vulnerable to this condition because of a decreased number of VDR in the intestine.

CONCLUSIONS

Supplementation with DFA III during the peripartum period appears to promote passive Ca transport in the intestine via the paracellular pathway during the early postpartum period. This effect holds promise for decreasing the incidence of hypocalcemia in older cows, which are vulnerable to hypocalcemia because they have fewer vitamin D receptors in their intestine.

Acknowledgments

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