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ORIGINAL CONTRIBUTION

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Ingestion of difructose anhydride III partially restores calcium absorption impaired by vitamin D and estrogen deficiency in rats

Summary *Background* Vitamin D (VD) and estrogen deficiencies impair Ca absorption and bone mineralization, and the relevance of the interaction between these factors has not been fully understood. *Aim of the study* The aim of the present study was to clarify the effects of a nondigestible saccharide, difructose anhydride III (DFAIII), on the interaction of VD and estrogen deficiencies involved in Ca malabsorption by assessing changes in intestinal Ca absorption and bone mineralization by feeding of DFAIII in rats with VD or estrogen deficiency or with a combined loss of VD and estrogen. *Methods* Three-week-old female Sprague-Dawley rats were divided into four groups: two groups were ovariectomized (OVX) and two were laparotomized (sham). One group each of OVX and sham rats were fed an AIN93G-based normal diet, and the other groups were fed a VD-deficient diet for 8-weeks. Rats from the four groups were divided into two subgroups and fed the normal or VD-deficient diet with or without DFAIII for next 4-weeks. *Results* VD deficiency decreased Ca absorption and bone mineralization with reductions in duodenal calbindin D9k mRNA and serum Ca levels. There were no additional reductions in these parameters in the OVX. The reductions in Ca absorption and femoral Ca were restored partially or fully by DFAIII. Recovery of Ca absorption rate by DFAIII was greater in the OVX than in the sham showing an interaction between OVX and VD deficiency in, at least, the DFAIII-fed groups. The cecal pH was lower and the level of short-chain fatty acids in the cecal contents was higher in all the DFAIII groups than those in the control groups. *Conclusions* VD deficiency impaired Ca absorption and bone mineralization, and feeding DFAIII partially restored Ca malabsorption and fully recovered bone Ca in VD-deficient rats. No additional reductions in these parameters with a combination of VD deficiency and OVX were noted. However, interactions were found between these factors in the DFAIII-induced increase in Ca absorption.

Key words DFAIII – calcium absorption – vitamin D deficiency – ovariectomy – rats

Abbreviations DFAIII: difructose anhydride III · GAPDH: glyceraldehydes-3-phosphate dehydrogenase · IGF-1: insulin-like growth factor 1 · OVX: ovariectomy · RT-PCR: reverse transcription-polymerase chain reaction · SCFA: short-chain fatty acid · VD: vitamin D · $1,25(OH)_2D_3$: 1,25-dihydroxyvitamin D_3

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Introduction

1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] is recognized as playing a role in transcellular calcium (Ca) absorption and maintenance of Ca homeostasis [1–4]. The active transcellular process involving vitamin D (VD) requires three groups of Ca transport proteins, Ca transport protein type 1, calbindin D9k (the intracellular Ca transporter), and Ca ATPase [1, 2]. Several reports show that VD increases the level of calbindin D9k in cells [1, 2] and that the calbindin-D9k mRNA level is decreased in the duodenum of VD

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receptor-knockout mice and VD-deficient animals [1–4]. Moreover, a previous study demonstrated that $1,25(OH)_2D_3$ also increases paracellular Ca flux through changes in the chemical structure of tight junctions [5], which is another important pathway for intestinal Ca absorption. Therefore, VD deficiency may decrease Ca absorption through both transcellular and paracellular pathways.

Estrogen also influences active intestinal Ca absorption. The presence of estrogen receptors in rat duodenal cells was confirmed [6, 7] and estrogen may have a direct physiological role via these estrogen receptors in regulating intestinal Ca absorption [8, 9]. It has been reported that estrogen deficiency reduced duodenal Ca transport protein type 1 mRNA expression [3]. However, some reports showed that estrogen deficiency decreases VD receptor and calbindin D9k mRNA levels in the intestinal mucosa, and these reductions are recovered by estrogen treatment [10, 11]. These results suggest that downregulation of the VD receptor contributes to the Ca malabsorption in estrogen deficiency.

Several reports have indicated that ingestion of oligosaccharides and fermentable dietary fibers increase Ca absorption in rats [12–15]. Difructose anhydride III (DFAIII) is a newly manufactured nondigestible saccharide prepared from inulin with *Arthrobacter* sp. H65-7 inulin fructotransferase (Inulinase II; EC 2. 4. 1. 93). Recent studies show that DFAIII promotes Ca absorption in in vivo, in situ and in vitro experiments [16–21]. The proposed mechanisms for the promotion of Ca absorption are that intact DFAIII stimulates paracellular Ca absorption in the small intestine [16, 17, 19], and DFAIII increases the large intestinal Ca absorption with enhancement of bacterial fermentation [20]. Our previous study demonstrated that feeding DFAIII restored the ovariectomy-induced reduction in Ca absorption and led to increases in femoral Ca content [22]. However, it is still not known whether DFAIII ingestion recovers impairment of Ca absorption associated with VD deficiency or whether DFAIII has an effect on the interactive actions of VD and estrogen deficiencies in Ca absorption. DFAIII promotes paracellular Ca transport and VD has a role in the paracellular transport process as mentioned above. It is not clear whether DFAIII promotes Ca absorption under when both transcellular and paracellular pathways are downregulated.

The aim of the present study was to clarify the effects of DFAIII on the interaction of VD and estrogen deficiencies involved in postmenopausal Ca malabsorption by assessing changes in intestinal Ca absorption and bone mineralization by feeding of DFAIII in rats with VD or estrogen deficiency or with a combined loss of VD and estrogen.

Materials and methods

Animals and diets

Three-week-old female Sprague-Dawley rats (Japan Clea, Tokyo, Japan) weighing about 50 g were housed in individual stainless-steel cages with wire-mesh bottoms. The cages were placed in a room with controlled temperature (22–24 °C), relative humidity (40–60%) and lighting (lights on 8:00–20:00 h). The rats had free access to water and the semipurified basal diets (Table 1) based on AIN93G formulation [23] for an acclimation period of 4 days. The final dietary contents of Ca, magnesium (Mg) and phosphate (P) were 3000 mg Ca/kg diet, 507 mg Mg/kg diet and 3000 mg P/kg diet. The recommended dietary amount is same except for Ca. The recommended amount of Ca is 5000 mg Ca/kg diet.

This study was approved by the Hokkaido University Animal Committee, and the rats were maintained in accordance with the Hokkaido Uni-

Table 1 Composition of basal and test diets (the basal diet was same as normal-control diet)

Ingredient	Normal diet (g/kg)		Vitamin D-deficient diet (g/kg)	
	Control	DFAIII	Control	DFAIII
Casein[a]	250	250	–	–
Vitamin-free casein[b]	–	–	250	250
Corn oil[c]	50	50	50	50
Mineral mixture[d]	35	35	35	35
Calcium carbonate[b]	7.5	7.5	7.5	7.5
Vitamin mixture[e]	10	10	–	–
Vitamin D-free Vitamin mixture[e]	–	–	10	10
Choline bitartrate[b]	2.5	2.5	2.5	2.5
Cellulose[f]	50	35	50	35
DFAIII[g]	–	15	–	15
Sucrose[g]	595	595	595	595

[a] ALACID; New Zealand Dairy Board, Wellington, New Zealand
[b] Waco Pure Chemical Industries, Osaka, Japan
[c] Ajinomoto Co. INC., Tokyo, Japan
[d] AIN-93G mixture except for Ca. It provided (mg/kg diet): Ca 3000, P 1561, K 3600, S 300, Na 1019, Cl 1571, Mg 507, Fe 35.0, Zn 30.0, Mn 10.0, Cu 6.0, I 0.2, Mo 0.15, Se 0.15, Si 5.0, Cr 1.0, F 1.0, Ni 0.5, B 0.5, Li 0.1, V 0.1 (Waco Pure Chemical Industries, Osaka, Japan)
[e] AIN-93 mixture with or without vitamin D. It provided (U/kg diet): Nicotinic acid 30 mg, Pantothenate 15 mg, Pyridoxine 6 mg, Thiamin 5 mg, Riboflavin 6 mg, Folic acid 2 mg, vitamin K 750 μg, D-Biotin 200 μg, vitamin B_{12} 25 μg, vitamin A 4000 IU, vitamin D_3 1000 IU, vitamin E 75 IU (Waco Pure Chemical Industries, Osaka, Japan)
[f] AVICEL; Asahi Chemical Industry Co. Ltd., Tokyo, Japan
[g] Nippon Beet Sugar Mfg. Co. Ltd., Obihiro, Japan

versity guidelines for the care and use of laboratory animals.

Experimental design

The 3-week-old rats were divided into four groups, two groups underwent bilateral ovariectomy (OVX) and the other two groups underwent bilateral laparotomy (sham). One group each of sham and OVX rats were fed an AIN93G-based normal diet, and rats of the other groups were fed a diet from which VD was excluded (VD-deficient) for 8 weeks (Table 1). Fluorescent lights were covered with sheets to cut ultraviolet radiation to inhibit light-synthesis VD. Food intake of rat in each group was adjusted to the average intake of the group with the lowest value in each day (pair-feeding). All rats were given free access to deionized water. When 11-weeks old, the four groups of rats were divided into two subgroups of eight rats, and fed a normal or VD-deficient diet with or without DFAIII (15 g/kg diet) as shown in Table 1 for 4 weeks (the control or DFAIII group), respectively.

The body weight and food intake were measured every day. Feces were collected during the last 4 days of the test period. On the last day, the rats were anesthetized (Nembutal: sodium pentobarbital, 50 mg/kg body weight, Abbott Laboratories, North Chicago, IL, USA), and then killed after aortic blood was taken. Blood was centrifuged (1,300 g for 10 min at 4 °C) to obtain the serum. The proximal duodenum (0–5 cm distal to the pylorus) was removed, washed in cold saline (0.9% NaCl solution), slit lengthwise, and the mucosa collected onto a glass microscope slide. The mucosa was quickly frozen with liquid nitrogen for analysis of calbindin mRNA. The uterus was removed from each rat and weighed to confirm the success of the ovariectomy. The cecum was removed with its contents and weighed. The contents were collected and stored at −40 °C until subsequent analyses. The femur was removed from each rat, carefully cleaned of adherent tissue, and freeze-dried for measurement of mineral contents.

Analytical methods

Total RNA in the duodenal mucosa was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) according to manufacturer's suggested procedure, and the concentration of RNA was determined by the absorbance at 260 nm. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect calbindin D9k and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Total RNA (10 μg) was subjected to cDNA synthesis using reverse transcriptase and a random primer. The cDNA was amplified in a PCR master mix (Promega, Madison, WI, USA) with specific primers. Primers and annealing temperature were: calbindin D9k forward AAGAGCATTTT-TCAAAAATA: reverse GTCTCAGAATTTGCTTTATT: annealing temperature = 42 °C: GAPDH forward TCCACCACCCTGTTGCTGTAG: reverse GACCACA-GTCCATGACATCACT: annealing temperature = 54 °C. Twenty cycles for calbindin D9k and 29 cycles for GAPDH were employed for semi-quantification. Ten microliters of PCR products were fractionated on a 1.5% agarose gel containing ethidium bromide and bands were visualized and quantified under UV light.

Freeze-dried feces were milled, and the powdered feces were wet-ashed with an acid mixture (16 mol/l HNO_3: 9 mol/l $HClO_4$ = 3:1) without drying. The amounts of Ca and Mg in the right femurs were measured after the samples had been wet-ashed in the same way as the feces. Ca and Mg concentrations in those solutions were measured by atomic absorption spectrophotometry (AA-6400F; Shimadzu Corporation, Kyoto, Japan) after appropriate dilution with 0.1 mol/l HCl. P was determined in the femoral solutions by the molybdovanadate method [24]. Serum Ca concentration was assayed with a commercial kit (Calcium-C test; Waco Pure Chemical Industries, Osaka, Japan).

The cecal contents were homogenized with nine volumes of deionized water. The pH values of these homogenates were measured with a semiconducting electrode (ISFET pH sensor 0010-15C, Horiba, Ltd., Kyoto, Japan). The organic acids in the cecal contents were measured by high-performance liquid chromatography (Organic Acid Analysis System, Shimadzu Corporation, Kyoto, Japan) as previously described [25].

Calculations and statistical analyses

Ca absorption was calculated by the following equation:

$$\text{Ca absorption rate (\%)} = 100 \times (\text{Ca intake} - \text{Ca excretion in feces})/\text{Ca intake}.$$

Values shown represent the means ± SEM. Statistical analyses were performed by three-way ANOVA (vitamin D deficiency × operation × diet). The significance of inter-group differences was evaluated by Duncan's multiple-range test [26] ($P < 0.05$). If the variance was unequal, log transformations of the data were performed before ANOVA. All statistical analyses were done using SPSS for Windows, Version 11.0 J (SPSS, Chicago, IL, USA).

Table 2 Final body weight, food intake and uterine weight of sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without difructose anhydride III (DFAIII) for 4 weeks (Values are means ± SEM, $n = 8$. Values in a column not sharing a common letter differ, $P < 0.05$)

	Final body weight (g)	Body weight gain (g/day)	Uterine weight (mg/100 g body weight)
Normal			
Sham-control	252 ± 5.3 bc	1.1 ± 0.08 ab	254 ± 28.2 a
Sham-DFAIII	242 ± 6.6 cd	0.7 ± 0.14 b	301 ± 25.3 a
OVX-control	267 ± 3.7 a	1.1 ± 0.10 ab	21.5 ± 2.6 b
OVX-DFAIII	263 ± 3.1 ab	0.9 ± 0.07 ab	20.2 ± 1.7 b
Vitamin D deficiency			
Sham-control	238 ± 4.3 d	0.9 ± 0.11 ab	268 ± 27.1 a
Sham-DFAIII	235 ± 3.9 d	0.8 ± 0.13 ab	301 ± 38.6 a
OVX-control	270 ± 3.8 a	1.3 ± 0.09 a	17.5 ± 0.6 b
OVX-DFAIII	268 ± 5.5 a	1.1 ± 0.12 ab	21.3 ± 1.9 b
P-*values*			
Vitamin D (V)	0.324	0.358	0.842
Operation (O)	0.001	0.020	0.001
DFAIII (D)	0.123	0.003	0.176
V×O	0.027	0.156	0.767
V×D	0.434	0.357	0.873
O×D	0.630	0.526	0.202
V×O×D	0.617	0.342	0.743

Results

The results of the three-way ANOVA showed that final body weight, body weight gain and uterine weight were affected by OVX. There was an interaction between VD deficiency and OVX for final body weight (Table 2). The *post hoc* test showed that the final body weights of the VD-deficient rats were lower than those of the VD-normal rats (normal diet groups) in sham rats, but not in OVX rats.

Serum Ca concentration (Table 3) and calbindin D9k mRNA level (Fig. 1) were affected by VD deficiency according to the results of three-way ANOVA. Neither operation nor diet influenced serum Ca concentration and calbindin D9k mRNA levels.

The ANOVA results show that VD deficiency and diet influenced Ca absorption rates (Fig. 2).

Table 3 Serum calcium concentration in sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without Difructose anhydride III (DFAIII) for 4 weeks (Values are means ± SEM, $n = 8$. Values in a column not sharing a common letter differ, $P < 0.05$)

	Serum calcium concentration
Normal	
Sham-control	2.54 ± 0.079 a
Sham-DFAIII	2.49 ± 0.064 a
OVX-control	2.49 ± 0.062 a
OVX-DFAIII	2.39 ± 0.065 a
Vitamin D deficiency	
Sham-control	2.02 ± 0.145 b
Sham-DFAIII	1.99 ± 0.031 b
OVX-control	1.83 ± 0.089 b
OVX-DFAIII	1.95 ± 0.059 b
P-*values*	
Vitamin D (V)	0.001
Operation (O)	0.110
DFAIII (D)	0.767
V×O	0.767
V×D	0.308
O×D	0.666
V×O×D	0.435

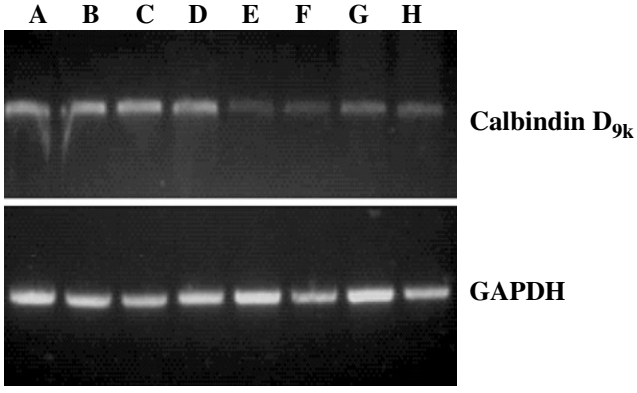

Fig. 1 Reverse transcription-polymerase chain reaction (RT-PCR) of duodenal calbindin D9k mRNA levels and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) in sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without DFAIII. Gel data of calbindin D9k mRNA were recorded using a NIH-imaging System. Normal diet sham-control (A): 221±14.4, sham-DFAIII (B): 215±12.2, OVX-control (C): 206±11.8, OVX-DFAIII (D): 211±12.8, vitamin D-deficient diet sham-control (E): 140±18.5, sham-DFAIII (F): 141±18.3, OVX-control (G): 143±15.9, and OVX-DFAIII (H): 141±18.0. Values are means±SEM, $n = 8$. *P*-values estimated by three-way ANOVA were <0.001 for vitamin D-deficiency (V), 0.712 for operation (O), 0.946 for DFAIII (D), 0.602 for V×O, 0.995 for V×D, 0.865 for O×D and 0.755 for V×O×D

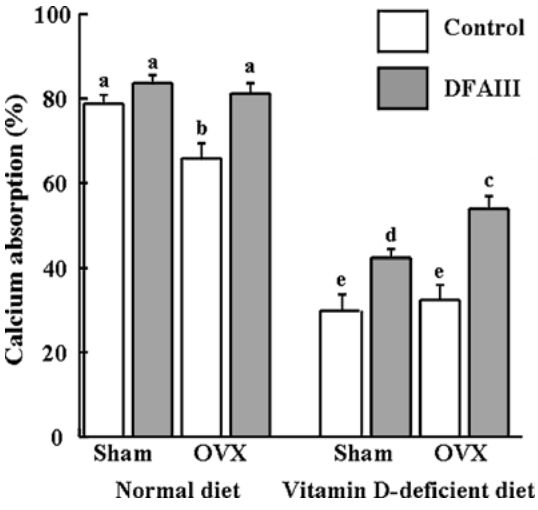

Fig. 2 Ca absorption rate in sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without DFAIII. Values are means±SEM, $n = 8$. *P*-values estimated by three-way ANOVA were <0.001 for vitamin D-deficiency (V), 0.864 for operation (O), <0.001 for DFAIII (D), <0.001 for V×O, 0.108 for V×D, 0.024 for O×D and 0.862 for V×O×D. Means not sharing a common letter differ, $P < 0.05$

Moreover, there were interactions between VD deficiency and operation, and between operation and diet. The Ca absorption rates were much lower in VD-deficient rats than in normal rats in the sham-control groups and also in the OVX-control groups by the results of post hoc test. OVX reduced Ca absorption in VD-normal rats, but the reduction was smaller than that in VD-deficient rats. OVX did not further reduce the absorption in VD-deficient rats. The absorption rate of Ca was higher in the DFAIII groups than in the control groups in both sham and OVX rats fed a normal or VD-deficient diet except for in the normal-sham rats. In the VD-deficient rat groups, Ca absorption rate in OVX rats fed the DFAIII diet was higher than in sham rats fed with the same.

The results of three-way ANOVA demonstrated that diet and OVX operation, but not VD deficiency influenced femoral Ca content, and there was a clear interaction between VD deficiency and DFAIII diet (Table 4). Femoral Ca content was lower in VD-deficient rats than in normal rats in the sham-control and OVX-control groups, and was higher in the DFAIII groups than in the control groups in VD-deficient sham and OVX rats. The results of ANOVA also showed that diet and OVX operation influenced femoral Mg content. The Mg content was higher in the DFAIII group than in the control group only in the VD-deficient sham rats. Femoral P contents were influenced by VD deficiency and OVX operation, but not by DFAIII.

Cecal wall weight was affected by VD deficiency, OVX, and DFAIII diet (Table 5). Cecal pH value and the pool of total SCFA in the cecal content were affected by the DFAIII diet. VD deficiency and OVX operation showed a clear interaction with regard to total SCFA pools.

Discussion

In the present study, we demonstrated that both VD and estrogen deficiencies impaired Ca absorption in

Table 4 Femoral mineral contents of sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without difructose anhydride III (DFAIII) for 4 weeks (Values are means ± SEM, $n = 8$. Values in a column not sharing a common letter differ, $P < 0.05$)

	Calcium (mmol/femur)	Magnesium (μmol/femur)	Phosphate (mmol/femur)
Normal			
Sham-control	1.57 ± 0.012 bc	156 ± 2.4 bc	1.50 ± 0.068 abcd
Sham-DFAIII	1.58 ± 0.004 bc	157 ± 1.0 ab	1.46 ± 0.043 bcd
OVX-control	1.60 ± 0.005 b	162 ± 0.7 a	1.67 ± 0.052 a
OVX-DFAIII	1.59 ± 0.003 b	162 ± 0.5 a	1.64 ± 0.089 ab
Vitamin D deficiency			
Sham-control	1.50 ± 0.031 d	153 ± 1.9 c	1.42 ± 0.065 cd
Sham-DFAIII	1.60 ± 0.009 b	157 ± 1.7 ab	1.35 ± 0.036 d
OVX-control	1.53 ± 0.020 cd	158 ± 0.8 ab	1.52 ± 0.052 abcd
OVX-DFAIII	1.66 ± 0.019 a	162 ± 1.8 a	1.55 ± 0.048 abc
P-*values*			
Vitamin D (V)	0.199	0.092	0.013
Operation (O)	0.010	0.001	0.001
DFAIII (D)	0.001	0.040	0.515
V×O	0.200	0.867	0.637
V×D	0.001	0.134	0.826
O×D	0.728	0.487	0.492
V×O×D	0.371	0.719	0.591

Table 5 Cecal wall weight, pH value, and short-chain fatty acid (SCFA) pool in the cecal contents of sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without Difructose anhydride III (DFAIII) for 4 weeks (Values are means ± SEM, $n = 8$. Values in a column not sharing a common letter differ, $P < 0.05$)

	Cecal wall weight (g/kg body wt)	pH	Total SCFA[a] (μmol/cecum)
Normal			
Sham-control	1.75 ± 0.155 cd	7.11 ± 0.123 a	45.8 ± 8.22 bc
Sham-DFAIII	2.30 ± 0.100 c	6.67 ± 0.081 b	78.0 ± 13.9 ab
OVX-control	2.16 ± 0.137 c	7.02 ± 0.092 a	27.2 ± 4.07 c
OVX-DFAIII	3.74 ± 0.369 a	6.98 ± 0.087 a	53.3 ± 6.79 abc
Vitamin D deficiency			
Sham-control	1.94 ± 0.164 cd	7.16 ± 0.053 a	44.2 ± 7.44 bc
Sham-DFAIII	2.15 ± 0.144 c	6.78 ± 0.084 b	56.9 ± 8.18 abc
OVX-control	1.59 ± 0.069 d	7.06 ± 0.087 a	61.3 ± 10.9 abc
OVX-DFAIII	2.96 ± 0.108 b	6.77 ± 0.059 b	84.8 ± 19.4 a
P-*values*			
Vitamin D (V)	0.012	0.350	0.169
Operation (O)	0.001	0.774	0.958
DFAIII (D)	0.001	0.001	0.003
V×O	0.008	0.566	0.006
V×D	0.282	0.903	0.474
O×D	0.001	0.263	0.876
V×O×D	0.794	0.467	0.582

[a] Total SCFA: sum of acetic, propionic and butyric acids in the cecal content

rats. However, the reduction in absorption was much greater in the VD-deficient rats. No combined effect of VD and estrogen deficiencies was found (Fig. 2). These results suggest that VD has a crucial role in both transcellular and paracellular Ca transport. It has been reported that VD receptor-knockout impairs Ca absorption and decreases calbindin D9 k mRNA levels in mice [3, 4, 27]. In the present study, we showed that the duodenal calbindin D9k mRNA level was clearly decreased in all VD-deficient rat groups without any inter-group differences among the VD-deficient rats (Fig. 1). These results confirm the induction of VD deficiency by our experimental conditions.

Estrogen deficiency with OVX impaired Ca absorption without any changes in calbindin D9k mRNA level (Figs. 1,2). Liel et al. reported that duodenal calbindin D9k mRNA was decreased by OVX [10]. Another report showed that estrogen receptor-α knockout mice displayed a significant reduction in duodenal Ca transport protein type 1 mRNA expression [3]. We used a semiquantitative method to estimate duodenal calbindin D9 k mRNA levels because we measured this mRNA level to confirm VD deficiency. A more quantitative method would likely show that OVX slightly but not significantly reduces calbindin D9k mRNA.

Ingestion of DFAIII was effective as a treatment for Ca malabsorption associated with estrogen deficiency, and partly restored Ca malabsorption associated with VD deficiency (Fig. 2). Previously, we reported that DFAIII promotes Ca absorption both in the small and large intestines [16–22]. In the large intestine, ingestion of DFAIII increases the SCFA pool and decreases the pH value, which may be associated with the increase in Ca absorption by DFAIII [20]. The active intracellular transport pathway may contribute to the enhanced absorption because there is a potential active Ca transport system in the large intestine [28] and DFAIII has a very limited effect on the paracellular Ca transport in the cecum (unpublished results). Our current study showed a decrease in cecal pH and a tendency toward an increased SCFA pool in the DFAIII groups (Table 5), which agrees with the previous study and shows stimulation of cecal fermentation. These findings suggest that DFAIII enhances Ca absorption in the large intestine via an active transport pathway.

In the small intestine, DFAIII promotes Ca absorption via the paracellular transport pathway through tight junctions [16, 17, 19]. Ingestion of DFAIII did not fully restore Ca malabsorption in the VD-deficient group (Fig. 2). VD plays a role in paracellular Ca transport [5], and VD deficiency may impair paracellular absorption. It is possible that the promotive effect of DFAIII on paracellular Ca transport is weak under VD-deficient conditions, which would explain the incomplete recovery of VD deficiency-induced Ca malabsorption with DFAIII feeding.

In the case of VD-deficient rats fed with DFAIII, Ca absorption rate was higher in OVX rats than in sham rats (Fig. 2), which was closely associated with the changes in bone Ca content (Table 4). Previous studies demonstrated that Ca homeostasis is also regulated by insulin-like growth factor 1 (IGF-1), and

that OVX elevates serum and duodenal levels of IGF-1 [29–31]. On the other hand, VD receptor-knockout reduces serum IGF-1 levels and inhibits normal growth in mice [32]. The current study showed the lowest final body weight in VD-deficient sham rats with the same food intakes as in the other groups (15.2 g/d, $P = 0.994$, $n = 64$), and that OVX improved the low body weight in VD-deficient rats (Table 2). This suggests that the lower level of IGF-1 is partly associated with the lower Ca absorption in VD-deficient animals, and the increase in IGF-1 level due to OVX is involved in the higher Ca absorption and higher body weight in OVX rats under VD-deficient conditions. This unexpected increase in Ca absorption by OVX is true only in the DFAIII groups. Some factors limiting Ca absorption may exist in the control diet groups, for example, low solubilization of the cecal Ca. Further studies will be necessary to determine the role of IGF-1 levels on Ca absorption and growth in VD- and estrogen-deficient rats.

We showed decreases in femoral Ca contents and serum Ca concentration in VD-deficient rats compared with VD-normal rats (Tables 3 and 4). And feeding DFAIII fully recovered bone Ca content in VD-deficient rats, but did not affect hypocalcemia. It has been reported that the primary cause for hypocalcemia in VD deficiency is reduction in the capacity of osteoclastic resorption [33]. Under the suppression of the bone resorption with VD deficiency, increase in bone formation with increasing calcium absorption by feeding DFAIII may prevent the reduction of bone calcium, but not hypocalcemia. Higher body weight may be a factor for bone recovery in the OVX rats. However, this was not true in the OVX-control group under VD-deficient conditions. In this group, no recovery in bone strength was noted in rats with higher body weight. Some factors influenced by DFAIII ingestion other than Ca absorption may also affect bone metabolism. Both VD and estrogen deficiencies are well known to be involved in osteoporosis [9, 10, 34, 35], and estrogen replacement therapy as well as VD treatment has been shown to be effective in preventing bone loss [34–37]. An adequate supply of Ca with improvement in Ca absorption by DFAIII may effectively prevent bone loss.

In conclusion, VD deficiency impaired Ca absorption and bone mineralization, and feeding DFAIII partially restored Ca malabsorption and fully recovered bone Ca in VD-deficient rats. No additional reductions in these parameters with a combination of VD deficiency and OVX were noted. However, interactions were found between these factors in the DFAIII-induced increase in Ca absorption. The large intestine may be partly involved in the beneficial effects of DFAIII.

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