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Ingestion of Raffinose Promotes Calcium Absorption in the Large Intestine of Rats

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We examined the effects of feeding raffinose on intestinal calcium absorption in ovariectomized rats by two separate experiments. In experiment 1, female Sprague-Dawley rats (6 wk old) were divided into two groups: sham operation and ovariectomy, and fed diets with or without raffinose (30 g/kg diet) for 4 wk. In experiment 2, ovariectomized rats with cecocolonectomy or transsection and reanastomosis (sham) were divided into two groups as in experiment 1 and fed the same diets for 3 wk. In experiment 1, calcium absorption was lower in the ovariectomized rats than in the sham rats but calcium absorption in rats fed the raffinose diet was higher than that in rats fed the raffinose-free diet. In experiment 2, increased calcium absorption in the raffinose group was abolished by cecocolonectomy. The impaired absorption in ovariectomized rats was restored by feeding raffinose. The large intestine is involved in the beneficial effects of raffinose.

Key words: raffinose; calcium absorption; ovariectomy; rats

Estrogen deficiency and declining calcium absorption are important factors in the pathogenesis of age-related bone loss. Guidelines for dietary calcium intake have been proposed for minimizing the risk of osteoporosis;[1,2] however, the calcium intake in Japan is lower than the dietary reference intake of calcium. [3,4] The recommended dietary allowance of calcium for Japanese adults is 600 mg/day, and the calcium requirement for elderly Japanese women was estimated by balance study to be 788 mg/day.[4] Mean intake of calcium in Japan (547 mg/day) has not reached the recommended dietary allowance. Moreover, women with postmenopausal osteoporosis are particularly susceptible to impaired calcium absorption.

It has been reported that calcium balance was decreased in ovariectomized (OVX) rats by increase in intestinal calcium secretion and by decrease in intestinal calcium absorption, and that these were the result of decreased levels of estrogen.[5] Holzherr *et al.*[6] and Kalu[7] reported similar observations in human and animals, and our previous report also agreed with these results that ovarian hormone deficiency impaired ability of calcium absorption in rats everted intestinal sacs.[8] The presence of estrogen receptors in rat duodenal cells were confirmed and estrogen stimulates intestinal calcium absorption via an estrogen receptor.[9] In cases with lower calcium intake and insufficient calcium absorption in the small intestine such as postmenopausal women, calcium absorption in the large intestine is important to maintain calcium homeostasis.[10]

Several reports have indicated that the ingestion of fermentable dietary fibers[11–14] and oligosaccharides[15,16] increased calcium absorption in rats, and that the large intestine was involved in this beneficial effect.[16,17] Basically, dietary fibers and oligosaccharaides are presumably not absorbed in the small intestine but metabolized by the bacterial flora in the large intestine. Some studies have suggested that microbial fermentation products of indigestible saccharides are responsible for the increase of calcium absorption in the large intestine.[18,19]

Raffinose, which is a trisaccharide composed of galactose, glucose, and fructose (β-D-fructofuranosyl-*O*-α-D-galactopyranosyl-(1,6)-α-D-glucopyranoside), is an indigestible oligosaccharide that is distributed in plants such as sugar beet and soybean. It has been reported that administration of raffinose increased the fecal bifidobacteria and improved defecation in humans.[20] On the other hand, raffinose has been fermented by colonic microorganisms to produce short-chain fatty acids (SCFA),[21] from which it is expected for raffinose to increase calcium absorption in the large intestine. However, little is known about effects of raffinose on calcium absorption. In a previous study, we reported that raffinose promoted calcium absorption in normal rats and *in vitro*.[21,22] However, it has not been clarified whether raffinose improves ovariectomy-induced calcium malabsorption in rats.

The aim of this study was to evaluate the promotive

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Abbreviations: OVX, ovariectomy; SCFA, short-chain fatty acids

Table 1. Composition of Stock and Test Diets[a]

	g/kg diet
Casein[b]	250
Corn oil	50
Mineral mixture[c]	35
Vitamin mixture[c]	10
Choline bitartrate	2.5
Cellulose[d]	50
Sucrose	To make 1 kg

[a] Raffinose (30 g/kg diet; Nippon Beet Sugar Mfg. Co., Ltd., Obihiro, Japan) was added to the test diets.
[b] Alacid (New Zealand Dairy Board, Wellington, New Zealand).
[c] Mineral and vitamin mixtures, except calcium, were prepared in accordance with the AIN-93G formulation.[23] The calcium concentration was 5.0 g Ca/kg diet in the stock diet. The test diets were replaced by a diet containing calcium carbonate at 7.5 g/kg diet (3.0 g Ca/kg diet).
[d] Crystallized cellulose (AVICEL; Asahi Chemical Industry Co., Ltd., Tokyo, Japan).

effects of raffinose on calcium absorption in OVX rats. We examined the effects of raffinose on calcium absorption in OVX rats (experiment 1), and evaluated the contribution of the large intestine to the effects of raffinose by using cecocolonectomy in OVX rats (experiment 2).

Materials and Methods

Animals and diets. Six-wk-old female Sprague-Dawley rats (Japan Clea, Tokyo, Japan) weighing about 150 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 0800–2000 h). The rats were given free access to water and the semi-purified stock diets shown in Table 1 for an acclimation period of 7 d.

In experiment 1, the acclimatized rats were divided into two groups, one group of rats underwent bilateral ovariectomy (OVX) and the other group underwent bilateral laparotomy (sham). All rats were given free access to deionized water and a stock diet for 5 d to recover from surgical damage. The rats in each group were divided into two subgroups of 10 rats, and then given either of two test diets, the control or raffinose (30 g/kg diet) diet, for 4 wk. The mineral and vitamin mixture of the test diets was prepared in accordance with the AIN-93[23] except for calcium. The calcium level in both test diets was 3.0 g Ca/kg diet. To prevent ovariectomy-associated hyperphagia, the OVX rats were given the average amount of each diet ingested by sham rats.

In experiment 2, all acclimatized rats underwent bilateral OVX operation. After a 7-d recovery period, two groups of OVX rats underwent cecocolonectomy or a sham operation. Briefly, the cecum and colon of rats in one group were removed after ligation of blood vessels, and end-to-end anastomosis was done between the cut edge of the ileum and the rectum (cecocolonectomy). Rats in the other group underwent transsection of the terminal ileum and reanastomosis (sham). All rats were deprived of food and water for 24 h after the operation. After a 7-d recovery period with a stock diet, both groups of rats were divided into two diet subgroups of 8 rats and given the control or raffinose diet for 3 wk.

In both experiments, body weight and food intake were measured every day. Feces were collected during the last 3 d of the test period. On the last day of the test period, rats were killed under pentobarbital anesthesia (Nembutal: sodium pentobarbital, 50 mg/kg body weight, Abbott Laboratories, North Chicago, IL, USA). The uterus was removed from each rat and weighed to confirm the success of the ovariectomy. In experiment 1, the ceca were removed with their contents and weighed. The contents were collected and stored at −40°C until subsequent analyses.

This study was approved by the Hokkaido University Animal Committee, and the rats were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

Analytical methods. 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); calcium and magnesium concentrations in the ashed solutions were measured by atomic absorption spectrometry (AA-6400F; Shimadzu Corporation, Kyoto, Japan). The cecal contents were homogenized with nine volumes of deionized water. The total calcium concentration in the homogenate was measured by the same method as that of the feces. The supernatant was obtained from the homogenate by centrifugation at 30,000 g for 20 min at 4°C to evaluate soluble calcium concentration in the cecal contents. The pH values of these homogenates were measured with a semiconducting electrode (ISFET pH sensor 0010-15C, Horiba, Ltd., Kyoto, Japan). The organic acids were measured by high-pressure liquid chromatography (Organic Acid Analysis System, Shimadzu Corporation, Kyoto, Japan) as previously described.[24]

Calculations and Statistical Analyses. Calcium and magnesium absorption rates were calculated by the following equations: Ca absorption rate (%) = 100 × (total Ca intake − Ca excretion in feces)/total Ca intake. Mg absorption rate (%) = 100 × (total Mg intake − Mg excretion in feces)/total Mg intake. The solubility of calcium in the cecal contents was estimated by the following equation: soluble Ca in the cecal contents (%) = 100 × (soluble Ca pool in the cecal contents/total Ca pool in the cecal contents).

Values shown represent the means ± standard error of the mean. The effects of treatment and diet were analyzed by two-way analysis of variance. When both treatment and diet were significantly different, Duncan's multiple-range test[25] was used to determine whether mean values were significantly different between groups

($P < 0.05$). All statistical analyses were done using SPSS for Windows, Version 10.0 J (SPSS, Chicago, IL).

Results

Experiment 1

The mean final body weight was greater in OVX rats than in the sham-operated rats despite there being no differences in food intake between groups (Table 2). The uterine weight was much lower in the OVX rats than in the sham rats (Table 2). The uterine weight of one rat in the OVX group was as large as that in the sham group and we excluded this rat from the OVX group.

The calcium absorption rate of the control diet group but not of the raffinose-fed group was lower in OVX rats than that in sham rats (Fig. 1A). As for the calcium absorption rate, we did a multiple-range test since both treatment and diet were significantly different. The absorption rates in the raffinose groups were higher than those in the control groups in both the sham and OVX rats. Moreover, the calcium absorption rate in OVX rats fed the raffinose diet was similar to those in the sham groups. Ovariectomy did not influence the magnesium absorption rate; however, the absorption rates were higher in the raffinose groups than in the control groups in both the sham and OVX rats (Fig. 1B).

In the OVX rats, the weight of the cecal wall and contents was greater than those in the sham rats (Table 3). In both the sham and OVX rats, the weight of the cecal wall and contents in the raffinose groups were much greater than those in the control groups. Cecal pH values in rats fed the raffinose diet were lower than those in rats fed the control diet. Ovariectomy did not influence the pH values. The total calcium pool in the cecal contents of OVX rats, especially in rats fed the control diet, was much higher than that of sham rats

Table 2. Final Body Weights, Food Intake and Uterine Weight (experiment 1)

	n	Final body weight	Food intake	Uterine weight
		g	*g/day*	*mg/100g body weight*
Sham				
Control	10	279±5.5	18.4±0.34	211±19.0
Raffinose	10	270±6.5	18.3±0.42	204±14.5
OVX				
Control	9	294±3.3	18.1±0.02	32±1.7
Raffinose	10	297±3.5	18.2±0.01	31±0.9
P values				
Operation		<0.001	0.493	<0.001
Diet		0.455	0.668	0.677
Operation × diet		0.176	0.856	0.701

Each value represents means±SEM.
OVX, ovariectomy.

Table 3. Weight of the Fresh Cecal Wall and Cecal Contents, and pH of the Cecal Contents (experiment 1)

	n	Cecal wall	Cecal contents	pH
		g/100g body weight	*g*	
Sham				
Control	10	0.178±0.017	1.69±0.111	7.50±0.077
Raffinose	10	0.257±0.020	3.11±0.314	6.43±0.116
OVX				
Control	9	0.198±0.015	2.60±0.216	7.50±0.085
Raffinose	10	0.318±0.017	4.05±0.303	6.70±0.096
P values				
Operation		0.027	<0.001	0.182
Diet		<0.001	<0.001	<0.001
Operation × diet		0.260	0.947	0.168

Each value represents means±SEM.
OVX, ovariectomy.

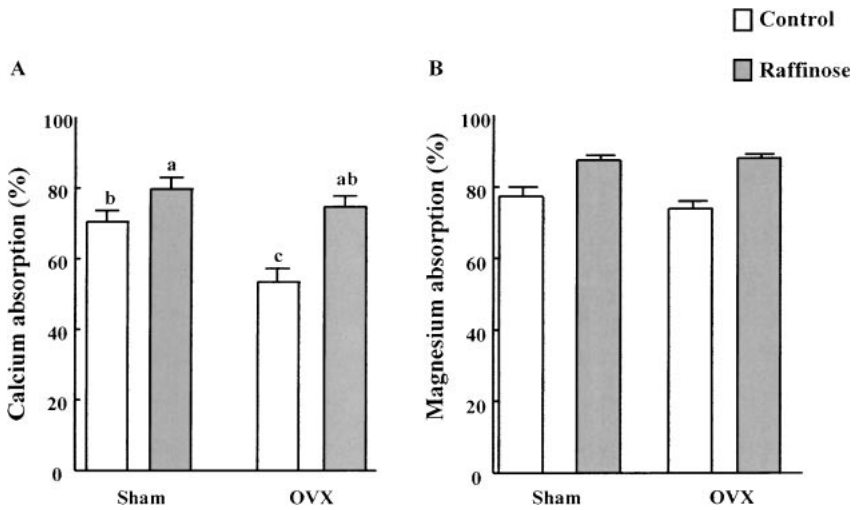

Fig. 1. Calcium Absorption Rate (A) and Magnesium Absorption Rate (B) in Sham-operated and OVX Rats (experiment 1).
Each value represents the mean±SEM ($n = 9$ in the ovariectomy and control group; $n = 10$ in the two sham groups and in the ovariectomy and raffinose group). *P*-values estimated by two-way analysis of variance were less than 0.001 (A), 0.419 (B) for operation, 0.001 (A, B) for diet, and 0.032 (A), 0.294 (B) for operation × diet. Values not sharing a common letter are significantly different, $P < 0.05$. OVX, ovariectomy.

Table 4. The Total and Soluble Calcium Pool and the Ratio of Soluble Calcium in the Cecal Contents (experiment 1)

	n	Total Ca pool	Soluble Ca pool	Soluble Ca
		μmol/cecal content		*%*
Sham				
Control	10	204±26.9	19.5±2.69	10.6±1.33
Raffinose	10	239±40.1	37.7±7.63	17.8±3.03
OVX				
Control	9	374±38.7	20.0±4.17	5.8±1.29
Raffinose	10	355±58.6	63.1±11.35	21.2±3.85
P values				
Operation		0.003	0.098	0.792
Diet		0.857	<0.001	<0.001
Operation × diet		0.544	0.111	0.140

Each value represents means±SEM.
Soluble Ca in the cecal content (%) = 100 × (soluble Ca pool/total Ca pool).
OVX, ovariectomy.

(Table 4). The soluble calcium pool and solubility rate of the raffinose diet group were higher than those of the control group. Concentrations of SCFA and other organic acids in the cecal contents are shown in Table 5. Butyrate and lactate concentrations were higher in rats fed the raffinose diet than in rats fed the control diet. In the sham rats, lactate concentration was 20-fold higher in rats fed the raffinose diet than in rats fed the control diet. There were no inter-group differences in acetate or succinate concentrations.

Experiment 2

There were no differences in mean final body weight or food intake between groups. The calcium absorption rate in the OVX-sham rats fed the raffinose diet was higher than that in the OVX-sham rats fed the control diet, as in experiment 1 (Fig. 2A). In the OVX-cecocolonectomized rats, the calcium absorption rate was not increased by feeding with the raffinose diet. In the raffinose-fed groups, the absorption rate was much lower in the cecocolonectomized rats than in the sham rats; however, there were no observed differences between in sham and cecocolonectomized rats fed the control diet. The magnesium absorption was lower in the OVX-cecocolonectomized rats than in the OVX-sham rats (Fig. 2B). There were no differences in magnesium absorption rates between the control and the raffinose diet in both the OVX-sham and OVX-cecocolonectomized rats.

Discussion

In this study, we demonstrated that ingestion of raffinose, an indigestible oligosaccharide, increased the calcium and magnesium absorption rates in OVX and sham-operated rats (Fig. 1). The observed decrease in calcium absorption caused by ovariectomy agrees with the results of previous studies, which have reported that estrogen stimulates duodenal calcium absorption via an estrogen receptor,[9] and estrogen deficiency impairs calcium absorption in the small intestine.[5,8,14] Calcium malabsorption has been implicated as a risk factor for postmenopausal osteoporosis.[1,2] Low dietary calcium levels also accelerate osteoporosis, and postmenopausal women tend toward a low calcium intake.[3,4] In this study, we adopted a 3.0 g Ca/kg diet, which is the minimum requirement of calcium carbonate in rats.[8] Under this condition, ingestion of raffinose promoted efficient calcium absorption. Especially, in the case of OVX rats, increasing calcium absorption by feeding raffinose was higher than that of sham rats. This result suggests that ingestion of raffinose prevented calcium malabsorption in OVX rats. In this study, there were considerable differences between calcium absorption rates of OVX rats in experiments 1 and 2. In experiment 2, OVX-sham rats underwent transsection of the terminal ileum and reanastomosis. This operation might affect calcium absorption in OVX-sham rats because if a part of the intestinal tract at the operation point becomes narrow, transit time of the chyme might be slowed and this alteration may increase in the calcium absorption. On the other hand, magnesium absorption was not influenced by ovariectomy (Fig. 1B). Magnesium is absorbed throughout the intestine and especially the small intestine is the main part, but the large intestine is

Table 5. Concentrations of SCFA and Other Organic Acids in the Cecal Contents (experiment 1)

	n	Acetate	Propionate	Butyrate	Succinate	Lactate
				μmol/g cecal contents		
Sham						
Control	10	29.5 ± 1.94	8.28 ± 0.93[a]	3.97 ± 0.61	4.14 ± 1.80	1.67 ± 0.22[b]
Raffinose	10	27.9 ± 4.46	2.62 ± 0.98[b]	6.54 ± 1.51	1.65 ± 0.79	32.9 ± 7.82[a]
OVX						
Control	9	24.4 ± 1.33	7.73 ± 0.62[a]	3.58 ± 0.23	4.38 ± 1.21	1.61 ± 0.51[b]
Raffinose	10	27.1 ± 1.18	7.75 ± 0.78[a]	9.59 ± 1.34	1.78 ± 0.66	14.2 ± 3.03[a]
P values						
Operation		0.258	0.011	0.220	0.879	0.033
Diet		0.821	0.002	<0.001	0.042	<0.001
Operation × diet		0.420	0.002	0.117	0.965	0.035

Each value represents means±SEM. Values in a column not sharing a superscript differ significantly, $P < 0.05$ by ANOVA.
SCFA, short-chain fatty acids; OVX, ovariectomy.

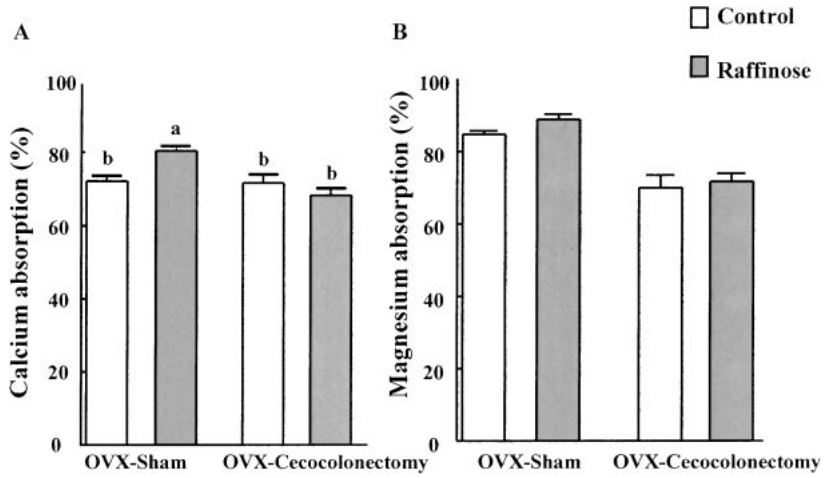

Fig. 2. Calcium Absorption Rate (A) and Magnesium Absorption Rate (B) in OVX Rats with or without Cecocolonectomy (experiment 2). Each value represents the mean±SEM ($n = 8$). *P*-values estimated by two-way analysis of variance were less than 0.001 (A, B) for operation, 0.146 (A), 0.130 (B) for diet, and 0.002 (A), 0.657 (B) for operation × diet. Values not sharing a common letter are significantly different, $P < 0.05$. OVX, ovariectomy.

also important for magnesium absorption in rats.[16,26] This study showed that the magnesium absorption rate was impaired by cecocolonectomy in rats, although it was small (Fig. 2B). This result suggests that magnesium is also absorbed efficiently in the distal parts of the intestine.

Canceling the promotive effect of raffinose by removal of the large intestine (Fig. 2) reveals that the small intestine does not contribute to the action of raffinose. Rather, the large intestine alone is involved in the beneficial effects of raffinose. In this study, we showed that ingestion of raffinose led to a lower cecal pH (Table 3), a greater pool of soluble calcium in the cecal contents (Table 4), and increased concentrations of SCFA, particularly butyrate and lactate, in rats (Table 5). These results suggest that ingestion of raffinose reduced cecal pH by production of organic acids, especially lactate. And that this acidification of the cecal contents induces the solubilization of calcium. An increase in the calcium solubility is involved in the increased calcium absorption. Moreover, the presence of SCFA is one of the promotive factors for calcium absorption in the large intestine.[18,19]

Calcium absorption in the small intestine is strictly regulated by endogenous factors such as parathyroid hormone and 1, 25-dihydroxycholecalciferol.[27] When small-intestinal calcium absorption was decreased, the large intestine compensated for the deficiency in calcium absorption.[10] In cases of lower calcium intake and insufficient calcium absorption in the small intestine, calcium absorption in the large intestine is important to maintain calcium homeostasis. This study demonstrates that ingestion of raffinose promotes calcium absorption in the large intestine in OVX rats. Guidelines for dietary calcium intake have been proposed for minimizing the risk of osteoporosis;[1,2] however, the calcium intake in Japan is lower than the dietary reference intake of calcium.[3,4] In this situation, the addition of raffinose as a natural food in the diet may effectively promote calcium absorption, especially in menopausal women who have impaired calcium absorption in the small intestine. Further studies are required to examine the effects of raffinose on calcium absorption in humans.

In conclusion, ingestion of raffinose increased calcium and magnesium absorption in OVX and sham-operated rats. In the case of OVX rats, raffinose feeding prevented calcium malabsorption completely. The promotive effect of raffinose on calcium absorption was abolished by cecocolonectomy. The large intestine is involved in the beneficial effects of raffinose.

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