Prolonged feeding of difructose anhydride III increases strength and mineral concentrations of the femur in ovariectomized rats

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This study demonstrates that feeding difructose anhydride III (DFAIII) improves bone strength and femoral mineral concentrations in a rat model of oestrogen deficiency. We showed the relationship between Ca, Mg and P absorption and bone characteristics in rats. Two groups of female Sprague-Dawley rats (6 weeks old) underwent bilateral ovariectomy (ovariectomized rats, OVX rats) or bilateral laparotomy (sham rats). At 10 weeks old, OVX and sham rats were divided into three subgroups and fed a control, 1·5 % DFAIII or 3 % DFAIII diet for 8 weeks, respectively. Ca but not Mg absorption rates were lowered by ovariectomy; however, ingestion of the 1·5 % and 3 % DFAIII diets similarly restored the reduced Ca absorption in OVX rats at 4 and 8 weeks after feeding of the test diets. DFAIII increased Mg absorption dose-dependently in sham and OVX rats. The bone strength, femoral Ca and Mg concentrations, and distal bone mineral density in the 3 % DFAIII group were higher than those in the control group in OVX rats. The absorption rates of Ca and Mg were significantly correlated with femoral Ca and Mg concentrations and strength, which suggests that increasing both Ca and Mg absorption improves bone characteristics in OVX rats. There were no differences in any of the variables in the femur between the 1·5 % and 3 % DFAIII groups in OVX rats. In conclusion, feeding of a low dose of DFAIII increased intestinal Ca and Mg absorption, and the promotive effect of DFAIII persisted for over 8 weeks. This effect was associated with prevention of ovariectomy-induced osteopenia.

Difructose anhydride III: Ca absorption: Bone strength: Ovariectomized rats

It is well known that ovarian hormone deficiency is involved in osteoporosis (Kanis, 1996; Qu *et al.* 2000). Oestrogen replacement therapy has been shown to be effective in preventing bone loss (Kanis, 1996); however, this therapy may induce serious side-effects (Eastell, 2003). There is considerable interest in dietary alternatives that include the consumption of phyto-oestrogen and increasing Ca intake (Breitman *et al.* 2003). Some studies have shown that oestrogen deficiency in post-menopausal or oophorectomized women impairs intestinal Ca absorption and decreases bone mineral density (BMD) (Holzherr *et al.* 2000; van den Heuvel *et al.* 2000), and these impairments were demonstrated in ovariectomized (OVX) rats (O'Loughlin & Morris, 1994; Kalu & Orhii, 1999; Mitamura *et al.* 2002). Moreover, Ca intake in East Asian people including Japanese is lower than the dietary reference intake of Ca (Ministry of Health, Labour and Welfare in Japan, 2002).

Absorption of Ca, Mg and P influences bone composition and structure as they are the main components of bone and contribute to bone strength with changes in bone metabolism (Ilich & Kerstetter, 2000; Eastell & Lambert, 2002). Bone collagen also contributes to bone toughness (Oxlund *et al.* 1995; Wang *et al.* 2002). Cu and Fe are also known to influence maturation of collagen as lysyl oxidase is a Cu-containing enzyme and Fe is a co-factor for lysyl hydroxylase (Medeiros *et al.* 1997, 2002). Among these dietary factors, the importance of Ca intake is well reported for the prevention of osteoporosis. A low Ca intake increases bone

resorption and induces bone loss (Ginty *et al.* 1998; Talbott *et al.* 1998). However, a previous study has shown that increasing dietary Ca intake above the recommended level had no effect on bone mineral composition in rats (Creedon & Cashman, 2001). Therefore, not only an increase in Ca intake but also improvement in mineral absorption may be necessary to prevent osteoporosis in post-menopausal women.

Several reports have indicated that ingestion of oligosaccharides and fermentable dietary fibres increases Ca absorption in rats (Ohta *et al.* 1995; Hara *et al.* 1999; Roberfroid, 1999; Mitamura *et al.* 2003). Difructose anhydride III (DFAIII) is a newly manufactured non-digestible saccharide prepared from inulin with *Arthrobacter* sp. H65-7 inulin fructotransferase (Inulinase II; EC 24.1.93). Recently, we have reported that DFAIII promotes Ca absorption in *in vivo* and *in vitro* experiments (Suzuki *et al.* 1998; Mineo *et al.* 2001, 2002; Mitamura *et al.* 2002; Shiga *et al.* 2003; Suzuki & Hara, 2004). The proposed mechanisms for the promotion of Ca absorption are reported that intact DFAIII stimulates paracellular Ca absorption in the small intestine (Mineo *et al.* 2001, 2002; Suzuki & Hara, 2004) and ingestion of DFAIII increases the SCFA pool and decreases the pH value of the caecal contents and this fermentation of DFAIII is involved in the increased Ca absorption in the large intestine in OVX rats (Mitamura *et al.* 2002). Our previous study also showed that feeding DFAIII restored the ovariectomy-induced reduction in Ca absorption and led to increases in bone strength and femoral Ca content (Mitamura *et al.* 2002).

However, the previous study conducted to determine the contribution of the small and large intestine to the promotive effects of DFAIII used caecocolonectomized rats. Therefore, the relationship between DFAIII feeding and the promotion of Ca absorption and bone characteristics has not been fully elucidated. Also, the 4-week feeding period in the previous study was relatively short for the observation of bone metabolism.

The aim of the present study was to clarify the relationship between Ca absorption and bone characteristics in OVX rats after feeding DFAIII. The rats were fed the stock diet without DFAIII for 4 weeks after ovariectomy to induce Ca malabsorption with oestrogen deficiency, and were then given the test diets for 8 weeks. We then assessed the effects of DFAIII on Ca absorption, and femur variables, such as bone mineral and collagen contents, bone-breaking force, regional BMD, and bone turnover, in both sham and OVX rats. Furthermore, we confirmed the dose-dependent effect of DFAIII.

Materials and methods

Animals and diets

Female Sprague-Dawley rats (6 weeks old; 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 08.00–20.00 hours). The rats had free access to deionized water and the stock diet shown in Table 1 for a 4 d adaptation period.

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.

Study design

At 6 weeks old, the rats were divided into two groups; rats of one group underwent bilateral ovariectomy (OVX rats) and those of the other group underwent bilateral laparotomy (sham rats). After the operation, all rats were fed the stock diet for 4 weeks. At 10 weeks, the rats in each group were divided into three subgroups of nine or ten rats, and then given one of the test diets (control, 1·5 % DFAIII (15 g/kg diet) or 3 % DFAIII (30 g/kg diet)) shown in Table 1 for 8 weeks. To prevent hyperphagia associated with ovariectomy, the OVX rats were given the average amount of

Table 1. Composition of stock and test diets*

Component	Amount (g/kg)
Casein†	250
Maize oil	50
Mineral mixture‡	35
Vitamin mixture‡	10
Choline bitartrate	2·5
Cellulose§	50
Sucrose	To make 1 kg

* Difructose anhydride III (15 or 30 g/kg diet; Nippon Beet Sugar, Obihiro, Japan) was added to the test diet.
† ALACID; New Zealand Dairy Board, Wellington, New Zealand.
‡ Mineral and vitamin mixtures were prepared in accordance with the AIN-93G formulation (Reeves et al. 1993) without Ca. The Ca concentration was 3·0 g Ca/kg diet.
§ AVICEL; Asahi Chemical Industry, Tokyo, Japan.

food ingested by the sham rats each day during the experimental period.

The body weight and food intake were measured each day. Faeces were collected three times; before feeding of the test diet (week 0), from day 23 to day 27 (week 4) or from day 51 to day 55 (week 8) after feeding of the test diets. From day 54, urine was collected for 24 h to measure deoxypyridinoline (DPD). On the last day, the rats were anaesthetized (Nembutal: sodium pentobarbital, 50 mg/kg body weight; Abbott Laboratories, North Chicago, IL, USA), then killed by withdrawal of the aortic blood. Both femurs were then removed, carefully cleaned of adherent tissue, and the left femurs were used to measure bone strength. The right femurs were used to measure BMD, the femurs were then freeze-dried to measure mineral and collagen concentrations. The uterus was removed and weighed to confirm the success of the ovariectomy in each rat.

Analytical methods

Freeze-dried faeces were milled, and the powdered samples (100 mg) 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 hydrolysed at 110°C for 24 h with 6 mol/l HCl. 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 concentration in the solutions of the ashed faeces and hydolysed femur was determined by the molybdovanadate method (Ueda & Wada, 1970). Hydroxyproline concentration in the acid hydrolysate of the femur was determined by a colorimetric method (Bergman & Loxley, 1970) in which the hydrolysed solution was oxidized by chloramine-T and applied to colorimetry by Ehrlich reaction at 558 nm.

The maximum breaking force of the left femoral diaphysis (the centre of the femur) was measured as the bone strength. A three-point bending test (Shiga et al. 2002) was performed with a rheometer (RE-3305 Rheoner; Yamaden, Tokyo, Japan) under the following conditions: sample space, 1·0 cm; pranger speed, 30 mm/min; load range, 20 kg. The whole BMD of the right femur was measured by dual-energy X-ray absorptiometry with a small animal high-resolution scan module (QDR-4500A; Hologic, Bedford, MA, USA). The regional BMD was estimated: 25 % proximal, 50 % middle (midshaft) and 25 % distal in the right femur.

Urinary DPD concentration was assessed by using a commercial kit (Osteolinks DPD; Sumitomo Pharmaceuticals, Osaka, Japan). Urinary creatinine concentration was measured by the Jaffe reaction (Lustgarten & Wenk, 1972). Values were expressed as nmol DPD/mmol creatinine.

Calculations and statistical analyses

Apparent Ca absorption rate was calculated with the following equation:

$$Ca\ absorption\ rate\ (\%) = 100 \times (Ca\ intake$$
$$- Ca\ excretion\ in\ faeces)/Ca\ intake.$$

Apparent Mg and P absorption rates were calculated in the same manner.

Values shown represent the means with their standard errors. Statistical analyses were performed by two-way ANOVA (treatment × diet). Duncan's multiple range test (Duncan, 1955) was used to determine whether mean values were significantly different between groups ($P < 0.05$). Pearson's correlation coefficients were calculated. All statistical analyses were done using SPSS for Windows, version 11·0 J (SPSS, Chicago, IL, USA).

Results

Body weight and food intake

Final body weight was greater in OVX rats than in sham-operated rats, while there were no differences in food intake (16·8 g/d, $P = 0.857$, n 58) between groups (Table 2). Body weight gain was also higher in OVX rats than in sham rats. The uterine weights of all OVX rats were much lower than those of the sham rats. There were no differences in final body weight, body weight gain and uterine weight among the three diet subgroups either in sham or OVX rats.

Mineral absorptions

As the result of two-way ANOVA in each term (0, 4 and 8 weeks), treatment (ovariectomy) influenced Ca absorption rate before feeding of test diets (0 week) and diet influenced the absorption rate after feeding of test diets (4 and 8 weeks) (Fig. 1). By the *post hoc* test within 4 or 8 weeks, the absorption rates of Ca were higher in the 1·5 % and 3 % DFAIII groups than those in the control group for both sham and OVX rats except for sham rats fed 1·5 % DFAIII diet at 4 weeks. Moreover, Ca absorption rates in both DFAIII diet groups of OVX rats were very similar to those of the sham DFAIII diet groups, respectively, at 4 and 8 weeks. There were no differences in Ca absorption rates between the 1·5 % and 3 % DFAIII groups in sham and OVX rats during feeding of the test diets.

The absorption rate of Mg was not influenced by ovariectomy, but was influenced by diet at 4 and 8 weeks according to the result of two-way ANOVA (Fig. 2). The absorption rates of Mg in the 1·5 % and 3 % DFAIII diet groups were higher than those in the control diet group in both sham and OVX rats at 4 and 8 weeks. The absorption rate of Mg was higher in the 3 % DFAIII group than in the 1·5 % DFAIII group at 4 weeks and tended to be higher in the 3 % DFAIII group than in the 1·5 % group in both sham and OVX rats at 8 weeks.

Ovariectomy and diet did not influence P absorption according to the result of two-way ANOVA (Fig. 3). There were no differences in P absorption rates among groups in all periods; however, the P absorption rate was decreased by ageing (week 0, 78·8 %; week 4, 72·1 %; week 8, 69·2 %; $P < 0.001$, n 174) according to the result of three-way ANOVA.

Bone strength and mineral status

The maximum breaking force of the femur was influenced by ovariectomy and diet according to the result of two-way ANOVA (Fig. 4). The values in the control group, but not in either of the DFAIII groups, were lower in OVX rats than in sham rats. In the OVX rats, the maximum breaking force was higher in rats fed the 1·5 % or 3 % DFAIII diet than that in rats fed the control diet. There were no differences in bone strength between the 1·5 % and 3 % DFAIII groups in OVX rats.

The BMD of the whole, proximal and distal femur evaluated by dual-energy X-ray absorptiometry was influenced by ovariectomy treatment, and distal BMD was influenced by diet according to the result of two-way ANOVA (Fig. 5). In OVX rats, distal BMD was higher in the 3 % DFAIII group and tended to be higher in the 1·5 % DFAIII group than in the control group. There were no differences in the midshaft BMD among the groups.

The hydroxyproline concentration of the femur was influenced by ovariectomy, and Ca, Mg and hydroxyproline concentrations were influenced by diet according to the result of two-way ANOVA (Table 3). The femoral Ca and Mg concentrations were higher in both the DFAIII groups than those in the control group in OVX rats. In sham rats, the femoral Ca concentrations changed in a similar manner without significant differences. The Mg concentration in the 3 % DFAIII group was higher than that in the control diet group in sham rats. The hydroxyproline concentrations in the 3 % DFAIII groups were higher compared

Table 2. Final body weight (g), body weight gain (g/d) and uterine weight (mg/100 g of body weight) of sham and ovariectomized (OVX) rats fed the control or difructose anhydride III (DFAIII) diet for 8 weeks*

(Mean values with their standard errors for nine or ten rats per group)

Diet	Final body weight Mean	SE	Body weight gain Mean	SE	Uterine weight Mean	SE
Sham						
Control	320[b]	7·4	1·2[b]	0·11	217[a]	18·1
1·5 % DFAIII	314[b]	9·3	1·1[b]	0·09	200[a]	20·8
3 % DFAIII	312[b]	7·3	1·1[b]	0·09	195[a]	13·6
OVX						
Control	359[a]	8·8	1·7[a]	0·12	27·0[b]	1·18
1·5 % DFAIII	371[a]	4·9	1·9[a]	0·07	25·2[b]	1·09
3 % DFAIII	356[a]	10·4	1·6[a]	0·12	27·5[b]	1·04
Significant effects as determined by two-way ANOVA						
Treatment (T)	$P < 0.001$		$P < 0.001$		$P < 0.001$	
Diet (D)	0·553		0·27		0·618	
T × D	0·496		0·286		0·628	

[a,b] Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).
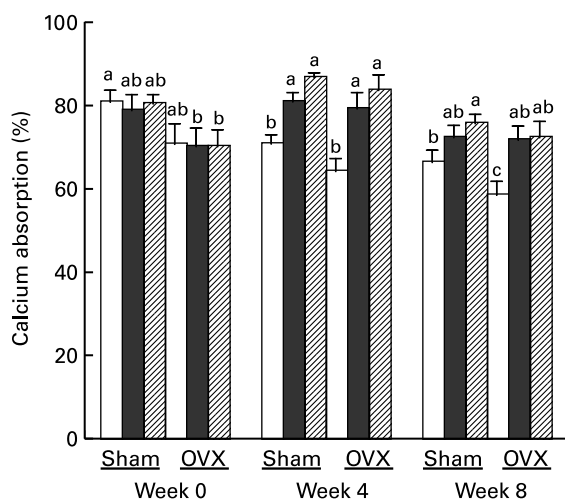* For details of diets and procedures, see Table 1 and p. 268.

Fig. 1. Calcium absorption rates in sham and ovariectomized (OVX) rats fed the control (□), 1·5 % DFAIII (■) or 3 % DFAIII (▨) diet at 0 week, 4 weeks and 8 weeks after starting the test diets. For details of diets and procedures, see Table 1 and p. 268. Values are means with their standard errors for nine or ten rats. P values estimated by two-way ANOVA were <0.001 (week 0), 0·086 (week 4), 0·049 (week 8) for treatment; 0·922 (week 0), <0.001 (weeks 4 and 8) for diet; and 0·964 (week 0), 0·641 (week 4), 0·374 (week 8) for treatment × diet. [a,b,c]Mean values with unlike superscript letters were significantly different ($P<0.05$).
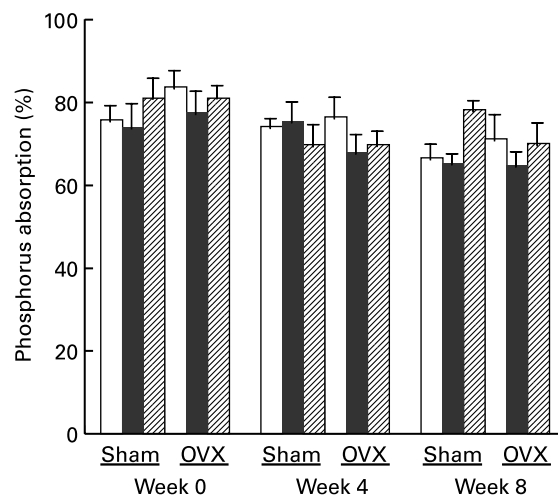

Fig. 3. Phosphorus absorption rates in sham and ovariectomized (OVX) rats fed the control (□), 1·5 % DFAIII (■) or 3 % DFAIII (▨) diet at 0 week, 4 weeks and 8 weeks after starting the test diets. For details of diets and procedures, see Table 1 and p. 268. Values are means with their standard errors for nine or ten rats. P values estimated by two-way ANOVA were 0·285 (week 0), 0·601 (week 4), 0·648 (week 8) for treatment; 0·431 (week 0), 0·369 (week 4), 0·081 (week 8) for diet; and 0·653 (week 0), 0·438 (week 4), 0·253 (week 8) for treatment × diet.

with those in the control groups in both sham and OVX rats. There were no differences in the femoral dry weight and P concentrations among groups.

Bone resorption marker

Urinary DPD levels were influenced by ovariectomy, but not by diet according to the result of two-way ANOVA (Fig. 6). In the

OVX rats, DPD levels in both the DFAIII groups tended to be lower than those in the control group.

Correlation

Femoral strength was positively correlated with Ca and Mg absorption and with femoral Ca, Mg and collagen concentrations (Table 4). Femoral strength was also positively correlated with the BMD of all femur segments. The absorption of Ca was
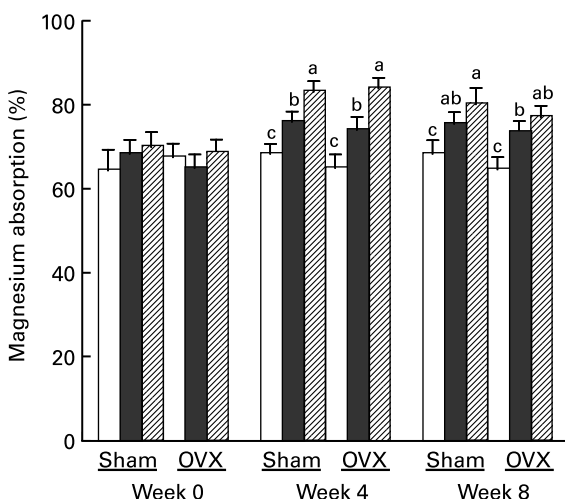

Fig. 2. Magnesium absorption rates in sham and ovariectomized (OVX) rats fed the control (□), 1·5 % DFAIII (■) or 3 % DFAIII (▨) diet at 0 week, 4 weeks and 8 weeks after starting the test diets. For details of diets and procedures, see Table 1 and p. 268. Values are means with their standard errors for nine or ten rats. P values estimated by two-way ANOVA were 0·855 (week 0), 0·277 (week 4), 0·055 (week 8) for treatment; 0·323 (week 0), <0.001 (weeks 4 and 8) for diet; and 0·443 (week 0), 0·463 (week 4), 0·882 (week 8) for treatment × diet. [a,b,c] Mean values with unlike superscript letters were significantly different ($P<0.05$).
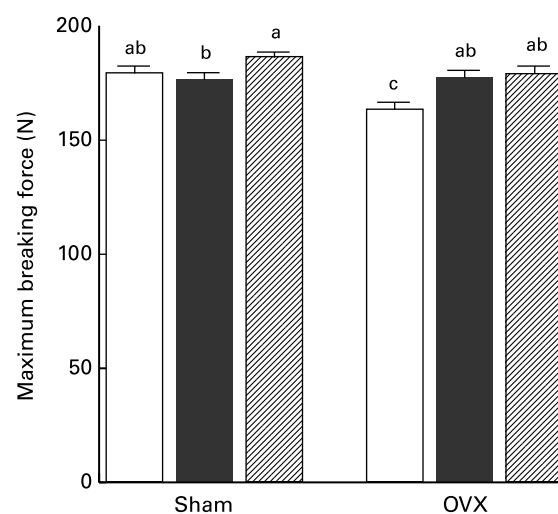

Fig. 4. Maximum breaking force of the femur in sham and ovariectomized (OVX) rats fed the control (□), 1·5 % DFAIII (■) or 3 % DFAIII (▨) diet at 8 weeks after starting the test diets. For details of diets and procedures, see Table 1 and p. 268. Values are means with their standard errors for nine or ten rats. P values estimated by two-way ANOVA were 0·003 for treatment, 0·001 for diet and 0·020 for treatment × diet. [a,b,c] Mean values with unlike superscript letters were significantly different ($P<0.05$).
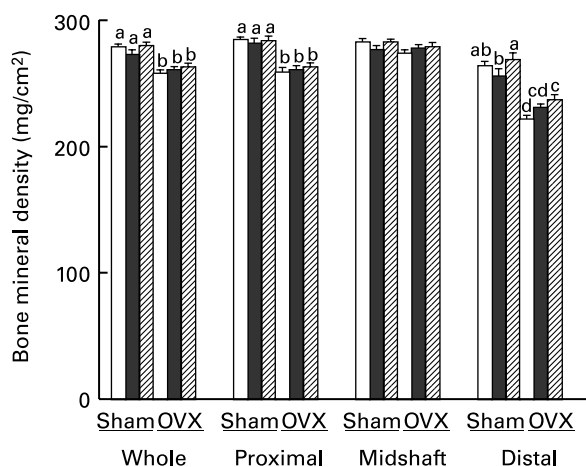
Fig. 5. Femoral bone mineral density of the whole femur and the proximal, midshaft and distal segments of the femur in sham and ovariectomized (OVX) rats fed the control (□), 1·5 % DFAIII (■) or 3 % DFAIII (▨) diet at 8 weeks after starting the test diets. For details of diets and procedures, see Table 1 and p. 268. Values are means with their standard errors for nine or ten rats. P values estimated by two-way ANOVA were <0·001 (whole, proximal and distal), 0·074 (midshaft) for treatment; 0·314 (whole), 0·751 (proximal), 0·425 (midshaft), 0·040 (distal) for diet; and 0·259 (whole), 0·681 (proximal), 0·233 (midshaft), 0·089 (distal) for treatment × diet. [a,b,c,d]Mean values with unlike superscript letters were significantly different ($P<0.05$).

strongly correlated with BMD, especially distal BMD, and Mg absorption correlated with only distal BMD. There were no correlations between P absorption and femoral strength or BMD.

Discussion

This study is the first report that the prolonged feeding of a non-digestible disaccharide, DFAIII, improves bone characteristics in OVX rats. We clearly demonstrated that 8 weeks of feeding of DFAIII increased femur Ca concentrations and strength in OVX rats (Table 3; Fig. 4), and the increase was associated with the enhancement of intestinal Ca absorption (Fig. 1). We previously demonstrated that DFAIII increases Ca absorption in both the small and large intestine (Suzuki *et al.* 1998; Mineo *et al.*

2001, 2002; Mitamura *et al.* 2002; Suzuki & Hara, 2004). To clarify the relationship between Ca absorption and bone characteristics, we calculated the correlations between mineral absorptions and bone parameter variables and observed positive correlations between Ca absorption rate and strength, BMD and Ca concentration of the femur (Table 4). These correlations indicate that an enhancement of Ca absorption with prolonged feeding of DFAIII may cause an increase in Ca concentration, BMD and bone strength. Ovarian hormone deficiency is well known to be involved in osteoporosis (Kanis, 1996; Qu *et al.* 2000) and an adequate supply of Ca with improvement in Ca absorption by DFAIII may prevent bone loss in post-menopausal women.

The present study showed that feeding of DFAIII increased Ca absorption over 8 weeks, which is the longest feeding period in experiments on DFAIII in rats. We used a 3·0 g Ca/kg diet level that is lower than the level of the standard diet in rats, AIN93G, which simulates a lower Ca intake than the dietary reference intake of Ca in East Asia including Japan (Ministry of Health, Labour and Welfare in Japan, 2002). We showed that rather high absorptive rates of Ca were maintained with feeding DFAIII over 8 weeks in OVX and also sham rats. The prolonged effects of DFAIII on Ca absorption and bone suggest a potential benefit to post-menopausal women with lower Ca intake. Moreover, the Ca content of the kidney did not differ among groups (data not shown), which suggests that feeding of DFAIII does not induce any renal side-effects even with prolonged increases in Ca absorption.

Mg is also an important factor for bone strength, though the content is low in the bone (0·5–1 % bone ash). Mg deficiency impairs bone growth and causes skeletal fragility (Rude *et al.* 2004) and several reports have shown that Mg homeostasis has a great influence on bone strength (Toba *et al.* 2000; Okuma, 2001). In the present study, Mg absorption was not influenced by ovariectomy (Fig. 2). However, the rate of Mg absorption in the DFAIII groups was higher than that in the control groups in both sham and OVX rats. The Mg absorption rate was also correlated with femur strength and Mg concentration (Table 4). These correlations indicate that an enhancement of Mg absorption by DFAIII may increase femoral Mg and may also promote bone strength in OVX rats. In this study, there were no differences in

Table 3. Femoral weight (mg), calcium (mmol/g femur), magnesium (μmol/g femur), phosphate (mmol/g femur) and hydroxyproline (μmol/g femur) concentration of sham and ovariectomized (OVX) rats fed the control or difructose anhydride III (DFAIII) diet for 8 weeks*

(Mean values with their standard errors for nine or ten rats per group)

Diet	Dry weight		Ca		Mg		Phosphate		Hydroxyproline	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Sham										
Control	0·515	0·006	7·11[ab]	0·109	236[bc]	7·36	3·84	0·206	166[b]	7·89
1·5 % DFAIII	0·497	0·008	7·68[a]	0·065	263[ab]	8·97	3·62	0·045	164[bc]	5·36
3 % DFAIII	0·506	0·012	7·83[a]	0·140	281[a]	8·60	3·91	0·180	188[a]	8·30
OVX										
Control	0·512	0·013	6·38[b]	0·401	210[c]	19·2	3·48	0·142	147[c]	3·10
1·5 % DFAIII	0·510	0·019	7·83[a]	0·329	269[ab]	14·6	3·90	0·222	156[bc]	7·65
3 % DFAIII	0·505	0·014	7·58[a]	0·302	259[ab]	12·4	4·00	0·161	171[ab]	2·88
Significant effects as determined by two-way ANOVA										
Treatment (T)	0·789		0·227		0·184		0·986		0·005	
Diet (D)	0·723		$P<0.001$		$P<0.001$		0·213		$P<0.001$	
T × D	0·797		0·284		0·409		0·157		0·644	

[a,b,c] Mean values within a column with unlike superscript letters were significantly different ($P<0.05$).
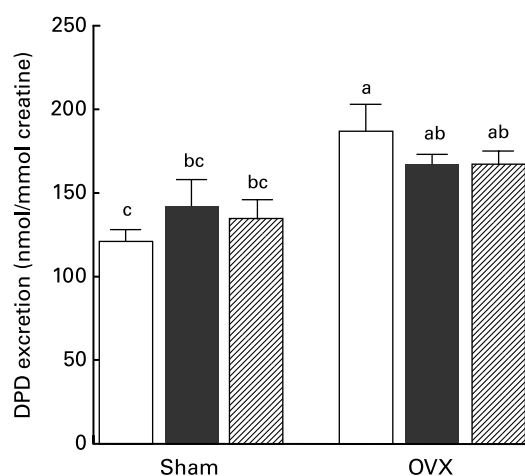* For details of diets and procedures, see Table 1 and p. 268.

Fig. 6. Urinary deoxypyridinoline (DPD) excretion of sham and ovariectomized (OVX) rats fed the control (□), 1·5 % DFAIII (■) or 3 % DFAIII (▨) diet at 8 weeks after starting the test diets. For details of diets and procedures, see Table 1 and p. 268. Values are means with their standard errors for nine or ten rats. P values estimated by two-way ANOVA were <0·001 for treatment, 0·912 for diet and 0·207 for treatment × diet. [a,b,c]Mean values with unlike superscript letters were significantly different ($P<0·05$).

P absorption rate between groups (Fig. 3). Further, diet did not influence femoral P concentrations and there were no correlations with any bone parameter variables (Tables 3 and 4). These results showed that DFAIII does not influence P absorption, and that the absorption of P has little influence on bone metabolism.

Ovariectomy reduced BMD in the whole, proximal and distal segments of the femur, and feeding of a 3 % DFAIII diet increased BMD only in the distal region of the femur, in which trabecular bone is predominant compared with the midshaft area (Fig. 5). Trabecular bone is more metabolically active than cortical bone (Breitman et al. 2003). In this study, the absorption rates of Ca and Mg were positively correlated with distal BMD. These results indicate that the enhancement of Ca and Mg absorption by DFAIII improved the trabecular bone area of the femur. The proximal region of the femur also contains trabecular bone. However, DFAIII did not augment BMD of this region. We found that the distal region was lower BMD compared with the other regions especially in OVX rats. This suggests that BMD in the distal femur is more sensitively influenced by ovariectomy, and increasing mineral absorption feeding with DFAIII.

In the present study, we also demonstrated that ingestion of 3 % DFAIII diet, but not 1·5 % DFAIII diet, prevented loss of

hydroxyproline (collagen) concentration in OVX rats (Table 3). It has been reported that minerals contribute to bone stiffness and collagen determines the toughness of bone (Oxlund et al. 1995; Wang et al. 2002). The femoral Ca, Mg and collagen concentrations were strongly correlated with the maximum breaking force of the femur (Table 4). Preventing bone collagen loss together with increasing bone minerals by ingesting DFAIII may effectively increase bone strength. However, in the case of the 1·5 % DFAIII group in OVX rats, bone strength was increased without prevention of collagen loss. The effect of DFAIII on collagen metabolism is not known. Further studies are needed to clarify the effects of DFAIII ingestion on bone strength and bone architecture and the possibility to improve bone metabolism independent of effects on mineral absorption.

Ovariectomy has been shown to induce osteopenia with an increase in bone turnover dominating bone resorption (Kaastad et al. 1997; Geng et al. 2000). Enhancement of bone resorption results in increases in collagen degradation products (Swaminathan, 2001). We measured urinary excretion of bone resorption marker, the collagen breakdown product DPD. Feeding of DFAIII tended to suppress urinary DPD excretion (Fig. 6); however, excretion was still higher than that in the sham control group and there was no difference between the 1·5 and 3 % DFAIII groups in OVX rats. The Ca concentrations in the femur of OVX rats fed DFAIII were comparable with those of the sham rats (Table 3). Also, as mentioned earlier, hydroxyproline (collagen) concentration in the 3 % DFAIII group was clearly higher than in the control group, and tended to be higher than in the 1·5 % DFAIII group. These results suggest that the effect of increased Ca absorption due to the DFAIII diet may more effectively promote bone formation with increasing collagen synthesis than suppression of bone resorption. In this study, we did not measure any bone formation marker. Further studies are required to examine the preventive effects of DFAIII on osteopenia.

We found that there were no differences in Ca absorption rate or bone variables between the 1·5 % and 3 % DFAIII groups in OVX rats in this study. In the previous studies, effective levels of other non-digestible saccharides in diets were 50–55 g/kg diet for the promotion of Ca absorption in rats (Ohta et al. 1995; Hara et al. 1999; Mitamura et al. 2003; Zafar et al. 2004), which is a much higher level than the effective level of DFAIII. These results demonstrated that a relatively low level of DFAIII in the diet (15 g DFAIII/kg diet) is sufficient to improve Ca absorption and bone metabolism.

Table 4. Correlations (r) between mineral absorptions and bone parameter variables in sham and ovariectomized rats fed the control or difructose anhydride III diet for 8 weeks

	Ca absorption	Mg absorption	P absorption	Femoral strength
Femoral strength	0·468**	0·450**	0·081	–
Femoral Ca	0·306*	–	–	0·525**
Femoral Mg	–	0·536**	–	0·489**
Femoral P	–	–	0·167	0·117
Femoral collagen	–	–	–	0·346**
Bone mineral density				
Whole	0·352**	0·253	0·099	0·510**
Proximal	0·305*	0·243	0·093	0·433**
Midshaft	0·316*	0·207	0·007	0·488**
Distal	0·459**	0·400**	0·064	0·555**

*,** Significant correlation (n 58, *$P<0·05$, **$P<0·01$).

In conclusion, prolonged feeding of a low level of DFAIII increased concentrations of bone minerals and collagen, and completely restored bone strength impaired by ovariectomy. We suggest that the enhancement of Ca and Mg absorption by DFAIII is involved in these beneficial effects.

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