The Effect of Fructooligosaccharides with Various Degrees of Polymerization on Calcium Bioavailability in the Growing Rat

MARLENA C. KRUGER,*,[1] KATHERINE E. BROWN,* GABRIELLE COLLETT,* LEE LAYTON,† AND LINDA M. SCHOLLUM‡

Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand; †AgResearch Grasslands, Palmerston North, New Zealand; and ‡Fonterra Research Centre, Palmerston North, New Zealand

Maximizing peak bone mass during adolescence may be the key to postponing and perhaps preventing bone fractures due to osteoporosis in later life. One mechanism to maximize peak bone mass is to maximize calcium absorption, and it has been suggested that inulin and oligofructose might be one of the ways of doing so. In this study, fructooligosaccharides with various degrees of polymerization have been compared in terms of impact on calcium absorption, bone density, and excretion of collagen cross-links in the young adult male rat. The various oligosaccharides were oligofructose (DP2-8), inulin (DP>23), and a mixture of 92% inulin and 8% short-chain oligofructose (DP2-8). Measuring *ex vivo* bone mineral density (BMD) and bone mineral content (BMC) showed that BMD was significantly higher in the group fed inulin (DP>23) in both femurs, whereas BMC was significantly higher in the spine. The excretion of fragments of Type 1 collagen decreased in all groups over the 4 weeks of feeding, but the decrease was most significant in the group fed inulin (DP>23). Several hypotheses have been offered to explain the effect of the fructooligosaccharides on calcium absorption and retention. These include the production of organic acids that would acidify the luminal contents and enhance solubility and hence absorption, or possibly a mechanism via calbindinD9k. This study is unique in that it compares the different fructooligosaccharides in the same model, and it clearly shows that the various fructans do not have the same effect. In our model, inulin (DP>23) had the most significant effect on calcium bioavailability. Exp Biol Med 228:683–688, 2003

Key words: rats; fructooligosaccharides; bone mineral density; markers of bone resorption

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[1] To whom requests for reprints should be addressed at Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11222, Palmerston North, New Zealand. E-mail: M.C.Kruger@massey.ac.nz

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Osteoporosis is increasing in the Western world and has been predicted to increase up to 300% until 2050 in Asia. It is strongly suggested that in addition to minimizing bone resorption in old age, maximizing peak bone mass during adolescence may be the key to postponing and even preventing bone fractures due to osteoporosis later in life. A key way to accomplish this is through increased calcium intake (1). Normally, only about 30% of the dietary calcium is absorbed by the body and deposited in the bones. Improved calcium absorption in the body would probably have important consequences on the occurrence of osteoporosis and bone fractures.

Among the components likely to be used in functional foods, prebiotics show interesting technological and nutritional properties. Chicory inulin and oligofructose are the most studied prebiotics (2) and offer new nutritional perspectives. Feeding rats a diet that includes poorly digestible or indigestible and fermentable carbohydrates can accelerate the absorption of both calcium and magnesium. These carbohydrates include lactose, oligosaccharides, or resistant starch. The main site of absorption of minerals such as calcium and magnesium was initially believed to be the small intestine. However, reports now indicate that Ca and Mg are also absorbed from the large intestine, namely the cecum and the colon (3).

Fructooligosaccharides (FOS) stimulate the growth of bifidobacteria. The luminal bacteria in the large intestine ferment indigestible carbohydrates, and various papers have suggested an important correlation between increases in the absorption of minerals and the fermentation of indigestible carbohydrates in the large intestine (4). Several hypotheses about the mechanisms of these effects have been proposed: indigestible oligosaccharides reach the large intestine intact and are fermented by bacteria in the intestinal lumen, resulting in the production of organic acids such as acetate, propionate, and butyrate. These acids create a localized drop in pH and may dissolve insoluble calcium salts in the lu-

minal content and accelerate the passive diffusion of minerals via the paracellular pathways; absorption of short-chain fatty acids is accompanied by absorption of minerals; and hypertrophy of the colon wall is noted during feeding of oligosaccharides, and is usually related to an enhanced capacity for absorption of minerals (5, 6). In addition, previous studies have shown that FOS, which is a mixture of fermentable and indigestible oligosaccharides, enhance not only intestinal calcium absorption, but also bone calcium stores in the rat (7, 8).

Inulin belongs to the fructan family, which are important storage carbohydrates. Fructans constitute a group of oligosaccharides derived from sucrose that are isolated from natural vegetable sources (1, 9). Generally, a product with a degree of polymerization (DP) from 2 to 60+ is labeled as inulin, whereas oligofructose, which is produced by partially enzymatic hydrolysis of inulin, is defined by a DP<10 (1).

The objective of this study was to assess the retention of calcium in rats fed diets supplemented with FOS with various degrees of polymerization. The retention of calcium was evaluated using calcium balance, parathyroid hormone levels, spine and femoral bone mineral density, bone calcium content, and excretion of collagen degradation products in the urine. Measuring fragments of Type 1 collagen in the urine or plasma is an indication of bone resorption, and a reduction due to feeding calcium or due to drug treatment indicates a reduction in the activity of the osteoclasts in resorbing bone. The markers for bone resorption do respond fast and can thus be measured in a short period of time (10).

Materials and Methods

Diets. The base diet given to the animals was a semisynthetic diet containing 0.5% calcium. The FOS was added to the diet at 5% replacing cornstarch, with 5% sucrose as the control (Table I). The FOS were manufactured by Orafti Active Food Ingredients (Tienen, Belgium) and were supplied by Terry Holdings (Auckland, New Zealand). The oligofructose used is a mixture of glycosyl (fructosyl)n-1 fructose (GFn) and homooligomers of fructose with a mean DP of 4.8 (FOS DP2-8). The inulin used has had the small molecular weight oligomers eliminated and it has an average DP of >23 (inulin DP>23). The third FOS was an enriched inulin powder containing 92% inulin and 8% short-chain oligofructose (DP2-8).

Animals. Forty 7-week-old male Sprague-Dawley rats were obtained from the Small Animal Production Unit (SAPU; Massey University), and were randomized into four groups ($n = 10$ per group). The animals were separately housed in shoebox cages and were kept in a temperature-(22°C ± 2°C) and light-controlled (12:12-hr day:night cycle) room in SAPU. Animals had *ad libitum* access to deionized water and diet. Animals were fed a milk powder semisynthetic base diet for 1 week, and test diets for 4 weeks. The three test diets consisted of a semisynthetic base diet with a 5% (w/w) addition of the various FOS. At the end of Week 1, animals were placed in metabolic cages for a metabolic balance study, and they were then tail-vein bled. At the completion of the trial, the animals were again placed in metabolism cages for a metabolic balance study, bled by heart puncture, and euthanized.

Table I. Composition of Diets Given as per 2 kg

Milk powder[a]	500			
Sucrose	100			
Milk powder		500		
DP2-8		100		
Milk powder			500	
DP>23			100	
Milk powder				500
Inulin +DP2-8				100
Vitamin mix[b]	100	100	100	100
Mineral mix[b]	100	100	100	100
Cellulose	100	100	100	100
Corn oil	100	100	100	100
Amino acid mix[c]	80.4	80.4	80.4	80.4
Starch	919.6	919.6	919.6	919.6
Total	2000	2000	2000	2000

Note. The milk powder was enriched with calcium and provided 5 g/kg calcium.
[a] High-calcium skim milk powder.
[b] Formulated to meet nutrient requirements for growth in young rats.
[c] Formulated by Crop & Food Research, New Zealand, according to the NRC nutrient requirements for laboratory animals (AIN 93G).

The Massey University Animal Ethics Committee approved this study.

Calcium-Balance Studies. Animals were placed into single metabolism cages for a continuous period of 72 hr, with *ad libitum* access to deionized water and diet. Daily feed intake was measured, and urine and feces were collected for further analysis. Urine samples were kept under dark conditions, measured, filtered, and then aliquoted for RatLaps (measurement of the urinary excretion of Type 1 collagen cross-links) and calcium analysis. Feces were weighed, freeze-dried, and ground for calcium content analysis.

Dual Energy X-Ray Spectrometry (DEXA) Scans. Bone mineral measurements were taken using a Hologic QDR4000 bone densitometer using a pencil beam unit (Bedford, MA). A daily Quality Control (QC) scan was taken to ensure precision. This scan was required to meet a standard coefficient of variation.

Regional high-resolution scans were performed using a 0.06-inch diameter collimator with a 0.0127-inch point resolution and a 0.0254-inch line spacing. The animal's right femur and spine were defrosted and dissected to a tissue depth of 0.5 cm, placed in a plastic dish, and covered with a uniform layer of 3 cm phosphate-buffered saline. Right femurs and spines were scanned using high-resolution software.

Bone Calcium Content. Left femurs were scraped and oven-dried for 12 hr at 105°C. Femur length was measured using calipers, and bones were weighed before preparation for calcium analysis. The femurs were then ground using an Industriesstr 8 6580 odar-Oberstein ham-

mer mill (Fritsch, Germany), weighed, and segregated into two duplicates.

Samples were then ashed in a muffled furnace at 600°C for 12 hr, and were weighed before and postashing. Duplicates were then dissolved in dilute nitric acid and analyzed using a Vista model Inductively Coupled Plasma Optical Emission Spectroscopy (ICPOES) machine (Varian) for calcium analysis.

Serum and Urine Assays. Blood was collected using tail vein blood or cardiac puncture at Weeks 0 and 4. Blood was centrifuged for 15 min and the resulting serum/plasma was aliquoted and snap-frozen in liquid nitrogen. All samples were stored at −70°C. Serum PTH was measured using the Rat Intact PTH ELISA Kit provided by Immutopics (San Clemente, CA). Serum calcium was measured on a Roche Cobas Fara II analyzer using commercial diagnostic kits supplied by Roche Diagnostics. The cresolphtalein-complexone method was used for calcium analysis and the Jaffe method (11) was used for creatinine analysis. Urinary RatLaps excretion was measured using the RatLaps ELISA kit provided by Osteometer BioTech A/S (Herlev, Denmark).

Statistical Analyses. Results were analyzed using Minitab, version 13. A P value of less than 0.05 was considered to be significant. Groups of animals were compared using analysis of variance, followed by *post hoc* testing (Tukey). Values and graphs are expressed and shown as mean ± SEM.

Results

There was no significant difference in body weight between the dietary groups after 4 weeks of feeding the test diets (Table II). Food intake was measured and is represented as total for 3 days. There was no significant difference in food intake, although the mixture of inulin + 8% DP2-8 showed a lower intake (Table II). Dry fecal weights were calculated during the balance studies, and the group fed inulin + 8% DP2-8 had a higher fecal weight compared with control. The inulin group had the lowest calcium content in the feces. Urinary calcium excretion was not significantly different between the diets, but there was a tendency for the excretion to be higher in the group fed inulin plus short-chain FOS.

After 28 days of feeding FOS, serum calcium, and parathyroid hormone levels did not change significantly (mean values at Week 4: PTH, 280 (30) pg/mL; calcium, 2.48 (0.22) m*M*). Measuring bone mineral density (BMD) and bone mineral content *ex vivo* showed that spine bone mineral content was significantly higher in the group fed inulin (DP>23; Fig. 1), but there was also an increase in the BMD of the groups fed inulin as well as oligofructose (DP2-8), although it did not reach statistical significance (Fig. 2). Right femur BMD was significantly increased in the group fed inulin (DP>23) compared with control, whereas feeding oligofructose (DP2-8) also increased density, but not to statistical significance (Fig. 3).

The excretion of fragments of Type 1 collagen in the urine was also measured (RatLaps). The excretion of collagen fragments decreased significantly in all groups over the feeding period of 4 weeks (Fig. 4). If the percentage change in excretion of fragments is calculated over time, the decrease in excretion was significant in the group fed inulin (DP>23) compared with the control (Fig. 5).

Discussion

Oligofructose is a mixture of oligosaccharides composed of fructose units linked together by β(2-1) linkages. Some of these molecules are terminated by glucose. The total number of fructose or glucose units in an oligofructose molecule generally ranges between 2 and 8 (1, 9). Inulin is a long chain unit containing more than 20 fructose or glucose units. In this study, three different types of FOS were compared: oligofructose (DP2-8), inulin (DP>23), and a mixture of 92% inulin plus 8% short-chain FOS (DP2-8).

The rats did grow at similar rates, but the group of rats fed a mixture of inulin plus 8% short-chain FOS (DP2-8) weighed less than the other groups (not statistically significant). These rats also had a lower food intake as measured using metabolic studies. Such an observation has been made previously by Levrat *et al*. (12) at much higher concentrations of inulin in the diet, more than 10%, and the reduced food intake could be ascribed to accumulation of fluid in the intestines. Inulin enhances the weight of the large intestine due to an osmotic effect, and such a phenomenon might have taken place in the inulin-fed groups, although colon mass was not determined. Fecal mass was also increased in

Table II. Final Body Weight and Balance Data Compared Between the Control and Various Groups Receiving FOS in the Diet

	Control	FOS (DP2-8)	Inulin (DP>23)	Inulin + FOS (DP2-8)
Final body weight (g)	473.6 (7.85)	463.4 (4.2)	453.1 (9.91)	448.0 (3.97)
Food intake/3 days (g)	64.09 (2.53)	68.58 (1.60)	65.41 (1.91)	63.57 (1.77)
Calcium intake/3 days (mg)	370 (13.7)	387 (10.4)	369 (11.2)	373 (11.2)
Faecal weight/3 days (g)	7.78 (0.279)	8.93 (0.333)	7.97 (0.406)	9.005 (0.514)
Faecal calcium/3 days (mg/g)	34.17 (6.98)	34.85 (7.89)	33.95 (4.97)	35.78 (5.24)
Urinary calcium/3 days (mg)	1.8 (0.24)	1.5 (0.224)	1.2 (0.133)	2.1 (0.482)
Calcium balance (mg/3 days)	93.8 (17.3)	70.3 (12.5)	88.3 (11.4)	73.14 (9.03)

Note. Values are expressed as mean (SEM).

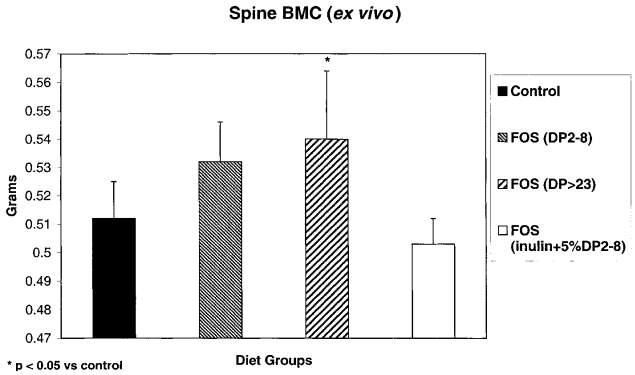

Figure 1. *Ex vivo* spine bone mineral content of the animals in the various diet groups containing fructooligosaccharides. Diets were fed for 4 weeks at 5% (*$P < 0.05$ versus control).

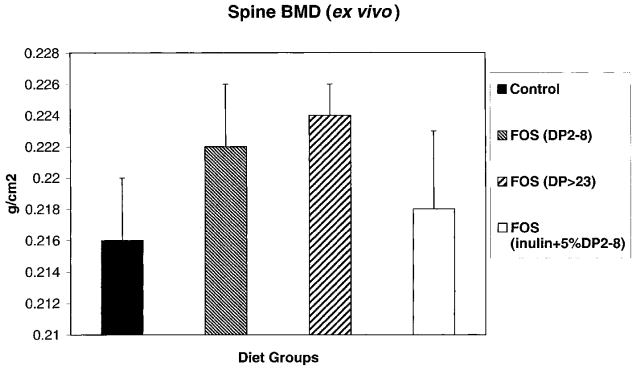

Figure 2. *Ex vivo* spine bone mineral density of the animals after being fed various fructooligosaccharides for 4 weeks.

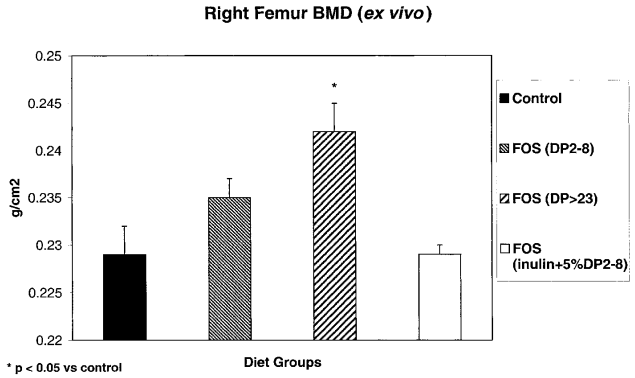

Figure 3. *Ex vivo* right femur bone mineral density of the animals after being fed various fructooligosaccharides for 4 weeks (*$P < 0.05$ versus control).

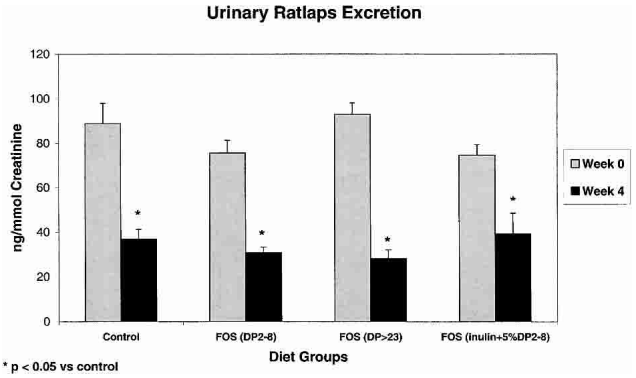

Figure 4. Urinary RatLaps excretion in the various groups of animals after being fed fructooligosaccharides for 4 weeks (*$P < 0.05$ versus Week 0).

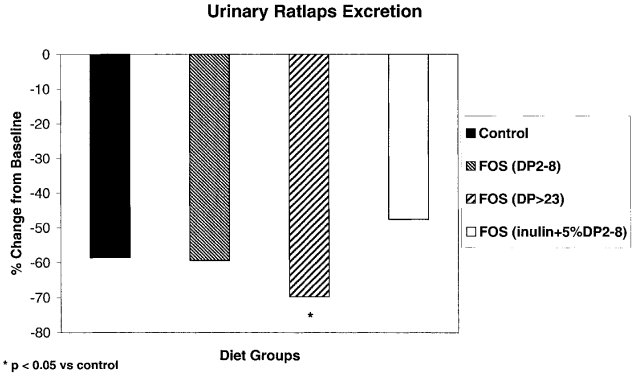

Figure 5. Change in urinary RatLaps excretion from Week 0 to Week 4 (*$P < 0.05$ versus control).

the groups fed oligofructose (DP2-8) and the mixture of inulin and 8% short-chain FOS, but this difference was not significant compared with the control. An increase in fecal bulk has been observed in rats (5) as well as humans (13) before, with no concomitant increase in intestinal transit time. Comparing fecal calcium content, it is clear that feeding inulin plus 8% short-chain FOS resulted in more calcium being excreted in the feces. The group of rats fed inulin (DP>23) had the lowest level of calcium in the feces as well as the lowest in the urine (Table II).

Unlike some other types of dietary fibers, inulin and oligofructose do not impair but rather improve the bioavailability of minerals such as calcium and magnesium. Several studies in rats showed that inulin type fructans increase intestinal calcium absorption (12, 14, 15). Delzenne *et al*. (16) compared the effects of 10% added FOS DP2-8 with that of inulin (DP2-60+) and found that both increased mineral absorption significantly compared with a control.

In this study, BMD as measured in the left and right femur and the spine bone mineral content increased significantly in the group of rats fed inulin (DP>23) as compared with the control group fed sucrose. Taguchi (17) showed that short-chain oligofructose (2.5 and 5% in the diet) prevented bone loss in the ovariectomized rat model. Scholtz-Ahrens *et al*. (8) observed a dose effect of oligofructose at three different doses on the increase in calcium absorption and bone mineralization expressed as bone calcium content. Lemort *et al*. (18) showed an increased BMD in the growing rat model supplemented by 5% or 10% inulin.

Measuring fragments of Type I collagen in the urine or plasma is an indication of bone resorption. The RatLaps assay is similar to the CTx assay used in human serum (10) and has been shown to be sensitive enough to detect changes in bone resorption in the ovariectomized rat model. During our feeding of 4 weeks, the excretion of the collagen cross-links decreased in all groups of rats.

This decrease could be due to a change in diet in comparison with the usual commercial rat diet or could possibly be ascribed to slowing of bone growth after puberty. Calculation of the change in collagen cross-link excretion over the 4 weeks of feeding FOS showed that the decrease in excretion was significant in the group of rats fed inulin (DP>23) compared with the control. Therefore, it can be expected that bone resorption was decreased in this group, and that as a consequence, the bone density and mineral content should be higher. This was indeed shown in the bone density results. Scholz-Ahrens (19, 20) also showed in the ovariectomized rat that various doses of inulin increased the amount of calcium absorbed and BMD. In this study, the femur BMD showed significance in density after 4 weeks, whereas the spine BMD increased in the inulin (DP>23) group, but not to significance. A longer feeding time is probably required to see changes in the spine. Scholz-Ahrens (8) observed a similar trend in a study using the ovariectomized females. In their study, animals were supplemented with FOS for 16 weeks. The effect of FOS was more pronounced in the femurs around 8 weeks, after which it lost significance. In the spine, the differences between control and supplemented groups became more obvious with time.

Several hypotheses have been presented to explain the effect of the fructans on calcium absorption. Fermentation of FOS results in the production of organic acids such as lactate, butyrate, propionate, and acetate. These acids lower the pH of the luminal contents and dissolve the insoluble calcium compounds such as phosphate and carbonate salts and, thereby, the luminal calcium content is increased. The increase in luminal calcium may enhance the diffusion of calcium into the cells of the intestinal mucosa (14, 20, 21). It is also possible that the short-chain fatty acids can directly stimulate calcium absorption in the rat colon (22), and that calcium could pass through the cell membrane more readily in the form of a less charged complex (calcium acetate), by a passive pathway (23). The increase in passive absorption in the colon could trigger a feedback mechanism that would inhibit duodenal active absorption, and this could possibly involve a decrease in Calbindin 9K (24, 25). Ohta (6, 24, 25) reported that the levels of calbindin-D9K in the small intestine are reduced by the oligofructans, whereas the levels of calbindin-D9K in the large intestine are increased. In a recent paper, Sakuma (26) also measured expression of CaBP protein and mRNA, and found that these were decreased by FOS in the small intestine and increased by FOS in the large intestine (26). An additional mechanism that should be considered is that an increase in osmotically active resistant sugars in the small intestine may increase the amount of fluid within the lumen to maintain isotonicity. Villus crypt height, number of epithelial cells per crypt, cecal vein flow, and mucosal-to-serosal calcium fluxes are all increased by FOS (20, 22, 27). The extent of these effects seems to be specific for the type of carbohydrate and may depend on the ingested dose.

The results of this study show that FOS with different degrees of polymerization have different effects on calcium absorption, retention, bone density, and collagen cross-link excretion. Our results show that inulin (DP>23) increased calcium absorption significantly compared with control, and this calcium was retained in the bone as shown by increased calcium balance and increased bone calcium content. The effect of FOS on collagen cross-link excretion has not been measured before. In this study, we show that feeding inulin (DP>23) significantly decreased Type I collagen cross-link excretion. The reduction in cross-link excretion as well as increased retention of calcium in the bones lead to increased bone mineral content and bone density as measured using bone densitometry. There are several hypotheses for the increase in calcium absorption by FOS but the mechanism of reducing cross-link excretion needs further investigation.

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