Effect of oligofructose or dietary calcium on repeated calcium and phosphorus balances, bone mineralization and trabecular structure in ovariectomized rats*

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We investigated the effects of dietary oligofructose and Ca on bone structure in ovariectomized rats, using microradiography and histomorphometry. Ninety-six animals were allocated to seven experimental groups: G1, sham-operated; G2–G7, ovariectomized. Semi-purified diets containing 5 g Ca/kg (recommended content) without oligofructose (G1, G2) or with 25, 50 or 100 g oligofructose/kg (G3, G4, G5) or 10 g Ca/kg (high content) without oligofructose (G6) or with 50 g oligofructose/kg (G7) were fed for 16 weeks. At the recommended level of Ca, high oligofructose (G5) increased femur mineral levels in ovariectomized rats, while medium oligofructose did so at high Ca. Increasing Ca in the absence of oligofructose did not increase femur mineral content. Trabecular bone area (%) analysed in the tibia was 10·3 (SEM 1·2) (G1), 7·7 (SEM 0·6) (G2), 9·3 (SEM 0·7) (G3), 9·4 (SEM 1·0) (G4), 9·5 (SEM 0·7) (G5), 10·2 (SEM 0·8) (G6), and 12·6 (SEM 0·8) (G7). At the recommended level of Ca, 25 g oligofructose/kg prevented loss of trabecular area due to increased trabecular thickness, while 50 or 100 g oligofructose/kg increased trabecular perimeter. At high Ca, oligofructose prevented loss of bone area due to increased trabecular number but similar thickness (G7 *v.* G6). When Ca was raised in the presence of oligofructose (G7), trabecular area and cortical thickness were highest, while loss of trabecular connectivity was lowest of all groups. At the same time, lumbar vertebra Ca was higher; 44·0 (SEM 0·8) (G7) compared with 41·6 (SEM 0·8) (G2), 41·4 (SEM 0·7) (G4), and 40·5 (SEM 1·0) mg (G6). We conclude that ovariectomy-induced loss of bone structure in the tibia was prevented but with different trabecular architecture, depending on whether dietary Ca was increased, oligofructose was incorporated, or both. Oligofructose was most effective when dietary Ca was high.

Oligofructose: Calcium and phosphorus balance: Osteoporosis: Trabecular structure: Bone quality

Certain groups of carbohydrates are not digested by gastrointestinal enzymes but are fermented by the microbial flora in the large intestine (Roberfroid *et al.* 1998). Non-digestible oligosaccharides (NDO), including inulin, oligofructose and fructo-oligosaccharides, and galacto-oligosaccharides, as well as lactulose, maltitol and resistant starch, were studied for their gastrointestinal effects. Several effects of NDO on the colonic milieu, composition of the microflora, production of microbial metabolites (Gibson *et al.* 1995) and mineral metabolism have been described (for reviews, see Scholz-Ahrens *et al.* 2001, 2002). A potential for benefits to the host's health was postulated. In this regard, consensus was achieved that there is promising evidence for positive effects on mineral metabolism and thus a potential for prevention of osteoporosis (van Loo *et al.* 1999).

Most of the scientific evidence for the effects of NDO is based on the results of experiments with rats, where these carbohydrates increase the availability of Ca (Delzenne *et al.* 1995; Ohta *et al.* 1995*a*, 1998*b,c*; Chonan & Watanuki, 1996), Mg (Ohta *et al.* 1994*b*, 1995*b*; Delzenne *et al.* 1995), Fe (Delzenne *et al.* 1995; Ohta *et al.* 1995*b*, 1998*c*) and Zn (Delzenne *et al.* 1995). Particularly Ca,

Mg, and Zn are important for bone mineralization and bone health (Heaney, 1996). In rats, Ca retention was greater after 11 d supplementation of NDO, but no longer after 25 d (Ohta *et al.* 1994*b*). This may explain why weight, total ash content and Ca content of the femur were not affected (Ohta *et al.* 1994*b*, 1997). In single-meal studies with human subjects, inulin, oligofructose or lactulose stimulated Ca absorption in some cases (Coudray *et al.* 1997; van den Heuvel *et al.* 1998*a*, 1999; Griffin *et al.* 2002) but not in all (van den Heuvel *et al.* 1998*b*; Griffin *et al.* 2002).

The significance of improved Ca absorption for postponing or preventing osteoporosis can be demonstrated only on the basis of long-term experiments including a focus on bone mineralization. Moreover, the stability of bone and thus bone quality may be associated more closely with the structure of the trabecular network than with bone mineral content alone (Kapadia *et al.* 1998), particularly under conditions comparable with those in postmenopausal women, i.e. oestrogen deficiency.

The aim of the present study was to investigate the long-term effects of low, medium, and high doses of oligofructose in the presence of the recommended dietary intake of Ca, and of medium-dose oligofructose in the presence of high dietary Ca on repeated Ca and P balances, bone mineralization and on bone quality, i.e. on tibia trabecular structure and mineral distribution between cortical and trabecular bone. The experiment was done in adult ovariectomized rats, an accepted animal model for human postmenopausal osteoporosis (Kimmel, 1996). Mineral content was analysed in femora and lumbar vertebrae as representing predominantly cortical and trabecular bone, respectively. Furthermore, these bones are characterised by different physical loading and mechanical anisotropy (Sugita *et al.* 1999), which may be important factors for determining the target of mineralization. The hypothesis was that oligofructose impedes ovariectomy-induced loss of bone mineral content and preserves bone architecture due to stimulated apparent Ca absorption.

Materials and methods

Animals and diets

The study was performed with virgin female, ovariectomized Fisher-344 rats. Ninety-six weanling rats were purchased from Harlan/Winkelmann (Borchen, Germany). Animals were fed *ad libitum* a standard rat diet with 8 g Ca/kg fresh matter and 5 g P/kg fresh matter, until the age of 5 months. The content of DM was 89·0 %. Then (at week 1) animals were divided into seven groups (G1–G7) as matched by body weights, and were sham-operated (G1) or ovariectomized (G2–G7; Table 1). At the same time, semi-purified diets with 5 g P/kg were fed that contained 5 g Ca/kg (recommended content) without oligofructose (G1 and G2), 5 g Ca/kg plus 25 g oligofructose/kg (G3, low dose), 50 g oligofructose/kg (G4, medium dose) and 100 g oligofructose/kg (G5, high dose) or 10 g Ca/kg (high content) either without oligofructose (G6) or with 50 g oligofructose/kg (G7). The content of DM was 91·0 %. Ca at 5 g/kg is equivalent to the recommended concentration for growth and maintenance in laboratory rats (National Research Council, 1995). The form of Ca used was tri-calciumdicitrate tetrahydrate ($C_{12}H_{10}Ca_3O_{14}.4H_2O$). Oligofructose (Raftilose® P95; Orafti, Tienen, Belgium) is a preparation of 95 % purity and is described in detail elsewhere (Roberfroid *et al.* 1998). Operations were done under anaesthesia with intra-peritoneal injection of xylazinhydrochloride and ketamin-hydrochloride (Rompun®/Ketavet®).

A restricted feeding regimen is needed to guarantee strict pair-feeding. Rats had free access to a feed reservoir throughout the 16 weeks. The reservoir was filled twice a week with 24 g of fresh feed matter for 3 d and 32 g for 4 d, respectively, equivalent to 8 g fresh matter/d. In earlier pilot studies, this amount of feed was found to represent a restricted feed intake for about 30 % and an *ad libitum* intake for 70 % of the animals, i.e. feed was consumed completely by all animals, by 30 % of the animals 12 h before the next feeding, and by 70 % of rats before the

Table 1. Experimental groups and body weights*

(Mean values and standard errors of the mean)

Group number	n	Operation	Ca (g/kg diet)	Oligofructose† (g/kg diet)	Week 0 (n 12–14) Mean	SEM	Week 8 (n 12–14) Mean	SEM	Week 16 (n 6–7) Mean	SEM
G1	12	Sham	5	0	159	2·6	145	1·7	171	2·1
G2	14	OVX	5	0	154	2·1	142	2·4	168	3·1
G3	14	OVX	5	25	161	2·9	145	1·8	164	2·0
G4	14	OVX	5	50	164	2·1	143	1·7	169	3·1
G5	14	OVX	5	100	164	2·4	142	1·5	169	2·7
G6	14	OVX	10	0	163	2·0	143	2·4	168	2·3
G7	14	OVX	10	50	162	3·4	142	1·7	163	3·6

Sham, sham-operated; OVX, ovariectomized.
* For details of animals and procedures, see p. 366.
† Raftilose® P95.

next feeding. The feed reservoirs and thus the feed consumption were inspected twice daily. The speed of feed consumption was not affected by the diet but reflected individual variation. The composition of the semi-synthetic diets for all groups was identical except for the Ca and oligofructose contents, which were given at the expense of maize starch (Table 2).

The animals were housed individually in stainless-steel wire mesh cages in a room with controlled temperature (20–21°C) and humidity (60–70 %), and a 12 h dark–light cycle. All animals had free access to demineralized water. Mineral balances were performed with 7 d collections after the rats had been on the experimental diets for 4, 8, and 16 weeks to investigate the short-term and long-term effect of treatments. Seven animals per group (except group 1, with n 6) were killed by desanguination under anaesthesia with intraperitoneal injection of xylazin-hydrochloride and ketaminhydrochloride (Rompun®/Keta-vet®) after 8 and 16 weeks on the experimental diets. Lumbar vertebrae, tibiae and left femora were collected and adherent soft tissue was removed carefully. Lumbar vertebrae and femora were stored at -20°C, tibia transferred to ethanol.

Analyses

Ca was measured by atomic absorption spectrometry (Perkin-Elmer 1100) using an air–acetylene flame at 2300°C. Ca in urine was measured directly after dilution with a lanthanum chloride solution. Ca in the diets was measured after ashing at 450°C in a muffle furnace for 16 h and solubilization in hydrochloric acid (2·3 mol/l)

Table 2. Composition of the semi-synthetic diets: constant components

Component	g/kg diet
Casein	160
Soya oil	50
Cellulose	50
Ca-free mineral premix (including P)*	118 (5)
Ca premix (including one of the levels of Ca)†	60 (5;10)
Vitamin premix‡	10
Oligofructose premix§	100
Maize starch	452
Total	1000

* Contained minerals equivalent to (g/kg diet): MgHPO$_4$.3H$_2$O, 5·31; NaHPO$_4$.2H$_2$O, 4·22; K$_2$HPO$_4$, 13·53; KCl, 2·52; MgSO$_4$.7H$_2$O, 1·59; C$_6$H$_8$O$_7$NFe, 338 mg; MnSO$_4$.H$_2$O, 162 mg; CuSO$_4$.5H$_2$O, 40 mg; KI, 1·56 mg; NaF, 10·2 mg; NH$_4$Al(SO$_4$)$_2$.12H$_2$O, 3·64 mg); ZnSO$_4$.7H$_2$O, 221 mg; Na$_2$SeO$_3$.5H$_2$O, 0·23 mg; KBr, 20·1 mg; NiSO$_4$.6H$_2$O, 8·5 mg; CoSO$_4$.7H$_2$O, 5·06 mg; Na$_2$MoO$_4$.2H$_2$O, 5·06 mg; NaAsO$_2$, 0·25 mg; Na$_2$B$_4$O$_7$.10H$_2$O, 5·06 mg; CrCl$_3$.6H$_2$O, 0·89 mg. All mineral components were from Merck (Darmstadt, Germany).
† Contained Ca as follows: G1–G5, 5 g/kg diet; or G6 and G7, 10 g/kg diet from C$_{12}$H$_{10}$Ca$_3$O$_{14}$.4H$_2$O at the expense of maize starch.
‡ Contained vitamins equivalent to (mg/kg diet): vitamin A (500 IE/mg), 9·1; vitamin D$_3$(500 IE/mg), 2·33; vitamin E (50 %), 68·4; vitamin K$_3$ (100 %), 11·4; choline (50 %), 2280; folic acid (100 %), 1·2; nicotinic acid (98 %), 23·3; pantothenic acid (98 %), 9·3; riboflavin (96 %), 3·6; thiamin (98 %), 4·7; pyridoxine (98 %), 7·0; cobalamin (0·10 %), 57·0; biotin (2 %), 22·8. Vitamins were purchased from Synopharm (Germany) except vitamins K$_3$ (Sigma, Deisenheim, Germany), and vitamins A, C, E (Deutsche Vilomix, Hessisch Oldendorf, Germany).
§ Contained one of the levels of oligofructose (0, 25, 50, or 100 g/kg diet at the expense of maize starch).

and nitric acid (1·3 mol/l). The within-day CV of diet samples was 0·73 %. The between-days CV was 1·22 %. Ca in faecal samples was measured after grinding and wet-ashing in a mixture of nitric acid (14·4 mol/l) and perchloric acid (11·8 mol/l) in a ratio 1:1·7 (v/v). A Ca standard solution was obtained from Merck (Titrisol; Merck, Darmstadt, Germany) and used as reference. The within-day CV of faecal samples was 0·97 %. The between-days CV was 1·50 %.

Apparent absorption and retention (mg/d) were calculated as follows:

$$\text{Apparent Ca absorption} = \text{Ca intake} - \text{faecal Ca},$$

$$\text{Ca retention} = \text{Ca intake} - (\text{faecal Ca} + \text{urinary Ca}).$$

Ca in bone was measured after ashing at 450°C in a muffle furnace for 16 h and solubilization in hydrochloric acid (6·0 mol/l). For analysis of lumbar vertebrae, lumbar vertebrae 1 + 4 were ashed together. The within-day CV of bone samples was 1·01 %. The between-days CV was 1·54 %.

P in urine was analysed as inorganic P with an automatic analyser (Cobas Bio; Hoffmann La-Roche, Basel, Switzerland) using the kit Unimate 7 PHOS (Hoffmann La-Roche, Basel, Switzerland). P in the diets, faeces and bones was measured in the solutions used for the measurement of Ca.

Microradiography and histomorphometry

This method has been applied to describe Ca bioavailability from different diets (Hein, 1997; Scholz-Ahrens *et al.* 1997) and to visualise sinus floor augmentation following dental implants (Terheyden *et al.* 1999). The proximal third of the left tibiae were cleaned of any adherent soft tissue before dehydration in ascending concentrations of ethanol for storage. Undecalcified tibia sections were embedded in methylmethacrylate, sawn, polished and ground as described (Donath, 1988). Semi-thick longitudinal sections of 100–150 μm were taken with a saw microtome with a diamond-tipped cutting blade (model 1600; Leica, Bensheim, Germany) and further polished to yield 80–90 μm sections (Micropolishing System; Exact, Norderstedt, Germany). Contact microradiographs (Fixitron X-Ray-Systems; Hewlett Packard, Manville, USA) were made under constant conditions (18 kV, 5 mA, 8 min exposure time) and documented on high-resolution plates (Microchrome Technology, San Jose, USA; Fig. 1). The quantitative assessment of trabecular structure was determined by computer-supported image analysis of video-scanned microradiographs (TK 1280 E; JVC, Berlin, Germany) connected to a light microscope (Mikrophot FXA; Nikon, Düsseldorf, Germany) and computer. The image was digitised into 256 different grey values and analysed with the software Leica Quantimet 500 MC (Leica, Bensheim, Germany). Calibration of the system was done (1 pixel was equivalent to 6·73 μm) and 'bone' was defined automatically according to a threshold grey value, the 'cut-off' line, which separated mineralised from non-mineralised bone tissue (Fig. 2). The intensity of grey-staining on high-resolution plates correlates with the degree of mineralization. Different parameters of bone structure were then analysed on the negative binary image by sequential interactive
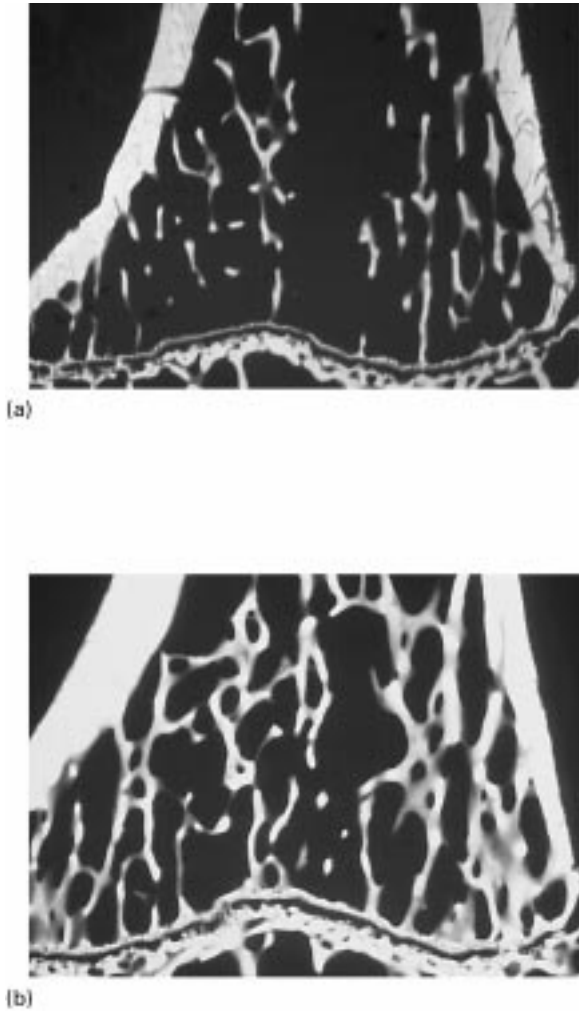
Fig. 1. Representative contact microradiographs of proximal tibia of ovariectomized rats. (a), Ovariectomy-induced loss of bone structure in an animal on a diet containing 10 g Ca/kg without oligofructose; (b) prevention of ovariectomy-induced loss of bone structure in an animal on a diet containing 10 g Ca/kg and 5 g oligofructose/kg.
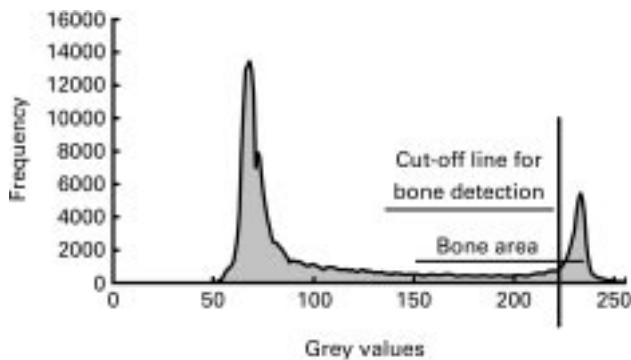
steps (Fig. 3 (a–d)). All analyses were done within a constant 13·75 mm^2 frame of measure and the values given are related to this defined area. Cortical bone thickness (μm) was analysed manually at 90° to the long axis of cortical bone as indicated in Fig. 3(a). Values are the mean distance of six interactive measurements at regular intervals. The epiphysis was excluded (Fig. 3(b)), and the remaining bone was 'filled' and referred to as bone tissue area (T.Ar, μm^2; Fig. 3(c)). Trabecular bone area (Tb.Ar, μm^2; Fig. 3(d)) was gained from Fig. 3(b) after cortical bone and primary trabeculae were removed. Fig. 3(d) was the basis for the following analyses, which were performed automatically: Trabecular number (Tb.N); trabecular bone area (Tb.Ar/T.Ar, %); trabecular perimeter (Tb.Pm, mm); anisotropy (a measure of directional orientation of the trabeculae). Mean trabecular thickness (Tb.Th, μm) was calculated using the formula presented by Olah (1974); namely, Tb.Ar/Tb.Pm $\times$ 2. The trabecular bone pattern factor (TBPf, /mm) was calculated as a measure of trabecular connectivity as described by Hahn *et al.* (1992). Briefly, two consecutive measurements of Tb.Ar and Tb.Pm were made, the second after computer-simulated dilatation of all structures by 1 pixel. Thereby, the area is enlarged but the perimeter may become smaller or larger, depending on the average shape of the trabeculae. A structure with more separated trabeculae (more convex surface area of particles) would enlarge the perimeter after dilatation, while more connected trabeculae (more concave surface area) would decrease the value. The following formula is then used for calculation: TBPf = (P1 − P2)/(A1 − A2), where P1 and A1 are perimeter and area before computer-simulated dilatation, and P2 and A2 are perimeter and area after dilatation. According to this definition, higher values for TBPf indicate a loss of connectivity. The within-day CV (and between-days CV) for some primary structure parameters were 1·15 % (1·91 %) for Tb.Ar/T.Ar, 4·84 % (5·92 %) for Tb.N, and 2·26 % (2·71 %) for Tb.Pm.

All analyses were done blinded and in duplicate.

Statistics

The program Statgraphics plus 4·1 was used. The dietary effects on bone mineral and bone structure were analysed by multifactorial analysis of variance (MANOVA) on pooled data (n 12–14) gained after 8 weeks (n 6–7) and 16 weeks (n 6–7), after testing for interaction between experimental groups (G1–G7) and time (8 and 16 weeks), which was not significant. Least-squares means with their pooled SEM are displayed in Figs. 4 and 5. Furthermore, ANOVA was performed separately for mineral balance after 4, 8 and 16 weeks and for bone mineral after 8 and 16 weeks to test whether effects were transient or developed gradually. These data are displayed in Tables 3 and 4 or, along with their least-squares means and individual SEM are described in the text. The following factors were analysed for significance after selection of the respective groups: ovariectomy, selecting G1 and G2; oligofructose with recommended Ca content of the diet, selecting G2, G3, G4, and G5; oligofructose with high Ca content of the diet, selecting G6 and G7; Ca content

Fig. 2. Frequency distribution of grey values as obtained on contact microradiographs. The relevant bone structure was defined as the area under the curve with high grey values. The cut-off line was set at the base of the peak representing high grey values, as indicated.
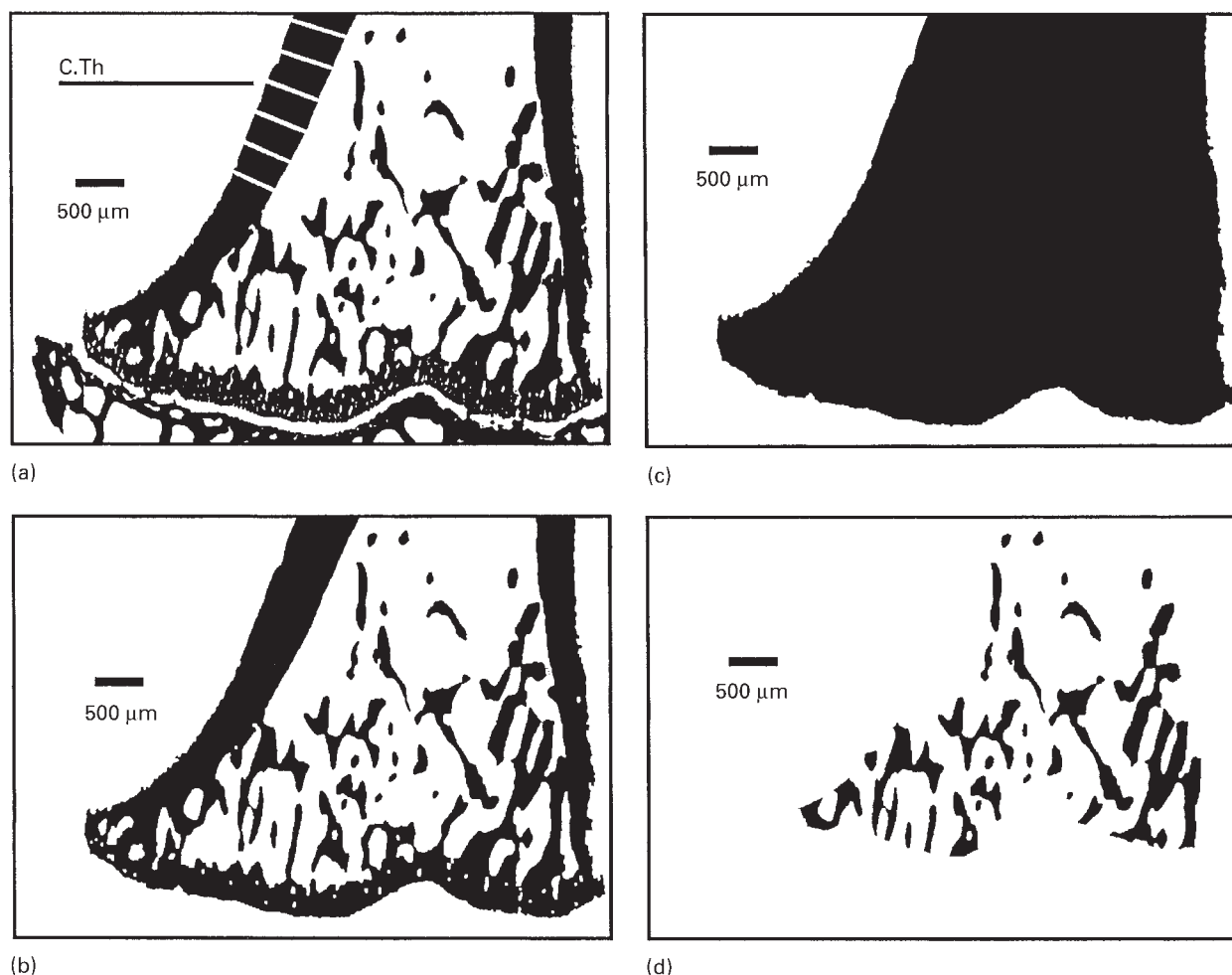
Fig. 3. Sequential interactive steps on the negative image of a contact microradiograph to assess different parameters of bone structure. (a), Cortical thickness (C.Th) was analysed manually as the mean distance of six interactive measurements at regular intervals on the tibia as indicated by white lines; (b), erasure of epiphysis; (c), the remaining bone was 'filled' and is referred to as bone tissue area; (d), trabecular bone area gained from (b) after erasure of cortical bone and primary trabeculae.

of the diet in the absence or in the presence of oligofructose, selecting G2, G4, G6 and G7. Significance was tested by the F test followed by a subsequent 2-tailed Newman–Keuls test. Differences were regarded as significant if P was <0.05.

The experiment was approved by the German institution for legalisation of animal experiments (Ministerium für Umwelt, Natur und Forsten des Landes Schleswig-Holstein).

Results

Body weight

The live body weight was not affected by ovariectomy, or by the level of oligofructose or Ca at any time (Table 1). The lower body weights in all groups after 8 weeks reflect the combined effect of operation and the switch from commercial rat chow fed *ad libitum* to restricted feeding (8·0 g feed/d) of semi-purified diets.

Effect of ovariectomy

Completeness of ovariectomy was confirmed by absence of

ovarian tissue and lower uterus weight with 0·09 (SEM 0·01) g compared with intact animals with 0·23 (SEM 0·01) g. Ovariectomy caused slightly lower femur Ca (Fig. 4) and significantly lower Tb.Ar/T.Ar and Tb.Pm in the tibia (Fig. 5, G2 v. G1). These effects developed gradually. Tb.Ar/T.Ar was 12·26 (SEM 2·05) % for sham-operated and 8·53 (SEM 1·04) % for ovariectomized rats after 8 weeks ($P>0.05$). Tb.Ar/T.Ar was 8·43 (SEM 0·36) % for sham-operated and 6·85 (SEM 0·28) % for ovariectomized rats after 16 weeks ($P<0.05$). Tb.Pm was 44·96 (SEM 4·68) % for sham-operated and 35·46 (SEM 3·26) % for ovariectomized rats after 8 weeks ($P>0.05$). Tb.Pm was 34·20 (SEM 2·14) % for sham-operated and 27·03 (SEM 1·55) % for ovariectomized rats after 16 weeks ($P<0.05$). Furthermore, oestrogen-deficient rats had higher values for TBPf; i.e. less trabecular connectivity with 22·89 (SEM 1·50)/mm compared with sham-operated rats with 17·33 (SEM 1·61)/mm ($P<0.05$).

Effects of oligofructose at the recommended level of dietary calcium

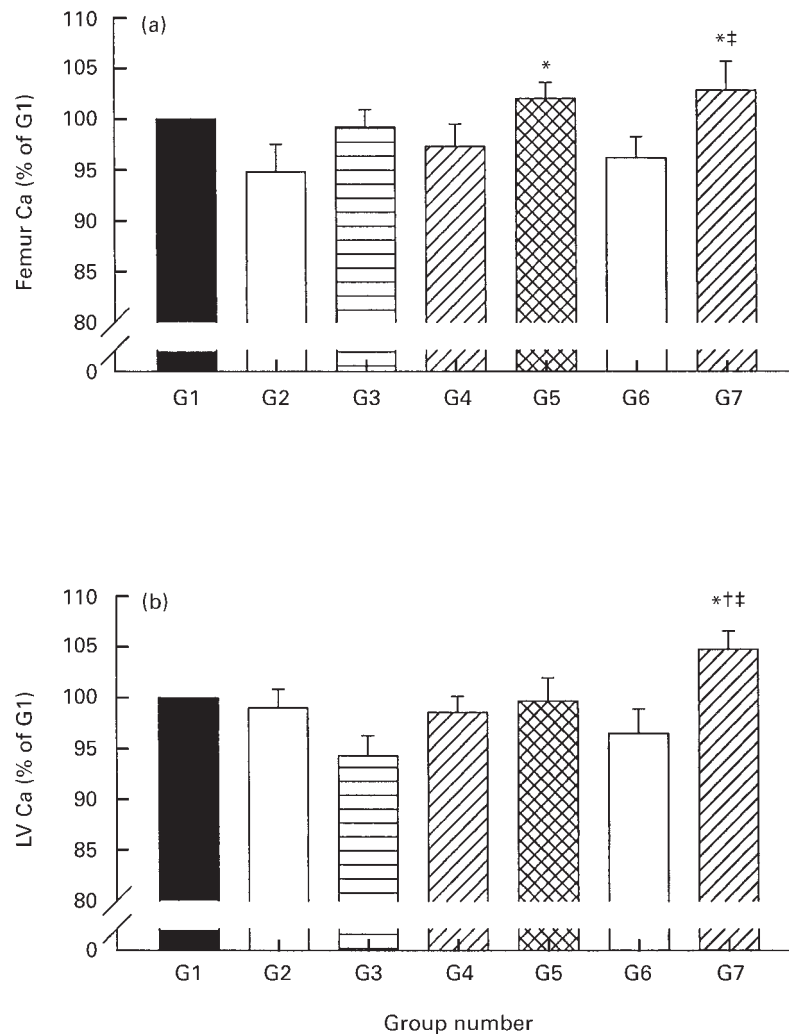Compared with the control group (G2), only the highest

Fig. 4. Effect of diets containing different concentrations of oligofructose with the recommended level of dietary Ca or a medium concentration of oligofructose with a high level of Ca on Ca content (mg/bone) of femur (a) or lumbar vertebrae (LV; (b)) in ovariectomized rats (G2–G7) relative to sham-operated (G1 = 100 %). Experimental groups G1–G7 studied were fed semi-purified diets with 5 g P/kg diet. Diets contained 5 g Ca/kg diet without oligofructose (G1 and G2), or with 25 g (G3), 50 g (G4) or 100 g oligofructose/kg diet (G5), or 10 g Ca/kg diet without oligofructose (G6) or with 50 g oligofructose/kg diet (G7). Values were obtained by multifactorial ANOVA, and are means and pooled SEM of all animals (8 weeks + 16 weeks, n 12–14). Mean values were significantly different ($P < 0.05$) : *different from G2; †different from G4; ‡ different from G6.

dose of oligofructose (100 g/kg, G5) increased Ca in femora significantly (Fig. 4), and P in femora after 16 weeks, when the P content was 38·61 (SEM 0·40) mg in the presence and 34·49 (SEM 1·48) mg in the absence of oligofructose ($P<0.05$). The Ca and P contents of lumbar vertebrae were not affected. The higher value for femur Ca was gained mainly between weeks 5 and 16, indicated by significantly higher apparent Ca absorption and retention in week 8 (Table 3). P retention was higher for 100 g of oligofructose after 4 weeks, which was due to reduced P excretion in urine (Table 4), and for 50 g of oligofructose after 8 weeks.

Low-dose (25 g/kg; G3) and high-dose oligofructose (100 g/kg; G5) given at the expense of starch in a diet with 5 g Ca/kg increased bone area significantly (Tb.Ar/T.Ar)

compared with the control group (G2), and thus prevented ovariectomy-induced loss of bone area (Fig. 5(a)). The effect of medium-dose oligofructose (50 g/kg; G4) on bone area was not significant because of larger variation within that group, while the means of Tb.Ar/T.Ar at different doses of oligofructose were almost the same. The larger Tb.Ar/T.Ar at a low dose of oligofructose was due to higher Tb.Th (Fig. 5(d)). At medium and high doses of oligofructose, the larger Tb.Ar/T.Ar was due to larger Tb.Pm (Fig. 5(c)). Anisotropy was not affected (results not shown).

Effects of oligofructose at a high level of dietary calcium

When 50 g oligofructose/kg was fed with a high level of dietary Ca, apparent Ca absorption was slightly higher

Table 3. Effect of diets containing different concentrations of oligofructose on repeated calcium balances in ovariectomized rats‖

(Least square means and standard errors of the means)

Dietary group…	G1 (Sham)		G2		G3		G4		G5		G6		G7	
Ca	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Intake (g Ca and oligofructose)	5; 0		5; 0		5; 25		5; 50		5; 100		10; 0		10; 50	
Intake (mg/7 d)	280		280		280		280		280		560§		560§	
Balance week 4 (n 12−14)														
Faecal (mg/7 d)	280·07	4·58	271·40	4·22	290·68*	3·51	280·03	3·79	268·03	4·30	543·79*	6·76	536·33*†	5·22
Urinary (mg/7 d)	7·72	0·27	8·17	0·38	8·14	0·46	9·47*	0·46	11·60*	0·63	13·57*	0·86	19·52*†‡	0·63
Absorption (mg/7 d)	−0·07	4·58	8·60	4·22	−10·68*	3·51	−0·03	3·79	11·97	4·30	16·21	6·76	23·67*†	5·22
Retention (mg/7 d)	−7·78	4·61	0·43	4·38	−18·81*	3·58	−9·50	3·69	0·38	4·43	2·64	6·71	4·16†	4·94
Balance week 8 (n 12−14)														
Faecal (mg/7 d)	266·56	17·00	273·99	10·36	246·95	10·72	254·23	12·42	236·57*	10·34	466·99*	13·97	439·04*†	15·44
Urinary (mg/7 d)	7·18	0·33	7·26	0·27	8·07	0·49	8·91*	0·34	11·43*	0·62	15·19*	1·06	22·29*†‡	1·09
Absorption (mg/7 d)	13·44	17·00	6·01	10·36	33·05	10·72	25·77	12·42	43·44*	10·34	93·01*	13·97	120·96*†	15·44
Retention (mg/7 d)	6·15	17·00	−1·25	10·26	24·98	10·97	16·86	12·49	32·01*	10·31	77·58*	14·05	98·67*†	15·79
Balance week 16 (n 6−7)														
Faecal (mg/7 d)	235·06	4·77	242·40	3·53	237·03	3·22	236·74	2·62	234·50	6·46	475·82*	3·82	458·27*†‡	6·17
Urinary (mg/7 d)	5·31	0·30	5·52	0·52	6·74	0·54	7·15*	0·41	11·44*	0·76	13·00*	0·79	18·69*†‡	0·95
Absorption (mg/7 d)	44·94	4·77	37·60	3·53	42·97	3·22	43·26	2·62	45·50	6·46	84·18*	3·82	101·73*†‡	6·17
Retention (mg/7 d)	39·63	4·62	32·08	3·55	36·22	3·55	36·11	2·52	34·07	5·82	71·18*	4·03	83·05*†	6·31

Mean values (by ANOVA) were significantly different ($P<0.05$): *different from G2; †different from G4; ‡different from G6; §different from G1-G5.
‖For details of diets and procedures, see Table 2 and p. 366

Table 4. Effect of diets containing different concentrations of oligofructose on repeated phosphorus balances in ovariectomized rats§

(Least square means and standard errors of the means)

Dietary group…	G1 (Sham)		G2		G3		G4		G5		G6		G7	
P	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Intake (g Ca and oligofructose/kg feed)	5; 0		5; 0		5; 25		5; 50		5; 100		10; 0		10; 50	
Intake (mg/7 d)	300		300		300		300		300		300		300	
Balance week 4 (n 12−14)														
Faecal (mg/7 d)	164·58	3·26	164·78	2·84	166·09	3·13	162·86	2·91	163·44	2·81	213·20*	2·47	224·75*†‡	2·45
Urinary (mg/7 d)	124·79	3·34	116·44	3·15	117·63	3·90	117·20	4·65	102·77*	3·69	69·11*	0·76	57·21*†‡	1·37
Absorption (mg/7 d)	136·42	3·26	136·23	2·84	134·91	3·13	138·14	2·91	137·56	2·81	87·80*	2·47	76·25*†‡	2·45
Retention (mg/7 d)	11·63	2·81	19·78	4·59	17·28	4·06	20·94	4·57	34·79*	4·34	18·69	2·26	19·04	2·71
Balance week 8 (n 12−14)														
Faecal (mg/7 d)	163·95	9·04	166·13	4·15	170·99	5·47	153·13	6·40	165·67	8·53	206·06*	7·36	210·69*†	9·96
Urinary (mg/7 d)	116·41	1·02	114·66	3·71	109·52	1·42	107·64	1·39	101·83*	1·27	61·01*	1·53	48·44*†‡	1·30
Absorption (mg/7 d)	137·05	9·04	134·87	4·15	130·01	5·47	147·87	6·40	135·33	8·53	94·94*	7·36	90·31*†	9·96
Retention (mg/7 d)	20·64	9·27	20·21	6·06	20·49	5·17	40·23*	6·69	33·50	8·87	33·93	7·31	41·87	10·45
Balance week 16 (n 6−7)														
Faecal (mg/7 d)	155·92	5·90	163·45	3·63	166·92	6·29	169·67	6·13	164·68	4·97	201·25*	4·20	210·91*†	3·39
Urinary (mg/7 d)	91·50	4·80	96·12	3·53	92·03	2·52	89·46	2·77	89·89	2·93	48·92*	6·51	43·51*†	1·55
Absorption (mg/7 d)	145·08	5·90	137·55	3·63	134·09	6·29	131·33	6·13	136·32	4·97	99·75*	4·20	90·09*†	3·39
Retention (mg/7)	53·58	8·31	41·43	5·84	42·06	8·44	41·87	7·93	46·44	6·75	50·83	10·27	46·58	3·53

Mean values (by ANOVA) were significantly different ($P<0.05$): *different from G2; †different from G4; ‡different from G6.
§For details of diets and procedures, see Table 2 and p. 366.
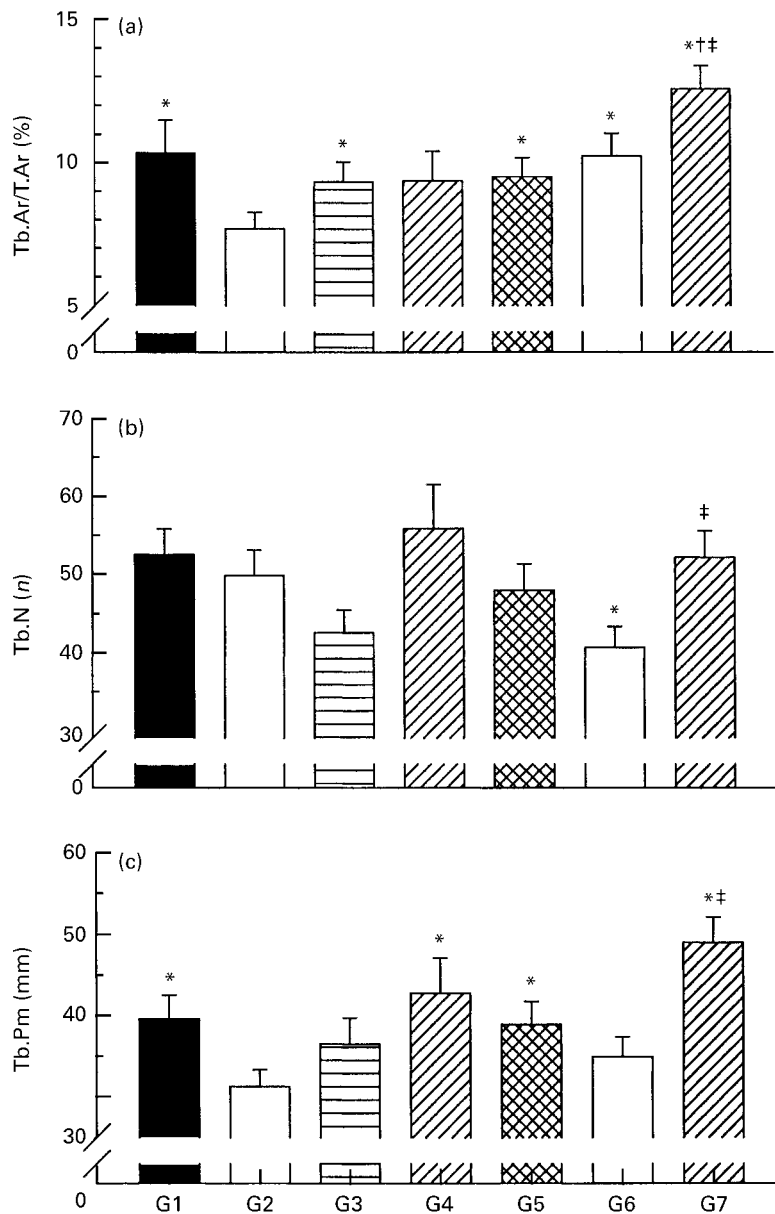
after 4 and 8 weeks and significantly higher after 16 weeks (Table 3). The Ca contents of femora and lumbar vertebrae were significantly higher (Fig. 4, G7 *v.* G6). The preservation of femur Ca occurred mainly within the first 8 weeks and, to a smaller extent, thereafter, while in lumbar vertebrae the preservation was moderate but persistent (results not shown). When dietary Ca was raised from 5 to 10 g/kg in the absence of oligofructose (G6 *v.* G2), apparent Ca absorption and retention were higher after 8 and 16 weeks (Table 3), but this was not associated with respective changes in bone mineral. However, if the increase in dietary Ca occurred in the presence of oligofructose (G7 *v.* G4), apparent Ca absorption and retention were stimulated persistently, but most at weeks 8 and 16 (Table 3). A greater Ca content was observed in lumbar vertebrae (Fig. 4).

When the diet contained 10 g Ca/kg, the exchange of starch for oligofructose raised Tb.Ar/T.Ar and Tb.Pm significantly

(Fig. 5(a) and (c), G7 *v.* G6). Both were due to significantly higher Tb.N (Fig. 5(b)) with similar or smaller size, since Tb.Th was slightly lower (Fig. 5(d)). There was a tendency for thicker corticalis (Fig. 5(f), $P=0.08$) and higher trabecular connectivity (Fig. 5(e), $P=0.07$).

When dietary Ca was increased from 5 to 10 g/kg in the absence of oligofructose (G6 *v.* G2), Tb.Ar/T.Ar was higher in spite of lower Tb.N, due to thicker trabecules (Fig. 5(a), (b) and (d)). However, if dietary Ca was increased in the presence of oligofructose (G7 *v.* G4), Tb.Ar/T.Ar was higher due to thicker trabecules (Fig. 5(a) and (d)), but without reduction in Tb.N (Fig. 5(b)). Only the diet containing a moderate dose of oligofructose and a high level of dietary Ca prevented loss of connectivity significantly (Fig. 5(e)), increased cortical bone thickness (Fig. 5(f)) and anisotropy with 1·93 (SEM 0·08) compared with G2 with 1·67 (SEM 0·07).
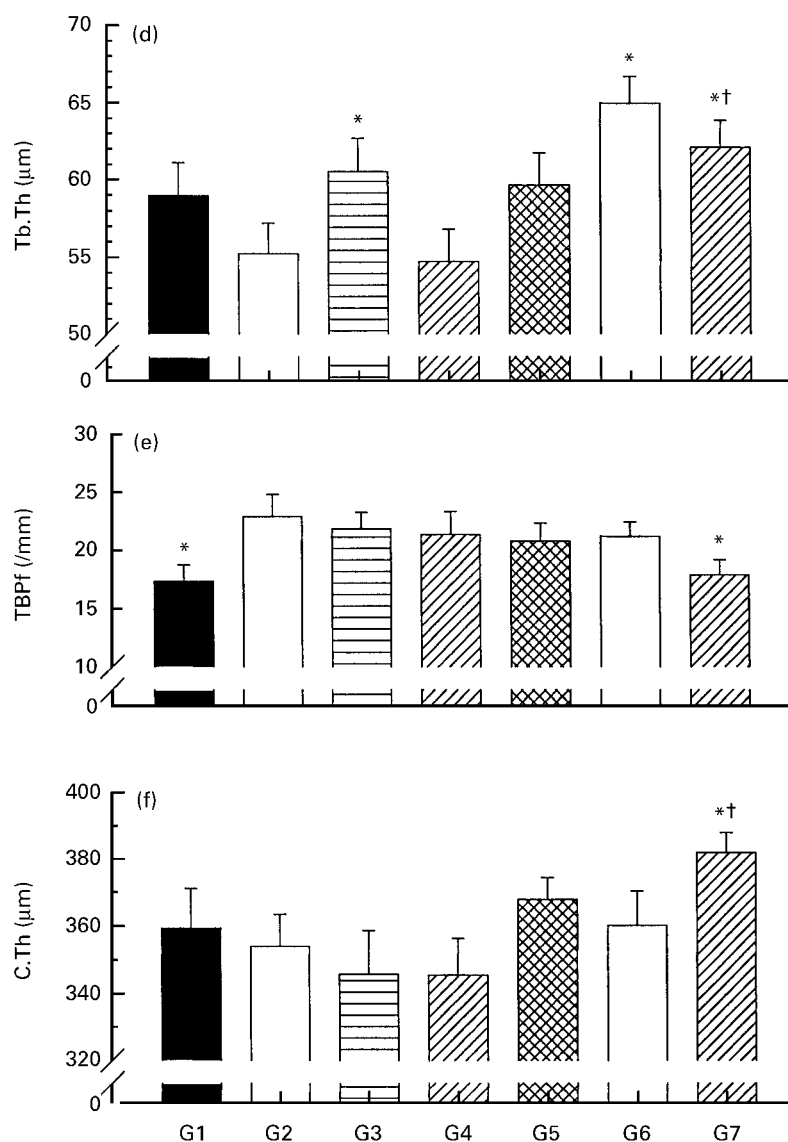
Fig. 5. Effect of a low, a medium and a high dose of oligofructose with the recommended level of dietary Ca or a medium dose of oligofructose with a high level of dietary Ca on (a), trabecular bone area as the percentage of bone tissue area (Tb.Ar/T.Ar); (b), trabecular number (Tb.N); (c), trabecular perimeter (Tb.Pm); (d), trabecular thickness (Tb.Th); (e), trabecular connectivity (TBPf); (f), cortical thickness (C.Th) in ovariectomized rats. Least-squares means and pooled SEM of all animals (8 weeks and 16 weeks on diets) were obtained by multifactorial ANOVA. The experimental groups were fed semi-purified diets with 5 g Ca/kg (recommended level) without oligofructose (G1 and G2), or with 25 g/kg (G3), 50 g/kg (G4) or 100 g oligofructose/kg (G5), or 10 g Ca/kg (high) either without oligofructose (G6) or with 50 g oligofructose/kg (G7). G1 animals were sham-operated, and G2–G7 were ovariectomized. Mean values were significantly different ($P < 0.05$): *different from G2; †different from G4; ‡different from G6.

Effects of time

In ovariectomized rats, the femur Ca content was significantly lower after 16 weeks (n 42), at 85·20 (SEM 0·96) mg compared with 90·11 (SEM 0·96) mg at 8 weeks (n 42; $P < 0.001$). This effect was mainly due to changes in G2, G4, and G7. No decrease was observed in sham-operated animals. The femur P content was significantly lower in ovariectomized rats after 16 weeks, at 36·64 (SEM 0·20) mg, compared with 41·43 (SEM 0·20) mg at 8 weeks ($P < 0.001$), and this occurred in all groups. Lower values were also observed in sham-operated animals. The weights of femora increased with time from 440 (SEM 10) mg after 8 weeks to 470 (SEM 10) mg after 16 weeks ($P < 0.05$), indicating higher values for non-mineralised organic matter with time, since there was no time-dependent

change of DM (results not shown). In contrast, no change occurred in Ca or P content of lumbar vertebrae, but weight decreased, from 270 (SEM 4) mg after 8 weeks to 240 (SEM 4) mg after 16 weeks, indicating higher mineral density in lumbar vertebrae with time.

In ovariectomized rats (mean ± pooled SEM, n 42), some parameters of bone structure were significantly lower after 16 weeks compared with 8 weeks. This was true for Tb.Ar/T.Ar (8·76 *v*. 11·17 (SEM 0·54) %; $P < 0·001$), Tb.N (42·50 *v*. 56·71 (SEM 2·44), $P < 0·01$), and Tb.Pm (33·51 mm *v*. 45·51 (SEM 1·92) mm, $P < 0·001$). Tb.Th became significantly higher with time (61·70 *v*. 57·46 (SEM 1·32) μm; $P < 0·05$).

Discussion

It is known from fluoride and bisphosphonate therapy that bone mineral content or bone density does not always correlate well with bone stability or fracture risk (Cummings *et al.* 1996; Meunier, 1996; Turner, 1996). Parameters other than density, particularly trabecular structure, may reflect bone quality or stability in a more valuable way (Kapadia *et al.* 1998). Therefore, we investigated the short- and long-term effects of low, medium, and high doses of oligofructose at the recommended level of dietary Ca, and of a medium dose of oligofructose in the presence of a high level of dietary Ca on Ca and P balances, and on bone mineralization, with the emphasis on trabecular structure and architecture.

Methods: animal model, effect of ovariectomy, microradiography and histomorphometry

In the present study, the lower femur Ca content in ovariectomized compared with sham-operated animals was not significant, presumably because cortical and trabecular bone are ashed and analysed together. However, the main changes following oestrogen deficiency occur at skeletal sites with high rates of bone turnover, i.e. in the trabecular bone (Gallagher, 1996). In young rats, trabecular bone metabolism is characterised predominantly by modelling, i.e. bone volume is still increasing. This process is parallel with that of ovariectomy-induced loss of bone mineral, making the interpretation of results more difficult than from adult rats, as they were used in the present study.

Ca retention was not significantly lower following ovariectomy, indicating a lack of sensitivity of the metabolic balance to the small effects of oestrogen deficiency in adult rats. Reports on the effect of ovariectomy on Ca absorption are equivocal (Thomas *et al.* 1988; O'Loughlin & Morris, 1994; Gaumet *et al.* 1997), depending on the study design. In general, a significant loss of cancellous bone area is associated with a loss of Tb.N in ovariectomized rats (Kimmel, 1996; Kapadia *et al.* 1998). In some cases, loss of bone area was not significant (Kalu & Orhii, 1998). In the present experiment, ovariectomized rats had 33 %, but non-significantly, lower trabecular area (Tb.Ar/T.Ar) after 8 weeks and 19 %, significantly, lower trabecular area after 16 weeks compared with sham-operated rats, demonstrating that the dietary intervention in

this experiment was done in an animal model with ovariectomy-induced loss of trabecular bone.

Baldock *et al.* (1999) reported a decrease of bone area of similar magnitude (about 35 %) for aged rats 8 weeks after ovariectomy, using the standard histomorphometry after van Kossa staining. We conclude that microradiography and histomorphometry is a valid method to detect ovariectomy-induced oestrogen deficiency. The loss of bone area was the result of decreased Tb.N at constant thickness (Baldock *et al.* 1999). The decrease in trabecular area after ovariectomy we observed was associated with significantly lower trabecular connectivity (higher TBPf) and of Tb.Pm as a result of slightly, but significantly, lower Tb.N and Tb.Th (Fig. 5). The reason for the equivocal results of different experiments may be due to differences in study protocol, like strain and the age of rats (Kimmel, 1996), time post ovariectomy (Abe *et al.* 1999), or skeletal site studied (Kimmel, 1996; Baldock *et al.* 1999). Cortical thickness was not affected by ovariectomy, which is in accord with observations made by Peng *et al.* (1999). It is assumed that in ovariectomized rats, mineral apposition rate and bone formation rate are increased compared with the rates in sham-operated rats, thus preventing a loss of cortical bone (Peng *et al.* 1999).

Effect of oligofructose at the recommended level of dietary calcium on mineral balance and bone mineral

In contrast to young growing rats (Ohta *et al.* 1994*b*), 50 g oligofructose/kg diet given with the recommended level of dietary Ca was too low a dose to stimulate apparent Ca absorption or retention in the adult ovariectomized rats used in the present experiment. Only the highest dose of oligofructose stimulated Ca retention. This effect became significant after 8 weeks in spite of higher urinary Ca, and persisted long enough to prevent the loss of Ca content in the appendicular skeleton. (Fig. 4). The better responsiveness of young, compared with aged rats may be explained by decreased intestinal and renal functions, known to be associated with ageing in the rat (Armbrecht *et al.* 1980; Gaumet *et al.* 1997), and by additional effects on mineral retention by increases in bone mass during growth. Urinary Ca increased with increasing dose of oligofructose, due to the higher apparent Ca absorption and not to increased bone resorption, as indicated by Ca balance (Table 3), femur Ca content (Fig. 4), and tibia Tb.Ar/T.Ar (Fig. 5). The lower sensitivity of metabolic Ca balance compared with bone mineral content is presumably due to the small daily effect of Ca retention compared with a prominent cumulative effect in bone over weeks. Oligofructose did not affect apparent P absorption, confirming results obtained in young rats after 1 or 3 weeks (Ohta *et al.* 1994*a*). The depression of urinary P on the highest dose of oligofructose lasted long enough to prevent the loss of P from the femur.

Effect of oligofructose at the recommended level of calcium on bone structure

To our knowledge, this is the first report on the long-term effect of oligofructose on parameters of bone architecture

in the adult ovariectomized rat, and therefore a wide variation of oligofructose was tested, although 10 % of oligofructose might be high for human nutrition. We observed that ovariectomy-induced loss of Tb.Ar/T.Ar was prevented by oligofructose at the recommended levels of dietary Ca, and was most prominent at high doses (Fig. 5(a)), confirming the results of the Ca balance, and the Ca and P content of femur. With respect to bone area and Tb.Th, it might be concluded that 25 g oligofructose/kg is a sufficient level to prevent loss of trabecular bone.

Effects of oligofructose at a high level of dietary calcium on mineral balance and bone mineral

In contrast to the small effect of a medium dose of oligofructose on bone Ca at the recommended level of dietary Ca, bone Ca was increased significantly by oligofructose with a high level of dietary Ca. Similar observations have been reported by others (Rémésy et al. 1993; Chonan & Watanuki, 1996). The moderate, although non-significant, effect of oligofructose on Ca retention was high enough and sufficiently persistent to reduce bone loss in the axial and appendicular skeleton (Fig. 4). Furthermore, we observed significantly higher femur Ca after 8 weeks and Ca in lumbar vertebrae after 16 weeks if dietary Ca was increased, but only in the presence of oligofructose. These results indicate that in adult oestrogen-deficient rats, a medium level of oligofructose was more effective if the dietary Ca level was high, and higher levels of oligofructose are needed for comparable results with diets providing the recommended level of Ca.

Effect of oligofructose at high levels of calcium on bone structure

Increasing the level of dietary Ca in the absence of oligofructose caused greater trabecular area, due to fewer but broadly thicker trabeculae. When a medium dose of oligofructose was added to the high-Ca diet, trabecular area and perimeter were increased further to the highest level of all groups, due to a greater number of trabeculae. Increasing dietary Ca in the presence of a medium dose of oligofructose caused the greatest trabecular area of all groups with thicker trabeculae but similar number compared with rats given a moderate dose of oligofructose with the recommended level of dietary Ca. Only with a combined intake of moderate oligofructose and high Ca (G7), were cortical thickness and anisotropy higher, and loss of connectivity (Fig. 5(e)) lower, compared with those seen with the recommended level of Ca without oligofructose (G2) or, for cortical thickness, with a medium dose of oligofructose (G4). Trabecular connectivity was found earlier to correlate with other variables of bone architecture (Croucher et al. 1996) and therefore may prove to be a good predictor of trabecular perforation, which was indicated as being lowest in G7. Thus, prevention of ovariectomy-induced loss of bone area was obviously associated with different bone architecture, depending on whether bone loss was prevented by increasing dietary Ca, by incorporation of oligofructose, or by both.

Mechanisms

The speed of feed consumption was not different between groups and thus cannot explain the differences in bone structure in the presence or in the absence of oligofructose. Microradiography-based histomorphometry of trabecular bone detects mineralised bone tissue with great sensitivity. Mineralised bone tissue reflects the combined effect of 'bulk' mineral incorporation and accumulation (like Ca, P and, to some extent, Mg) into bone matrix and of functional properties of trace minerals in the matrix and thus structure formation. Zn, Mn and Cu are essential metallic cofactors of enzymes involved in the synthesis of various bone matrix constituents and the cross-linking of collagen (Heaney, 1996). Absorption of Zn and Cu was stimulated by the β(2-1) type fructans (Delzenne et al. 1995; Lopez et al. 2000). This may explain the different bone architecture gained after increasing solely Ca, after adding oligofructose, or both. Increasing the level of dietary Ca delivers 'bulk mineral' but can impair trace mineral absorption (Wood & Zheng, 1997). Oligofructose may counteract this negative effect, especially in diets with high levels of Ca. Consequently, low concentrations of oligofructose can affect trabecular architecture significantly, while the effect on total bone mineral is borderline and even smaller on Ca balance.

The potential of fructo-oligosaccharides to stimulate bone mineralization or to preserve bone mineral content is due to increased apparent Ca absorption, and might be favoured if solubility of Ca is limited, as at a high dietary concentration (Brommage et al. 1993). The underlying mechanism was described in detail elsewhere (Scholz-Ahrens et al. 2001, 2002). In brief, under these conditions, a higher proportion of insoluble Ca would reach the colon. Fructo-oligosaccharides, such as oligofructose, are fermented in the caecum and colon to short-chain fatty acids with a consequent luminal acidification (Roberfroid et al. 1998). At lower pH, the solubility of Ca is higher and therefore its absorption is facilitated (Levrat et al. 1991). Limitation of solubility may explain the effect of oligofructose on bone Ca in rats on low levels of dietary Ca (0·3 %) if rats were gastrectomized (Ohta et al. 1998b). Gastrectomy depresses gastric acid production and thus solubility of Ca.

Fructo-oligosaccharides are known to increase caecal and colonic weight, villus height and the number of mucosal cells (Levrat et al. 1991; Rémésy et al. 1993; Campbell et al. 1997), and thus resorptive surface, probably by direct stimulation through short-chain fatty acids, especially butyrate. The production in the caecum of butyrate, a favoured candidate to stimulate colonic epithelial cell proliferation (Lupton & Kurtz, 1993), was persistently higher after 27 weeks on oligofructose (Le Blay et al. 1999). A direct effect of short-chain fatty acids on colonic Ca absorption was observed (Trinidad et al. 1993). Calcium acetate and propionate pass across cell membranes even more readily than ionised Ca alone (Trinidad et al. 1996). Moreover, an increased expression of mucosal Ca-binding protein (calbindin-D9k) in the large intestine may be involved (Ohta et al. 1998a).

We conclude that supplementing diets with oligofructose increases bone mineral and impedes ovariectomy-induced

loss of bone structure due to stimulated apparent absorption of macro and micro minerals and thus may help to improve bone health in periods with high demands, such as after menopause, when bone loss occurs following oestrogen deficiency.

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