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Fish bone calcium and bone density in the ovariectomised female rat.

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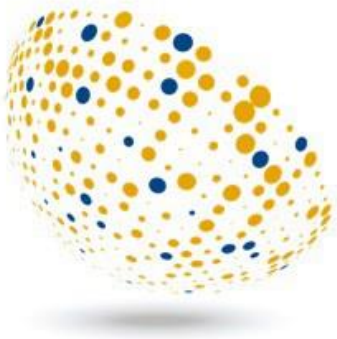

IANZ Accredited Laboratory

new zealand accredited laboratory

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The following results presented in this report were conducted under IANZ accredited protocols:

Dual-energy X-ray Absorptiometry (DEXA): KPI Dr W H Chua
Dietary analysis*: KPIs under F Jackson

* tests marked with an asterisk were conducted by the IANZ accredited Massey University Nutrition Laboratory.

SUMMARY

A proprietary fish bone powder product (FBP) high in calcium was assessed using an ovariectomised rat model to provide preliminary data regarding the health benefits of the FBP in menopause-induced osteoporosis. Adult female rats were fed a nutritionally complete diet in which the dietary calcium was sourced solely from FBP versus solely from calcium carbonate (CCC) for two months, ovariectomised or sham-treated, and then continued on the diets for an additional three months.

As expected, ovariectomy induced menopause and a resultant decrease in uterus weight, increase in blood level of the bone turnover marker CTX-1, and loss of bone mineral density. Ovariectomised rats also had slightly higher food intake and substantially greater weight gain, as well as a significant increase in lymphocyte counts in the peripheral blood.

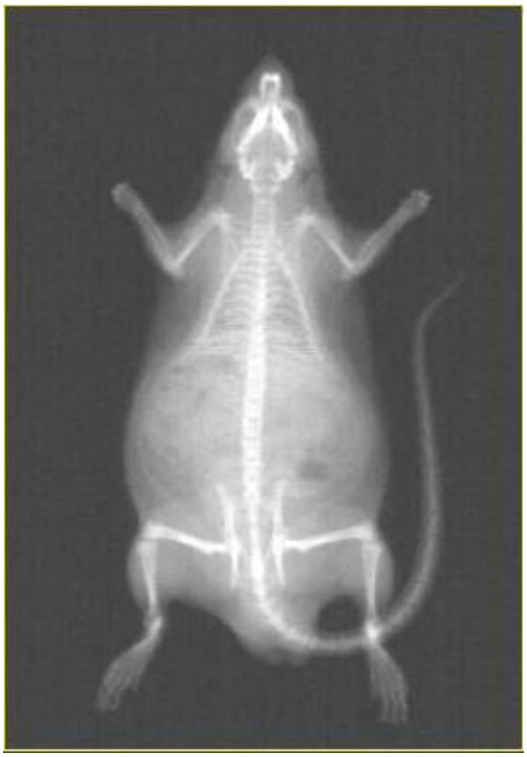
FBP did not have any detrimental effects and did not exacerbate any of the effects of ovariectomy compared to CCC. FBC did reduce the level of body weight and fat mass gain in ovariectomised rats while increasing lean mass gain. The results of this study demonstrate that FBP is an acceptable source of dietary calcium as an alternative to calcium carbonate and may protect against the changes in energy metabolism that occur after menopause.

Effect of ovariectomy (vs sham)	**Effect of FBP (vs CCC)**	**Recommendations**
Hormonal: ⬆ CTX-1; ⬇ oestrogen, uterine weight	No difference	No differences expected
Body weight: ⬆ food intake, body mass, fat mass, lean mass, BMI	Decreased fat mass gain; significantly improved lean mass gain	Assess FPB in diet-induced obesity model on lean/fat mass, glucose tolerance, metabolic syndrome
Blood: ⬆ haematocrit, red cell volume, platelet volume, white cell count, neutrophil count, lymphocyte count	Increased platelet volume in sham rats	Assess FPB in non-obese animals on inflammation and platelet activation
Bone: ⬇ bone mineral density and bone elasticity	No difference	Assess calcium-depleted FBP (collagen and protein) on bone and joint health

OBJECTIVES

The objectives of this study were to assess the effects of feeding a nutritionally complete diet containing calcium sourced from FBP versus calcium carbonate in adult female rats in which osteoporosis had been induced by ovariectomy. The primary parameters to be assessed were:

1. Bone mineral density
2. Bone turnover biomarkers
3. Bone breaking strength



Secondary objectives identified by Massey University as being of scientific interest were added to the study design and carried out at Massey University's expense:

1. Energy intake
2. Body weight gain
3. Feed conversion efficiency
4. Fat:lean body ratio
5. Complete blood count

INTRODUCTION

Bone provides structural support for the body. Calcium is the most abundant mineral in the human body, and 99% of the body's calcium is held in the bone. Bone tissue is metabolically active and undergoes continuous change throughout life via a series of tightly regulated processes. Bone is destroyed and resorbed by osteoclasts, then replaced through the formation of new bone by osteoblasts.[1] Mineralisation is laid down by osteoblasts under the direction of osteocytes and osteoclast activity.

An imbalance between bone resorption and bone formation results in a decrease in bone mineralisation termed osteopenia. Osteoporosis, which follows on from osteopenia, is a generalised skeletal disorder characterised by decreased bone mass and deteriorated bone architecture. Osteoporosis results in an increased susceptibility to bone fractures and increased post-fracture mortality risk.[2] Osteoporosis is most prevalent in post-menopausal women.[3]

As calcium is the key component of bone, calcium deficiency is strongly associated with osteoporosis. Similarly, vitamin D, which is required for calcium absorption, is implicated in bone health, and vitamin D deficiency correlates with osteoporosis. Thus, adequate intake of calcium and vitamin D are considered key factors to both prevent and ameliorate osteoporosis.[4,5] However, calcium supplementation in tablet form has proven unsuccessful and, instead, calcium and vitamin D from dietary sources are recommended.[4, 6-8]

The role of dietary calcium and vitamin D in preventing or ameliorating existing osteoporosis post-menopause has been established as being very similar in humans and rats.[9] Ovariectomy is a commonly-used rat model to induce menopause, which results in the development of osteoporosis that can be exacerbated if dietary micronutrients are deficient.[9-14]

The current study was designed to compare fish bone powder and calcium carbonate in an ovariectomised rat model and their effects on the loss of bone mineralisation associated with the surgery.

MATERIALS AND METHODS

Animals and housing:

Conventional female Sprague-Dawley rats aged 3 months were purchased from the Massey University Small Animal Production Unit (SAPU). Rats were individually housed in plastic cages with wire lids and bedded with sterile wood shavings. All rats had access to water *ad libitum.* From age 3 months to 5 months, rats were fed a powdered casein-based diet containing 0.35% calcium from calcium carbonate. From age 5 months to age 8 months, rats were fed a powdered casein-based diet containing 0.5% calcium from either the control source of calcium (calcium carbonate; CCC), or the test source (fish bone powder; FBP). Food intake was limited to 20 grams per day per rat.

Daily feeding, care, and measurements

The feeding studies were carried out as described elsewhere.[15] Briefly, rats were randomly allocated to one of the two diets with each group being balanced for body weight. Powdered feed was replaced with fresh feed once daily. For each rat, the feeder was removed from the cage and its weight recorded before it was emptied and cleaned. The feeder was then filled with fresh diet, its weight recorded, and the feeder placed back into the rat cage. Daily food intake was thus measured as food offered minus food remaining 24 hours later. Rats were limited to 20 grams of food per day to reduce the weight gain that is known to occur post-ovariectomy.

At the time of feeding, each rat's general health was also checked. Water bottles were checked daily to ensure rats had sufficient water. Rats were weighed weekly using a one decimal point balance. Cages, stainless steel feeders, and water bottles were cleaned and replaced once weekly.

Food efficiency ratios for the CCC and FBP diets were calculated[16] with modifications as:

FER = gain in body weight (g)/food consumed (kg)

Ovariectomy surgery

Rats within each diet group were allocated by body weight into sham or ovariectomy treatment. Surgeries were carried out on 5 month old rats as described elsewhere [15]. Briefly, rats were anaesthetised with an intraperitoneal injection of anaesthesic containing acepromazine, ketamine and xylazine. The surgical site was shaved and cleaned with an iodine scrub, and the rat then fully anaesthetised with an

isoflurane/air mixture via a nose-cone. Ovaries were exteriorized through a dorsal incision and removed at the distal uterine horn. The incision was closed with surgical clips, and saline and analgesic administered.

Dietary components

Sodium caseinate (CAS) was purchased from Tatua (Morrinsville, NZ). Cysteine, methionine, glutamic acid, glycine, lysine, calcium carbonate, potassium phosphate, potassium sulphate, potassium citrate, and magnesium oxide were purchased from Merck (Darmstadt, Germany). Fish bone powder was provided by United Fisheries with the specifications of shown in Table 1.

Table 1. Proximate analysis of Fish Bone Powder (MCHC).

Component	
Protein	24.75%
Calcium	25.14%
Magnesium	0.45%
Phosphorus	12.86%
Moisture	1.9%
Collagen	14.27%
Mercury	<0.07 ppm
Manganese	16 ppm
Copper	0.19 ppm
Iron	6.2 ppm
Potassium	0.04%
Selenium	0.73 ppm
Zinc	61 ppm
Sulphur	0.25%
Boron	<5 ppm

Tryptophan, ferric citrate, manganous sulphate, zinc oxide, cupric carbonate, chromic potassium sulphate, sodium selenite, cobaltous chloride, potassium iodate, and ammonium molybdate were purchased from Sigma-Aldrich (Auckland, NZ). Cellulose was purchased from Hawkins Watts (Auckland, NZ). Cornstarch, soy oil, and sucrose were purchased from Davis Trading (Palmerston North, New Zealand). Vitamin mix specifically designed to conform to AIN-93M was purchased from Plant & Food (Palmerston North, NZ). Trace salt and mineral mixes were prepared at Massey University. The composition of the mineral mix, including the trace salt mix, is shown in Table 2.

All diets were formulated to meet AIN-93M specifications for protein and fat, and to meet amino acid and micronutrient requirements as specified in the Nutrient Requirements of Laboratory Animals (4^{th} revised edition, 1995). The diets were matched for macronutrients, micronutrients, and energy, with the sole difference being the source of calcium in the diet. Powdered diets were made up and blended in 10 kilo batches as shown in Table 3. Diets were labelled, colour-coded, and stored at -20°C.

Table 2. Components of mineral mix. Amounts are shown in g/kg of mix.

Mineral Mix	(g)
Potassium phosphate monobasic	196
Potassium sulphate	46.6
Potassium citrate tri-K monohydrate	70.8
Magnesium oxide	24.0
Trace salt mix	40.0
Cellulose	622.6

Trace Salt Mix	(g)
Ferric citrate	756.7
Manganous sulfate	80.0
Zinc oxide	20.0
Cupric carbonate	6.7
Chromic potassium sulphate	6.3
Sodium selenite	0.11
Cobaltous chloride	0.039
Potassium iodate	0.085
Ammonium molybdate	0.93
Cellulose	129.973

Table 3. Components per kilo of diet. Amounts are shown in grams.

	Pre-study base diet	Control (CCC)	Test (FBP)
Sodium caseinate	150	150	145
Cysteine	2.7	2.7	2.7
Glycine	3.3	3.3	3.3
Methionine	1.5	1.5	1.5
Glutamine	7	7	7
Cellulose	50	50	50
Vitamin mix	10	10	10
Sucrose	40	40	40
Mineral mix*	50	50	50
Corn oil	50	50	50
Cornstarch	626.75	623	620.6
$CaCO_3$	8.75	12.5	0
Fish Bone Powder	0	0	19.9

**See Table 2.*

Diet proximate analyses were carried out by an IANZ-accredited testing facility (Nutrition Laboratory, MIFST, Massey University, NZ). Protein was measured using the Leco total combustion method (AOAC 968.06). Fat was measured using Soxtec extraction (AOAC 991.36). Moisture was measured by convection oven drying at 105ºC (AOAC 930.15, 925.10). Ash was measured using a 550ºC furnace (AOAC 942.05). Gross energy was measured by bomb calorimetry. Based on the laboratory proximate analysis of compounds and the information provided by the supplier or available in the literature, the predicted and true levels of individual components in each diet design were found to be comparable (Table 4).

Dual Energy X-ray Absorptiometry (DEXA) measurements

Rats were anaesthetised and scanned for body composition using a Dual-Energy X-ray Absorptiometry scanner (DEXA) as described elsewhere[15] at the ages of 5, 6, 7 and 8 months. Femurs and spines collected post-euthanasia were stored frozen at -20°C, and later thawed and scanned using the same DEXA machine for bone mineral density.

Euthanasia and dissection

At the age of 8 months, fasted rats were given a lethal dose of the anaesthesia cocktail described above prior to blood sampling. Euthanasia was carried out by exsanguination and pneumothorax induction. Blood was collected from the superior vena cava through a 19 gauge 1½ inch needle into a heparin-flushed 10 mL syringe, then aliquoted into heparin- or EDTA-containing vacutainer tubes. Each uterus was collected and weighed to verify the success of the ovariectomy or sham surgery. Spine and femurs were removed, and gross pathology of the internal organs assessed.

Table 4. Predicted and final levels of key diet components.

	Predicted in base diet (from formulation/ mineral mix)	**Present in base diet (proximate analysis)**	***Predicted in CCC and FBP diets (from formulation/ mineral mix)***	**Present in CCC diet (proximate analysis)**	**Present in FBP diet (proximate analysis)**
Protein (%)	*15.0%*	15.6%	*15.0%*	14.7%	15.6%
Fat (%)	*5.0%*	5.1%	*5.0%*	5.0%	6.2%
Energy (kJ/gram)	*17.2*	17.3	*17.2*	17.2 kJ/g	17.3
Calcium (mg/kg)	*3500*	3500	*5000*	4800	4700
Phosphorus (mg/kg)			*2162*	4700	4800
Magnesium (mg/kg)			*720*	770	730
Potassium (mg/kg)			*5063*	4500	4500
Zinc (mg/kg)			*32*	39	37

Bone strength

Bone flexibility and tensile breaking strength were measured using a Shimadzu texture analyser, as described elsewhere.[15]

Blood cell and haemoglobin parameters

Complete blood count (CBC) analyses on EDTA-anticoagulated whole blood was carried out using a Beckman Coulter Sysmex XE5000 haematology analyser.

Plasma markers

Plasma oestrogen (estradiol) and carboxyl-terminal cross-linking telopeptides of type 1 collagen (CTX-1) were measured by ELISA using commercial kits.

Statistical analyses

Means and standard deviations, two-tailed Student t-tests and Pearson's correlation coefficient calculations were carried out using Microsoft Excel software. Multivariate ANOVA and mixed-model repeated measures ANOVA analyses were carried out using SAS/STAT 9.4 (SAS Institute Inc., Cary, NC, USA) by Dr W-H Chua.

RESULTS

Uterus weight and oestrogen levels

Adult female rats were randomised into one of two diet groups and balanced for body weight (Figure 1): control diet containing calcium from calcium carbonate (CCC sham) or test diet containing calcium from fish bone powder (FBP). Rats within each diet group were further randomised to undergo either a sham operation or ovariectomy (OVX).

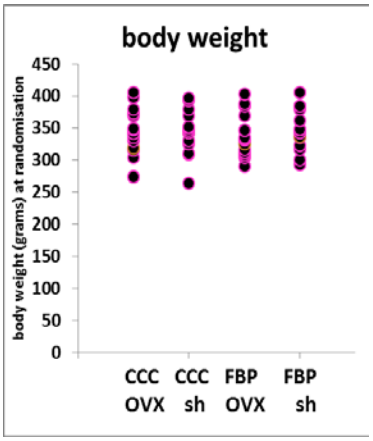


Figure 1: Initial body weight. Rats were randomised into control (CCC) or fish bone powder (FBP) diet groups, and further assigned to undergo a sham (sh) or ovariectomy (OVX) operation. Each dot represents the starting body weight of an individual rat (N ≥ 15 rats per group).

Three rats (#26 FBP-OVX, #30 FBP-sham, and #44 CCC-sham) had minor calcification in the mesenteric adipose tissue. One rat (#62 CCC-OVX) had an injured crucial ligament in the right hind knee and the presence of scar tissue and bone damage in the foot, likely due to an injury caused by the paw being caught in the cage lid wire. One rat (# 61 CCC-sham) had a non-palpable subcutaneous mammary tumour, well circumscribed, located over the left ribs but not penetrating the fascia into the dermis or abdominal wall. As none of these pathologies were likely to affect the primary study outcomes, the data from these rats were included in this report.

Ovariectomy results in atrophy of the uterine horns due to a reduction in oestrogen production. Surgery success was verified by dissecting out the uterus from each rat after euthanasia at the end of the study and weighing the organ, and by measuring oestrogen levels in the blood.

As expected, diet did not significantly affect uterine weight, but surgery type did (Figure 2). The mean uterine weights of the OVX CCC and FBP rats were 0.297 ± 0.098 grams and 0.255 ± 0.061 grams, respectively. The mean uterine weights of the sham CCC and FBP rats were 1.006 ± 0.178 grams and 0.885 ± 0.163 grams, respectively. One rat that underwent OVX surgery had a uterine weight of 0.915 grams, indicating that her ovaries had not been completely excised and the organ had regenerated due to the presence of sufficient oestrogen to retain the full size of the uterus. This individual animal was discarded from all data sets and her results are not included in this report.

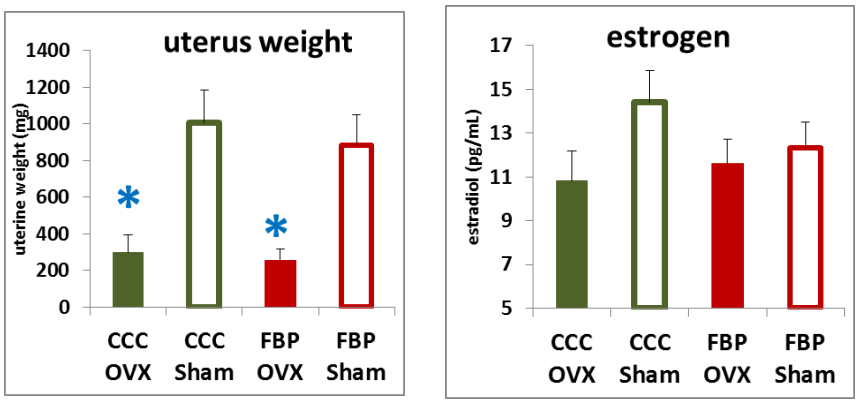


Figure 2: Uterus weights and estrogen levels. The uterus of each rat was collected at the end of the study (left). Blood serum was collected and assessed for oestrogen (right). Data are shown as mean + SEM of N = 15 rats per group. *p < 0.001 by ANOVA compared to sham rats by ANOVA using body weight as a covariate.

Also as expected, all OVX rats in both test groups had low oestrogen levels. In the sham groups, most rats had low oestrogen but a few individuals, who would have been ovulating, had higher oestrogen. Oestrogen levels are low (<15 pg/mL) in ovariectomised rats and in normal rats who are not in oestrus on the day the blood is sampled. Sprague-Dawley rats ovulate once every five days and during this oestrus period the blood level of oestrogen rises.

Food intake and body weight

Rats were fed a base diet from age 3 months to 5 months. At age 5 months, the rats were DEXA scanned and then underwent ovariectomy (OVX) or sham surgery. Following surgery, rats were placed on the control or test diet. Food intake, which was limited to the recommended maintenance level of 20 grams per day, was monitored daily.

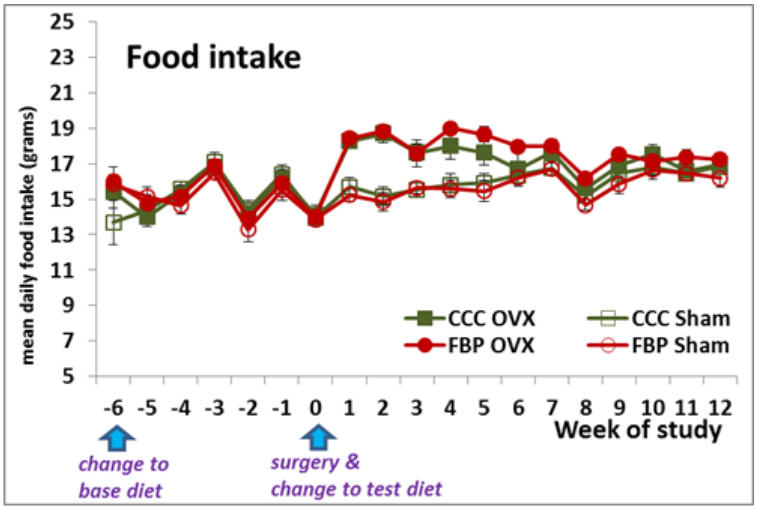


Figure 3: Food intake. Rats were fed one the CCC or FPB powdered diet and food intake was measured daily. Mean daily intake per week is shown as mean + SEM of N ≥ 15 rats per group.

Body weight of the rats was measured weekly. The adult rats that underwent a sham surgery gained approximately 6% of their initial body weight over 16 weeks (Figure 4), whereas the rats that underwent ovariectomy gained approximately 22% of their initial body weight.

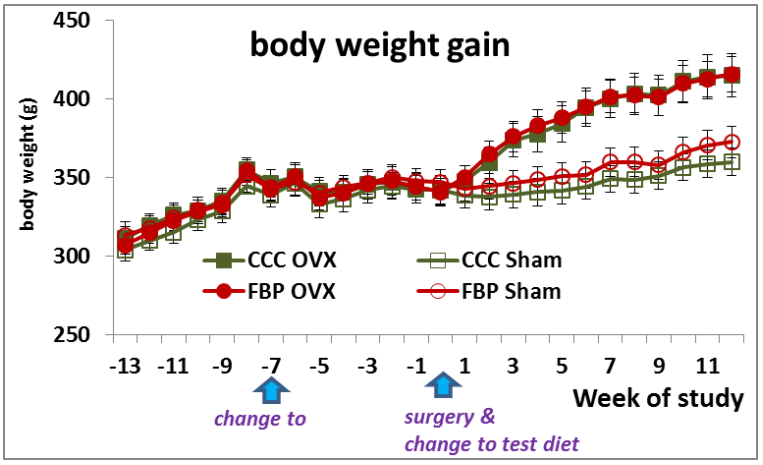


Figure 4: Weekly body weight gain. Body weight (BW) was measured weekly. Weight gain as the percent gain normalised to starting body weight was calculated for each rat. Data are shown as mean + SD of N ≥ 15 rats per group.

Body weights were further examined (Table 5). There was no difference in starting weight at week 0 of the study by body weight. However, in both diet groups, OVX rats weighed significantly ($p<0.01$) more than sham rats at week 13.

As the rats did differ significantly by surgery group in both food intake and weight gain, the food efficiency ratio was calculated as grams of body weight gained per kilogram of food consumed. OVX rats tended to eat all of the food offered (20 grams per day) while sham rats often left part of their food uneaten. As shown in Table 5, OVX rats in both diet groups ate significantly more food ($p<0.03$) during the study than sham rats. Similarly, OVX rats in both diet groups gained significantly more weight ($p<0.0001$) during the study than sham rats.

Table 5. Body weights (BW), food intake, weight gain, and feed conversion ratio (mean + SD of N ≥ 15 rats per group) over a 13 week period.

	CCC-sham	CCC-OVX	FBP-sham	FBP-OVX
Wk 0 BW (g)	344 + 34	342 + 37	347 + 32	341 + 33 *
Wk 13 BW (g)	363 + 35	418 + 58 *	374 + 39	418 + 48
Food intake (kg)	1.45 + 0.15	1.57 + 0.16 *	1.42 + 0.13	1.59 + 0.08 *
Weight gain (g)	19 + 18	76 + 26 **	27 + 14	79 + 21 **
Food efficiency	13 + 12	49 + 13 **	18 + 10	50 + 13 **
Weight gain (g BW per 100 kJ "excess" intake)	0.22 + 0.06	0.72 + 0.05 **	0.30 + 0.04	0.69 + 0.05 **

*$p<0.05$ and **$p<0.0001$ compared to diet-matched sham group by Student's t test.*

However, weight gain did not correlate with the amount of food eaten. Sham rats gained 13 – 18 grams of body weight for each kilo of food consumed; OVX rats gained 49 – 50 grams of body weight for each kilo of food consumed. That is, OVX

rats ate 10% more food but gained >200% more weight than sham rats, even though the diets were within 1% of each other for energy (caloric) content.

Total energy intake during the study in these rats ranged from 18,890 kJ to 30,400 kJ. The rat that ate the least did gain 28 grams of body weight during the study, indicating that this amount of food was more than adequate for maintenance. Therefore, weight gain was recalculated as grams of body weight gained per 100 kJ 'excess' energy consumed above an estimated 'maintenance' intake of 16,000 kJ in total. Again, diet had no effect, but ovariectomy more than doubled the amount of weight gain per 100 kJ consumed above the maintenance level ($p<0.0001$).

Body composition

DEXA scanning measured total lean and total fat masses of the rats, from which each rat's body composition was calculated from the percent fat mass; this is commonly equated to body mass index (BMI). All four groups were very similar in body weight, body mass index, fat mass, and lean mass at the time of surgery, at which time they began the CCC or FBP diets (Figure 5).

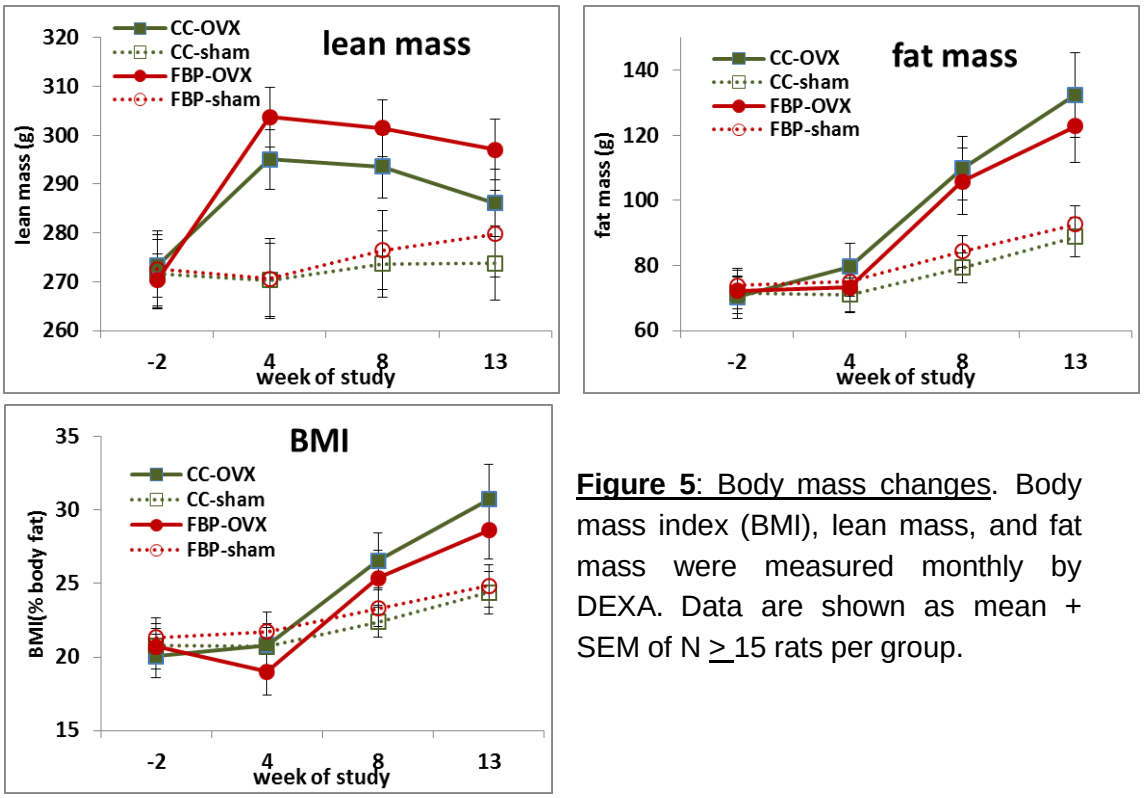


Figure 5: Body mass changes. Body mass index (BMI), lean mass, and fat mass were measured monthly by DEXA. Data are shown as mean + SEM of N ≥ 15 rats per group.

As shown in Figure 5, OVX rats gained more lean mass, fat mass and BMI than sham rats. Even when analysed with body weight as a covariate factor, OVX rats made significant gains in both lean and fat mass during the study, whereas sham rats did not. However, CCC-OVX rats did not gain significant lean mass from baseline to week 4, whereas FBP-OVX rats gained significant lean mass in each of

the periods measured (weeks -2 to 4, 4 to 8, and 8 to 13). Table 6 shows proportional gains of fat, lean, and BMI relative to each animal's baseline measurement.

Table 6. DEXA body scan data (mean ± SD). The % change depicts the proportion gained from baseline to week 14.

	CCC-sham	CCC-OVX	FBP-sham	FBP-OVX
Total mass (g)				
Baseline	343 ± 32	343 ± 39	346 ± 30	341 ± 34
Week 4	341 ± 34	374 ± 41	346 ± 33	377 ± 41
Week 9	353 ± 34	399 ± 53	361 ± 36	407 ± 47
Week 14	363 ± 34	419 ± 56	372 ± 37	420 ± 49
% change	*6 ± 5*	*22 ± 7* *	*7 ± 4*	*23 ± 6* *
Fat mass (g)				
Baseline	71 ± 19	71 ± 24	74 ± 19	71 ± 26
Week 4	71 ± 21	79 ± 27	75 ± 18	73 ± 31
Week 9	79 ± 18	107 ± 40	84 ± 20	106 ± 41
Week 14	89 ± 24	132 ± 52	93 ± 23	123 ± 45
% change	*25 ± 19*	*89 ± 35* *	*27 ± 15*	*73 ± 34* *
Lean mass (g)				
Baseline	272 ± 28	272 ± 25	273 ± 31	270 ± 20
Week 4	270 ± 29	295 ± 23	271 ± 32	304 ± 23
Week 9	274 ± 27	292 ± 25	276 ± 31	302 ± 23
Week 14	274 ± 29	286 ± 27	280 ± 34	297 ± 24
% change	*1± 5*	*5 ± 6* *	*3 ± 4*	*10 ± 6* * †
Fat (% of mass)				
Baseline	21 ± 4	20 ± 6	21 ± 5	21 ± 6
Week 4	21 ± 5	21 ± 6	22 ± 5	19 ± 6
Week 9	22 ± 4	26 ± 7	23 ± 4	25 ± 7
Week 14	24 ± 6	31 ± 9	25 ± 6	29 ± 7
% change	*18 ± 15*	*55 ± 24* *	*18 ± 12*	*41 ± 22* *

**p<0.05 compared to sham surgery within the same diet; †p < 0.05 compared to CCC-OVX.*

Blood cells and iron levels

As diet can affect iron uptake, blood samples were assessed for complete blood counts (CBC) at the time of cull. Data were collected for all but three rats, whose blood was too clotted to permit analysis; this is an inherent problem as rat blood clots very rapidly compared to humans.[17, 18]

OVX surgery significantly impacted several CBC parameters in both diet groups (Table 7). Rats that underwent ovariectomy regardless of diet had significantly higher haematocrit and mean red cell volume, as well as significantly higher white blood cell counts; the latter finding was reflected particularly in a higher lymphocyte count. Overall, CBC data matched the expected values reported elsewhere for similarly-aged Sprague-Dawley female rats.[19, 20]

In FBP but not CCC rats, ovariectomised rats also had a significantly higher haemoglobin content and higher neutrophil count. Within the sham rats, the FBP diet resulted in a significantly lower platelet count. Within the ovariectomised rats, the

FBP also resulted in a lower platelet count, though this did not reach statistical significance (p=0.053). These differences, and the large standard deviations observed in the platelet counts, likely reflect a low and inconsistent level of sample clotting prior to analysis, which rat blood is prone to do.

Table 7. CBC analysis of whole blood from 36 week old female rats. Data are shown as mean ± standard deviation of N ≥ 14 rats per group.

	CCC sham	CCC OVX	FBP sham	FBP OVX	*Expected* [Δ]
RBC count (x 10^{12}/L)	6.85 ± 0.25	6.93 ± 0.28	6.79 ± 0.28	6.91 ± 0.36	*8.96 ± 0.27*
Hg (g/L)	136 ± 6	138 ± 4	135 ± 5	142 ± 8 *	*131 + 9*
Hct (L/L)	0.37 ± 0.02	0.40 ± 0.04 *	0.38 ± 0.02	0.39 ± 0.02 *	*0.44 + 0.04*
MCV (fL)	54.7 ± 1.1	56.2 ± 2.0 *	55.3 ± 1.9	56.6 ± 1.2 *	*55.7 ± 0.3*
MCH (pg)	19.9 ± 0.4	20.0 ± 0.8	19.9 ± 0.7	20.4 ± 0.5 *	*18.3 ± 0.2*
MCHC (g/L)	364 ± 5	355 ± 8 *	351 ± 5	361 ± 7	*328 ± 4*
RDW (%)	13.0 ± 0.6	13.1 ± 0.8	13.1 ± 0.7	12.6 ± 0.5	*n/a*
Platelet count (x 10^9/L)	706 ± 144	628 ± 83 *	586 ± 153[@]	570 ± 65	*830 ± 50*
MPV (fL)	7.22 ± 0.29	7.72 ± 0.41 *	7.51 ± 0.37 †	7.85 ± 0.24 *	
WBC count (x 10^9/L)	4.63 + 1.00	7.33 ± 1.39 *	5.02 + 1.99	7.28 ± 1.55 *	*5.78 ± 1.99*
% Neutrophils	28 ± 14	23 ± 12	27 ± 16	30 ± 15	*30 + 11*
Neutrophils (x 10^9/L)	1.34 ± 0.92	1.62 ± 0.99 *	1.23 ± 1.03	2.15 ± 1.25 *	*1.37 ± 0.16*
% Lymphocytes	69 ± 19	72 ± 22	71 ± 15	70 ± 15	*66 + 12*
Lymphocytes (x 10^9/L)	3.27 ± 0.84	5.71 ± 1.70	3.66 ± 2.05	5.14 ± 1.79 *	*3.18 ± 0.39*

**p<0.05 by Student's t test compared to sham control; †p < 0.05 compared to CCC-sham; [Δ]mean values reported elsewhere [19, 20]. RBC, red blood cell; Hb, haemoglobin; Hct, haematocrit; MCV, mean corpuscular volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin content; MPV, mean platelet volume; RDW, red cell deviation of width; WBC, white blood cell; n/a, not available.*

Bone turnover markers and bone mineralisation

During osteoporosis, caused by ovariectomy in rats or by menopause in women, bone breakdown results in the release of measurable metabolites in the blood, including CTX-1 (carboxyl-terminal crosslinking telopeptides of type I collagen). The level of CTX-1 was measured in the blood of all rats at the study end.

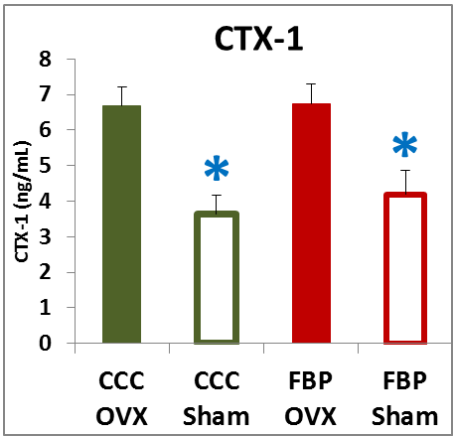


Figure 5: CTX-1. The bone turnover biomarker CTX-1 in rat plasma was measured by ELISA. Data are shown as mean + SEM of N = 15 rats per group. *p < 0.001 by ANOVA compared to sham rats by ANOVA using body weight as a covariate.

Diet did not impact the level; however, induction of osteoporosis through ovariectomy was confirmed by a significant rise in CTX-1 in OVX rats of both diet groups (Figure 5). Uterine weight, the parameter used to verify ovariectomy success, correlated negatively, as expected, with CTX-1 levels (R = - 0.55) in the rat cohort.

Bone area (BA), bone mineral content (BMC), and bone mineral density (BMD) were measured by DEXA scanning prior to OVX/sham surgery and at then monthly thereafter. Whole body BMD was similar between all groups prior to surgery. There was more variability in spine and femur BMD in rats at week -2 (Figure 6). BMD was reduced in the whole body, lumbar spine, and femur in OVX rats within the first 6 weeks post-surgery, as expected, although this did not reach statistical significance. BMD did not fall in the sham rats after surgery.

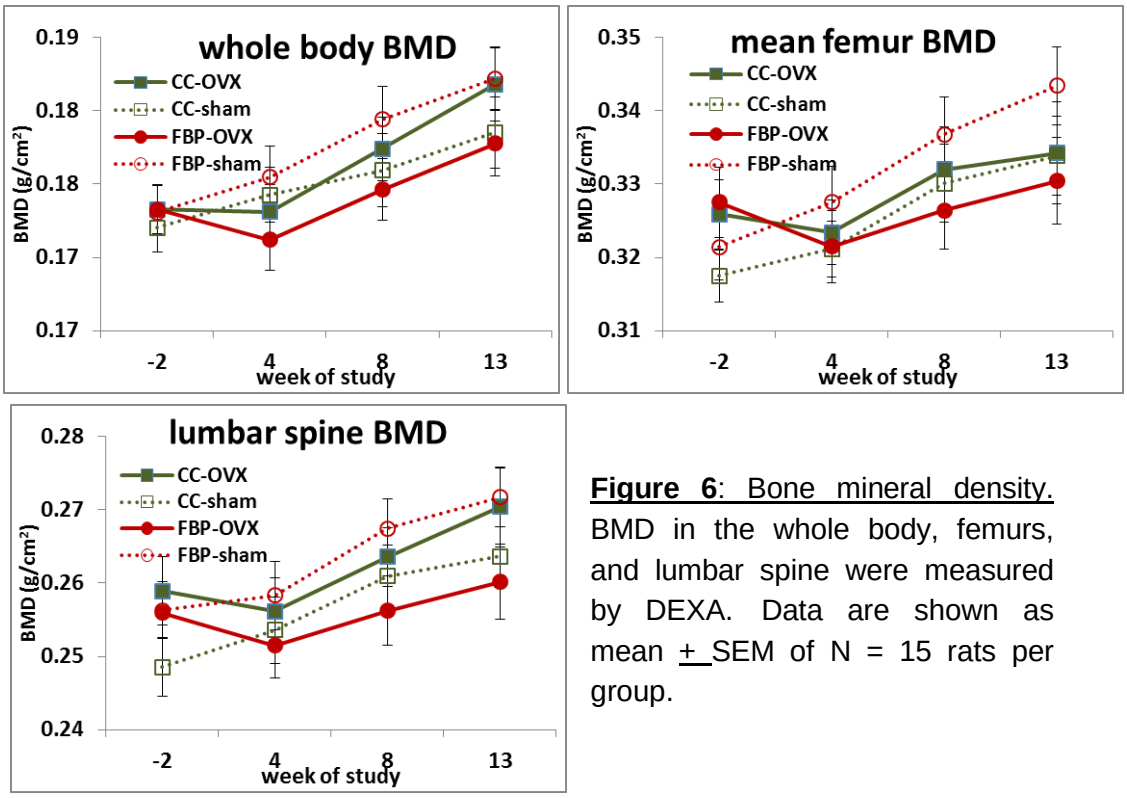


Figure 6: Bone mineral density. BMD in the whole body, femurs, and lumbar spine were measured by DEXA. Data are shown as mean ± SEM of N = 15 rats per group.

Because of the variability of starting BMD, measurements for each individual rat over time were normalised to its baseline reading. OVX caused an approximate 2% reduction in BMD in the lumbar spine and femur within the first month. OVX rats then gradually rose to or above baseline levels, with OVX rats fed the CCC diet recovering more quickly than those fed the FBP diet. Sham rats gradually increased BMD regardless of diet (Figure 7).

BA, BMC, and BMD changes between the time of surgery (week -2) and the end of the study (week 13) were further analysed by repeated-measures ANOVA with body weight as a covariate factor, since bone will naturally grow to support an increase in body weight. Sham rats experienced significant increases in BA of the lumbar spine, femur, and whole body, irrespective of body weight gain, whereas OVX

rats did not. Likewise, sham rats experienced significant increases in lumbar spine and femur BMC, irrespective of body weight gain, whereas OVX rats did not. Finally, sham rats demonstrated significant increases in lumbar spine and femur BMD. In addition, whole body BMD in sham rats increased from weeks -2 to 4, whereas this did not occur in OVX rats. These findings are not unexpected, as OVX surgery reduces the body's ability to build bone in these areas of the skeleton. However, diet did not affect these parameters.

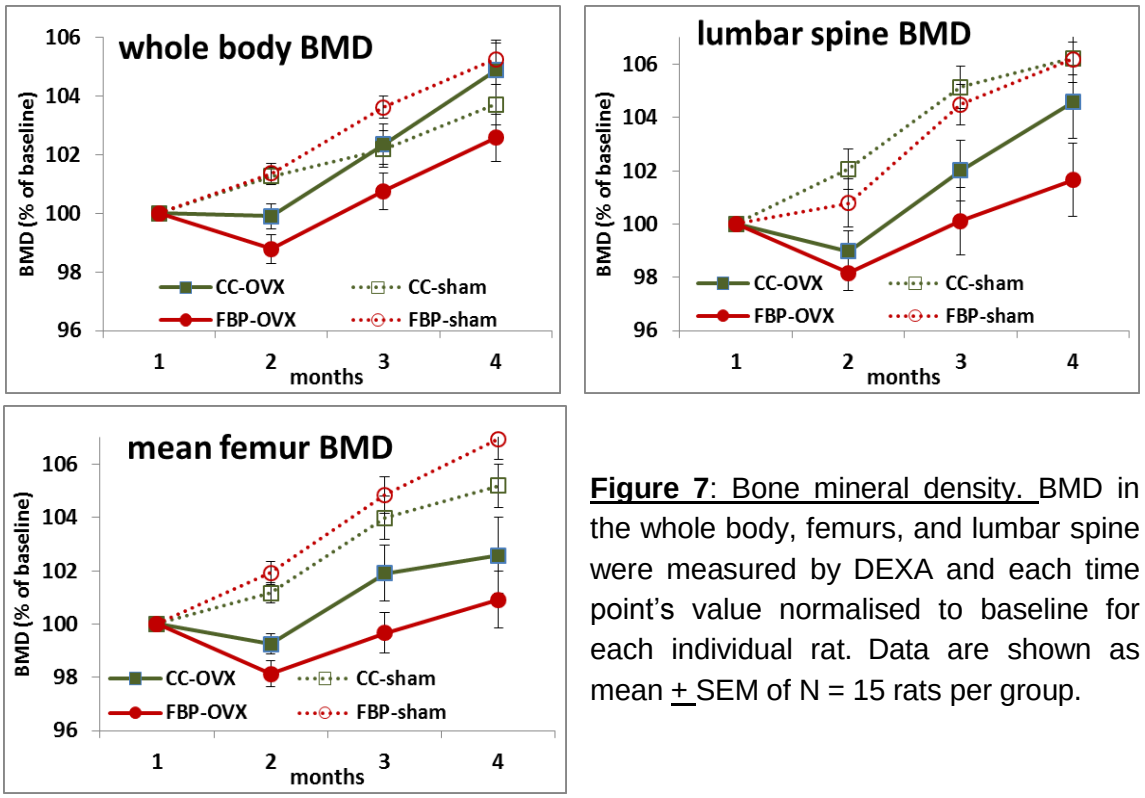


Figure 7: Bone mineral density. BMD in the whole body, femurs, and lumbar spine were measured by DEXA and each time point's value normalised to baseline for each individual rat. Data are shown as mean ± SEM of N = 15 rats per group.

Right femurs and spines were excised from the carcasses after euthanasia at 14 weeks and re-scanned as individual bones (ex vivo; Table 9). As expected, the matched in vivo and ex vivo scanning data for right femur and lumbar spine correlated very strongly (R^2>0.90).

Table 9. Individual spine and femur bone mineralisation.

	CCC-sham	CCC-OVX	FBP-sham	FBP-OVX
ex vivo DEXA				
Right femur BMC (g)	0.59 ± 0.10	0.58 ± 0.07	0.60 ± 0.08	0.58 ± 0.07
Right femur bone area (cm^2)	2.04 ± 0.41	2.21 ± 0.15	2.18 ± 0.16	2.24 ± 0.13
Right femur BMD (g/cm^2)	**0.28 ± 0.07**	**0.26 ± 0.02**	**0.27 ± 0.02**	**0.26 ± 0.02**
Lumbar spine BMC (g)	0.53 ± 0.10	0.57 ± 0.09	0.58 ± 0.06	0.54 ± 0.06 *
Lumbar spine bone area (cm^2)	2.18 ± 0.17	2.22 ± 0.17	2.21 ± 0.13	2.19 ± 0.11 *
Lumbar spine BMD (g/cm^2)	**0.25 ± 0.02**	**0.25 ± 0.02**	**0.26 ± 0.01**	**0.24 ± 0.02 ***

** $p \leq 0.05$ by ANOVA and Tukey's pairwise difference using body weight as a covariate, compared to sham treatment in the same diet group.*

As described above, the ex vivo scan data were also analysed with body weight as a covariate factor to determine whether changes occurred in bone that were not due to weight gain. Sham rats on the FBP diet had significantly ($p<0.05$) greater lumbar spine BA, BMC, and BMD compared to the FBP-OVX rats. This was not observed in the sham versus OVX rats fed the CCC diet. Similarly, FBP-sham rats had significantly higher BMC and BMD in the right femur compared to FBP-OVX rats, while there was no significant difference in the CCC-fed groups. Diet did not have a significant effect between the two OVX groups or between the two sham groups.

Bone biomechanics

Finally, the right femurs were subjected to a biomechanical strength test by breaking the bones under controlled conditions after measuring the bone's size with digital callipers. Break stress is the most relevant biomechanical parameter, with a higher break stress correlating with stronger, more resilient bone. These data were assessed statistically using body weight as a covariate, to exclude the effect of body weight on bone size and density. There were no significant differences in biomechanical properties between groups, including break stress, when the effect of body weight was removed. Elasticity was reduced in OVX bones, as expected, although this did not reach statistical significance due to the high standard deviation between individual animals. There were significant differences in sham versus OVX rats only for femur length, but the differences were too small to be physiologically relevant.

Table 10. Parameters and biomechanics of right femur. Data are shown as mean ± SD.

	CCC-sham	**CCC-OVX**	**FBP-sham**	**FBP-OVX**
Femur weight (g)	1.20± 0.13	1.26 ± 0.14	1.26 ± 0.14	1.26 ± 0.10
Femur length (mm)	37.85 ± 1.17	36.86 ± 1.14*	38.30 ± 1.20	39.04 ± 0.82*
Femur thickness (mm)	3.39 ± 0.23	3.44 ± 0.22	3.38 ± 0.27	3.37 ± 0.122
Femur width (mm)	4.50 ± 0.19	4.68 ± 0.24	4.52 ± 0.36	4.67 ± 0.31
Break force (N)	183 ± 30	203 ± 25	185 ± 32	186 ± 23
Break stroke (mm)	1.55 ± 0.26	1.63 ± 0.16	1.47 ± 0.27	1.59 ± 0.22
Break stress (N/mm^2)	**79.6 ± 7.9**	**83.5 ± 12.8**	**82.4 ± 17.4**	**80.7 ± 17.1**
Break strain (%)	14.06 ± 2.99	15.00 ± 2.17	13.22 ± 2.58	14.27 ± 2.46
Elasticity (N/mm^2)	**873 ± 159**	**853 ± 197**	**908 ± 194**	**875 ± 184**
Energy (J)	0.18 ± 0.05	0.20 ± 0.04	0.17 ± 0.06	0.19 ± 0.04

** $p \leq 0.05$ by ANOVA and Tukey's pairwise difference using body weight as a covariate, compared to sham treatment in the same diet group.*

DISCUSSION & CONCLUSIONS

The current study examined the effect in normal or ovariectomised rats of replacing calcium carbonate (CCC) in the rat diet with calcium sourced from fish bone powder (FBP). Bone mineralisation has been shown to vary depending on the level and source of dietary calcium.[21-24] Bone mineralisation is also partially dependent on other dietary factors. The source and content of protein,[25] fiber,[26] micronutrients,[27] and caffeinated beverages[28] all affect bone loss. The diets used in the current study were matched for energy, protein, and fibre; therefore, differences in calcium absorption and utilisation would be due solely to the calcium source.

As expected, ovariectomised (OVX) rats had significantly lower uterus weights, significantly higher CTX-1 blood levels, and lower oestrogen levels than rats that underwent sham surgery. These are all direct effects of the surgery, which induces the equivalent of human menopause. The bone turnover marker CTX-1 appears in the blood at levels corresponding with the rate of bone breakdown by osteoclasts. As expected, CTX-1 levels in the blood of OVX rats three months after surgery were significantly elevated to almost twice the levels in sham rats. The source of dietary calcium did not affect CTX-1 levels in either sham or OVX rats.

In addition, ovariectomised rats had a significant increase in food intake in the eight weeks following surgery, compared to sham rats. This resulted, as expected, in an increase in body weight, as has been observed elsewhere.[29] As in humans, female rats reduce food intake at ovulation; conversely, food intake peaks at diestrus.[30] This explains in part the increase in overall food intake in OVX rats, as they would not experience fluctuations in oestrogen or in food intake. Food intake after this period then normalised between the groups, as has been observed elsewhere.[10]

The OVX rats also had a significantly higher rate of weight gain than sham rats, which was not due solely to their increased energy intake. Indeed, OVX rats experienced more than double the amount of weight gain per 100 kJ consumed above the maintenance level as that seen in sham rats. The excess weight was largely in the form of adipose tissue, with only a small gain in lean mass: at the end of the study, the body mass index (body fat as % of body weight) of sham rats was approximately 18, while that of OVX rats was >40. These changes in energy metabolism were likely due in part to an oestrogen decrease in the OVX rats,[31] reflected in the lower uterus weights in the OVX rats. Oestrogen is linked to energy expenditure and thermogenesis, so the lower oestrogen levels in OVX rats would facilitate energy retention.

Interestingly, the effect of OVX on energy metabolism was less pronounced in FBP-fed rats compared to CCC-fed rats. FBP-fed rats gained a lower proportion of their body fat ($p = 0.10$) whereas their proportional lean mass increase was double that of CCC rats ($p = 0.025$). A similar finding was observed in an OVX mouse

model, in which weight gain, percent body fat, and insulin resistance were lower in mice receiving dairy calcium compared to calcium carbonate.[32] It cannot be determined from the current study whether this effect was due to unique peptides in the protein portion of FBP, its collagen content, or other components. It may be of interest to further explore the effect of FBP on long-term changes in lean mass and fat mass, to determine whether it only occurs with OVX-induced weight gain or whether it also occurs in rats with diet-induced obesity, and to explore its effect on insulin resistance and glucose tolerance.

Rats that underwent ovariectomy had slightly higher haematocrit and mean red cell volume, significantly larger platelets, and markedly higher white blood cell counts. In particular, the lymphocyte count in OVX rats was approximately 50% higher than that of sham rats. This elevation in lymphocytes and total white blood cells following ovariectomy has also been observed in other rodent models[33, 34] and is likely due to the increase in fat mass and subsequent leptin-driven lymphopoiesis. The only significant diet-specific effect observed on haematological parameters measured was an increase in platelet volume in FBP-fed sham rats compared to CCC-sham. Increased platelet volume is a marker of platelet activation and is commonly observed with obesity and non-obese fatty liver disease[35] as well as atherosclerosis.[36] It is unclear why FBP should induce this change in platelets in the absence of obesity; however, a near-significant (p=0.052) increase in platelet volume in FBP-fed rats was also observed in the male growing rat study.

Clinically, the gold standard for measuring bone quality is dual energy x-ray absorptiometry (DEXA).[3, 37] DEXA measures the area of bone in the body and the amount of mineral in the bone, from which the bone mineral density (BMD) of the individual bone sections can be determined. Femur and lumbar spine are the two bone areas known to be most strongly affected by OVX.

The BMD values in the current study were similar to those reported by other groups. Mean lumbar spine bone mineral densities at 8 weeks post-surgery in FBP-fed groups were 0.262 g/cm^2 in sham rats vs 0.251 g/cm^2 in OVX rats; these match well with 8-week lumbar spine BMD data (sham 0.26 vs OVX 0.23 g/cm^2) reported in a similar recent study.[38] Likewise, femur BMC at age 8 months in rats fed FBP (sham 0.545 g vs OVX 0.529 g) were similar to those reported elsewhere[39] (sham 0.608 g vs OVX 0.510 g). A loss in femur and lumbar spine BMD 3 – 4 weeks was observed, as expected, in OVX but not sham rats. An increase in BMD was observed over the following 2 months in the current study, as opposed to the more commonly reported steady-state retention with little change between 4 weeks post-OVX and 12 weeks post-OVX. However, similar gains occurred in both OVX and sham rats in the current study, demonstrating the value of the control groups.

In both sham and OVX cohorts, FBP and CCC produced similar results with regards to bone mineral content, bone mineral density, and bone breaking biomechanics. Calcium from FBP and CCC were equally effective in maintaining

bone health under normal or post-menopausal circumstances in a calcium-replete diet. This matches a recent finding in which hake fish bone as a source of dietary calcium was similar to Lithotame calcium supplement in providing bone mineralisation in young growing rats under conditions of calcium deficiency, although the hake fish bone appeared superior to the control in in vitro experiments.[40]

The results of this study suggest that FBP is an acceptable source of dietary calcium and has no significant detrimental effects on health. Moreover, FBC may reduce the amount of body fat and body weight gained post-menopause. In light of the observed effects of FBP on body weight and composition as well as platelet volume, further assessment of FBP as a source of protein, collagen, and micronutrients other than calcium may be warranted.

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