The effect of whey acidic protein fractions on bone loss in the ovariectomised rat

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Bovine milk has been shown to contain bioactive components with bone-protective properties. Earlier studies on bovine milk whey protein showed that it suppressed bone resorption in the female ovariectomised rat. A new osteotropic component was subsequently identified in the whey basic protein fraction, but bone bioactivity may also be associated with other whey fractions. In the present study, we investigated whether acidic protein fractions isolated from bovine milk whey could prevent bone loss in mature ovariectomised female rats. Six-month-old female rats were ovariectomised (OVX) or left intact (sham). The OVX rats were randomised into four groups. One group remained the control (OVX), whereas three groups were fed various whey acidic protein fractions from milk whey as 3g/kg diet for 4 months. Outcomes were bone mineral density, bone biomechanics and markers of bone turnover. Bone mineral density of the femurs indicated that one of the whey AF over time caused a recovery of bone lost from OVX. Plasma C-telopeptide of type I collagen decreased significantly in all groups except OVX control over time, indicating an anti-resorptive effect of whey acidic protein. Biomechanical data showed that the AF may affect bone architecture as elasticity was increased by one of the whey AF. The femurs of AF-supplemented rats all showed an increase in organic matter. This is the first report of an acidic whey protein fraction isolated from milk whey that may support the recovery of bone loss *in vivo*.

Ovariectomised rat: Whey acidic protein: Bone density: Bone breaking strength: Biochemical markers of bone turnover

Osteoporosis is one of the most critical disorders occurring in women with advancing age. During menopause, there is a change in bone turnover, with an imbalance between bone formation and bone resorption resulting in net bone loss. Bone loss as a result of oestrogen deficiency is primarily caused by an increase in the activity of the osteoclast (Omi & Ezawa, 1995).

Nutritional components with potential anti-resorptive activity generally include only Ca and to some extent cholecalciferol. The Ca found in milk has an anti-resorptive effect and can help to prevent bone loss (Heaney, 2000). Milk (and colostrum) also contains a number of other components that may be bone-active. These include whey protein components, lipids and a large number of caseins, all of which are phosphoproteins (Delisle *et al.* 1995). In particular, milk contains a highly phosphorylated protein, osteopontin, which has been implicated in bone mineralisation (Bayless *et al.* 1997). Milk also contains bioactive peptides with an anti-resorptive activity (Neeser *et al.* 2000). Similarly, whey may contain certain growth factors affecting bone (Takada *et al.* 1993).

Takada *et al.* (1997*a,b*) showed that milk whey protein suppressed bone resorption in the female ovariectomised rat. Toba *et al.* (1999, 2000) subsequently described a new osteotropic component in the whey basic protein fraction of milk (later called milk basic protein), and animal studies showed that it suppressed bone resorption. Results also showed that the milk basic protein had no effect on Ca absorption, as calculated using Ca balance data, but that it enhanced bone strength in the ovariectomised rat model (Takada *et al.* 1997*a,b*).

A recent study in young growing male rats showed that feeding whey protein increased alkaline phosphatase activity and insulin-like growth factor-I (IGF-I) mRNA levels in ectopic bone (Kelly *et al.* 2003). As alkaline phosphatase is a marker for bone formation, whereas IGF-I is a known potent anabolic agent (Price *et al.* 1994), this further indicated a possible effect of whey protein on bone formation.

In contrast to the basic whey protein fractions, little is known of the possible benefits of the acidic protein peptide fractions from milk. The acidic (low isoelectric point) protein component of whey is known to contain phosphorylated proteins and peptides (Sorensen & Petersen, 1993; Reid *et al.* 2004), some of which might play a role in Ca absorption. Additionally, the whey acidic protein (WAP) complement contains osteopontin (and likely fragments thereof; K Palmano, unpublished data), which is essential to bone mineralisation (Bayless *et al.* 1997; Denhardt & Noda 1998). We have recently shown that an acidic protein fraction (AF) isolated from mineral acid whey protein concentrate (WPC) had anti-resorptive effects *in vitro* (Reid *et al.* 2004). It was therefore of immediate interest to establish whether such fractions had bone bioactivity *in vivo*.

In the present study, we investigated whether WAP fractions (AF) isolated from milk could prevent bone loss and bone resorption in mature ovariectomised female rats. We compared three different fractions prepared from mineral acid WPC, lactose-reduced mineral acid WPC and lactic acid WPC, respectively. Our primary outcome was *in vivo* bone mineral density (BMD). Secondary outcomes included bone biomechanics and markers of bone resorption and turnover.

Materials and methods

Animals

Seventy-five 5·5-month-old female Sprague–Dawley rats were obtained from the Small Animal Production Unit, Massey University, Palmerston North, New Zealand. The animals were sham-operated or ovariectomised (OVX) at age 6 months (week 0 of the study). Sham-operated animals (*n* 15) were anaesthetised and an incision was made, but the ovaries were left intact, whereas in the OVX animals the ovaries were removed. These OVX animals were randomised into four groups (fifteen per group).

The animals were housed separately in shoebox cages, and kept in a temperature (22 °C ± 2 °C) and light-controlled (12 h day–night cycle) room in the Small Animal Production Unit at Massey University. Animals had access *ad libitum* to deionised water. Animals were fed a casein-based semi-synthetic diet to which the milk fractions had been added as 3 kg/kg (Table 1). The sham control group and the OVX control group received the base diet with no fractions added. The daily intake of the animals was measured, and their intake was adjusted weekly according to the sham group's body weight in order to prevent excessive body weight gain in the OVX groups. The mean food intake over the trial was 20 g/d per animal. The trial ran for 4 months, with monthly measurements. Massey University Animal Ethics Committee approved the study (01/70)

Diets

The animals were fed a balanced semi-synthetic diet consisting of 15 % caseinate, 5 % cellulose, 5 % corn oil, 0·5 % Ca, 62 % starch and added vitamins and minerals as needed from week −2 until week 0, the week that they were ovariectomised. From week 0,

animals were fed either the control diet (sham and OVX control) or an experimental diet in which the casein content had been adjusted to allow the incorporation of 3 g/kg acidic whey protein fraction to the diets. The diet formulation was a modification on AIN-76A. The mineral and vitamin mixes were formulated according to AIN-93M to ensure optimal levels of minerals and trace minerals (National Research Council, 1995) (Table 1).

The dose of the fraction was selected referring to published studies on the milk basic fraction, which ranged between 0·1 % and 1 % of the diet (Takada *et al.* 1993; Toba *et al.* 1999, 2000). The following fractions were screened:

mineral acid WPC (AF1);
mineral acid WPC (lactose-reduced) (AF2);
lactic acid WPC (AF3).

Purification of fractions

Materials. The following whey WPC were obtained from Fonterra Cooperative Group Ltd, Auckland, New Zealand: ALACEN 342 (mineral acid WPC), ALACEN 163 (lactose-reduced mineral acid WPC) and ALACEN 312 (lactic acid WPC), Q Sepharose Big Beads were purchased from Amersham Biosciences (Uppsala, Sweden). Dialysis tubing (Spectra/Por, nominal molecular weight cut-off 3 kDa) was from Spectrum Companies (Ranco Dominguez, CA, USA). Ultrafiltration was carried out using a Prep/Scale spiral-wound membrane cartridge with a nominal molecular weight cut-off of 3 kDa obtained from Millipore (Bedford, MA, USA).

Methods. WAP fractions were prepared from each of the WPC using the same method. Briefly, WPC (2000 g) was reconstituted in 20 litres deionised water and adjusted to pH 4·5 with 10 % HCl. The acidified WPC solution was then loaded on to a 4 litre column packed with Q Sepharose Big Beads. The column was sufficiently washed with deionised water, and the bound protein was eluted with 1 M NaCl. The eluted fraction (6 litres) was then concentrated to 500 ml by ultrafiltration, and the WAP fraction was obtained by freeze-drying, after exhaustive dialysis of the ultrafiltration fraction. The yield of WAP was approximately 100 g.

Tail-vein sampling

At weeks −1, 4, 8, 12 and 16, rats were placed in a purpose-built restrainer, which was then placed on top of a heat pad and under a heat lamp. A tourniquet was placed around the base of the tail. A single blood sample of approximately 1 ml was withdrawn from the lateral tail vein, using a 23 G×3/4 inch hypodermic needle and 1 ml syringe. Blood samples were transferred immediately into vacutainers containing heparin and then centrifuged at 1000 *g* for 10 min. The plasma was removed, snap-frozen with liquid N_2 and stored at −85 °C.

Blood was collected after an overnight fast and between 08.00 and 10.00 hours in the absence of anaesthesia. Plasma collected at weeks −1, 8 and 16 was used to measure bone biochemical markers, and blood collected at weeks 4 and 12 was used to measure plasma oestrogen levels.

Dual-energy X-ray spectrometry scans

Animals were scanned for baseline measurements at week −1, before being ovariectomised at week 0. Animals were then

Table 1. Dietary composition (g/kg)

	Control	Experimental
Sodium caseinate	150	147
Acidic whey protein fraction		3
Cystine	2·7	2·7
Glycine	3·3	3·3
Methionine	1·5	1·5
Glutamine	7	7
Cellulose	50	50
Vitamin mix*	50	50
Mineral mix*	50	50
Corn oil	50	50
$CaCO_3$	12·5	12·5
Starch	623	623
Total	1000	1000

* Formulated by Crop & Food Research, New Zealand, according to the National Research Council (1995) nutrient requirements for laboratory animals (AIN 93M).

scanned every 4 weeks under anaesthesia up to week 16 after OVX. Animals were weighed and anaesthetised with an appropriate dose of anaesthetic (0·05 ml/100 g body weight). The anaesthetic was a mixture of 2 ml/10 ml acepromazine +5 ml/10 ml ketamine +1 ml/10 ml xylazine +2 ml/10 ml sterile H_2O, and was administered via an intraperitoneal injection using a 25G × 5/8 inch needle and 1 ml syringe. The animals attained a suitable level of anaesthesia approximately 5–10 min after injection and remained under anaesthesia for 2 h.

BMD was measured with a Hologic QDR4000 bone densitometer using a pencil beam unit (Hologic, Bedford, USA). A daily quality control scan was taken to ensure that precision met the required coefficient of variance. The coefficient of variance for the quality control data was 0·98–1·01. Regional high-resolution scans were performed using a 0·06 inch diameter collimator with 0·0127 inch point resolution and 0·0254 inch line-spacing. Rats were placed on an acrylic platform of uniform 1·5 inch thickness. Each rat underwent three regional high-resolution scans of the spine (L1–L4) and left and right femurs. Rats were positioned supine with right angles between the spine and femur, and between the femur and tibia.

Coefficient of variance for the femurs ranged between 0·92 % and 0·85 % with and without repositioning between scans. These values ranged between 1 % and 0·98 % for the spine.

Sampling

After 16 weeks, animals were weighed and anaesthetised with an appropriate dose of anaesthetic (0·1 ml/100 g body weight). A 19 G × 1·5 inch needle and 5 ml syringe were used to withdraw blood directly from the heart. Animals were then killed by exposure to 100 % CO_2 and dissected.

Blood was placed immediately into heparin-filled tubes and centrifuged at 1000 *g* for 10 min; the plasma was then removed, divided into aliquots and snap-frozen. Both the right and left femur and spine were excised with some flesh remaining and frozen in PBS for further analysis. The uteri were removed and weighed to confirm that ovariectomy had been successful.

Bone biomechanics

The right femurs were scraped clean of adhering flesh and stored in PBS at −20°C. Before biomechanical testing, the bones were thawed. The length of the femurs was measured between the proximal end of the intercondylar notch and the proximal limit of the base of the femoral neck using an electronic calliper. The midpoint was marked with a waterproof pen, and the width and thickness at this midpoint were also recorded. The femurs were then incubated at 23°C to be at room temperature before and during the test. The femurs were placed in a testing jig constructed for a three-point bending test. The distance between the

supporting rods had a fixed length of 12 mm. Load was applied at a constant deformation rate of 50 mm/min. Maximum load (N), elasticity (N/mm^2) and energy (J) were measured using a Shimadzu Ezi-test (Kyoto, Japan).

Bone biochemical markers and plasma oestrogen

The plasma levels of telopeptides of type I collagen and osteocalcin were measured using the RatLaps and osteocalcin ELISA kits provided by Osteometer BioTech A/S (Herlev, Denmark). Inter-assay and intra-assay coefficient of variance for both kits were 10 % and 6–7 %, respectively. Plasma oestrogen was measured with a double-antibody radioimmunoassay for oestradiol -2 (Diasorin, Stillwater, MN, USA).

Bone ashing

The left femurs were thawed, scraped clean of remaining flesh and dried overnight at 105°C. After being weighed, the femurs were ashed overnight at 660°C and weighed again.

Statistical analyses

A total of seventy-five rats (fifteen per group) were used in the trial. Within-group variation is such that a minimum of fourteen animals is needed per group: on calculating the power and sample size (one-way ANOVA) on bone density measurements, a sample size of fourteen is necessary for a power of 0·8. The expected effect was set at 0·02 g/cm^2 for BMD measurements. Results were analysed using Minitab version 13. A *P* value <0·05 was considered to be significant. Groups of animals were compared using repeated measures ANOVA, followed by *post hoc* testing (Tukey). Values and graphs are expressed and shown as means with their standard error.

Results

The mean food intake for all the animals was between 18 and 20 g/d. Table 2 shows the final mean body weights and uterus weights for all groups at week 16 of supplementation (age 10 months). There were no significant differences in the final body weights. The uteri from all the OVX groups were significantly smaller than those of the sham group. Sequential measurement of plasma oestrogen also confirmed that the ovariectomy had been successful (data not shown).

Fig. 1 shows the respective changes in femur and lumbar spine BMD over time. BMD was measured monthly from week −1 to week 16, and the results reflect the changes over time caused by ovariectomy and the effects of feeding the various milk fractions. There was a rapid loss in BMD over the first 4 weeks

Table 2. Final body weights and uterus weights of all groups of rats at week 16
(Mean values and standard errors of the mean)

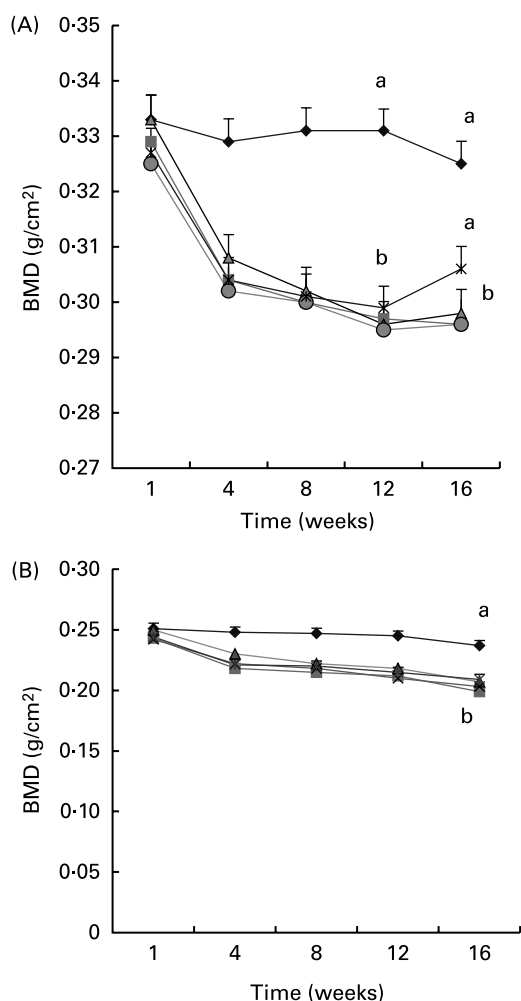|	Sham		Ovariectomised control		AF1		AF2		AF3	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Body weight (g)	375·5	26·7	403·0	13·8	417·8	13·3	423·9	14·5	407·1	12·3
Uterus weight (g)	0·98	0·11	0·28	0·08*	0·21	0·03*	0·20	0·02*	0·25	0·06*

* *P*<0·05 *v.* sham.

Fig. 1. Sequential bone loss from the femur (A) and lumbar spine (B) shown as bone mineral density (BMD) from week −1 to week 16 for sham operated and ovariectomised rats while being fed acidic whey protein fractions AF1−3 for 16 weeks. (◆, Sham, not ovariectomsed; ■, ovariectomised control; x, ovariectomised + AF3; ◆ ovariectomised + AF1; ■ ovariectomised + AF2) The whey fraction was added as 3g/kg diet. Values are given as mean and SEM. Note that all OVX groups were different from the sham group at level $P<0.05$ for all weeks measured except for the femurs of the AF3 group at week 16. Different superscripts (a, b) denote significant differences at $P<0.05$.

Fig. 2. *In vivo* (A) spine bone mineral content (BMC) and (B) bone mineral density (BMD) of various groups of rats after being fed acidic whey protein fractions (at 3g/kg diet) for 16 weeks after ovariectomy. The various groups are Sham (ovaries intact), OVX (ovaries removed) and AF1−3 (ovariectomised rats fed acidic whey fractions from milk). Values are means with standard errors shown by veritical bars. *Mean value was significantly different to that of the Sham ($P<0.05$).

following ovariectomy for all groups except sham, and this was followed by a more gradual loss over the next 4 weeks. However, the femur BMD for the AF1 and AF3 groups showed an upward trend between weeks 12 and 16, this trend being more prominent for the AF3 group. The lumbar spine did not seem to respond to the same extent as the femur in that all groups except sham lost BMD with no obvious improvement over time.

Fig. 2 shows the *in vivo* spine bone mineral content (BMC) and bone mineral density (BMD) at 16 weeks. From these data, it appears that all OVX groups lost bone mineral in comparison to the sham control. However, the groups fed AF1 and AF3 had BMC values that were not statistically different from sham animals.

The values for the left and right femurs were averaged for each animal for statistical analysis. Fig. 3 shows the *in vivo* femur BMC and BMD, respectively. The femur BMC of the AF1 and AF3 groups was not statistically different from that of the sham control, whereas the other OVX groups lost a significant quantity of
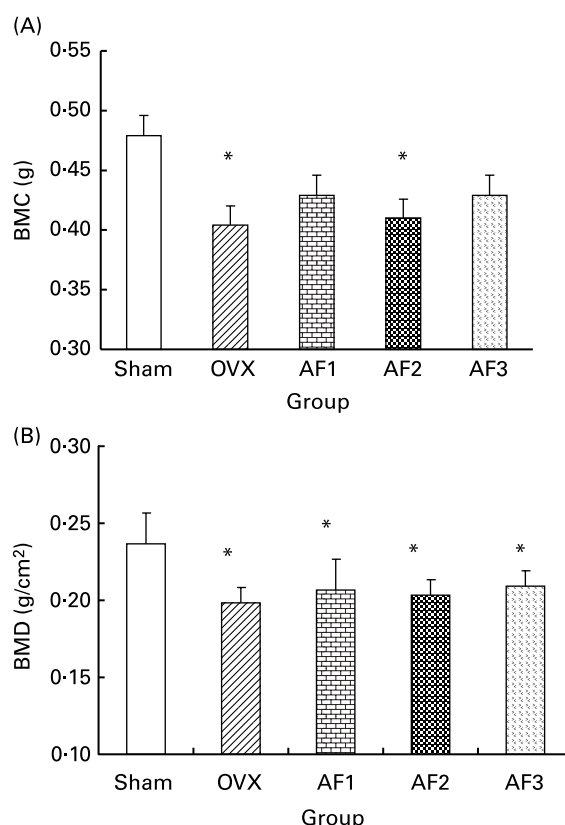
bone compared with the sham control animals ($P<0.05$). The *in vivo* femur BMD for all the OVX groups was significantly lower than that of the sham controls, with the exception of the group fed AF3, which had a value not significantly different from that of the sham group and significantly higher than that of OVX controls.

Tables 3 and 4 show the results for the markers of bone resorption (RatLaps; C telopeptide of type I collagen) and bone turnover (osteocalcin), respectively. RatLaps did not change significantly from week −1 to week 8 in all groups. Bone resorption decreased significantly in all the experimental groups as well as in the sham group from week 8 to 16, but not in the OVX control group.

Osteocalcin, a marker of bone turnover, increased significantly between weeks −1 and 8 in all the OVX groups, whereas it was slightly reduced in the sham group. The increase observed in the OVX groups would be caused by the loss of oestrogen, leading to an increase in bone turnover. At week 16, osteocalcin remained at the same level in the sham group but decreased significantly in all the AF OVX groups. This decrease was more pronounced in the animals fed AF2 and AF3. The percentage change for the various OVX groups between weeks 8 and 16 was 16 % (OVX), 10 % (AF1), 36 % (AF2) and 24 % (AF3). The osteocalcin level for AF3 was significantly higher than that of the sham and OVX controls at week 16.

Table 5 shows the biomechanical information from the right femurs harvested from the animals at week 16 of supplementation. Femurs harvested from the AF1 group have
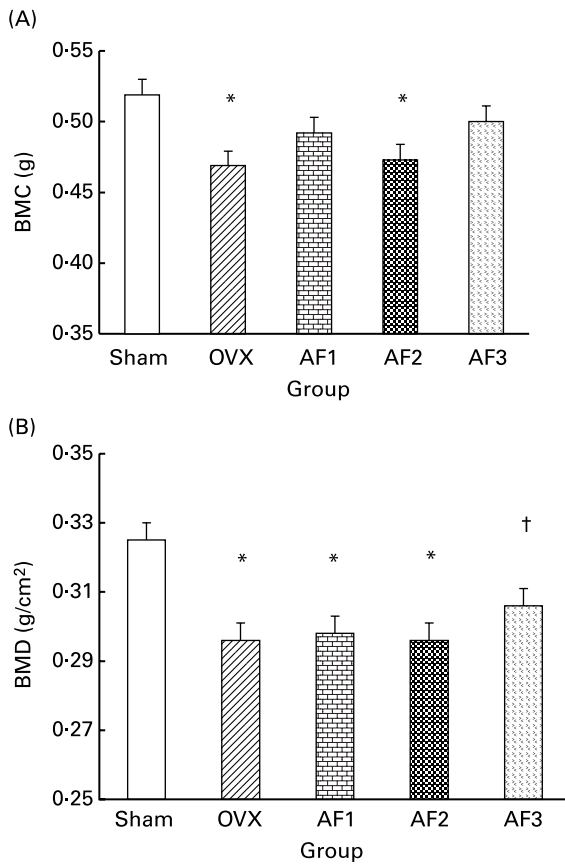
Fig. 3. *In vivo* (A) femur bone mineral content (BMC) and (B) bone mineral density (BMD) of sham operated and ovariectomised rats after being fed acidic whey protein fractions (3g/kg diet) for 16 weeks. The various groups are Sham (ovaries intact), OVX (ovaries removed) and AF1–3 (ovariectomised rats fed acidic whey fractions from milk). Values are means with standard errors shown by vertical bars. Mean values were significantly different to that of the Sham (*$P < 0.05$) and ovx ($^{\dagger}P < 0.05$).

indices that demonstrate that the bones were more stiff than the sham control (break stress and elasticity). Femurs harvested from the AF1 animals were also significantly thicker than the femurs derived from the other groups.

Table 6 summarises further measurements of bone physical properties performed on the left femurs at 16 weeks of dietary intervention. The left femurs harvested from the AF1 group were heavier than those from all other groups. The weights were significantly higher than those of the sham and OVX controls. There was no significant difference in femur ash weight between the five groups. A calculation of organic matter (femur weight – ash weight) showed that the femurs from the supplemented groups had a significantly higher portion of organic matter. This is also reflected in the calculated ratio of ash to organic matter, which was also significantly lower in all the supplemented groups.

Discussion

In this study, we evaluated three whey AF isolated from bovine milk whey for bone-protective activity. Our model was the aged female OVX rat, and our primary outcome was *in vivo* BMD. Our results showed that two of the whey AF led to a recovery of some of the bone lost due to ovariectomy. Results also indicated that all three whey AF preserved bone organic matter to a large extent.

There were no significant differences in body weight between the animals at week 16. However, owing to the fact that BMD is influenced by body weight, all statistical analyses on *in vivo* dual-energy X-ray spectrometry were individually corrected for body weight.

In vivo dual-energy X-ray spectrometry

Femur data show that, between weeks 12 and 16, the BMD started to rise towards that of the sham group for all three supplemented groups, the AF3 group not being significantly different from the sham group at week 16. Although this effect was relatively

Table 3. RatLaps (C telopeptide of type I collagen) values for weeks 0, 8 and 16 of rats supplemented with various acidic whey protein fractions from milk
Mean values and standard errors of the mean

RatLaps Mean (ng/ml)	Sham		Ovariectomised		AF1		AF2		AF3	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Week −1	8·72	1·38	10·14	1·74	11·16	1·38	11·46	1·17	8·63	1·38
Week 8	8·35	1·18	9·23	1·49	12·31	1·18	13·02	1·10	12·10	1·18
Week 16	4·42	0·90†	8·79	1·14	7·20	0·90†	7·75	0·77†	6·94	0·90†

† $P < 0.05$ *v.* week 8 within groups.

Table 4. Osteocalcin (marker for bone turnover) values for weeks 0, 8 and 16 of rats supplemented with various acidic whey protein fractions (AF) from milk.
Mean values and standard errors of the mean

Osteocalcin Means (ng/ml)	Sham		Ovariectomised		AF1		AF2		AF3	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Week −1	143·1	19·2	136·6	36·9	192·4	16·9	183·4	19·5	167·8	27·1
Week 8	126·4	8·9	205·6	26·6‡	222·9	26·3‡	281·2	22·0‡	281·8	30·9‡
Week 16	124·3	5·4	172·3	14·9*	201·3	13·5*	180·1	8·3*†	216·9	11·7*†§

Values with different superscripts are significantly different ($P < 0.05$) between groups at the time points as well as within a group over the 16 weeks.
* $P < 0.05$ *v.* sham at week 16.
† $P < 0.05$ *v.* week 8 within groups.
‡ $P < 0.05$ *v.* week −1 within groups, as well as versus sham for all groups at week 8.
§ $P < 0.05$ *v.* ovariectomised at week 16.

Table 5. Biomechanical data for the right femurs harvested from rats after 16 weeks of feeding various acidic whey protein fractions (AF)
(Mean values and standard errors of the mean)

| | Max | | | | Break | | | | | | | | | | | | | Width (mm) | | Thickness (mm) | | Femur length (mm) | |
| | Load (N) | | Stroke (mm) | | Load (N) | | Stress (N/mm²) | | Stroke (mm) | | Strain (%) | | Elastic (N/mm²) | | Energy (J) | | | | | | | | |
Diet	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Sham	208·7	10·41	1·65	0·061	204·8	11·21	99·41	4·23	1·67	0·06	21·63	0·99	561·5	41·62	0·17	0·01	3·88	0·05	3·09	0·05	37·50	0·17
Ovariectomised	201·4	8·33	1·49	0·04	200·8	8·32	92·02	5·28	1·50	0·04	19·94	0·53	550·6	27·12	0·16	0·001	3·90	0·04	3·19	0·04	38·33	0·19
AF1	200·1	14·12	1·46	0·08	199·3	13·90	72·29	4·57*§	1·47	0·08	21·63	1·69	425·0	39·32*	0·16	0·01	4·08	0·06	3·50	0·12*§	39·06	0·39*
AF2	198·3	5·92	1·54	0·03	194·2	6·04	87·84	4·05	1·56	0·03	20·88	0·68	543·3	31·32	0·17	0·01	3·95	0·04	3·19	0·06	38·70	0·24
AF3	208·1	5·1	1·59	0·04	207·6	5·18	90·38	4·64	1·59	0·04	21·27	0·64	525·2	32·6	0·18	0·01	4·09	0·05	3·21	0·05	38·49	0·39

* $P < 0.05$ v. sham.
§ $P < 0.05$ v. ovariectomised.

small, the trend was present for all three groups, and it is likely that feeding for a period longer than 16 weeks might have elicited a more pronounced effect. No effect was observed in the spine. Several nutritional trials investigating the effect of nutrients on bone density of the femur and spine have indicated that the effect on bone mineralisation is dependent on the part of the skeleton measured (Scholz-Ahrens *et al.* 2001).

Additionally, at week 16, the lumbar spine BMC of rats fed AF3 was also not significantly different from sham. The tendency towards an improvement in bone mineralisation following the feeding of AF3 diets was also mirrored in the femoral BMC. In this respect (BMC), it appeared that the AF1 diet was also instrumental in decreasing bone mineral loss resulting from ovariectomy. The bones of the OVX rat continue to grow until the age of 6 months, with significant changes in Ca and density. From the age of 6 months, changes in femoral density and Ca are minimal but still present (Kalu, 1991). Increased growth and larger bone size may result in the measurement of an increased BMC as BMC is expressed as Ca per gram bone (Moskilde, 1995). Since the bone lengths and widths of the femurs from the AF1 group were significantly higher than those of the sham and OVX control groups, it is possible that the significantly higher BMC for AF1 was due to a larger bone size.

Ca absorption was not measured in the present study. However, the whey AF is composed of highly phosphorylated proteins and peptides, some of which are derived from caseins (Sorensen & Petersen, 1993; Reid *et al.* 2004). Prevalent among these is proteose peptone 5, a polypeptide released from β-casein owing to milk plasmin activity. Phosphorylated peptides from casein have been shown to improve Ca absorption by binding to the Ca and maintaining the cation in a soluble form (Camara-Martos & Amaro-Lopez, 2002). Prior work has also shown that rats fed with casein phosphopeptides had significantly higher femoral and humeral weights than a control group (Tsuchita *et al.* 1995). It is therefore important to establish whether the presence of phosphorylated casein (and other) peptides in the whey fractions used in this study increased Ca bioavailability, thereby contributing to an observed improvement in bone density.

In respect of composition of the whey AF, it is likely that the fraction from lactose-reduced mineral acid WPC (AF2) contained fewer (phosphorylated) peptides than that derived from mineral acid WPC (AF1). The lactose reduction of mineral acid whey requires repeated ultrafiltration, which would lead to some losses of peptides and small-protein components. On the other hand, in lactic acid whey, the proteins are subjected to proteolysis by lactic acid bacterial enzymes, thus producing more peptide fragments. The tendency for a somewhat greater improvement in bone density indices in the AF3-fed animals may reflect the more proteolysed nature of this fraction. Although caseinoglycomacropeptide, an acidic phosphorylated peptide, has been implicated in the bone health-promoting effects of milk (Neeser *et al.* 2000), it is most unlikely that that there would have been significant quantities in the fractions studied. Caseinoglycomacropeptide is only prevalent in cheese whey, where it is produced by the specific action of chymosin during the cheese-making process.

Bone markers

RatLaps is a marker of bone resorption. It measures the C-telopeptide of type I collagen found in urine or circulating in the blood in greater concentration during bone resorption

Table 6. Bone parameters for the left femurs for all groups after 16 weeks of feeding various acidic whey protein fractions (AF)

(Mean values and standard erros of the mean)

Parameter	Sham		Ovariectomised		AF1		AF2		AF3	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Left femur weight (g)	0·66	0·02	0·66	0·01	0·70	0·01†	0·67	0·01	0·68	0·02
Ashed weight (g)	0·36	0·01	0·34	0·01	0·35	0·01	0·34	0·01	0·34	0·01
Organic matter (g)‡	0·30	0·01	0·31	0·01	0·34	0·01†	0·34	0·01*	0·34	0·01†
Ash/organic	1·18	0·03	1·11	0·03	1·01	0·03*	1·03	0·03*	1·03	0·03†

* $P < 0.05$ *v.* sham.
† $P < 0.05$ *v.* ovariectomised.
‡ Organic matter = left femur weight − ashed weight.

(Swaminathan, 2001; Kruger *et al.* 2003). The levels of RatLaps between the various groups were not statistically different at baseline. Between weeks −1 and 16 of supplementation, RatLaps decreased in all groups, possibly owing to the feeding of a high-Ca diet, which may to a limited extent be protective against bone resorption. However, the decreases in resorption in the AF-supplemented groups were greater than that of the OVX control group and also statistically significant, thus indicating an anti-resorptive effect of the whey AF. An anti-resorptive effect of whey protein as well as an effect on bone formation has been shown before in rat studies using the milk basic protein (Toba *et al.* 1999, 2000).

Changes in the bone turnover marker osteocalcin are more difficult to interpret as there are no clear-cut trends other than increased bone turnover in the OVX groups as a response to oestrogen deficiency. However, the more dramatic changes in the AF2 and AF3 groups with respect to both the increase and subsequent decrease in serum osteocalcin suggest that these fractions affected bone remodelling in some way.

Whole bovine milk has also been shown to have cell growth-promoting activity in human lymphocytic cells (Klagsbrun & Neumann, 1979). Several mitogenic factors have been identified in bovine colostrum and milk, such as insulin, IGF-I, IGF-II and transforming growth factor-β (Ballard *et al.* 1982; Cox & Burk, 1991). In the present study, the WAP exhibited some bone-protective effects and may therefore contain components that promote osteoblast growth. It is unlikely, however, that these effects can be attributed to growth factors such as IGF-I, IGF-II and transforming growth factor-β as they were not detectable by ELISA in the whey acidic fractions (results not shown). Growth factors are also generally not absorbed by the adult human intestine (Thomson & Keelan, 1986; Pacha, 2000). Other potentially bone active proteins include osteopontin and bone sialoprotein (Ito, 1991; Takada *et al.* 1996).

Osteopontin is a highly phosphorylated and glycosylated protein found in milk and in the extracellular matrix of mineralised tissue (Bayless *et al.* 1997; Gravallese, 2003). It facilitates the attachment of osteoclasts to the bone matrix via an interaction with cell surface integrins, and it is essential for bone remodelling. Being a highly acidic protein associated with the whey fraction of milk, it is captured in the whey AF from anion exchange (Sorensen & Petersen, 1993; Reid *et al.* 2004). Osteopontin in milk would be subject to post-ingestion digestive processes that could release peptides containing the RGD (Arg–Gly–Asp) integrin recognition sequence. RGD peptides and their analogues have been shown to inhibit osteo-

clast activity via a competing interaction with integrins (Horton *et al.* 1991). If RGD peptides released from osteopontin during digestion (or indeed present in the whey either naturally or as a result of processing) are absorbed through the gut, they might well have an effect on osteoclast activity.

Biomechanics

Bone breaking strength is the maximum power that is required to break bone by the three-point bending method; breaking energy is an integration value of power that is required to make the break. Breaking strength reflects the mineral content of bone as well as the protein component, whereas breaking energy is thought to reflect bone collagen content (Moskilde, 1995; Thompson *et al.* 1995).

None of the measured parameters in the present study were significantly different, except for the break stress and elasticity (stiffness) of the AF1-fed group. The lack of significant differences except for those two parameters is an indication that, overall, the acidic fractions were not detrimental to bone. Differences in breaking strength are linearly related to femur Ca content (Moskilde, 1995; Thompson *et al.* 1995). However, correcting the biomechanical data for bone Ca content in the present study did not change the final outcomes or conclusions. Previous work indicated that feeding whey basic protein increased the amount of bone protein such as collagen in rats, thereby enhancing bone breaking force (Takada *et al.* 1997a,b). Some effect on breaking force due to feeding AF3 was observed in this study; the result was, however, not statistically significant.

Further investigation into bone weight, and the ratio between ash content of the bones and the organic matter, indicated that AF1 increased bone weight towards sham-treated levels. A calculation of the organic matter in the bones showed that the AF groups all had a significantly higher quantity of organic matter compared with the sham group, whereas that of the AF1 group was significantly higher than the value for the OVX group as well.

Recalcification of bone takes place with bone collagen as a scaffold (Termine & Robey, 1996). It is possible that in the groups fed WAP, bone loss was slowed by first building the organic phase of the bone, with calcification following later. The AF diets had an anti-resorptive effect resulting in lower levels of type I collagen fragments in the circulation at the termination of the study. Bone resorption occurs in two phases, being initiated by matrix metalloproteinases, which release the cross-linked carboxy-terminal telopeptide of type I collagen into the circulation. This initial phase is then followed by further destruction by cathepsin K, a cysteine proteinase that releases the car-

boxy-terminal cross-linked peptide of type I collagen into the circulation. The latter is measured by the RatLaps assay. From the results of the present study, it is therefore possible that the whey protein acidic fraction might also have exerted an effect through the cysteine proteinases (Parikka et al. 2001). Although milk does contain cystatin C, a naturally ocurring cathepsin K inhibitor (Lerner et al.1997; Toba et al. 2000), it is unlikely to be associated with the whey AF from milk (Toba et al. 2000).

Conclusion

The results of this study suggest that the AF from both mineral acid whey and lactic acid whey may lead to a remediation of the bone loss associated with ovariectomy. Femoral bone density was significantly increased in the animals fed the lactic whey acidic fraction after 4 months of feeding. Biomechanical data showed that the AF may affect bone collagen. Further measurements and calculation confirmed that the organic phase of the bones was affected. The whey AF may contain components that may act on bone, such as osteopontin or cysteine proteinase inhibitors.

The present study was undertaken in a high-Ca (0.5%) environment compared with previous studies, in which a Ca level of 0.1–0.3% was used (Toba et al. 1999, 2000). It is therefore possible that the high Ca level has masked some of the effects of the whey proteins. Further work is being undertaken to identify any bone-active factors that may be present in the whey protein acidic fraction.

From a human dietary perspective, the mean food intake of the rats, at 50 g/kg per d (calculated from the mean weight of the rats and the average daily intake), equates to an intake of 0.15 g acid protein fraction/kg per d. In human terms, this would equate to an intake of 9 g acid protein fraction/d for an average female weighing 60 kg. The AF from whey constitutes approximately 3% of the total milk protein and occurs at a level of about 1 g/l (Tremblay et al. 2003). Thus, the above intake of WAP would be the equivalent of 9 litres of milk. However, with respect to protein, the WAP constituted 2% of the protein in the rat diet. Given the daily recommended protein intake of 1.7 g/kg per d for adult human females, a similar percentage would give an intake requirement of 2.04 g WAP/day, or the equivalent of 2 litres of milk. In terms of dietary bone prophylaxis, the incorporation of small amounts of WAP into the diet would therefore present a more attractive option than the consumption of large volumes of milk.

Acknowledgements

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