Amino Acids (2013) 44:1409–1418
DOI 10.1007/s00726-011-1160-3

ORIGINAL ARTICLE

Effects of dietary bread crust Maillard reaction products on calcium and bone metabolism in rats

Irene Roncero-Ramos · Cristina Delgado-Andrade · Ana Haro · Beatriz Ruiz-Roca · Francisco J. Morales · María Pilar Navarro

Received: 4 July 2011 / Accepted: 14 November 2011 / Published online: 23 November 2011

Abstract Maillard reaction products (MRP) consumption has been related with the development of bone degenerative disorders, probably linked to changes in calcium metabolism. We aimed to investigate the effects of MRP intake from bread crust on calcium balance and its distribution, and bone metabolism. During 88 days, rats were fed control diet or diets containing bread crust as source of MRP, or its soluble high molecular weight, soluble low molecular weight or insoluble fractions (bread crust, HMW, LMW and insoluble diets, respectively). In the final week, a calcium balance was performed, then animals were sacrified and some organs removed to analyse calcium levels. A second balance was carried out throughout the experimental period to calculate global calcium retention. Biochemical parameters and bone metabolism markers were measured in serum or urine. Global calcium bioavailability was unmodified by consumption of bread crust or its isolate fractions, corroborating the previously described low affinity of MRP to bind calcium. Despite this, a higher calcium concentration was found in femur due to smaller bones having a lower relative density. The isolate consumption of the fractions altered some bone markers, reflecting a situation of increased bone resorption or higher turnover; this did not take place in the animals fed the bread crust diet. Thus, the bread crust intake does not affect negatively calcium bioavailability and bone metabolism.

Keywords Advanced glycation end-products · Bone markers · Calcium bioavailability · Maillard reaction products intake

I. Roncero-Ramos · C. Delgado-Andrade (✉) · A. Haro · B. Ruiz-Roca · M. P. Navarro
Instituto en Formación de Nutrición Animal, EEZ-CSIC, Camino del Jueves s/n, 18100 Armilla, Granada, Spain
e-mail: cdelgado@eez.csic.es

F. J. Morales
Instituto de Ciencia y Tecnología de Alimentos y Nutrición ICTAN-CSIC, 28040 Madrid, Spain

Introduction

An adequate calcium intake is necessary for healthy bone mass, at all stages of life. Calcium plays an important role in bone mineral structure and in the prevention of age-related bone diseases. Many intervention and observational studies have demonstrated the connection between calcium intake and bone health (Heaney 2000). Calcium is the most abundant cation in the human body and is a nutritionally essential mineral. Over 99% of body calcium is in the skeleton, while less than 1% is in an ionised form or bound to proteins in the extracellular fluid (Anderson and Garner 1996). Calcium is responsible for the mechanical and structural functions of bone and teeth as well as many metabolic functions involving neuromuscular transmission, enzyme activation, muscle contraction, signal transduction, etc.

Food consumption habits of the population are changing: the intake of processed food is increasing, while consumption of dairy products, the major source of dietary calcium, is decreasing (Seiquer et al. 2006; Fernández San Juan 2006), such that during childhood and adolescence calcium requirements are not completely met (Elmadfa and Weichselbaum 2005). Thermal treatment of food during industrial and/or home processing promotes the development of the Maillard reaction, which occurs between amino acids and reducing sugars (van Boekel et al. 2010).

Processes such as roasting, baking or frying rely on favourable effects of the Maillard reaction, especially colour and flavour formation. These quality attributes mainly occur on the surface of the food, e.g. in bread crust, which represents one of the major Maillard reaction products (MRP) inputs to the diet, since bread contributes a high proportion of the daily energy intake (Westerterp-Plantega 2004). Therefore, for many people, important amounts of MRP are ingested in their normal diet (Vlassara 1996), which promotes the increase of advanced glycation end-products (AGEs) in plasma (Somoza 2005). Numerous studies have related AGEs accumulation with osteoporosis and aging, leading to structural and functional changes in bone, in which calcium metabolism could be involved (Hein 2006).

In in vitro trials, Rendleman (1987) was one of the first to report the formation of calcium complexes with different soluble and insoluble melanoidins. Thus, the kind of pigments formed during the toasting of bread had very little ability to bind this mineral, which was also the case with the high molecular weight soluble non-dialyzable compounds in coffee.

The very few studies made on the in vivo effects of MRP consumption on calcium balance, mainly carried out using model systems, describe non-significant changes in calcium absorption or retention (Navarro 2003). Studies in rats fed a heated casein–glucose–fructose mixture have reported increased urinary calcium excretion with no modifications in calcium balance (Seiquer et al. 2001). These results are in agreement with preceding studies in rats fed diets containing MRP from glucose/glycine (Andrieux and Saquet 1984) and glucose/glutamate (O′Brien et al. 1986). Assays carried out in our laboratory reported an increase in calcium absorption and digestibility in rats given glucose–methionine MRP diets, although no significant changes were found in calcium balance (Delgado-Andrade et al. 2006). Nevertheless, Sarriá et al. (2001) observed a decrease in calcium bioavailability in rats that consumed liquid infant formulas including MRP.

In the above-mentioned study by Delgado-Andrade et al. (2006), a reduction in bone calcium concentration was detected in 3-week-old rats consuming MRP derived from glucose–methionine and glucose–lysine mixtures heated for 30 and 90 min, respectively. Lower calcium content in rat bones was also described by Yuan and Kitts (1994) after the rats consumed a diet containing heat-damaged casein.

The goal of the present study was to investigate the effects of the consumption of MRP from bread crust, as one of the major sources of glycotoxins in the diet, on calcium balance and its distribution, and bone metabolism. Apart from bread crust, we also evaluated the effects of diets containing its different soluble and insoluble fractions.

Materials and methods

Chemicals

All chemicals used were of analytical grade and were obtained from Merck (Darmstadt, Germany), unless stated otherwise. Pronase E (4,000,000 PU/g) was also purchased from this company.

Extraction of bread crust and its soluble and insoluble fractions

The bread crust was supplied by a Spanish manufacturer of cereal-derived food products. Once the attached bread crumbs had been manually removed, the sample was weighted, lyophilised, powdered and homogenised. A fraction of the bread crust was then stored at −20°C until diet formulation. In order to access the MRP and melanoproteins linked to proteins, another important fraction was isolated, via enzymatic hydrolysis with pronase E. The results obtained from previous studies by our research group indicated the most appropriate pronase E concentration and incubation time (Delgado-Andrade et al. 2008). Briefly, 125 g of bread crust were digested with 750 mL of a 0.100-mg/mL pronase E solution (400 U/mL in 1 M phosphate buffer, pH 8.2) in stoppered test recipients at 37°C for 72 h in a water bath under shaking. This procedure was scaled up and repeated as many times as necessary to obtain sample enough for the diets formulation. After cooling, sample was treated with 15 mL of 40% trichloroacetic acid solution (w/v), and centrifuged at 4,500*g* for 10 min at 4°C to separate soluble and insoluble fraction. Afterwards, the insoluble fraction was weighted, lyophilised, homogenised and stored at −20°C until used for diet formulation. The soluble fraction was subjected to ultrafiltration employing a Pellicon Ultrafiltrate cassette connected to a cassette-style tangential flow filtration device (Millipore, MA, USA) and a flow variable peristaltic pump. A Biomax polyethersulphone membrane (0.5 m^2 size, 17.8 cm width × 21 cm length, Millipore, MA, USA) with 5 kDa NMWL was used. Fraction constituted by compounds with a molecular mass higher than 5 kDa were retained (retentate, high molecular weight, HMW) while fraction possessing compounds with a mass below 5 kDa were filtered (filtrate, low molecular weight, LMW). Both fractions, retentate and filtrate, were lyophilised, properly powdered and homogenised and stored at −20°C until used for diets formulation.

Preparation of diets

The AIN-93G purified diet for laboratory rodents (Dyets Inc, Bethlehem, PA, USA) was used as the control diet

(Reeves et al. 1993). The bread crust was added to the AIN-93G diet to reach a final concentration of 10%. This one was named bread crust (BC) diet. In order to determine the compounds responsible for the possible effects observed in the trial, the LMW, HMW and insoluble fractions were also individually added to the diet in the same proportion as they were present in the 10% of bread crust, which was calculated based on the recovery of each fraction after pronase E digestion. These diets were named LMW, HMW and insoluble, respectively. Bread crust is a sodium source, and so, to maintain the concentration of this element at adequate levels, these diets were prepared mixing appropriate proportions of AIN-93G and low-sodium AIN-93G diets. Calcium carbonate was added when necessary to reach the values present originally in the AIN-93G diet.

The individual analysis of the different diets revealed no modification of the overall nutrient composition, compared with the control diet (AIN-93G). The mean ± SD nutrient content of the diets was: moisture (%) 7.9 ± 0.4; protein (g/kg) 168.4 ± 4.0; fat (g/kg) 77.9 ± 1.6; Na (g/kg) 1.34 ± 0.02; and Ca (g/kg) 4.86 ± 0.05.

The highest MRP content in the prepared diets, with respect to the control diet, was established by analysing the furosine and hydroxymethylfurfural (HMF) contents following the procedures described by Delgado-Andrade et al. (2010). The data obtained for furosine (mean ± SD) were as follows: 28.8 ± 0.5, 49.5 ± 0.3, 39.7 ± 1.4, 39.4 ± 1.0 and 34.7 ± 0.8 mg/kg diet for control, bread crust, LMW, HMW and insoluble diets, respectively. The results for HMF (mean ± SD) were as follows: 0.44 ± 0.06, 4.26 ± 0.02, 0.47 ± 0.04, 0.47 ± 0.01 and 0.89 ± 0.01 mg/kg diet for control, bread crust, LMW, HMW and insoluble diets, respectively.

Biological assays

Seventy weanling Wistar rats weighing 41.02 ± 0.16 g (mean ± SE) were involved in the study. Sixty were randomly distributed into five groups (12 animals per group) and each group was assigned to one of the dietary treatments. The animals were individually housed in metabolic cages in an environmentally controlled room under standard conditions (temperature: 20–22°C with a 12-h light–dark cycle and 55–70% humidity). Rats had ad libitum access to their diets and demineralised water (Milli-Q Ultrapure Water System, Millipore Corps., Bedford, MA, USA). The remaining ten animals were killed by anaesthesia overdose at day 0 to analyse their initial calcium body content.

The animals were fed different diets for 88 days. Excluding the insoluble group, in which the accidental death of four animals limited the number of trials, two different balances were carried out during the experimental period. The calcium balance for the entire experimental period, termed the 'Global balance', was calculated from the difference between the final body weight of each animal and the initial mean calcium body content (375.4 ± 2.8 mg Ca). Calcium intake was monitored during this period. Six animals from each group were sacrified by anaesthesia overdose on day 88 to calculate their final calcium body content. None of their organs were extracted. The animals in the insoluble group were excluded from this global balance; in this case, the balance technique described below was applied.

In the last week of the experimental period (days 82–88) another calcium balance was performed on all the animals. This test involved a preliminary 81-day period during which solid food intake and body weight changes were weekly monitored, followed by a 7-day period in which a calcium balance was performed. In this last week, faeces and urine from each animal were collected daily and stored separately as a 1-week pool. The faeces were weighed, lyophilised and then homogenised. The urine was collected on 0.5% HCl (vol/vol), filtered (Whatman Filter Paper No. 40, ashless, Whatman, England) and diluted to an appropriate volume. To control for possible environmental contamination during the collection of urine and faeces, empty cages were manipulated in the same way as those used for the animals. On day 88, after an overnight fast, six animals in each group were anaesthetised with sodium pentobarbital (5 mg per 100 g of body weight) (Abbott Laboratories, Granada, Spain) and terminal exsanguination was performed by a cannulation of the carotid artery. Blood was drawn to obtain serum, and the liver, right kidney, spleen, small intestine and right femur were removed, weighed and frozen at −80°C until calcium analysis.

All management and experimental procedures carried out in this study were in strict accordance with the current European regulations (86/609 E.E.C.) regarding laboratory animals. The Bioethics Committee for Animal Experimentation at our institution (EEZ-CSIC) approved the study protocol.

Analytical techniques

After sacrifice of the animals to determine the global balance, whole cadavers were weighed, lyophilised and then homogenised. The liver, spleen, kidney, small intestine and femur were dry-ashed in a muffle furnace (Selecta, Mod.366, Barcelona, Spain) at 450°C and the white ashes obtained were dissolved with $HCl/HNO_3/H_2O$ (1:1:2). Aliquots of the remaining samples (urine, faeces, diets and whole cadavers) were completely digested by the addition of concentrated HNO_3, $HClO_4$ and by heating at high temperatures (210–220°C) in a sand beaker. All samples were diluted with milli-Q water to an appropriate volume for calcium measurement.

The calcium analyses in all samples were carried out by flame atomic absorption spectroscopy (AAS) in a Perkin-Elmer Analyst 700 Spectrophotometer (Norwalk, Conn., USA). Standard solutions were prepared from a stock Tritisol solution of calcium (Cl_2Ca in 6.5% HCl, 1,000 mg Ca). Lanthanum chloride was added to samples and standards to reach a final concentration of 0.3%.

Pools of faeces, urine and diet were used as an internal control to assess precision. The inter-assay coefficient of variation was 1.80% in faeces, 1.08% in urine and 2.07% in the diet. Milk powder (certified reference material CRM 063; Community Bureau of Reference, Brussels, Belgium) was used to quantify accuracy, yielding a value of 13.48 ± 0.04 mg/g (mean ± SD; certified value: 13.49 ± 0.10 mg/g).

All glassware and polyethylene sample bottles were washed with 10 N nitric acid, and milli-Q water was used throughout the study.

The following indices were calculated using the data for calcium intake and faecal and urinary excretion obtained in the last week of the assay: apparent absorption (ingested Ca − faecal Ca); apparent retention or balance (apparent absorption − urinary Ca); apparent absorption efficiency or digestibility (%A/I) = apparent absorption/ingested Ca × 100; apparent retention efficiency (%R/A) = apparent retention/apparent absorption × 100; and bioavailability (%R/I) = apparent retention/ingested Ca × 100. Since all the indices were calculated in apparent form, henceforth, the term 'apparent' will be omitted.

The parameters calculated for the global balance were global retention (final Ca body content − initial Ca body content) and global %R/I (global retention/total Ca intake × 100).

Before being dry-ashed in the muffle furnace, the relative density of each femur (at room temperature, 23°C) was determined in triplicate, using a water picnometer (25 cm^3, Pobel, Madrid, Spain). Briefly, this value was calculated gravimetrically, as the ratio between the femur weight in air and in water.

Biochemical parameters and bone markers

Parathyroid hormone (PTH) was determined in serum (Rat Intact PTH ELISA kit, Immunotopics Inc., San Clemente, CA, USA). Serum alkaline phosphatase (AP) was measured enzymatically by standard techniques, using para-nitrophenyl phosphate as the substrate. Osteocalcin (OC) and procollagen type I N-terminal propeptide (PINP) were analysed in serum using an enzyme immunoassay kit (Rat-MID™ Osteocalcin EIA, IDS Ltd, Boldon, UK; Rat/Mouse PINP EIA, IDS Ltd., Boldon, UK). C-telopeptide degradation products from type I collagen (β-CTX) were also measured in serum, using an enzyme immunoassay kit (RatLaps™ EIA, IDS Ltd., Boldon, UK) and desoxypyridinoline (DPD) was analysed in urine, using an immunoassay kit (Metra DPD EIA (Quidel Corp., San Diego, CA, USA). Urine creatinine was measured using a chemical analyser (Shimadzu UV-1700, model TCC-240A, Duisburg, Germany).

Statistical analysis

All data were statistically tested by one-way analysis of the variance (ANOVA), followed by Duncan's test to compare means that showed a significant variation ($P < 0.05$). Analyses were performed using Statgraphics Plus, version 5.1, 2001. Evaluation of the relationship between the different variables was carried out by computing the relevant correlation coefficient (Pearson's linear correlation) at the $P < 0.05$ confidence level.

Results and discussion

Food intake and body weight

Food intake over the whole experimental period was significantly lower in all groups than in the control group, and particularly in the insoluble group, the lowest of all (Table 1). During the balance week, the food intake only

Table 1 Food intake in the whole experimental period and in the balance week and initial and final body weight after feeding rats the different diets

Diets	Global intake (g)	Intake in the balance week (g/day)	Initial weight (g)	Final weight (g)
Control	1,311.6 ± 35.4^{a}	14.2 ± 0.5^{a}	40.8 ± 0.3	247.6 ± 5.1^{a}
BC	1,220.8 ± 18.2^{b}	14.1 ± 0.3^{a}	41.0 ± 0.4	235.0 ± 4.4^{ab}
LMW	1,163.3 ± 25.1^{b}	13.9 ± 0.3^{ab}	41.1 ± 0.4	236.3 ± 7.2^{ab}
HMW	1,159.1 ± 27.5^{b}	13.6 ± 0.3^{ab}	41.2 ± 0.4	227.5 ± 5.8^{b}
Insoluble	1,050.1 ± 24.9^{c}	12.9 ± 0.4^{b}	41.0 ± 0.4	220.7 ± 4.0^{b}

Values are means ± SE, $n = 12$ except in insoluble group ($n = 8$). Different letters within a column indicate significant differences between groups ($P < 0.05$)

decreased significantly in the insoluble group, which points to a possible adaptative process to different dietary treatments. The decrease could be associated with MRP consumption, as has been described by many authors. Furniss et al. (1989) detected such a decrease after feeding rats with a glucose–casein heated mixture. More recently, Sarriá et al. (2001) obtained similar results with liquid infant formulas containing Maillard derivates. The cause of the decrease in food intake could be related to a slower digestive process; some time ago, Tanaka et al. (1975) and Kimiagar et al. (1980) described a slower rate of stomach emptying, leading to an increased sensation of satiety in rats fed diets containing MRP.

From similar initial body weights, the final weight of all animals decreased moderately with respect to the controls, but this was only statistically significant in the HMW and insoluble groups (Table 1). In the same line, O'Brien and Walker (1988) observed a weight decrease in the rats that consumed MRPs from model systems; this was subsequently confirmed by Seiquer et al. (2010), who compared the intake of diets based on overheated or UHT milk in rats. Nevertheless, Sebeková et al. (2005) described a weight gain in rats fed a diet containing 25% bread crust, the same food used in the diet formulation in our assay.

The lower weights recorded in the present study appear to be derived from a reduction in food intake, as demonstrated by the statistically significant correlation between these two parameters ($r = 0.61$; $P < 0.001$). However, the reduction in body weight was not as severe as that in food intake, and in fact it was only significant in the animals fed the HMW and insoluble fractions, where the indigestible compounds would be more abundant. Larger and more indigestible particles are known to be retained in the stomach for longer periods (Kutchai 1998) and this situation could equally apply to the presence of MRP, as they are less digestible (Rèrat et al. 2002), especially those with higher molecular weight.

Calcium balance

Calcium intake in the balance week followed the same behaviour as that of food consumption, since daily calcium ingested decreased significantly in the insoluble group with respect to the control and BC groups, while in the HMW and LMW groups only tended to decline. Faecal calcium excretion decreased in rats fed the HMW and insoluble diets (Table 2). Thus, calcium absorption was unmodified and its digestibility even improved in the above-mentioned groups. It is well-known that MRP can form metal complexes, although it seems that the ability to bind calcium is weaker than with respect to other cations (Rendleman 1987; Delgado-Andrade et al. 2004). In our experimental conditions, MRP from the bread included in the diets might not have chelated calcium or formed soluble and absorbable chelates, since calcium absorption did not vary. Rendleman (1987) showed that other high molecular weight soluble compounds derived from coffee or from toasted bread have a very low affinity to bind calcium. Even if an MRP–calcium insoluble complex was formed, intestinal microflora would play an important fermentative role on MRP, improving mineral absorption (Andrieux and Saquet 1984). Melanoidins, the final and high molecular weight compounds of the Maillard reaction, may be degraded by microbial action in the intestine (Dell'Aquila 2003).

In vitro and in vivo studies revealed no changes in calcium absorption efficiency associated with MRP consumption. Assays by our research group using the Caco-2 cell line have shown no effects on the percentage of calcium transported in the presence of MRP from the glucose-lysine model system heated for 30 min at 150°C (Delgado-Andrade et al. 2006). However, when the MRP come from a more complex system, casein–glucose–fructose heated for 60 min at 150°C, the amount of transported calcium increased (Seiquer et al. 2001), which is in accordance with the higher calcium digestibility observed in the HMW and insoluble groups (Fig. 1). The older in vivo studies performed by Adrian and Boisselot-Lefebvres (1977) reported an increase in calcium digestibility when products obtained from xylose–lysine and glucose–glycine systems were added to the normal diet of rats, but recent works have indicated no changes in rats fed a glucose–lysine heated mixture (Delgado-Andrade et al. 2006). Studies of MRP have shown decreases in calcium

Table 2 Calcium final week balance in rats fed different diets

Diets	Intake (mg/day)	Faeces (mg/day)	Urine (mg/day)	Absorption (mg/day)	Retention (mg/day)	Ca × P urine	Ca/creatinine urine
Control	69.1 ± 2.2^{a}	52.1 ± 1.7^{a}	2.7 ± 0.2	17.0 ± 1.6	14.4 ± 1.6^{a}	18.1 ± 2.4^{a}	0.38 ± 0.02
BC	69.7 ± 1.2^{a}	50.4 ± 0.7^{a}	2.5 ± 0.2	19.3 ± 1.2	16.9 ± 1.1^{ab}	10.9 ± 1.7^{b}	0.36 ± 0.03
LMW	67.1 ± 1.6^{ab}	50.1 ± 1.4^{a}	2.7 ± 0.2	17.1 ± 1.2	14.3 ± 1.2^{a}	15.9 ± 1.5^{ab}	0.39 ± 0.03
HMW	66.0 ± 1.6^{ab}	44.4 ± 1.5^{b}	2.6 ± 0.2	21.6 ± 1.3	19.0 ± 1.2^{b}	15.3 ± 2.1^{ab}	0.37 ± 0.03
Insoluble	62.2 ± 2.1^{b}	43.2 ± 1.9^{b}	2.3 ± 0.2	19.0 ± 2.1	16.7 ± 2.0^{ab}	11.4 ± 2.0^{b}	0.35 ± 0.03

Values are means ± SE, $n = 12$ except in insoluble group ($n = 8$). Different letters within a column indicate significant differences between groups ($P < 0.05$)

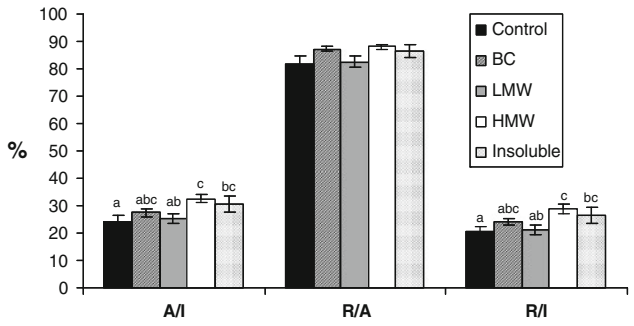

Fig. 1 Calcium biological indices in rats fed different diets (mean ± SE, $n = 12$, except in the insoluble group, $n = 8$). *Different letters within an index* indicate significant differences between groups ($P < 0.05$)

absorption and increases in calcium digestibility in rats consuming in-bottle-sterilized infant formulas compared with powder formulas (Sarriá et al. 2001), but no effects on calcium digestibility after feeding rats a diet with overheated milk compared with a UHT milk diet (Seiquer et al. 2010). In the same line, in a study of male adolescents, intake of an MRP-rich diet was not found to modify calcium absorption or its digestibility (Mesias et al. 2009).

Although other studies have reported an increased urinary elimination of minerals, especially trace elements, after MRP consumption (Furniss et al. 1989; Fairweather-Tait et al. 1989; O'Brien et al. 1994; Seiquer et al. 2001), in the present assay, urinary calcium remained unchanged during the last week balance (Table 2), even when expressed as a creatinine function (Table 2); this finding is in agreement with the non-modification of calcium excretion in urine observed by Delgado-Andrade et al. (2006).

Calcium retention in the last week balance was higher in the HMW group (Table 2), but over the whole experimental period, this retention did not vary (Fig. 2), although it was observed to be a trend towards increased body content and retention, in all groups with respect to the controls. Taking into account the animals' body weight, the retention (mean ± SE; mg Ca/g body weight) was as

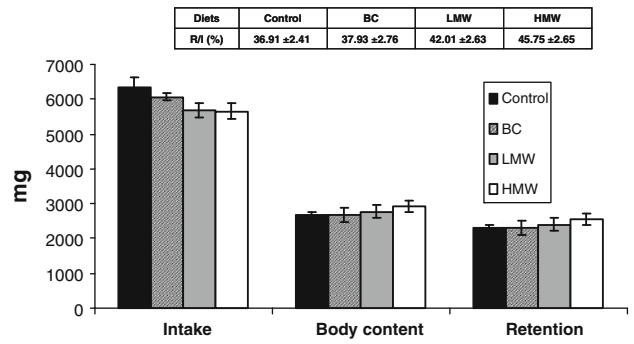

Fig. 2 Calcium global balance in rats fed different diets (mean ± SE, $n = 6$). No significant differences were found

follows: 9.37 ± 0.25, 9.82 ± 0.69, 9.97 ± 0.57 and 10.65 ± 0.56 for control, BC, LMW and HMW groups, respectively; with a P value = 0.06 in the latter group versus the control one.

As the retention efficiency (% R/A) remained unchanged in the last week of the assay (Fig. 1), the higher calcium bioavailability in animals fed the HMW and insoluble diets could be a direct consequence of improved calcium digestibility (Fig. 1), probably as an adaptive response to a lower food intake. But this increase does not seem particularly significant since it is not reflected in the global balance (Fig. 2), although once again the highest value was found in the HMW group.

The bioavailability values obtained in the two kinds of balance should be evaluated in relation to the different periods considered. In the last week balance, the rats are adult, have reached maturity and their calcium needs are stable. Considering the whole experimental period, in the first weeks calcium necessities would be higher, for growth and bone mass accumulation. These weeks of increased requirements decisively influence the greater calcium bioavailability values found during the 3 months. Accordingly, experiments carried out feeding rats the AIN-93G diet for 3 weeks after weanling have reported a bioavailability value of around 65% (Delgado-Andrade et al. 2006).

Different studies have reported decreases (Sarriá et al. 2001) as well as no changes (Seiquer et al. 2001; Delgado-Andrade et al. 2006) in calcium bioavailability in rats consuming diets supplemented with different MRPs, as was the case in our own experiment in rats fed the BC diet. Human assays using MRP-rich or poor diets also report stability in this respect (Mesias et al. 2009).

Calcium content in different organs

The weights of the liver, spleen and small intestine did not differ with respect to the control group (Table 3). Nevertheless, a general reduction, statistically significant in the BC group, was observed in kidney, and femur weight in all the animals fed diets containing BC or its fractions. Similarly, reductions have been documented in kidney, liver and femur weights after the consumption of different MRP (Delgado-Andrade et al. 2006; Seiquer et al. 2010); on the other hand, there are also many reports of increased weights of rats' liver and kidney (Lee et al. 1982; O'Brien and Walker 1988). Sebeková et al. (2005), also working with a rat diet added 25% of bread crust reported a significant increase in both liver and kidney weights.

The results for calcium content and concentration in the organs varied considerably (Table 3). Consumption of bread crust increased hepatic calcium concentration, mainly due to higher levels of calcium deposit in the organ.

Table 3 Calcium content and concentration in organs of rats fed different diets

Sample	Control	BC	LMW	HMW	Insoluble
Liver					
Weight (g)	6.15 ± 0.29	6.05 ± 0.23	6.30 ± 0.27	6.00 ± 0.21	5.81 ± 0.17
Ca (μg)	331.8 ± 22.7^{ab}	389.4 ± 43.8^{b}	251.7 ± 16.5^{c}	286.5 ± 9.7^{ac}	270.9 ± 10.2^{ac}
Ca (μg/g)	54.5 ± 4.1^{a}	64.1 ± 6.0^{b}	40.3 ± 2.2^{c}	47.8 ± 1.0^{ac}	46.6 ± 1.4^{ac}
Kidney					
Weight (g)	0.77 ± 0.03^{a}	0.68 ± 0.01^{b}	0.71 ± 0.03^{ab}	0.71 ± 0.03^{ab}	0.69 ± 0.03^{ab}
Ca (μg)	77.3 ± 4.2	72.7 ± 4.8	81.3 ± 6.3	81.8 ± 5.0	69.2 ± 4.5
Ca (μg/g)	100.2 ± 3.3^{a}	107.0 ± 5.8^{ab}	113.9 ± 4.1^{b}	114.3 ± 3.6^{b}	99.5 ± 3.0^{a}
Spleen					
Weight (g)	0.50 ± 0.02	0.49 ± 0.02	0.48 ± 0.03	0.49 ± 0.03	0.46 ± 0.03
Ca (μg)	15.2 ± 0.8^{a}	17.4 ± 1.2^{a}	27.5 ± 2.1^{b}	30.9 ± 1.5^{b}	26.8 ± 1.5^{b}
Ca (μg/g)	30.3 ± 1.7^{a}	35.8 ± 2.1^{a}	58.7 ± 7.1^{b}	64.5 ± 5.6^{b}	59.6 ± 4.4^{b}
Small intestine					
Weight (g)	4.72 ± 0.21	4.58 ± 0.19	4.95 ± 0.30	4.37 ± 0.19	4.60 ± 0.11
Ca (μg)	550.5 ± 42.8	636.3 ± 85.7	462.4 ± 49.9	472.5 ± 82.3	534.6 ± 70.7
Ca (μg/g)	116.8 ± 7.3^{ab}	139.6 ± 19.8^{a}	93.0 ± 8.0^{b}	106.8 ± 16.1^{ab}	114.8 ± 13.9^{ab}
Femur					
Weight (g)	0.59 ± 0.05^{a}	0.51 ± 0.02^{b}	0.51 ± 0.02^{b}	0.51 ± 0.02^{b}	0.49 ± 0.01^{b}
Relative density	1.50 ± 0.03^{a}	1.44 ± 0.04^{ab}	1.37 ± 0.03^{b}	1.37 ± 0.04^{b}	1.38 ± 0.02^{b}
Ca (mg)	119.9 ± 6.8	115.6 ± 3.9	116.8 ± 6.0	115.1 ± 3.9	112.0 ± 1.7
Ca (mg/g)	207.2 ± 5.2^{a}	227.4 ± 3.4^{b}	230.2 ± 2.2^{b}	225.2 ± 2.9^{b}	229.8 ± 1.3^{b}
Ca/P ratio	2.13 ± 0.03^{a}	2.23 ± 0.02^{b}	2.21 ± 0.04^{ab}	2.17 ± 0.01^{ab}	2.14 ± 0.03^{a}

Values are means ± SE, $n = 6$ except in insoluble group ($n = 8$). Different letters within a row indicate significant differences between groups ($P < 0.05$)

This is in agreement with results reported by Delgado-Andrade et al. (2005), and those of others, for whom the liver is an important organ in the metabolisation of MRP and a target for their accumulation (Homma and Fujimaki 1981; O'Brien and Morrissey 1989). However, when bread crust fractions were consumed separately, the effect was opposite and more prominent in the LMW group. Therefore, the increase in hepatic calcium concentration must be a joint action of all the bread crust components.

Renal calcium concentration was higher after consumption of the LMW and HMW diets, but not in the case of the BC diet. Therefore, in some way, the compounds incorporated in the LMW and HMW diets increased the amount of calcium in the kidney, another preferential target organ for MRPs (Tuohy et al. 2006). The Ca × P product in urine (Table 2) was significantly lower or tended to decrease in all groups respect to the controls, as a result of decreased urinary phosphorus excretion (data not shown). Similar results were observed in the study by Delgado-Andrade (2002).

Splenic calcium content and concentration were significantly higher after consumption of the bread crust fractions, but this hyperconcentration was not present in the animals fed the BC diet, in which all the fractions were present. Therefore, in a realistic situation, the consumption of bread crust must not affect splenic calcium deposit. No previous data have been found in the literature regarding the effect of MRP on calcium concentration in the spleen.

In the small intestine, the calcium concentration was lower in the LMW group than in the BC group. The effects of MRP on calcium concentration in the small intestine have been very little studied; to the best of our knowledge, the only reports available are from Seiquer et al. (2010), who, in disagreement with our data, described a marked rise in intestinal calcium after feeding rats a diet based on overheated milk.

Bone effects

The most interesting results were observed in the femur. The lower femur weight was strongly correlated with the final body weight of the animals ($r = 0.65$; $P = 0.0001$). In this respect, the literature describes reductions in bone strength and a decreased ash content in animals consuming diets including MRP, but these authors report simultaneous decreases in calcium content (Gregor and Emery 1987; Yuan and Kitts 1994). However, in the present study total femur calcium was unchanged and the concentration significantly enhanced in all the groups respect to the control one. But the higher calcium concentration should form part of the normal bone structure, since the Ca/P ratio remained stable, except in the case of the BC group, in which it increased slightly. Previous research by our team revealed

diminished Ca/P ratios when different MRP from model systems or from milk were included in rats' diets (Delgado-Andrade et al. 2006; Seiquer et al. 2010), but these assays were developed in shorter experimental times (21 and 28 days, respectively) in weaning rats.

The data obtained lead us to believe that the consumption of bread crust or its isolate fractions does not seriously affect bioavailability or the bone deposit of calcium, since mineralisation appeared to be adequate. Nevertheless, the relative density decrease in all bones compared with controls, pointing to a possible effect by MRP on the organic matrix, suggests the beginning of changes in the constitution of the bone framework. The relation between MRP, usually named AGEs in vivo, and the development and progress of degenerative bone disorders is well described (Hein 2006; Saito 2009).

The accumulation of AGEs in bone has been linked with bone adynamia, since the presence of these products, both in vivo and in vitro, causes inhibition or depression in the production of bone procollagen and osteocalcin (Yamamoto et al. 2001). In diabetes-induced rats, as well as in in vitro tests on primary cultures of bone, AP and osteocalcin decrease as AGEs level increase in collagen (Katayama et al. 1996). In the same line, Sanguineti et al. (2008) reported that pentosidine, a fluorescent AGE, exerted a negative effect on bone AP expression, although without modifications in the expression of osteocalcin.

In view of our results, a situation of bone adynamia, as described by Franke et al. (2007), cannot be supported, since AP and PINP, a bone formation marker, did not decrease, even the last one increased in the HMW group. This fact together with a trend to increase the DPD value could indicate a situation of higher turnover. On the other hand, osteocalcin levels, also marker of formation, only decreased in the LMW group (Fig. 3), where DPD was also greater. Both facts would indicate the predominance of the bone resorption. Accordingly, the relative density measured in the femurs of the animals given the LMW diet was among the lowest values, and a statistically significant positive correlation was found between osteocalcin and relative density ($r = 0.42$; $P = 0.0288$), suggesting a lower bone matrix in this group.

Pyridinoline and DPD are enzymatic mature cross-linking markers in bone. The enzymatic and non-enzymatic cross-linking seems to occur simultaneously and competitively, such that AGEs deposit in bone is the result of a mechanism of competitive inhibition with the enzymatic cross-linking formation (Saito et al. 2006). Accordingly, these authors report lower levels of pyridinoline and DPD in fractured bones compared with healthy bones, with a parallel increase in the accumulation of pentosidine and higher urinary excretion of DPD.

As described, the groups that consumed the bread crust fractions presented urinary DPD levels that were about 50% higher than the controls (Fig. 3). Since DPD comes almost exclusively from bone, a urinary increase suggests a situation of increased bone resorption or perhaps higher turnover. In the contrary, in animals consuming the whole bread crust, the only realistic diet of those tested, this effect did not appear, despite the fact that bread crust consumption supposes the intake of the HMW, LMW and insoluble fractions. However, fractions naturally present in the bread crust can be different from those artificially obtained after pronase digestion. It is possible that the action of pronase and subsequent gastrointestinal digestion generates products with a more negative effect than those resulting from

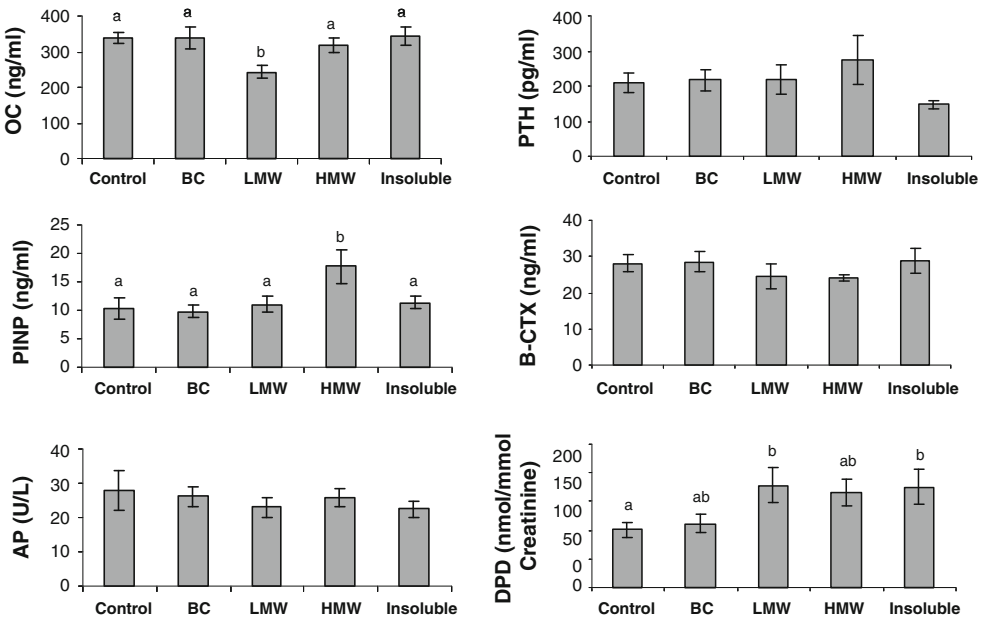

Fig. 3 Biochemical parameters and bone metabolism markers in urine or serum in rats fed different diets (mean ± SE, $n = 6$, except in the insoluble group, $n = 8$). *Different letters within a parameter* indicate significant differences between groups ($P < 0.05$)

the normal digestion of the bread crust in vivo. In this normal digestion a variety of products that modulate each other are originated and the isolate effect of each fraction is counteracted.

The bone resorption marker (Fig. 3), β-CTX, which together with α-CTX indicate isomerisation phenomena, and reflect maturation and aging in the collagen (Viguet-Carrin et al. 2006; Forwood and Vashishth 2009), did not present any modifications in our assay. This fact seems logical, since in the scientific literature focused on collagen glycation this marker is not among those most commonly studied to assess the effects of AGEs on bone health.

Finally, PTH, the main regulator of calcium metabolism, did not vary among the groups (Fig. 3), which is in accordance with the stability observed in serum calcium levels.

Conclusion

In our study MRP from bread, consumed as bread crust or its different fractions, did not modify calcium bioavailability in the diet considering the whole experimental period. This fact supports the well-established idea that MRP have a low affinity for calcium compared with other divalent cations. Despite none effect was found in calcium bioavailability, a higher calcium concentration was shown in femur of all animals due to lighter bones having a lower relative density. The isolate consumption of bread crust fractions slightly altered some bone markers, suggesting a situation of increased bone resorption or higher turnover. In the contrary, whole bread crust consumption did not modify bone features. Therefore, the results obtained using a food submitted to a physiological digestive process are not similar to the intake of its fractions isolated in the laboratory. These findings lead us to believe that studies carried out in model systems should always be completed with real-food systems, in the same way that they are usually processed and consumed, in order to derive realistic data that really help to understand the in vivo effects of AGEs.

Acknowledgments This work was supported by a project of the Spanish Ministry of Science and Innovation. The authors thank Grupo Siro, a Spanish manufacturer of cereal-derived food products, for supplying the bread crust samples.

Conflict of interest Authors declare no conflict of interest.

References

Adrian J, Boisselot-Lefebvres J (1977) Interrelations entre les produits de la reaction de Maillard et le metabolisme calcique. Cahiers Nutr Diet 12:233–234

Anderson JJB, Garner SC (1996) Dietary issues of calcium and phosphorus. In: John JB (ed) Calcium and phosphorus in health and disease. CRC Press, Florida

Andrieux C, Saquet E (1984) Effects of Maillard's reaction products on apparent mineral absorption in different parts of the digestive tract. The role of microflora. Reprod Nutr Develop 23:379–386

Delgado-Andrade C (2002) Reacción de Maillard: influencia sobre la biodisponibilidad mineral (Doctoral thesis), Granada, Spain

Delgado-Andrade C, Seiquer I, Nieto R, Navarro MP (2004) Effects of heated glucose-lysine and glucose–methionine model systems on mineral solubility. Food Chem 87:329–337

Delgado-Andrade C, Seiquer I, Navarro MP (2005) Comparative effects of glucose–lysine versus glucose–methionine Maillard reaction products consumption: in vitro and in vivo calcium availability. Mol Nutr Food Res 49:679–684

Delgado-Andrade C, Seiquer I, Navarro MP (2006) Changes in calcium absorption and subsequent tissue distribution induced by Maillard reaction products: in vitro and in vivo assays. J Sci Food Agric 86:271–278

Delgado-Andrade C, Rufián-Henares JA, Morales FJ (2008) Optimised procedure to analyse Maillard reaction-related fluorescence in cereal-based products. Czech J Food Sci 26:339–346

Delgado-Andrade C, Seiquer I, Haro A, Castellano R, Navarro MP (2010) Development of the Maillard reaction in foods cooked by different techniques. Intake of Maillard-derived compounds. Food Chem 122:145–153

Dell'Aquila C (2003) Microbial degradation of heated gluten-glucose systems. Implications for gut health. In: Vegarud G., Morales F. (eds) Melanoidins in food and health, EC Directorate for Research, vol 4, pp 181–183

Elmadfa I, Weichselbaum E (2005) Energy and nutrient intake in the European Union. European Nutrition and Health Report 2004. Forum Nutr 58:19–46

Fairweather-Tait SJ, Portwood DE, Symss LL, Eagle SJ, Minski MJ (1989) Iron and zinc absorption in human subjects from a mixed meal of extruded and nonextruded wheat bran and flour. Am J Clin Nutr 49(1):151–155

Fernández San Juan PM (2006) Dietary habits and nutritional status of school aged children in Spain. Nutr Hosp 21(3):374–378

Forwood MR, Vashishth D (2009) Translational aspects of bone quality–vertebral fractures, cortical shell, microdamage and glycation: a tribute to Pierre D. Delmas. Osteoporos Int 20(3):247–253

Franke S, Siggelkow H, Wolf G, Hein G (2007) Advanced glycation endproducts influence the mRNA expression of RAGE, RANKL and various osteoblastic genes in human osteoblasts. Arch Physiol Biochem 113(3):154–161

Furniss DE, Vuichoud J, Finot PA, Hurrell RF (1989) The effect of Maillard reaction products on zinc metabolism in the rat. Brit J Nutr 62:739–749

Gregor JL, Emery SM (1987) Mineral metabolism and bone strength of rats fed coffee and decaffeinated coffee. J Agric Food Chem 35:551–556

Heaney RP (2000) Calcium, dairy products and osteoporosis. J Am Coll Nutr 19(2):83–99

Hein GE (2006) Glycation endproducts in osteoporosis—is there a pathophysiologic importance? Clin Chim Acta 371:32–36

Homma S, Fujimaki M (1981) Growth response of rats fed a diet containing nondialyzable melanoidins. Prog Food Nutr Sci 5:209

Katayama Y, Akatsu T, Yamamoto M, Kugai N, Nagata N (1996) Role of nonenzymatic glycosylation of type I collagen in diabetic osteopenia. J Bone Miner Res 11(7):931–937

Kimiagar M, Lee TC, Chichester CO (1980) Long-term feeding effects of browned egg albumin to rats. J Agric Food Chem 28:150–155

Kutchai HC (1998) Gastrointestinal secretion. In: Berne RM, Levy MN, Koeppen BM, Stanton BA (eds) Physiology, 4th edn. Mosby Inc., St. Louis, pp 617–646

Lee TC, Pintauro SJ, Chichester CO (1982) Nutritional and toxicological effects of nonenzymatic Maillard browning. Diabetes 31:37–46

Mesias M, Seiquer I, Navarro MP (2009) Influence of diets rich in Maillard reaction products on calcium bioavailability. Assays in male adolescents and in Caco-2 cells. J Agric Food Chem 57:9532–9538

Navarro MP (2003) Impact of Maillard reaction products on mineral bioavailability. In: Vaquero MP, García-Arias T, Carbajal A, Sánchez-Muniz FJ (eds) Bioavailability of micronutrients and minor dietary compounds. Metabolic and technological aspects, research signpost. Kerala, India, pp 133–145

O'Brien J, Morrissey PA (1989) Nutritional and Toxicological aspects of Maillard Browning reaction in foods. Crit Rev Food Sci Nutr 28:211–248

O'Brien J, Walker R (1988) Toxicological effects of dietary Maillard reaction products in the rat. Food Chem Toxicol 26(9):775–783

O'Brien J, Morrissey PA, Flynn A (1994) Alterations of mineral metabolism and secondary pathology in rats fed Maillard reaction products. In: Labuza TP, Reineccius GA, Monnier VM, O'Brien J, Baynes JW (eds) Maillard Reactions in chemistry, food and health. Royal Society of Chemistry, Cambridge, pp 393–401

O′Brien J, Morrissey PA, Flynn A (1986) The effect of Maillard reaction products on mineral homeostasis in the rat. In: Proceedings of EURO-FOOD TOX II. Institute of Toxicology, Swiss Federal Institute of Technology, Zurich

Reeves PG, Nielsen FH, Fahey GC (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. J Nutr 123:1939–1951

Rendleman JA (1987) Complexation of calcium by the melanoidin and its role in determining bioavailability. J Food Sci 52:1699–1705

Rèrat A, Calmes R, Vaissade P, Finot PA (2002) Nutritional and metabolic consequences of the early Maillard reaction of heat treated milk in the pig, significance for man. Eur J Nutr 41:1–11

Saito M (2009) Biochemical markers of bone turnover. New aspect. Bone collagen metabolism: new biological markers for estimation of bone quality. Clin Calcium 19(8):1110–1117

Saito M, Fujii K, Soshi S, Tanaka T (2006) Reductions in degree of mineralization and enzymatic collagen cross-links and increases in glycation-induced pentosidine in the femoral neck cortex in cases of femoral neck fracture. Osteoporos Int 17:986–995

Sanguineti R, Storace D, Monacelli F, Federici A, Odetti P (2008) Pentosidine effects on human osteoblasts in vitro. In Maillard reaction: recent advances in food and biomedical sciences, Schleicher E, Somoza V, Shieberle P. Ann NY Acad Sci 1126:166–172

Sarriá B, López-Fandino R, Vaquero MP (2001) Does processing of a powder or in-bottle-sterilized liquid infant formula affect calcium bioavailability? Nutrition 17:326–331

Sebeková K, Hofmann T, Boor P, KJr Sebeková, Ulicná O, Erbersdobler HF, Baynes JW, Thorpe SR, Heidland A, Somoza V (2005) Renal effects of oral Maillard reaction product load in the form of bread crusts in healthy and subtotally nephrectomized rats. Ann NY Acad Sci 1043:482–491

Seiquer I, Aspe T, Vaquero P, Navarro P (2001) Effects of heat treatment of casein in the presence of reducing sugars on calcium bioavailability: in vitro and in vivo assays. J Agric Food Chem 49:1049–1055

Seiquer I, López-Frías M, Muñoz-Hoyos A, Galdó G, Delgado-Andrade C, Mesías M, Navarro MP (2006) Dietary calcium utilization among a group of Spanish boys aged 11–14 years on their usual diets. J Physiol Biochem 62(1):9–16

Seiquer I, Delgado-Andrade C, Haro A, Navarro MP (2010) Assessing the effects of severe heat treatment of milk on calcium bioavailability: in vitro and in vivo studies. J Dairy Sci 93:5635–5643

Somoza V (2005) Five years of research on health risks and benefits of Maillard reaction products: An update. Mol Nutr Food Res 49:663–672

Tanaka M, Lee TC, Chichester CO (1975) Nutritional consequences of the Maillard reaction. The absorption of fructose-L-tryptophan in the large intestine of the rat. J Nutr 105:989

Tuohy KM, Hinton DJS, Davies SJ, Crabbe JC, Gibson GR, Ames JM (2006) Metabolism of Maillard reaction products by the human gut microbiota—implications for health. Mol Nutr Food Res 50:847–857

van Boekel M, Fogliano V, Pellegrini N, Stanton C, Scholz G, Lalljie S, Somoza V, Knorr D, Jasti PR, Eisenbrand G (2010) A review on the beneficial aspects of food processing. Mol Nutr Food Res 54:1215–1247

Viguet-Carrin S, Roux JP, Arlot ME, Merabet Z, Leeming DJ, Byrjalsen I, Delmas PD, Bouxsein ML (2006) Contribution of the advanced glycation end product pentosidine and of maturation of type I collagen to compressive biomechanical properties of human lumbar vertebrae. Bone 39:1073–1079

Vlassara H (1996) Advanced glycosylation in nephropathy of diabetes and aging. Adv Nephrol 25:303–307

Westerterp-Plantega MS (2004) Effects of energy, density of daily food intake on long term energy intake. Physiol Behav 81:765–771

Yamamoto T, Ozono K, Miyauchi A, Kasayama S, Kojima Y, Shima M, Okada S (2001) Role of advanced glycation end products in adynamic bone disease in patients with diabetic nephropathy. Am J Kidney Dis 38(4):161–164

Yuan YV, Kitts DD (1994) Calcium absorption and bone utilization in spontaneously hypertensive rats fed on native and heat-damaged casein and soya-bean protein. Br J Nutr 71:583–603