Health

Bone loss prevention in ovariectomized rats using stable amorphous calcium carbonate[*]

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ABSTRACT

In assessing the relationship between calcium supplementation and maintaining bone mass or reducing the risk of fracture, the effectiveness of calcium supplementation has never been decisive. Freshwater crayfish rely on amorphous calcium carbonate (ACC), an instable polymorph of calcium carbonate, as the main mineral in the exoskeleton and in the temporary storage organ, the gastrolith. Inspired by the crayfish model, we have previously shown an increase in calcium bioavailability in rats administered with synthetic stable ACC vs. crystalline calcium carbonate (CCC). The current study compared the effects of amorphous calcium derived from either gastrolith or synthetic ACC with those of crystalline calcium, found in commercial CCC or calcium-citrate supplements, in a bone loss prevention model. Rats were subjected to either sham or ovariectomy (OVX) operation (n~20/ group) followed by administration of food pellets supplemented with 0.5% calcium from either source over 12 weeks. Micro-computed tomography (μCT) and histomorphometric analyses revealed bone loss prevention by both gastrolith and ACC treatments, manifested by an increase in morphometric bone parameters, compared to both CCC- and calcium citrate-treated groups. Both gastrolith and ACC treatments resulted in bone formation in the tibia cancellous bone, indicated by dynamic histomorphometry parameters, compared to either the CCC or calcium citrate treatments. Levels of urine deoxypyridinoline (DPD), suggested an anti-resorptive effect of ACC, which was also the only treatment that led to a significant increase in vertebral mechanical strength, as supported by μCT analysis of topology and orientation parameters of the vertebral trabeculae. To our knowledge, such levels of bone loss prevention by calcium supplements have never been reported. These findings thus suggest the potential of both natural (crayfish gastrolith) and, to a greater extent, synthetic ACC sources for the prevention of metabolic bone disorders and possibly of osteoporotic processes.

Keywords: Amorphous Calcium Carbonate; Bone Metabolism; Calcium Carbonate; Calcium Citrate; Gastrolith; Osteoporosis; Ovariectomized Rats

1. INTRODUCTION

Strategies for preventing osteoporotic processes include a balanced diet rich in calcium, vitamin-D and regular exercise [1]. Calcium supplementation with and without vitamin-D has become a widely recognized strategy for realizing adequate calcium intake. The calcium currently used in dietary supplements is derived from

several origins, including natural sources, such as oyster shells, coral calcium and dolomite minerals, and synthetic precipitates, comprising both organic and inorganic calcium salts [2]. Calcium from carbonate and citrate salts are by far the most commonly used forms of calcium supplements [2]. Still, the effectiveness of calcium supplementation for the prevention of progressive skeletal disease has never been decisive. The relatively limited absorbability of calcium has resulted in an ongoing scientific debate as to the superior bioavailability of different calcium supplements [2]. Yet, sensitive isotopic tracer methods revealed comparable bioavailability of all calcium salts [2,3]. The effectiveness of calcium supplements with or without vitamin-D in reducing bone loss and preventing fractures has also been the focus of many clinical trials and several meta-analyses [4-8]. Most of these studies reported a modest but significant effect of calcium supplementation in reducing bone loss [4,7,9, 10], although the effectiveness of calcium supplementation on the prevention of fractures was less conclusive [5,6,11-13] probably due to the fact that it is simply so modest that significance is not found in every study. According to an extensive meta-analysis, calcium combined with vitamin-D was associated with a 12% reduction of fracture risk [4].

Despite the documented benefits of calcium supplementation in combination with vitamin-D as an over the counter first-line management strategy in the prevention of osteoporosis, the effectiveness of available calcium forms is still very moderate. Calcium carbonate, one of the most abundant minerals in nature, has six known polymorphs of which the most thermodynamically stable form is calcite. The least stable polymorph is the amorphous form, termed amorphous calcium carbonate (ACC) [14]. Stabilization of synthetic ACC can be a challenging task. Indeed, of the several attempts at stabilizing synthetic ACC reported [14-17], all used either toxic materials or various organic polymers to enable the stabilization of ACC, yet for no more than 3 days [14,15].

Freshwater crayfish construct specialized transient mineral storage sites, gastroliths, that are composed of stabilized amorphous calcium carbonate embedded into an organic matrix comprising dense chitin fibers and proteins [18]. When needed, the thermodynamic instability of ACC is exploited to offer a highly bioavailable calcium source that enables fast and effective transport of the mineral across the intestinal epithelium and into the hardening exoskeleton. Inspired by the molecular mechanism used by crayfish to stabilize ACC, a novel method for synthetic production of stabilized ACC using phospho-amino acids was developed [19].

The superior bioavailability of ACC over crystalline calcium carbonate (CCC) was recently confirmed using an intrinsic radioactive labeling rat model, where in-creased bioavailability of up to 40% and 30% were seen in the serum and bone, respectively, while a 26.5% increase in retention levels was measured [20]. In the scope of the current study, we compared the effects of the above synthetic ACC and of the gastrolith to those of leading commercially-available calcium carbonate and calcium citrate supplements on bone loss prevention, bone formation and mechanical strength in an ovariectomized (OVX) estrogen-deficiency bone loss rat model.

2. MATERIALS AND METHODS

2.1. Diet Preparation

For administration of the different calcium sources, low calcium rodent pellets containing 0.02% calcium (Rat chow #TD95027, Harlan, Jerusalem, Israel) were finely grounded and separately mixed with the following four different calcium sources (**Table 1**): Calcium carbonate as contained in a commercial supplement (carbonate; Caltrate, Pfizer, Madison, NJ); gastrolith powder harvested from the Australian crayfish, *Cherax quadricarinatus* (Gastrolith, Ben's farm, Beer-Tzofar, Israel; see composition in [18,21]), stable ACC (Batch #ETS001, Amorphical, Beer-sheva, Israel; see composition in [19, 20]) and calcium citrate from a commercial supplement (citrate; GNC, Pittsburgh, PA). Each mix was separately pelleted and kept in a sealed bag at RT. Pellets from all four mixtures contained ~0.5% elemental calcium (as generally used in EU and correlates to 250 mg calcium/kg body weight/day; a standard calcium requirement [22]), as confirmed by an atomic absorption (Varian AA240, Palo Alto, CA; **Table 1**) with no added vitamin-D, except the amounts found in the original food pellets (22 IU/g).

2.2. Animal Treatment

One hundred and one 16 - 17 weeks old female Sprague-Dawley rats (Harlan) with an average body weight of 250 ± 16 g were group-housed (~3 rats/cage) and acclimated under controlled room conditions (21°C ± 2°C

Table 1. Elemental calcium content in calcium sources and specialized pellets.

	Pelleted mix			
	CaCO$_3$	Gastrolith	ACC	Ca-Citrate
Ca^{2+} in calcium source (%)	34.5	28.1	33.0	18.3
Ca^{2+} in pellets (%)	0.54	0.54	0.53	0.55

Calcium content (measured by atomic absorption) in commercial crystalline CaCO$_3$ (CaCO$_3$), gastrolith powder from the Australian crayfish, Cherax quadricarinatus (gastrolith), synthetic stable amorphous calcium carbonate (ACC) and calcium citrate from a commercial supplement (Ca-citrate). Values are represented as mean weight %.

and a 12-hour dark-light cycle). During the 7-day of acclimation, the rats were fed a standard laboratory diet (#2018SC rat chow, Harlan) and de-ionized water *ad libitum*.

Following this period, the rats were individually housed in metabolic cages and deprived of food and water for 18 h. Urine was collected in the dark and stored at −80˚C for bone resorption markers analysis at baseline (time 0). Immediately after, the rats were anesthetized by intraperitoneal (IP) injection of ketamine (75 mg/kg body weight; Fort Dodge. Laboratories, Fort Dodge, IA) and xylazine (5 mg/kg body weight; EuroVet AH, Bladel, UK) solution. Blood was sampled from the rats' tails (~180 µl) and centrifuged for 10 minutes at 3000 g (Hettich Zentrifugen, Bach, Switzerland) for serum extraction. Serum samples were stored at −80˚C for bone formation markers analysis at baseline (time 0).

Rats were randomly assigned to five treatment groups, namely a sham-operated group that underwent sham operation and four other groups that were ovariectomized by a bilateral dorsal approach [23] and subsequently divided into OVX-control, OVX-gastrolith, OVX-ACC and OVX-citrate treatment groups (see below). Following surgery, the rats were given a 7-days recovery period, allowing a washout of estrogen, during which they were administered standard laboratory diet *ad libitum* and de-ionized water containing 1.5 ml/400ml dipyrone (Vitamed, Binyamina, Israel) and 1 mg/kg enrofloxacin (Bayer, Leverkusen, Germany).

At the end of the recovery period, the rats were group-housed (2 rats/cage) according to the treatment assignment and fed the following different food mixes (**Table 1**): Sham (n = 19) and OVX-control (n = 19) rats were fed the CCC-containing mix, OVX-gastrolith rats (n = 18) were fed the gastrolith-containing mix, OVX-ACC rats (n = 20) were fed the ACC-containing mix and OVX-citrate rats (n = 18) were fed the calcium citrate-containing mix. The supplemental pellets and de-ionized water were provided *ad libitum* throughout the entire treatment period (90 days). Food consumption and body weight were recorded weekly (**Table 2**).

For dynamic histomorphometric analysis, the fluoro-chromatic dye, calcein (20 mg/kg body weight; Sigma-Aldrich, St. Louis, MO), was IP-injected into all rats on days 76 and 83. All 94 animals had calcein double labeling, demonstrated by two distinguished labeling lines along the trabecular bone.

On day 90, the rats were individually housed in metabolic cages and deprived of food and water for 18 h. Urine was collected and stored at −80˚C for deoxypyridinoline (DPD) bone resorption marker analysis at the end of the experiment (day 90). Serum samples were extracted for osteocalcin (OC) bone formation markers evaluation and stored as described for day 0 samples.

All rats were sacrificed through inhalation of CO_2. Right femurs and 4th- and 5th-lumbar vertebrae were dissected, wrapped in gauze pads soaked with saline buffer and stored −80˚C for micro-computed tomography (μCT) scanning or mechanical testing. Right tibias were dissected and immediately placed in 3.7% formaldehyde solution (F1636, Sigma-Aldrich) at 4˚C for 24 h, and processed as elaborated below in the structural and dynamic histomorphometry section. All procedures were approved by Ben-Gurion Animal Care and Use Committee, Ben-Gurion University, Beer-Sheva, Israel.

2.3. Micro-Computed Tomography (μCT) Scanning

Trabecular and cortical bone microarchitecture of right femurs and 4th-lumbar vertebrae were analyzed using a Skyscan 1174 μCT scanner (Skyscan, Kontich, Belgium). Bone mineral density (BMD) calibration was performed according to the manufacturer's instructions, using designated rat phantom rods with densities of 0.25 and 0.75 g/cm^3 as standards (Skyscan). Femurs and vertebrae were removed from storage at −80˚C, transferred to new gauze pads soaked with 70% ethanol and placed in plastic vials containing 70% ethanol. The distal region of each femur was scanned for trabecular and cortical bone parameters at an isotropic resolution of 13.8 µm. Reconstruction was carried out employing a modified algorithm using Nrecon software, ver. 1.6.4 (Skyscan). The volume of interest (VOI) was set to 0.5 mm below the growth plate

Table 2. Food consumption and total body weight.

		OVX			
	Sham	**Control (CaCO₃)**	**Gastrolith**	**ACC**	**Ca-Citrate**
Rat body weight (Day 0)	253 (3.5)	249 (3.3)	249 (5.2)	251 (4)	253 (4.7)
Rat body weight (Day 90)	310 (8.3)	345 (5.8)	370 (6.4)	359 (4.2)	354 (5.6)
*Weight gain (%)	22.5[a]	38.5[b]	48.5[b]	43.0[b]	40.0[b]
Mean food consumption (g/day)	15.2 (0.07)	15.2 (0.07)	15.2 (0.07)	15.1 (0.02)	15.2 (0.05)

Weight gain represents the rate of weigh increase over the course of the experiment. Mean daily food consumption was calculated per animal, by averaging the weekly food intake per cage over the treatment period and dividing it by 14. Values correspond to means (±SD). One-way ANOVA: *p < 0.01. Letters represents Fisher's LSD post-hoc comparison tests.

(most proximal boundary with the metaphysis) and extended proximally for 1.5 mm [24,25]. Trabecular and cortical segments within the VOI were extracted by depicting ellipsoid contours every ~5 slices using CTan analysis software, ver. 1.11 (Skyscan) [26]. The trabecular and cortical BMD, bone volume/tissue volume (BV/TV, %), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, μm), and trabecular separation (Tb.Sp, μm) were calculated by CT analyzer software, ver. 1.11 (Skyscan). Vertebrae were scanned at a 13.8 μm isotropic resolution. Reconstruction and analysis were performed as described above for the femurs. Trabecular and cortical bone parameters were evaluated in the vertebral body, set to cover a VOI of a 2-mm thick cross-section [25], starting 0.14 mm below the cranial growth plate. In addition to the same trabecular and cortical bone parameters as studied for the distal femur, the topology parameters: *i.e.*, trabecular bone pattern factor (TBPf) and structural model index (SMI); and the trabecular orientation parameter, degree of anisotropy (DA) were also measured in the vertebral body.

2.4. Histomorphometry

Right tibias were removed from the formaldehyde solution and dehydrated through an ethanol gradient, cleared using xylene (24250521, Bio-Lab, Haifa, Israel), and infiltrated with 80% methylmethacrylate (MMA; M5599, Sigma Aldrich) and 20% dibutyl phthalate (DBP; 61-5062, Merck, Hohenbrunn, Germany) for 3 days at 4˚C. Specimens were then transferred to 80% MMA containing 20% DBP and 2.5% benozoyl peroxide (617008, Merck) solution for an additional 6 days of incubation at 4˚C. The specimens were next embedded in 80% MMA, 20% DBP and 2.5% benozoyl peroxide under a temperature gradient (38˚C - 50˚C) over a period of 4 days. Mid-sagittal un-decalcified sections (4 μm) from each specimen were prepared using a microtome (RM2165 Leica Microsystems). Sections were either stained with McNeal's tetrachrome for structural histomorphometry, or left unstained for dynamic histomorphometry. Structural and dynamic histomorphometry analyses of the proximal tibia metaphysis were performed using a semi-automatic analysis system (Bioquant OSTEO, ver. 7.20.10, BIOQUANT Image Analysis, Nashville, TN) connected to an Optiphot 2 fluorescent microscope (Nikon, Kingston, England). Histomorphometric parameters, *i.e.* BV/TV, Tb.N, Tb.Th, Tb.Sp, bone formation rate/bone surface (BFR/BS) ratio, mineralizing surface/bone surface (MS/BS) ratio and mineral apposition rate (MAR), were calculated in an ROI that was selected to cover 1000 μm × 1000 μm, 0.5 mm below the tibia growth plate and 0.5mm inwards from the cortical bone in a magnitude of X4.

2.5. Evaluation of Bone Turnover Biochemical Markers

Serum OC levels were determined using an enzyme-linked immunoassay (EIA) kit (#BT-490, Biomedical Technologies, Stoughtone, MA) following a 1:10 dilution of the sample. Urinary DPD levels were determined using an EIA kit (#8007, Quidel, San Diego, CA) following a 1:50 dilution of the sample. DPD levels were normalized to creatinine levels, measured with the appropriate EIA kit (#8009, Quidel).

2.6. Evaluation of the Mechanical Strength of the 5th-Lumbar Vertebra

5th-lumbar vertebral biomechanical properties were measured after soft tissue removal. The transverse and posterior processes of the vertebra were removed and the ends of the centrum were rendered parallel using a diamond wafering saw (Buehler Isomet, Lake Bluff, IL). The vertebral bodies were then broken by compression along the S-I axis at a speed of 0.5 mm/min using the Modular Test Machine 100P255 (Test Resources, Shakopee, MN). Force and displacement measurements were collected every 0.1 s. From the force versus displacement curves, structural (extrinsic) properties included ultimate force [maximum force obtained during testing ultimate load (UL, N)], energy (E) to UL (mJ) were calculated using standard procedures [27]. Toughness (material level energy to fracture, E to UL/TotBV) was calculated using total bone volume as measured by μCT.

2.7. Statistical Analysis

Results are expressed as means (±SD or SEM). One-way ANOVA followed by Fisher-LSD post-hoc comparison tests were performed using STATISTICA 6.1 software (StaSoft, Tulsa, OK). A p value < 0.01 was deemed significant.

3. RESULTS

3.1. Femoral and Vertebral μCT Morphometric Analysis

Three-dimensional μCT-based schematic microarchitectural reconstructions of a representative right distal femur and 4th-lumbar vertebra from each group are presented in **Figure 1**. Femoral and vertebral (**Figures 2A** and **B**, respectively) Tb.BMD of the sham group was significantly higher than that of all four OVX groups (one-way ANOVA; p < 0.001). Within the various OVX groups, the femoral and vertebral Tb.BMD of OVX-gastrolith and OVX-ACC groups were significantly higher than those of the OVX-control and OVX-citrate groups. No significant differences were observed in the femoral
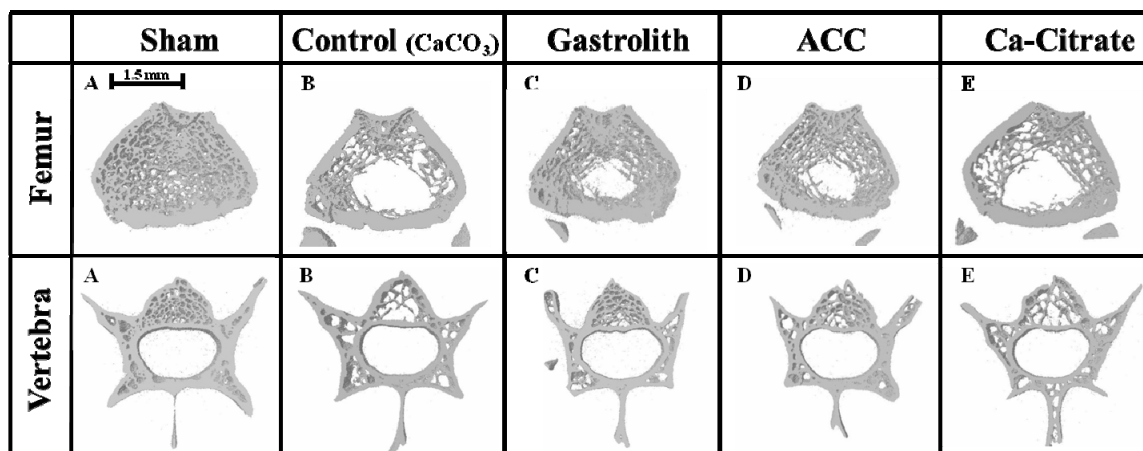
Figure 1. Three-dimensional reconstruction of representative distal femoral and 4th-lumbar vertebral cross sections. The trabecular bone region from the (A) sham-operated group treated with crystalline CaCO₃ (sham). OVX-treated groups treated with: (B) crystalline CaCO₃ (control), (C) gastrolith powder, (D) amorphous calcium carbonate (ACC) or (E) calcium citrate (Ca-citrate). Scale bar represents 1.5 mm. For the illustration, the femoral volume of interest (VOI) was set to 1 mm below the growth plate (most proximal boundary with the metaphysis) and extended proximally for 0.4 mm. The vertebral VOI was set to a 0.4 mm thick cross-section starting 0.14 mm below the cranial growth plate.
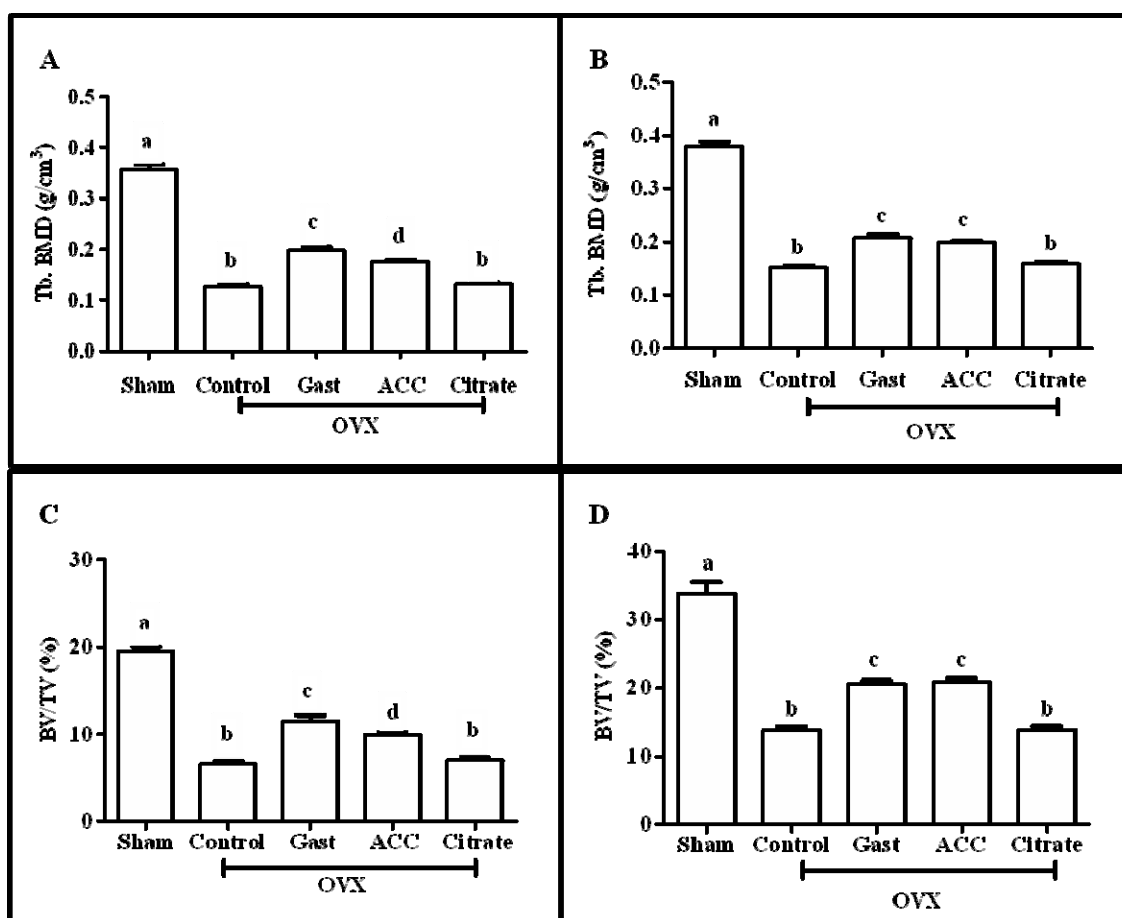

Figure 2. Trabecular bone mineral density (Tb.BMD) and bone volume from total bone tissue (BV/TV) assessed by μCT. (A) Tb. BMD in Distal femur. (B) Tb.BMD in 4th-lumbar vertebra. (C) BV/TV in Distal femur. (D) BV/TV in 4th-lumbar vertebra. Sham-operated group treated with crystalline CaCO₃ (Sham). OVX-treated groups treated with: crystalline CaCO₃ (Control), gastrolith powder (Gast), amorphous calcium carbonate (ACC) or calcium citrate (Citrate). One-way ANOVA: $p < 0.001$. Letters represent Fisher's LSD post-hoc comparison. Bars represent SEM.

and vertebral Tb.BMD of the OVX-citrate group compared to the OVX-control group. Comparing the OVX-gastrolith and OVX-ACC groups revealed a 13% increase in the femoral Tb.BMD of the former, relative to the latter (**Figure 2A**), although no difference in the vertebral Tb.BMD was noted (**Figure 2B**). In contrast to what was observed with the Tb.BMD, no significant differences were observed in the cortical BMD of either femurs or vertebrae across all groups (**Tables 3(A)** and (**B**)).

Femoral and vertebral μCT morphometric analysis (**Figures 2C** and **D** and **Tables 3(A)** and (**B**)) revealed a significant decrease in BV/TV (p < 0.01) and Tb.N (p < 0.01) in all OVX groups, compared to the sham. Within the femurs and vertebras of the OVX groups, OVX-gastrolith and OVX-ACC group members presented a significantly higher BV/TV and Tb.N, compared to the OVX-control and OVX-citrate groups, while no significant differences were observed in BV/TV and Tb.N between the OVX-citrate and OVX-control groups. Comparison of femurs from OVX-gastrolith and OVX-ACC group members revealed a significant difference in BV/TV, Tb.Th and Tb.Sp (**Figure 2C** and **Table 3A**), while no significant differences in any of the morphometric parameters considered were observed between the vertebrae of these two groups (**Figure 2D** and **Table 3B**).

3.2. Structural and Dynamic Histomorphometry Analysis of the Proximal Tibia

The above differences observed in microarchitectural structure were further emphasized in the structural histomorphometry analysis of the proximal tibia, as presented in **Table 3(C)**. All OVX groups showed a significantly lower BV/TV (p < 0.01) and Tb.N (p < 0.01) relative to the sham group, and Tb.Sp was significantly higher (p < 0.01) in all OVX groups, compared to the sham group. Within the OVX groups, the OVX-gastrolith and OVX-ACC groups presented a significantly higher BV/TV and Tb.N together with a lower Tb.Sp compared to the OVX-control group, and a significantly higher Tb.Th in the OVX-ACC group, compared to the same value in the OVX-control group. The OVX-citrate group presented significantly higher Tb.Th compared to the OVX-control group. Examination of the OVX-gastrolith and OVX-ACC groups revealed a significantly higher BV/TV and Tb.N along with lower Tb.Sp in the OVX-gastrolith group than measured in the OVX-ACC group.

Proximal tibia dynamic histomorphometric analysis (**Table 3C**) revealed a 13% elevation of BFR/BS between OVX-control and OVX-citrate groups compared to the sham group; however, this did not reach a statistic-

Table 3. Bone morphometric parameters and biochemical markers.

			OVX			
		Sham	**Control (CaCO$_3$)**	**Gastrolith**	**ACC**	**Ca-Citrate**
(A) Femur	*Tb.N, mm^{-1}	6.1^a (0.9)	2.2^b (0.6)	3.5^c (0.6)	3.3^c (0.5)	2.4^b (0.5)
	*Tb.Sp, µm	111^a (28)	248^b (83)	158^c (46)	209^d (74)	226^{bd} (47)
	Tb.Th, µm	31.5^{ac} (5.3)	29.0^b (0.8)	33.6^a (3.5)	30.9^{cb} (1.4)	29.1^b (2.0)
	Ct. BMD, g/cm^3	963 (51)	980 (57)	983 (62)	952 (53)	970 (53)
(B) Vertebra	*Tb.N, mm^{-1}	3.98^a (0.7)	1.57^b (0.3)	2.29^c (0.3)	2.24^c (0.3)	1.59^b (0.2)
	*Tb.Sp, µm	153^a (28)	254^b (74)	197^c (54)	229^{bc} (36)	262^b (60)
	Tb.Th, µm	84.6 (7.3)	87.5 (6.4)	83.8 (20.3)	93.5 (6.9)	86.6 (8.5)
	Ct. BMD, g/cm^3	933 (55)	950 (50)	959 (56)	961 (57)	943 (68)
(C) Tibia	*BV/TV, %	28.5^a (4.1)	12.8^b (2.1)	23^c (3.3)	19.4^d (2.8)	12.8^b (1.4)
	*Tb.N, mm^{-1}	5.6^a (0.6)	2.5^b (0.5)	4.5^c (0.4)	3.7^d (0.8)	2.3^b (0.3)
	*Tb.Sp, µm	128^a (25)	296^b (44)	166^c (15)	205^d (43)	318^b (42)
	Tb.Th, µm	51.2^{ab} (6.2)	47.1^a (6.0)	51.6^{ab} (7.1)	54.8^b (7.7)	53^b (9.9)
	*BFR/BS, µm^2/µm/day	0.15^a (0.03)	0.16^a (0.02)	0.21^b (0.03)	0.21^b (0.03)	0.16^a (0.02)
	*MS/BS, %	8.04^a (1.5)	8.31^a (1.4)	11.6^b (1.4)	11.1^b (1.2)	8.25^a (1 4)
	MAR, µm/day	1.26 (0.3)	1.30 (0.2)	1.34 (0.3)	1.29 (0.2)	1.40 (0.2)
(D) Biochemical markers (Day 0)	DPD, nmol/mmol Cr	28.7 (14.3)	24.9 (14.2)	31.7 (16.7)	28.6 (13.8)	28.0 (14.2)
	OC, ng/ml	9.9 (4.0)	10.00 (3.3)	9.4 (4.13)	8.8 (3.6)	9.2 (3.6)
(E) Biochemical markers (Day 90)	*DPD, nmol/mmol Cr	13.8^a (5.4)	36.7^{be} (31.2)	29.8^{bd} (16.56)	22.6^{ad} (11.09)	43.6^e (22.3)
	OC, ng/ml	10.0 (4.8)	14.8 (6.4)	14.7 (8.7)	12.7 (6.8)	15.7 (8.4)

(A) Distal femurs metaphysic; (B) 4th-lumbar vertebra; (C) Proximal tibia structural parameters (analyzed in 4 µm-thick MacNealstained sections) and dynamic parameters (analyzed in un-decalcified sections double-labeled with calcein); (D) Biochemical markers. Trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), cortical bone mineral density (Ct. BMD), bone formation rate/bone surface (BFR/BS), mineral surface/bone surface (MS/BS), mineral apposition rate (MAR), Dihydropyrimidine (DPD), osteocelain (OC). Values correspond to means (±SD). One-way ANOVA: *p < 0.01. Letters represents Fisher's LSD post-hoc comparison tests.

cal significance. Nevertheless, the BFR/BS ratios of the OVX-gastrolith and OVX-ACC groups were significantly higher than were the same parameters in the sham group (p < 0.01). MS/BS ratios between the OVX-control and OVX-citrate groups, compared to the sham group were not altered, while the MS/BS ratios of the OVX-gastrolith and OVX-ACC were elevated relative to the sham (p < 0.01). Within the OVX groups, the OVX-gastrolith and OVX-ACC groups presented significantly higher BFR/BS and MS/BS ratios compared to OVX-control. The BFR/BS and MS/BS ratios of the OVX-citrate group were similar to that measured for the OVX-control group, and significantly lower from the value determined for the OVX-gastrolith and OVX-ACC groups, while MAR showed no significant differences among all treatment groups.

3.3. Biochemical Markers Analyses

Evaluation of bone turnover in all treatment groups at day 0 and 90, as reflected by the levels of OC, a formation marker, and urine DPD, a resorption marker, is presented in **Tables 3(D)** and **(E)**. When both markers were evaluated at baseline (*i.e*. day 0) prior to randomization, no statistical differences among the treatment groups were found (**Table 3(D)**). At day 90 (**Table 3(E)**), however, significantly higher DPD levels were observed in the OVX-control, OVX-gastrolith and OVX-citrate groups, compared to the sham group (p < 0.001), while the DPD levels in the OVX-ACC group were not statistically different than that of the sham group, yet were significantly lower than in the OVX-control and OVX-citrate groups. DPD levels in the OVX-gastrolith group were significantly lower than that of OVX-citrate group, yet were not different from that of the OVX-control or OVX-ACC group. Serum OC marker levels showed no significant difference across the various experimental groups.

3.4. Vertebral Mechanical Strength Analysis

Mechanical strength analysis of the 5th-lumbar vertebra exhibited significantly lower values, namely 40% for the OVX-control, OVX-gastrolith and OVX-citrate groups, on average, relative to the values measured with the sham group (**Figures 3A-C**; p < 0.01). At the same time, most of the mechanical strength values of the OVX-ACC group were lower only by an average of 17% than were the values determined for the sham group. Interestingly, the ultimate force parameter of the OVX-ACC group was not significantly different from that of the sham group. Within the OVX groups, the OVX-ACC group presented significantly higher values in comparison to all other OVX groups in every tested parameter, including ultimate force (**Figure 3A**), energy to ultimate force (**Figure 3B**) and toughness (**Figure 3C**).

3.5. Vertebral Topology and Orientation Parameters

Due to the notion that compression tests are not only affected by bone density but also by microarchitecture topology and orientation of the vertebral body [28-30], we further sought to determine trabecular topology and orientation parameters within the vertebral body of these rats. Micro-CT analysis of the 4th-lumbar vertebra showed that TBPf was lower in the sham compared to all OVX groups. Within the OVX groups, the TBPf of the OVX-ACC group was significantly lower than that of the OVX-control, OVX-citrate and OVX-gastrolith groups (**Figure 3D**; p < 0.001). SMI evaluation points to the same trend, being significantly lower in the sham group, compared to all OVX groups. Here, values obtained with the OVX-ACC group were significantly lower that what was measured for the OVX-control and OVX-citrate groups. At the same time, SMI values obtained with the OVX-ACC group were reduced relative to the index measured with the OVX-gastrolith group (**Figure 3E**; p < 0.001). The last micro-architectural parameter measured, namely DA, was lower in the sham group, compared to the OVX-control, OVX-gastrolith and OVX-citrate groups, by 22% on average. No significant differences in DA was observed between the sham and OVX-ACC groups, or among all OVX groups, although OVX-ACC showed lower values that measured with the other OVX groups (**Figure 3F**; p < 0.001).

4. DISCUSSION

The results of this study point on a significant prevention of OVX-induced bone loss by both natural ACC (*i.e*. gastrolith powder of crayfish origin) and synthetic ACC treatments compared to that of two commercially available calcium supplements (*i.e*. calcium carbonate and calcium citrate). A significant increase in morphometric bone parameters, studied by μCT and structural histomorphometry was observed for the gastrolith and ACC compared to CCC and calcium citrate treatments. Both the gastrolith and ACC treatments displayed increased bone formation rate as indicated by dynamic histomorphometry, compared to the other treatments, with no effect on serum OC levels. Intriguingly, urinary DPD levels suggested an anti-resorptive effect for the synthetic ACC compared to the other treatments, and this was also the only treatment that prevented the loss of vertebral mechanical strength following OVX, supported by microarchitectural analysis of topology and orientation parameters.

The results of this study are consistent with our basic hypothesis, that both natural and synthetic ACC have a beneficial effect on bone, derived from the peculiar thermodynamic properties of ACC in both its analytic and
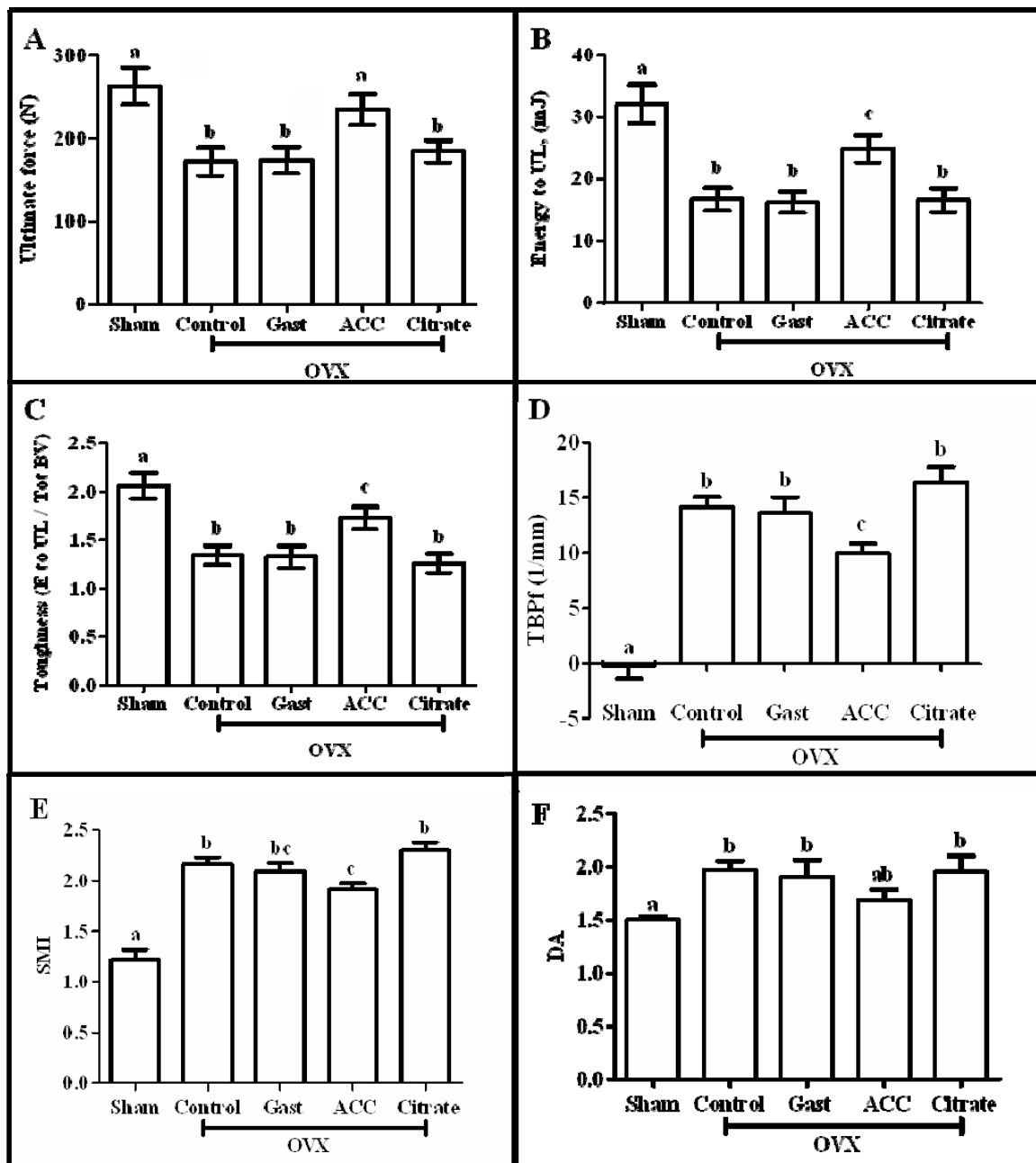
Figure 3. Lumbar vertebrae mechanical strength and microarchitectiral properties. (A) Ultimate Force; (B) Energy to ultimate force; (C) Toughness; (D) TBPf; (E) SMI; (F) DA. Sham-operated group treated with crystalline $CaCO_3$ (Sham). OVX-treated groups treated with: crystalline $CaCO_3$ (Control), gastrolith powder (Gast), amorphous calcium carbonate (ACC) or calcium citrate (Citrate). One-way ANOVA: $p < 0.01$. Letters represent Fisher's LSD post-hoc comparison. Bars represent SEM.

natural forms and further support our previous report, showing the enhanced bioavailability of synthetic stable ACC over that of CCC [20].

In the current study we observed a significant bone loss in the trabecular area of the distal femur, proximal tibia and vertebrae in all four OVX groups, relative to the sham-operated group, as reflected in almost all of the μCT and histomorphometry parameters considered. The lack of change in cortical BMD between the sham and any of the OVX groups is presumably due to the fact that this study is shorter than others who relied on longer periods and who reported cortical BMD changes [31]. The most significant bone loss was observed in the OVX-control and OVX-citrate groups, demonstrating the limited ability of currently available calcium supplements to prevent osteoporotic processes. In almost all of the

parameters evaluated in this study, no significant differences were observed upon treatment with either of these two supplements, supporting the observation that both calcium carbonate and calcium citrate, when administered with food, have similar absorption and effects on bone [2,3]. Interestingly, both the gastrolith and synthetic ACC treatments resulted in significantly less bone loss in the femur, tibia and vertebrae, compared to control or citrate treatments.

In most of the μCT-analyzed parameters of the distal femur and in the histo-morphometric analysis of the proximal tibia, the gastrolith presented a slightly enhanced preventative effect than did ACC. On the other hand, μCT analyses of the vertebrae revealed insignificant differences following the two treatments. The variability of bone loss rate induced by estrogen depletion in different bones is well documented [32], with the vertebrae being less affected than are the femur and tibia.

The use of gastrolith in traditional medicines dates back several hundreds of years [33]. Both gastrolith and synthetic ACC contain stable ACC; yet, the enhanced performance of the gastrolith observed in some of the μCT and histo-morphometric analyses reported here suggests that organic and inorganic components present in the gastrolith, other than calcium and carbonate, such as the indigestible oligosaccharide chitin in the scaffold, proteins, inorganic phosphate or other metabolites [19,21], may enhance the positive effect of the ACC mineral in this compound. Chitooligosaccharides converted from the derivative of chitin were previously suggested to enhance calcium absorption, suppress bone resorption and affect bone strength in OVX rats [34]. Nevertheless, in our previous report [20], such calcium absorption-related advantages were not observed upon treatment with ACC with chitosan. The nanometric particle size of ACC in both the gastrolith and synthetic ACC might also contribute to the increased bioavailability and mineral bone density observed, as was suggested in OVX mice and rat models [16,35]. However, further findings suggest that diet calcium levels rather than particle size are responsible for the effects of this mineral on bone density and mechanical properties [36].

The higher trabecular bone fraction measured in the OVX-gastrolith and OVX-ACC groups could be a result of reduced bone resorption, as reflected in urinary DPD marker levels in both groups. Previous reports of the effects of calcium supplements on urinary DPD markers following OVX were inconsistent [37,38]. In our study, a significant decrease in DPD levels, relative to those measured in the OVX-control group was only observed with the OVX-ACC group. In the OVX-gastrolith group, the degree of DPD reduction was not significant, relative to the OVX-control group, but only compared to the OVX-citrate group.

Calcium supplements are not thought to induce anabolic effects on bone [39]. Dynamic histomorphometric analysis of the proximal tibia trabecular bone revealed only a 13% increase of BFR/BS in the OVX-control and OVX-citrate groups compared to the sham, an effect that did not reach a statistical significance. BFR/BS is almost always elevated in OVX-control rats [25], yet there are studies showing no effect and even reduction of this parameter following OVX intervention [40,41]. Interestingly, both the OVX-gastrolith and OVX-ACC groups presented significant increased BFR/BS and MS/BS ratios compared to OVX-control rats, reflecting bone formation during bone remodeling [41,42]. The active mineralization that apparently transpires upon treatment with gastrolith and ACC can most probably be attributed to the mineral component of these substances, as well as by the presence of phosphoserine [19,21,43], previously reported to induce bone formation *in-vitro* and *in-vivo* [44]. Nevertheless, MAR and the OC serum marker levels following both gastrolith and the synthetic ACC treatments did not coincide with a possible anabolic effect, showing insignificant differences from the sham, OVX-control and OVX-citrate groups. Yet, a trend of reduction in the Sham and OVX-ACC group is seen in the serum OC.

In contrast to most of the morphometric data obtained in this study, treatment with the synthetic ACC resulted in significantly higher bone strength compared to all other OVX groups, including the OVX-gastrolith group, and in some of the parameters considered, such as ultimate force, even presented no statistical differences from the sham. Bone strength can only be partially predicted using BMD and morphometric parameters, and discrepancies between mechanical strength and parameters such as BMD and trabecular bone morphology were reported by others [45]. We, therefore, further analyzed specific topology and orientation microarchitectural parameters previously shown to be useful for the discovery of minimal modifications to the spongy lattice, affecting mechanical properties of bones [28]. Intriguingly, in contrast to the bone volume morphometric parameters and in good agreement with our mechanical strength findings, ACC had a beneficial effect on the topology and orientation parameters. Studies presenting changes in topology parameters with no effect on BV/TV were already reported by others [28], suggesting that the surface contour of the trabecular bone structure, plays a critical role in the loss of bone strength [29]. These results might propose a different mechanism of action for the gastrolith versus synthetic ACC that might be attributed to elements from the extracellular matrix of the gastrolith and that are absent in the synthetic ACC [19,21].

To conclude, our findings point to the superior effects of ACC-containing compounds over two of the most

common calcium supplements in terms of bone loss prevention. In particular, this study highlights the advantage of synthetic ACC with respect to mechanical bone strength. In future, the ability of ACC-containing compounds to reduce, prevent or even treat bone deterioration in human metabolic bone disorders should be tested.

REFERENCES

[1] Grossman, J.M. (2011) Osteoporosis prevention. *Current Opinion in Rheumatology*, **23**, 203-210. doi:10.1097/BOR.0b013e3283439426

[2] Straub, D.A. (2007) Calcium supplementation in clinical practice: A review of forms, doses, and indications. *Nutrition in Clinical Practice*, **22**, 286-296. doi:10.1177/0115426507022003286

[3] Weaver, C. and Heaney, R. (2006) Calcium in human health. *Nutrition Health*, Humana Press Inc., New Jersey, 450.

[4] Tang, B.M., Eslick, G.D., Nowson, C., Smith, C. and Bensoussan, A. (2007) Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: A meta-analysis. *Lancet*, **370**, 657-666. doi:10.1016/S0140-6736(07)61342-7

[5] Boonen, S., Wahl, D.A., Nauroy, L., Brandi, M.L., Bouxsein, M.L., Goldhahn, J., Lewiecki, E.M., Lyritis, G.P., Marsh, D., Obrant, K., Silverman, S., Siris, E. and Akesson, K. (2011) Balloon kyphoplasty and vertebroplasty in the management of vertebral compression fractures. *Osteoporosis International*, **22**, 2915-2934. doi:10.1007/s00198-011-1639-5

[6] Grant, A.M., Avenell, A., Campbell, M.K., McDonald, A.M., MacLennan, G.S., McPherson, G.C., Anderson, F.H., Cooper, C., Francis, R.M., Donaldson, C., Gillespie, W.J., Robinson, C.M., Torgerson, D.J. and Wallace, W.A. (2005) Oral vitamin D3 and calcium for secondary prevention of low-trauma fractures in elderly people (Randomised evaluation of calcium or vitamin D, RECORD): A randomised placebo-controlled trial. *Lancet*, **365**, 1621-1628. doi:10.1016/S0140-6736(05)63013-9

[7] Shea, B., Wells, G., Cranney, A., Zytaruk, N., Robinson, V., Griffith, L., Ortiz, Z., Peterson, J., Adachi, J., Tugwell, P. and Guyatt, G. (2002) Meta-analyses of therapies for postmenopausal osteoporosis. VII. Meta-analysis of calcium supplementation for the prevention of postmenopausal osteoporosis. *Endocrine Reviews*, **23**, 552-559. doi:10.1210/er.2001-7002

[8] Dawson-Hughes, B., Dallal, G.E., Krall, E.A., Sadowski, L., Sahyoun, N. and Tannenbaum, S. (1990) A controlled trial of the effect of calcium supplementation on bone density in postmenopausal women. *The New England Journal of Medicine*, **323**, 878-883. doi:10.1056/NEJM199009273231305

[9] Dawson-Hughes, B. (1991) Calcium supplementation and bone loss: A review of controlled clinical trials. *The American Journal of Clinical Nutrition*, **54**, 274S-280S.

[10] Dawson-Hughes, B., Harris, S.S., Krall, E.A. and Dallal, G.E. (1997) Effect of calcium and vitamin D supplementation on bone density in men and women 65 years of age or older. *The New England Journal of Medicine*, **337**, 670-676. doi:10.1056/NEJM199709043371003

[11] Bischoff-Ferrari, H.A., Dawson-Hughes, B., Baron, J.A., Burckhardt, P., Li, R., Spiegelman, D., Specker, B., Orav, J.E., Wong, J.B., Staehelin, H.B., O'Reilly, E., Kiel, D.P. and Willett, W.C. (2007) Calcium intake and hip fracture risk in men and women: A meta-analysis of prospective cohort studies and randomized controlled trials. *The American Journal of Clinical Nutrition*, **86**, 1780-1790.

[12] Jackson, R.D., LaCroix, A.Z., Gass, M., Wallace, R.B., Robbins, J., Lewis, C.E., Bassford, T., Beresford, S.A., Black, H.R., Blanchette, P., Bonds, D.E., Brunner, R.L., Brzyski, R.G., Caan, B., Cauley, J.A., Chlebowski, R.T., Cummings, S.R., Granek, I., Hays, J., Heiss, G., Hendrix, S.L., Howard, B.V., Hsia, J., Hubbell, F.A., Johnson, K.C., Judd, H., Kotchen, J.M., Kuller, L.H., Langer, R.D., Lasser, N.L., Limacher, M.C., Ludlam, S., Manson, J.E., Margolis, K.L., McGowan, J., Ockene, J.K., O'Sullivan, M.J., Phillips, L., Prentice, R.L., Sarto, G.E., Stefanick, M.L., Van Horn, L., Wactawski-Wende, J., Whitlock, E., Anderson, G.L., Assaf, A.R. and Barad, D. (2006) Calcium plus vitamin D supplementation and the risk of fractures. *The New England Journal of Medicine*, **354**, 669-683. doi:10.1056/NEJMoa055218

[13] Porthouse, J., Cockayne, S., King, C., Saxon, L., Steele, E., Aspray, T., Baverstock, M., Birks, Y., Dumville, J., Francis, R., Iglesias, C., Puffer, S., Sutcliffe, A., Watt, I. and Torgerson, D.J. (2005) Randomised controlled trial of calcium and supplementation with cholecalciferol (vitamin D3) for prevention of fractures in primary care. *British Medical Journal*, **330**, 1003. doi:10.1136/bmj.330.7498.1003

[14] Nebel, H., Neumann, M., Mayer, C. and Epple, M. (2008) On the structure of amorphous calcium carbonate—A detailed study by solid-state NMR spectroscopy. *Inorganic Chemistry*, **47**, 7874-7879. doi:10.1021/ic8007409

[15] Donners, J.J.J.M., Heywood, B.R. and Sommerdijk, N.A. J.M. (2000) Amorphous calcium carbonate stabilised by poly(propylene imine) dendrimers. *Chemical Communications*, 1937-1938. doi:10.1039/b004867o

[16] Huang, S.C., Naka, K. and Chujo, Y. (2007) A carbonate controlled-addition method for amorphous calcium carbonate spheres stabilized by poly(acrylic acid)s. *Langmuir*, **23**, 12086-12095. doi:10.1021/la701972n

[17] Guillemet, B., Faatz, M., Grohn, F., Wegner, G. and Gnanou, Y. (2006) Nanosized amorphous calcium carbonate stabilized by poly(ethylene oxide)-b-poly(acrylic acid) block copolymers. *Langmuir*, **22**, 1875-1879. doi:10.1021/la052419e

[18] Shechter, A., Berman, A., Singer, A., Freiman, A., Grinstein, M., Erez, J., Aflalo, E.D. and Sagi, A. (2008) Reciprocal changes in calcification of the gastrolith and cuticle during the molt cycle of the red claw crayfish *Cherax quadricarinatus*. *Biological Bulletin*, **214**, 122-134. doi:10.2307/25066669

[19] Bentov, S., Weil, S., Glazer, L., Sagi, A. and Berman, A. (2010) Stabilization of amorphous calcium carbonate by phosphate rich organic matrix proteins and by single

phosphoamino acids. *Journal of Structural Biology*, **171**, 207-215. doi:10.1016/j.jsb.2010.04.007

[20] Meiron, O.E., Bar-David, E., Aflalo, E.D., Shechter, A., Stepensky, D., Berman, A. and Sagi, A. (2011) Solubility and bioavailability of stabilized amorphous calcium carbonate. *Journal of Bone and Mineral Research*, **26**, 364-372. doi:10.1002/jbmr.196

[21] Glazer, L. and Sagi, A. (2012) On the involvement of proteins in the assembly of the crayfish gastrolith extracellular matrix. *Invertebrate Reproduction & Development*, **56**, 57-65. doi:10.1080/07924259.2011.588010

[22] Welch, J.M., Turner, C.H., Devareddy, L., Arjmandi, B.H. and Weaver, C.M. (2008) High impact exercise is more beneficial than dietary calcium for building bone strength in the growing rat skeleton. *Bone*, **42**, 660-668. doi:10.1016/j.bone.2007.12.220

[23] Lasota, A. and Danowska-Klonowska, D. (2004) Experimental osteoporosis—Different methods of ovariectomy in female white rats. *Roczniki Akademii Medycznej Bialymstoku*, **49**, 129-131.

[24] Ogawa, K., Hori, M., Takao, R. and Sakurada, T. (2005) Effects of combined elcatonin and alendronate treatment on the architecture and strength of bone in ovariectomized rats. *Journal of Bone and Mineral Metabolism*, **23**, 351-358. doi:10.1007/s00774-005-0612-9

[25] Shahnazari, M., Yao, W., Wang, B., Panganiban, B., Ritchie, R.O., Hagar, Y. and Lane, N.E. (2011) Differential maintenance of cortical and cancellous bone strength following discontinuation of bone-active agents. *Journal of Bone and Mineral Research*, **26**, 569-581. doi:10.1002/jbmr.249

[26] Trivedi, R., Kumar, A., Gupta, V., Kumar, S., Nagar, G.K., Romero, J.R., Dwivedi, A.K. and Chattopadhyay, N. (2009) Effects of Egb 761 on bone mineral density, bone microstructure, and osteoblast function: Possible roles of quercetin and kaempferol. *Molecular and Cellular Endocrinology*, **302**, 86-91. doi:10.1016/j.mce.2009.01.011

[27] Turner, C.H. and Burr, D.B. (1993) Basic biomechanical measurements of bone: A tutorial. *Bone*, **14**, 595-608. doi:10.1016/8756-3282(93)90081-K

[28] Hahn, M., Vogel, M., Pompesius-Kempa, M. and Delling, G. (1992) Trabecular bone pattern factor—A new parameter for simple quantification of bone microarchitecture. *Bone*, **13**, 327-330. doi:10.1016/8756-3282(92)90078-B

[29] Ikeda, S., Tsurukami, H., Ito, M., Sakai, A., Sakata, T., Nishida, S., Takeda, S., Shiraishi, A. and Nakamura, T. (2001) Effect of trabecular bone contour on ultimate strength of lumbar vertebra after bilateral ovariectomy in rats. *Bone*, **28**, 625-633. doi:10.1016/S8756-3282(01)00462-8

[30] Odgaard, A. (1997) Three-dimensional methods for quantification of cancellous bone architecture. *Bone*, **20**, 315-328. doi:10.1016/S8756-3282(97)00007-0

[31] Lelovas, P.P., Xanthos, T.T., Thoma, S.E., Lyritis, G.P. and Dontas, I.A. (2008) The laboratory rat as an animal model for osteoporosis research. *Comparative Medicine*, **58**, 424-430.

[32] Komm, B.S., Vlasseros, F., Samadfam, R., Chouinard, L.

and Smith, S.Y. (2011) Skeletal effects of bazedoxifene paired with conjugated estrogens in ovariectomized rats. *Bone*, **49**, 376-386. doi:10.1016/j.bone.2011.05.024

[33] Huxley, T.H. (1880) The crayfish. D. Appleton and Company, Cambridge.

[34] Jung, W.K., Moon, S.H. and Kim, S.K. (2006) Effect of chitooligosaccharides on calcium bioavailability and bone strength in ovariectomized rats. *Life Sciences*, **78**, 970-976. doi:10.1016/j.lfs.2005.06.006

[35] Park, H.S., Ahn, J. and Kwak, H.S. (2008) Effect of nano-calcium-enriched milk on calcium metabolism in ovariectomized rats. *Journal of Medicinal Food*, **11**, 454-459. doi:10.1089/jmf.2007.0086

[36] Shahnazari, M., Martin, B.R., Legette, L.L., Lachcik, P.J., Welch, J. and Weaver, C.M. (2009) Diet calcium level but not calcium supplement particle size affects bone density and mechanical properties in ovariectomized rats. *The Journal of Nutrition*, **139**, 1308-1314. doi:10.3945/jn.108.101071

[37] Koshihara, M., Masuyama, R., Uehara, M. and Suzuki, K. (2004) Effect of dietary calcium: Phosphorus ratio on bone mineralization and intestinal calcium absorption in ovariectomized rats. *Biofactors*, **22**, 39-42. doi:10.1002/biof.5520220107

[38] Shiraishi, A., Ito, M., Hayakawa, N., Kubota, N., Kubodera, N. and Ogata, E. (2006) Calcium supplementation does not reproduce the pharmacological efficacy of alfacalcidol for the treatment of osteoporosis in rats. *Calcified Tissue International*, **78**, 152-161. doi:10.1007/s00223-005-0014-y

[39] Erben, R.G., Bromm, S. and Stangassinger, M. (1998) Therapeutic efficacy of 1alpha,25-dihydroxyvitamin D3 and calcium in osteopenic ovariectomized rats: evidence for a direct anabolic effect of 1alpha,25-dihydroxyvitamin D3 on bone. *Endocrinology*, **139**, 4319-4328. doi:10.1210/en.139.10.4319

[40] Dai, R., Ma, Y., Sheng, Z., Jin, Y., Zhang, Y., Fang, L., Fan, H. and Liao, E. (2008) Effects of genistein on vertebral trabecular bone microstructure, bone mineral density, microcracks, osteocyte density, and bone strength in ovariectomized rats. *Journal of Bone and Mineral Metabolism*, **26**, 342-349. doi:10.1007/s00774-007-0830-4

[41] Shoji, K., Elsubeihi, E.S. and Heersche, J.N. (2011) Effects of ovariectomy on turnover of alveolar bone in the healed extraction socket in rat edentulous mandible. *Archives of Oral Biology*, **56**, 114-120. doi:10.1016/j.archoralbio.2010.09.013

[42] Parfitt, A.M., Drezner, M.K., Glorieux, F.H., Kanis, J.A., Malluche, H., Meunier, P.J., Ott, S.M. and Recker, R.R. (1987) Bone histomorphometry: Standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *Journal of Bone and Mineral Research*, **2**, 595-610. doi:10.1002/jbmr.5650020617

[43] Sato, A., Nagasaka, S., Furihata, K., Nagata, S., Arai, I., Saruwatari, K., Kogure, T., Sakuda, S. and Nagasawa, H. (2011) Glycolytic intermediates induce amorphous calcium carbonate formation in crustaceans. *Nature Chemical Biology*, **7**, 197-199. doi:10.1038/nchembio.532

[44] Park, J.W., Kim, Y.J., Jang, J.H. and An, C.H. (2011) MC3T3-E1 cell differentiation and *in vivo* bone formation induced by phosphoserine. *Biotechnology Letters*, **33**, 1473-1480. doi:10.1007/s10529-011-0565-0

[45] Goldstein, S.A., Goulet, R. and McCubbrey, D. (1993) Measurement and significance of three-dimensional architecture to the mechanical integrity of trabecular bone. *Calcified Tissue International*, **53**, S127-S132.