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Biochemical and Histological Assessment of Alkali Therapy During High Animal Protein Intake in the Rat^A

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Introduction

Osteoporosis is a major public health problem in the Western World and is gaining attention in the developing world [1]. Although the role of adequate nutritional calcium and vitamin D on bone health has been extensively studied, there is growing awareness that other nutrients also have effects on the balance between bone formation and resorption. For example, the typical Western diet that is high in animal protein generates a daily acid load due to the oxidation of sulfur-containing amino acids to sulfuric acid [2]. To maintain acid-base balance, prior to excretion of the acid load by the kidney, it must be buffered to protect plasma pH. An increase in bone resorption is one possible buffering mechanism [3]. *In vitro* studies have shown that under culture conditions simulating metabolic acidosis, there is a net calcium efflux from bone secondary to increased osteoclast and decreased osteoblast activities [4,5]. Even a small pH decrease (7.25 to 7.15) results in a 6-fold increase in the number of resorption pits [6]. We have previously demonstrated in humans that a diet high in animal protein significantly increases urinary net acid and calcium excretion, while decreasing citrate excretion, and in an animal model of dietary protein excess, changes in urinary parameters are associated with a marked increase in bone resorption on histomorphometric analysis [7,8].

Dietary alkali is a potentially effective mechanism to buffer metabolically-generated acid, without impacting bone. *In vitro*, simulated metabolic alkalosis (increased medium pH and bicarbonate) decreases bone calcium efflux by suppressing osteoclasts while stimulating osteoblastic type I collagen production [9]. In clinical studies, potassium bicarbonate administration improved calcium balance in a small number of postmenopausal women with only 18 days of treatment [10]; similar findings have been observed with potassium citrate [11–14]. Other population-based studies have demonstrated beneficial effects of diets rich in fruits and vegetables, indicative of potassium alkali intake, as assessed from changes in biochemical markers of bone turnover, BMD, or both [15,16]. To our knowledge, there has been no direct assessment of the effect of alkali on bone turnover via bone histomorphometry

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under conditions of high dietary animal protein intake. Therefore, we undertook an assessment of the role of potassium alkali therapy on bone physiology by performing both static and dynamic bone histomorphometry studies in rats following 8 weeks of either a low or high animal protein diet, with or without supplemental alkali treatment.

Materials and Methods

Experimental protocol

Thirty-six male Sprague-Dawley rats, (8 weeks old, average weight 272 ± 6 g) (Charles River, Boston, MA, USA) were randomized into 4 groups ($n=9$ per group). Two groups were randomized to either a low casein (138 g casein/kg chow) (LC) or high casein (552 g casein/kg chow) (HC) diet x 8 wks. The diets were matched for Na^+ , K^+ , phosphorus, Ca^{+2} , and Mg^{+2} . The major difference in the two diets was in the sulfur content (1.57 g per kg of low casein chow and 3.34 g per kg of high casein chow). Each of the two groups was further randomized for dietary supplementation with either KCl (LC-KCl and HC-KCl, 4 mEq K per day) or KCitrate (LC-KCit and HC-KCit, 4 mEq K per day). All animals were given distilled water ad lib for the duration of the study. One rat from the HC-KCit group was dropped because of failure to eat and consequently a slow decline in body weight. Thus, $n=8$ for that group.

Animals were initially housed in large shoebox cages, but moved to individual metabolic cages for at least one week prior to sacrifice. During the last three days of the study, three 24-hour urine collections were done for each animal. The urine was collected under mineral oil and thymol crystals placed in the urine container to prevent bacterial overgrowth. After analysis of the three urine collections, the values were averaged and reported as an $n=1$ for each rat. The last day of the study, rats were anesthetized and blood obtained from the aorta for biochemical and blood gas analysis. All studies were approved by the UT Southwestern Institutional Animal Care and Use Committee.

Harvesting of bone

All rats received two intraperitoneal tetracycline injections (30 mg/kg body weight) either 8 or 10 days apart and 4–7 days before sacrifice. On day 56 at sacrifice, femora were harvested and stored in 70% ethanol for bone histomorphometric analysis.

Analytical methods

Blood gases were measured using a Radiometer America, Inc. Model ABL5 blood gas machine (Westlake, OH). Urinary sodium and potassium concentrations were measured by flame photometry, and calcium and magnesium concentrations by atomic absorption spectrophotometry. Phosphorus, creatinine, ammonium and citrate concentrations were measured by autoanalyzer (Cobas MIRA, Roche Diagnostics, Inc., Montclair, NJ, USA). Oxalate and sulfate concentrations were assayed by ion chromatography (Dionex 2001, Dionex, Sunnydale, CA, USA). Titratable acidity was determined by titrating the urine sample to pH 7.4 with 0.1 N NaOH. Serum chemistries were assayed on the Roche autoanalyzer (Indianapolis, IN). Net acid excretion was obtained by calculation as follows: [titratable acidity + ammonium] – [bicarbonate + citrate $^{2-/3-}$]. The bicarbonate concentration was calculated using the Henderson-Hasselbach equation from the measured urine pH and pCO_2 . Urinary citrate is included in this calculation because citrate $^{2-/3-}$ is a legitimate and significant urinary base [17]. All urine parameters are normalized to creatinine to correct for any incomplete 24-hour urine collections.

Histomorphometric analysis

Bone histomorphometry was performed on the distal metaphysis of the left femur from each rat after double tetracycline labeling. After removing the entire femur, the distal half was placed in Villanueva osteochrome (Polysciences, Warrington, PA, USA) for 72 h. The specimen was then dehydrated in a graded series of alcohol, and processed undecalcified in methylmethacrylate as previously described [8,18]. Ten μm sections were obtained in the longitudinal plane using a Reichert Polycut E microtome (Leica Microsystems, Wetzlar, Germany). Histomorphometric examination was performed via computer monitor on images captured from an Aus Jena microscope equipped with an Optronics video camera (Galeta, CA). Using bone histomorphometry software (Bioquant Nova II, R & M Biometrics, Nashville, TN, USA) permitted quantitation of areas, lengths, and individual distances. Static measurements of cortical and trabecular thickness, cancellous bone volume, osteoid indices and cellular parameters were performed on toluidine-blue stained sections. Trabecular number/mm was calculated as the product of $(\text{BV/TV}) \times (10/\text{Tb.Th})$ according to Parfitt et al. [19]. Measurements of cancellous bone volume were taken at a distance of at least 1 mm from the growth plate to prevent inclusion of primary spongiosa. Fluorochrome-based indices of bone formation were measured in unstained sections. The terminology used to define the measured parameters is that recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [20].

Statistical analysis

Data are expressed as the mean $\pm$ SEM. One-way analysis of variance (ANOVA) with multiple comparisons analysis was used to determine significant differences between the four experimental groups (Systat Software, San Jose, CA).

Results

Body weight and food intake

Table 1 summarizes the initial, ending, and change in body weight and food intake for the 4 diet regimens. There are no differences in any of these parameters between the 4 dietary groups.

Serum chemistry

Serum biochemistries and arterial blood gases are summarized in Table 2. All values, except for blood urea nitrogen (BUN) are within normal limits and not significantly different between the 4 groups. Statistical analysis disclosed a significant dietary effect on serum BUN and, thus, the BUN/creatinine ratio. The higher BUN in the rats consuming the high casein diet is a direct reflection of the increased dietary nitrogen load.

Urine chemistry

Table 3 summarizes the urine biochemistry data for the 4 study groups. All values are normalized to creatinine to control for any incomplete 24 h urine collections. Mean values for Na, K, Cl, uric acid, phosphorus, magnesium, and oxalate are not significantly different among the 4 study groups. Urine SO_4^{2-} /creatinine excretion is significantly increased in both HC groups, reflective of the higher animal protein content of the diet. Urine Ca/creatinine is significantly higher in the HC-KCl group, as is ammonium and net acid excretion. Addition of alkali to the low casein diet (LC-KCit) significantly increases citrate and lowers ammonium excretion. Addition of alkali to the high casein diet (HC-KCit) returns calcium, citrate, ammonium, and NAE to values not different than observed in the LC-KCl group. Neither urine pH or titratable acid/creatinine excretion are significantly different between the 4 groups, although both parameters change in the direction that reflects a higher acid load in the HC-KCl

group (lower urine pH and higher TTA/Cr) and higher base load in the LC-KCIt group (higher urine pH and lower TTA/Cr).

Bone histomorphometry

Table 4 summarizes the bone histomorphometric findings. There is no significant effect of diet or the supplement on cortical, trabecular, or osteoid thickness, the percent of osteoid volume/total bone volume, osteoid surface/total bone surface, osteoblast surface/total bone surface, or mineral apposition rate. However, the HC-KCl diet increases the percent of eroded surface/total bone surface 2.5-fold and increases osteoclast surface/total bone surface 3.5-fold, effects that are significantly different from the other 3 groups (LC-KCl, LC-KCIt, and HC-KCIt). In addition, the percent of mineralized surface/total bone surface and bone formation rate are significantly different between the LC-KCl and HC-KCL groups, but neither is significantly different from either of the alkali treated groups. Lastly, the percent of double-labeled surface/total bone surface in the LC-KCl group is significantly lower than in either the HC-KCl or LC-KCIt group. Alkali addition to the low casein diet increases the percent of tetracycline labeled bone while alkali addition to the high casein diet decreases tetracycline labeling back toward baseline levels.

Discussion

The present studies demonstrate that a high animal protein diet that generates a substantial acid load increases the percentage of eroded bone, osteoclast, mineralized, and double-labeled surfaces/total cancellous bone surface, and the bone formation rate, and decreases the percentage of cancellous bone volume/total bone volume, all parameters demonstrating increased bone turnover. Addition of dietary alkali sufficient to neutralize about 75% of the dietary acid load returns all of the above bone parameters toward normal, with none being statistically different than when a low animal protein diet is ingested. Supplementing a low animal protein diet with alkali increases the percent of tetracycline labeled surface. Thus, in the presence of a low animal protein diet, alkali stimulates bone mineralization, while in the presence of a high animal protein diet it minimizes the stimulatory effect of the acid load.

The typical 'high animal protein' Western diet is proposed as a risk factor for osteoporosis, possibly secondary to the need to buffer the acid generated by animal protein metabolism. When compared to our ancestral diet, the current Western diet imposes an acid load on the body and an accompanying decrease in potassium and organic anion (alkali) intake [21,22]. Previous studies in rat models of high protein intake have demonstrated mixed results. Chronic acid loads lead to decreased bone calcium or 'wet weight' in some [23,24] but not all animal studies [25–29]. More recently, a high protein diet in rats did not increase urinary pyridinoline cross-link excretion, a marker of increased bone resorption [30]. In a similar study, rats treated for up to 21 months with a high protein diet (26%) have no differences in femoral bone mineral density, biomechanical properties, or bone biochemical turnover markers from rats on a normal protein diet (13%) [31]. In contrast, Amanzadeh et al. [8] observed hypercalciuria, hypocitraturia, and bone loss by histomorphometry in rats fed a 48% casein diet during 8 weeks of treatment. Differences in study design such as type and amount of protein given, length of treatment, and age of the rats at initiation of therapy may explain some of the different results between these studies. The adverse skeletal effect of acidogenic diets is further supported from studies of alkali supplementation. Neutralization of diet-induced acid loads during short-term oral intake of alkali salts reverses bone loss, as suggested by changes in biochemical markers of bone turnover, in both postmenopausal women and healthy young adults [10–14,32–34]. However, there has been only one previous study describing the specific effects of alkali intake on the skeleton in rodents [31]. In that study, potassium citrate reversed the acid-base abnormalities associated with a high protein diet but had no effect on femoral bone mineral

density, biomechanical properties of bone, and bone biochemical turnover markers. Bone histomorphometry was not performed in that study. Although the treatment period in that study was long enough to observe any potential skeletal changes, the casein content of the high protein diet was approximately one-half of that used in the present study and may have been insufficient for use in a rodent model.

Using a high animal protein diet that we have shown previously to deliver a substantial acid load without inducing a measurable acidemia [8], urinary calcium increases significantly compared to animals ingesting a low animal protein diet. A major source of acid-induced increases in urinary calcium is increased bone resorption [7,8,35,36]. Increased bone resorption is supported by the histomorphometric data in the current study. Cancellous bone volume decreases significantly in rats consuming the HC-KCl diet, as does trabecular number, likely due to increased bone turnover as confirmed by significant increases in the indices of both bone resorption (ES/BS and Oc.S/BS) and bone formation (MS/BS, dLS/BS, and BFR). Osteoid parameters were not significantly different between groups. In addition, osteoid surface did not change and is less than the tetracycline labeled surfaces for all four treatment groups, a situation not normally encountered during normal bone turnover. We do not know the reason for this discrepancy. We have ruled out failure to identify toluidine-blue stained osteoid since additional sections of bone stained by the Masson-Goldner trichrome method give nearly identical results for osteoid surface (data not presented). Because these rats were tetracycline naïve until labeling for bone histomorphometry, and because tetracycline localization in bone is very objective, we feel confident that the values for mineralized surfaces are correct.

Although the majority of studies support increases in bone resorption as the most likely mechanism for high animal protein induced hypercalciuria, intestinal and renal factors may also contribute to the hypercalciuria [27–29,37,38]. The renal contribution to the hypercalciuria caused by the high animal protein diet is probably secondary to direct gating of TRPV5 and calbindin-D_{28K} in the renal distal tubule [39–42]. On the other hand, the mechanism of high protein-induced increases in intestinal calcium absorption is not known [43,44]. Although our results are best explained by a skeletal contribution to the observed hypercalciuria, some studies in rodents during high protein feeding are consistent with increased intestinal calcium absorption as a major contributor to hypercalciuria [27–29].

Administration of alkali as KCit returns most of the HC-induced changes in bone turnover and mineral homeostasis close to baseline. Although KCit did not significantly affect serum parameters, alkali administration significantly attenuates the HC-induced increases in urinary ammonium and net acid excretion. KCit also attenuates the hypercalciuric response to the HC diet. Overall, the most marked effect of alkali administration is on bone histomorphometry. HC-KCit animals have preserved cancellous bone volume and trabecular number, reduced bone resorption, and decreased rates of bone formation compared to the HC-KCl group. As none of the HC-KCit bone histomorphometric parameters are significantly different from the LC-KCit group, it is clear that dietary KCit supplementation, sufficient to reduce the HC-induced increase in ammonium excretion by 73% and net acid excretion by 81%, is effective in neutralizing the increased dietary acid load enough to relieve the need for bone buffering.

To our knowledge, this is the first detailed examination utilizing bone histomorphometry in rats of the effects of alkali therapy on bone during high animal protein intake. A previous clinical study reported on bone histological assessments after correction of distal renal tubular acidosis (dRTA) with potassium citrate [45]. However, baseline bone physiology differs from the current investigation. In the dRTA study, the subjects displayed a bone histological picture more consistent with osteomalacia and defective bone mineralization prior to treatment. Thus, unlike the current high animal protein model, bone turnover was not increased prior to treatment with alkali therapy. Yet alkali therapy produced a significant increase in mineral apposition

rate and bone formation rate with no change in bone resorption parameters, suggesting that the alkali therapy selectively stimulated bone formation in the setting of normal bone turnover. This observation is consistent with the effect of dietary alkali supplementation in the low animal protein group in the present study, where double-labeled tetracycline surface is significantly increased compared to the LC-KCl group.

The signaling mechanism(s) mediating the effect of acid on bone is(are) not entirely known. Acutely, the physicochemical dissolution of bone to liberate calcium, phosphate, and carbonate could serve to attenuate the effect of the acid load on whole body acid-base homeostasis. Under chronic conditions acid-induced stimulation of bone resorption is associated with cyclooxygenase 2 (COX-2) mediated prostaglandin E₂ synthesis [46], leading to activation of the RANK/RANKL signaling pathway and increases in osteoclast numbers and activity [47, 48]. More recently, identification of acid-sensing ion channels in osteoclasts, as well as in osteoblasts [49], provides another potential mechanism by which bone can sense and respond to differences in extracellular pH. A role for the non-receptor tyrosine kinases Pyk2 and c-Src in bone resorption has been recently shown, where it was demonstrated that Pyk2 kinase is required for formation of the actin ring at the adhesion site where osteoclasts attach to bone and for normal bone resorption [50]. c-Src kinase is also required, but alone is not sufficient to mediate ring formation and bone resorption.

An unresolved physiological question is how cells respond to acid loads in the absence of changes in extracellular pH, as is the situation when high animal protein diets are ingested. In the renal proximal tubule, the response to an acid load is mediated by changes in intracellular, and not extracellular pH [51]. In this system, Pyk2 has been shown to be a pH-sensor with kinase activity increasing in response to physiological decreases in pH and being required for acid-activation of c-Src kinase [52]. It is possible that this acid-activated pathway is involved in mediating the effects of acid on bone resorption and turnover also. Additional studies are required to determine if one or more of these mechanisms contribute to the increased bone turnover observed during high animal protein intake.

In summary, utilizing an animal model of a typical Western diet (high animal protein intake), we have shown that the major effect of dietary acid on the skeleton is an increase in bone resorption and commensurate increase in bone formation activity. Over a lifetime, this acid-induced high turnover of bone may contribute to age-related loss of bone mass. Alkali addition to the diet attenuates these acidosis-induced changes by preventing the increase in bone resorption with little or no effect on bone formation. These findings may offer an explanation for epidemiological studies that have observed increases in bone mineral density in subjects with the greatest dietary alkali intake.

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Table 1
Beginning and ending body weight, average weight gain and average food consumption.

Parameter	LC-KCl (n=9)	HC-KCl (n=9)	LC-KCit (n=9)	HC-KCit (n=8)	ANOVA
Body Weight, g Beginning of Study	267 ± 11	272 ± 12	275 ± 13	272 ± 14	NS
Body Weight, g End of Study	355 ± 8	358 ± 6	355 ± 4	343 ± 6	NS
Average Weight Gain, g	87 ± 11	85 ± 9	80 ± 11	71 ± 13	NS
Average Food Consumption, g/d	14.8 ± 0.8	14.0 ± 0.9	13.4 ± 0.6	13.1 ± 0.7	NS

LC = low casein diet, HC = high casein diet, KCl = potassium chloride, KCit = potassium citrate. NS = not significant by one way ANOVA.

Table 2
Serum parameters in rats after 8 weeks of either a low or high casein intake and KCl or KCitrate.

Parameter	LC-KCl (n=9)	HC-KCl (n=9)	LC-KCit (n=9)	HC-KCit (n=8)	ANOVA
Na, mEq/L	137 ± 3	138 ± 1	140 ± 1	139 ± 1	NS
K, mEq/L	4.3 ± 0.3	5.0 ± 0.3	4.6 ± 0.3	4.9 ± 0.4	NS
Cl, mEq/L	105 ± 2	105 ± 1	107 ± 1	106 ± 1	NS
CO ₂ , mEq/L	21 ± 2	24 ± 1	23 ± 1	25 ± 1	NS
Anion gap	14.5 ± 0.8	14.4 ± 0.8	15.6 ± 1.1	13.8 ± 1.3	NS
Ca, mg/dL	9.2 ± 0.1	9.3 ± 0.2	9.7 ± 0.2	9.6 ± 0.1	NS
P, mg/dL*	5.6 ± 0.5	5.7 ± 0.4	6.8 ± 0.1	6.9 ± 0.3	NS
BUN, mg/dL	12 ± 1	30 ± 2 ^a	13 ± 1	30 ± 1 ^a	^a p<10 ⁻⁹ vs LC groups
Creatinine, mg/dL	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	NS
BUN/Creatinine	24.8 ± 2.1	67.8 ± 5.4 ^a	26 ± 1.6	61.2 ± 3.8 ^a	^a p<10 ⁻⁸ vs LC groups
pH*	7.36 ± 0.02	7.38 ± 0.01	7.33 ± 0.02	7.32 ± 0.01	NS
pCO ₂ , mmHg*	42 ± 2	44 ± 2	48 ± 8	46 ± 6	NS
HCO ₃ , mEq/L*	23 ± 2	25 ± 1	24 ± 3	23 ± 3	NS

NS = not significant by one way ANOVA.

* N=3 for these serum parameters.

Table 3
Urine biochemistries in rats after 8 weeks of either a low or high casein intake and KCl or KClCitrate.

Parameter	LC-KCl (n=9)	HC-KCl (n=9)	LC-KCit (n=9)	HC-KCit (n=8)	ANOVA
pH	6.2 ± 0.1	6.1 ± 0.1	6.4 ± 0.2	6.3 ± 0.1	NS
Na ⁺ /Creatinine (μmol/mg)	65 ± 9	67 ± 9	45 ± 14	67 ± 21	NS
K ⁺ /Creatinine (μmol/mg)	117 ± 17	136 ± 13	107 ± 12	134 ± 26	NS
Cl ⁻ /Creatinine (μmol/mg)	161 ± 22	185 ± 26	103 ± 30	124 ± 26	NS
Mg ²⁺ /Creatinine (μmol/mg)	5.6 ± 2.3	6.9 ± 1.0	4.8 ± 2.2	8.2 ± 0.5	NS
Ca ²⁺ /Creatinine (μmol/mg)	3.4 ± 0.4 n=9	10.1 ± 1.4 ^a n=9	2.8 ± 0.5 n=9	4.3 ± 1.2 N=8	^a p<0.001 vs other groups
Phos/Creatinine (μmol/mg)	48 ± 9	41 ± 4	34 ± 11	41 ± 1	NS
Uric acid/Creatinine (μmol/mg)	1.5 ± 0.2	1.5 ± 0.1	1.4 ± 0.1	1.7 ± 0.2	NS
Oxalate/Creatinine (μmol/mg)	0.31 ± 0.03	0.21 ± 0.01	0.26 ± 0.03	0.32 ± 0.02	NS
Citrate/Creatinine (μmol/mg)	4.7 ± 1.0 n=9	1.5 ± 0.4 n=9	12.9 ± 2.8 ^a n=9	4.5 ± 1.3 n=8	^a p<0.01 vs other groups
NH ₄ ⁺ /Creatinine (μmol/mg)	102 ± 6	210 ± 2 ^a	59 ± 18 ^b	127 ± 14	^a p<0.01 vs other groups; ^b p<0.05 vs HC KCit
SO ₄ ²⁻ /Creatinine (μmole/mg)	15.6 ± 2.5	52.1 ± 1.3 ^c	18.1 ± 4.4	56.1 ± 10.6 ^c	^c p<0.05 vs LC groups
TTA/Creatinine (μmol/mg)	26 ± 6	29 ± 4	16 ± 6	23 ± 2	NS
NAE/Creatinine (μmol/mg)	124 ± 5	232 ± 16 ^a	66 ± 20 ^a	144 ± 6	^a p<0.01 vs other groups;

NS = not significant by one way ANOVA. TTA: Total titratable acidity; NAE: Net acid excretion. N = 3 per group unless otherwise noted. Conversions: μmol = μEq for solutes with a valence =1 (Na, K, Cl, NH₄, TTA, and NAE); (μmol × MW = μg (MW = Ca: 40, Phos: 31, Mg: 24, Citrate⁻³: 189, Oxalate⁻²: 88, Uric Acid: 168).

Bone histomorphometry in rats after 8 weeks of either a low or high casein intake and KCl or KCltrate.

Table 4

Parameter	LC-KCl (n=9)	HC-KCl (n=9)	LC-KCl (n=9)	HC-KCl (n=9)	ANOVA
C.Th, μm	436 $\pm$ 60	282 $\pm$ 46	327 $\pm$ 33	341 $\pm$ 62	NS
BV/TV, %	19.3 $\pm$ 2.9	10.1 $\pm$ 1.7 ^a	13.3 $\pm$ 1.5	18.4 $\pm$ 2.4	^a p<0.05 vs LC-KCl
Tb.Th, μm	49 $\pm$ 3	44 $\pm$ 2	48 $\pm$ 2	48 $\pm$ 2	NS
Tb.N, mm^{-1}	3.9 $\pm$ 0.5	2.2 $\pm$ 0.3 ^a	2.8 $\pm$ 0.3	3.7 $\pm$ 0.4	^a p<0.02 vs LC-KCl
OV/BV, %	0.34 $\pm$ 0.11	0.27 $\pm$ 0.03	0.17 $\pm$ 0.04	0.39 $\pm$ 0.15	NS
O.Th, μm	2.1 $\pm$ 0.1	2.1 $\pm$ 0.2	2.0 $\pm$ 0.1	2.2 $\pm$ 0.3	NS
OS/BS, %	5.4 $\pm$ 1.0	4.2 $\pm$ 0.7	4.2 $\pm$ 0.4	4.5 $\pm$ 0.5	NS
Ob.S/BS, %	4.3 $\pm$ 0.7	3.0 $\pm$ 0.5	2.7 $\pm$ 0.5	3.3 $\pm$ 0.4	NS
ES/BS, %	6.0 $\pm$ 0.7	14.9 $\pm$ 1.3 ^b	9.1 $\pm$ 0.7	9.5 $\pm$ 1.0	^b p<0.01 vs other groups
Oc.S/BS, %	1.2 $\pm$ 0.3	4.2 $\pm$ 0.6 ^b	1.9 $\pm$ 0.2	2.3 $\pm$ 0.3	^b p<0.01 vs other groups
MS/BS, %	17.5 $\pm$ 1.7	26.6 $\pm$ 1.5 ^a	22.8 $\pm$ 2.2	23.3 $\pm$ 1.6	^a p<0.01 vs LC-KCl
dl.S/BS, %	7.0 $\pm$ 0.8	15.7 $\pm$ 1.6 ^a	14.0 $\pm$ 2.3 ^a	11.8 $\pm$ 1.9	^a p<0.05 vs LC-KCl
MAR, $\mu\text{m}/\text{d}$	1.1 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.04	1.1 $\pm$ 0.1	NS
BFR, $\mu\text{m}^3/\mu\text{m}^2/\text{yr}$	50 $\pm$ 7	91 $\pm$ 7 ^a	80 $\pm$ 12	69 $\pm$ 11	^a p<0.05 vs LC-KCl

C.Th: cortical thickness; BV/TV: cancellous bone volume/total bone volume; Tb.Th: trabecular thickness; Tb.N: trabecular number; OV/BV: osteoid volume/total bone volume; O.Th: Osteoid thickness; OS/BS: osteoid surface/total bone surface; Ob.S/BS: osteoblast surface/total bone surface; ES/BS: eroded surface/total bone surface; Oc.S/BS: osteoclast surface/total bone surface; MS/BS: mineralized surface/total bone surface; dl.S/BS: double-labeled surface/total bone surface; MAR: mineral apposition rate; BFR: bone formation rate. NS = not significant by one way ANOVA.