

Bone Density and Tissue Lead Accretion in Growing Rats Fed Low High Calcium With or Without Supplemental Clinoptilolite

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The toxicity of lead in animals and humans is well documented (Aub et al, 1962; Underwood, 1971; Quarterman, 1986; Church and Pond, 1988). The naturally occurring zeolite, clinoptilolite, is known to offer protection against ammonium ion toxicity in rats (Pond et al, 1981) and sheep (Pond, 1984) and to counteract cadmium-induced iron deficiency anemia in rats and swine (Pond and Yen, 1983a, 1983b). The cation-exchange and adsorption properties of clinoptilolite suggest its possible role in reducing tissue uptake of ingested lead by animals. Evidence supporting this role was reported in growing pigs whose liver and kidney concentrations of lead were significantly reduced by the addition of 1.0 % clinoptilolite to diets containing 500 or 1000ppm of lead (Pond et al, 1993). The basal diet was a highly fortified milk-replacer containing about 1% calcium supplied by milk constituents. High dietary calcium is known to reduce tissue uptake of lead and protect the pig from the tissue pathology associated with lead ingestion (Hsu et al, 1975). The objective of this experiment was to test the hypothesis that dietary clinoptilolite and calcium levels affect the growth, tissue uptake and bone morphology of growing rats fed diets containing toxic levels of lead.

MATERIALS AND METHODS

Seventy-two male weanling Sprague-Dawley rats weighing 45 ± 5 g were assigned within 4 replicates based on litter to 18 groups of 4 animals each in a $2 \times 3 \times 3$ factorial arrangement of dietary treatments (2 levels of Ca, 3 levels of Pb, and 3 levels of clinoptilolite). The levels of Ca, Pb and clinoptilolite in each diet are described in Table 1. The basal low Ca and high Ca diets contained 74.0 % ground corn, 19.0% soybean meal (44% protein), 0.4% sodium chloride, 0.2% choline chloride, and 0.2% each of a complete vitamin premix and trace mineral element

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premix. The low Ca diet contained 1.0% monocalcium phosphate and zero limestone; the high Ca diet contained 2.4% monocalcium phosphate and 0.5% ground limestone. Cornstarch was added at levels of 5.0, 3.5 and 2.0% of the low Ca diet and 3.1, 1.6 and 0.1% of the high Ca diet to replace 0, 1.5 and 3.5% clinoptilolite, respectively, in each diet series.

Table 1. Diet designations and levels of Ca, Pb and clinoptilolite in each diet.

Diet		Calcium (Ca), %	Clinoptilolite (Clin), %	Lead (Pb), ppm
Number	Description			
1	LLL	0.29	0	0
2	LLM	0.29	0	200
3	LLH	0.29	0	600
4	HLL	0.96	0	0
5	HLM	0.96	0	200
6	HLH	0.96	0	600
7	LML	0.29	1.5	0
8	LMM	0.29	1.5	200
9	LMH	0.29	1.5	600
10	HML	0.96	1.5	0
11	HMM	0.96	1.5	200
12	HMH	0.96	1.5	600
13	LHL	0.29	3.0	0
14	LHM	0.29	3.0	200
15	LHH	0.29	3.0	600
16	HHL	0.96	3.0	0
17	HHM	0.96	3.0	200
18	HHH	0.96	3.0	600

*L=low, M=medium, H=high levels of each dietary variables, e.g., LLL=low Ca, low clin and low Pb, respectively.

Within each level of dietary Ca and clinoptilolite, Pb was added to the diet at levels of 0, 200, and 600 ppm as lead acetate. The clinoptilolite was mined from Castle Creek, Idaho and reduced to <200 mesh for incorporation into the diets. It contained, by analysis (Midwest Laboratories, Inc., Omaha, NE 68144), the following concentrations of mineral elements: Pb 12.7 ppm, Fe 8321 ppm, Mn 159 ppm, Ca 23 ppm, Zn 50 ppm, S 0.31%, P 0.01%, K 1.09%, Mg 0.54% and Ca 0.33%. The basal diet contained 1.04 ppm Pb, 90 ppm Fe, 8 ppm Mn, 0.175 ppm Cu, 50 ppm Zn, 0.389% S, 0.76% P, 1.57% K, and 0.13% Mg.

The rats were kept in individual stainless steel wire-bottom cages in a temperature- and light-controlled room (21 ± 1 degrees C; light/dark cycle of 12 h light/ 12 h dark). They were fed their respective diets *ad libitum* throughout the 8-week experiment; tap water was available at all times via a nipple waterer in each cage. the concentrations of Ca and Pb in the tap water were 21 and <0.015 ppm (below the detectable level) for Ca and Pb, respectively. These amounts were unimportant contributors to total Ca and Pb ingestion by the rats. body weight of each rat was recorded on day 0 and weekly; feed consumed was recorded every second day throughout the 8 weeks.

After 8 weeks, all rats were killed in a carbon dioxide chamber, and cerebrum, liver, kidneys, and left and right femur were removed and weighted. A section of liver, kidney, and the right femur were fixed in 10% buffered formalin for histopathology. Femurs were split in the distal median plane and the medial condyle; the distal half of the bone was demineralized in 10% formic acid buffered to pH 4.5 with sodium citrate. It was sliced through the midplane of the medial condyle, embedded in paraffin, sectioned at 4 micrometers and stained with hematoxylin and eosin (H&E) and with carbol fuchsin and methylene blue according to Ziehl-Neelsen (ZN) for detection of lead inclusion bodies. The secondary spongiosa region of H&E sections as photographed in color; the field excluded the primary spongiosa and did not extend beyond the secondary spongiosa. The sagittally sectioned slides were projected onto an 8.5 ± 11 inch paper marked with 2000 dots equally spaced in 40 X 50 rows. "Hits" in bone tissue were recorded and the percentage of bone tissue in the field was calculated.

Liver and kidney (minus sections for histopathology) were frozen at -5 degrees C and later used for determination of Pb concentration by ICAP (Midwest Laboratories, Inc., Omaha, NE). Tissues fixed in formalin were sectioned at 6 micrometers and stained with H&E.

All data were analyzed in a randomized block design analysis of variance (Minitab, 1989) in a 2 x 3 x 3 factorial arrangement with levels of dietary Ca, Pb, and clinoptilolite as main effects. Main effects of block and dietary treatment and all interactions were tested in the model.

RESULTS AND DISCUSSION

There was no effect of diet on average daily weight gain or feed consumed. Overall mean initial body weight was 43.2 ± 3.1 g; overall mean daily weight gain was 6.5 ± 0.6 g and overall mean daily feed consumed was 24.5 ± 2.2 g. The presence of clinoptilolite in the diet was associated with an increase in gain to feed ratio ($P < 0.03$). Mean gain to feed ratio for rats fed zero clinoptilolite was 0.259 ± 0.019 versus a mean of 0.270 ± 0.019 for rats fed diets containing clinoptilolite. The failure of dietary Pb to depress feed intake and weight gain, even at the level of 600 ppm in the diet, indicates the insensitivity of short-term body weight gain and feed conversion in growing animals to levels of Pb exposure known to be toxic when chronically ingested. Infant pigs fed diets containing 500 or 1000 ppm Pb also showed no depression in growth over a 5 week experiment despite significant tissue accretion of Pb (Pond et al, 1993). Histologic examination of livers and kidneys of all rats in the present study revealed no pathological changes. The data indicate that the ingestion of toxic levels of Pb throughout the major portion of the postweaning growth period is tolerated by growing rats without producing clinical or histopathologic changes. The onset of pathologic changes associated with chronic ingestion of these levels of Pb probably varies with animal species, age, diet composition, source and chemical form (organic or inorganic) of the lead and other unknown variables.

The effects of dietary Ca, Pb, and clinoptilolite concentrations on the concentration of Pb in liver and kidneys are summarised in Table 2. Mean squares and probability levels indicating main effects of dietary variables and all interactions on daily weight gain, daily feed consumed, gain to feed ratio, and on Pb concentration in liver and kidney and on percentage of bone in the femur are shown in Table 3. Concentration of Pb in liver and kidneys was lower ($P < 0.01$) in rats fed high Ca than in those fed low Ca, in agreement with previous reports (Shields and Mitchell, 1941; Hsu et al, 1975, and Six and Goyer, 1970). The magnitude of the decrease in Pb accretion in both liver and kidney tissue as a result of increasing dietary Ca from 0.29 to 0.96% of the diet was profound. The overall mean concentration of Pb in livers of rats fed high Ca was 29% of that of rats fed low Ca; concentration of Pb in kidneys of rats fed high Ca was 38.5% of that of rats fed low Ca (Table 2). The percentage of bone in the femur, as assessed by measurement of the bone tissue in the sagittally sectioned area of an H&E stained histological section, was higher ($P < 0.01$) in rats fed high Ca than in those fed low Ca, as expected (Tables 2 and 3).

The addition of clinoptilolite to the diet at either 1.5 or 3.0 % was not associated with a reduction in tissue concentration of Pb in rats fed diets contained Pb (Table 2), in contrast to the observation reported in pigs fed clinoptilolite (Pond et al, 1993), in which supplemental dietary clinoptilolite reduced liver and kidney Pb concentration in the presence of dietary Pb. The protective effect of dietary clinoptilolite against tissue Pb accumulation in growing pigs, but not in growing rats may represent a species difference in response related to fundamental differences in eating behavior and digestive physiology.

Table 2. Means for kidney and liver Pb concentrations and distal femur metaphysis bone percentage.

Diet		Kidney ppm	Pb, Liver ppm	Pb, Femur tissue, %
Number	Description			
1	LLL	<0.10	<0.10	32.18
2	LLM	9.31	0.94	24.60
3	LLH	20.13	2.58	24.56
4	HLL	<0.10	0.40	47.89
5	HLM	2.95	0.36	43.81
6	HLH	8.19	0.38	51.23
7	LML	<0.10	0.58	45.14
8	LMM	10.73	0.92	43.30
9	LMH	20.40	1.74	43.38
10	HML	<0.10	0.58	45.14
11	HMM	3.95	0.35	45.26
12	HMH	8.43	0.57	44.36
13	LHC	<0.10	<0.10	42.98
14	LHM	6.29	0.55	41.43
15	LHH	18.99	3.53	46.99
16	HHL	1.55	<0.10	42.98
17	HHM	3.07	<0.10	39.25
18	HHH	6.51	1.56	46.16
Overall mean ± SD		6.723	0.830	41.353

Previous work (Pond et al, 1985) suggested the possibility of clinoptilolite protecting growing rats against the adverse effects of Pb ingestion, but tissue Pb concentrations were not determined. The rat normally practices coprophagy, resulting in recycling

of dietary constituents and an increased opportunity for absorption of cations such as Pb whose transfer across the intestinal cell into the blood is less efficiently controlled than that of cations such as Ca, the absorption of which is highly regulated (National Research Council, 1980). Thus, the cation exchange capacity of clinoptilolite may not effectively reduce the net absorption of a high level of ingested Pb in the coprophagic rat.

Table 3. Mean squares and probability levels of variance components from analyses of variance of trait means.

Source of variation	df	Daily gain	Daily feed	G/F	Kidney Pb	Liver Pb	Femur bone tissue
Replicate	3	2.6156***	21.2 **	0.00219***	19.49	4.270**	31.93
Ca	1	1.2310*	2.09	0.00100*	584.82***	8.371**	1021.97***
Clin	2	0.4853	0.95	0.00131**	8.73	0.496	285.67**
Pb	2	0.7233	1.21	0.00055	1090.52***	14.986***	62.16
Ca × Clin	2	0.1599	8.50	0.00031	4.13	0.317	176.58***
Ca × Pb	2	0.1749	2.38	0.00020	238.95***	6.335***	10.39
Clin × Pb	4	0.4611	2.49	0.00094**	6.64	2.052*	47.25
Ca × Clin × Pb	4	0.2977	1.13	0.00018	2.55	0.197	33.60
Error	51	0.4053	4.95	0.00035	22.32	0.865	55.37

***p < 0.001

** p < 0.05

* p < 0.1

Dietary supplementation with clinoptilolite significantly increased the percentage of bone in femur ($P < 0.01$) (Table 3). Other evidence supports the improved utilization of dietary Ca in poultry by synthetic dietary zeolites; e.g., zeolite A in the diet of laying hens improves egg shell thickness (Frost and Roland, 1992) and improves Ca absorption and retention in broiler chickens (Frost and Roland, 1992); Watkins and Southern, 1992). The mechanism of action of the physiological effect is unknown, but the increased bone tissue accretion in animals fed clinoptilolite in the present experiment suggests a beneficial effect on both Ca and P retention. Corroboration of this finding in food animal species would have important implications for animal production with respect to Ca and P and environmental quality. The improvement in efficiency of feed utilization (gain to feed ratio) obtained in rats fed clinoptilolite suggests some metabolic effect of this natural zeolite in the growing rat. Pond et al (1981) showed that clinoptilolite decreased ammonia toxicity in rats dose orally with ammonia; it was suggested (Pond and Yen, 1984) that the improved growth and feed utilization often reported in swine

fed clinoptilolite may be due to the ammonium ion adsorption properties of this zeolite in the lumen of the gastrointestinal tract, resulting in reduced concentrations of ammonia, a known cell toxicant. The mechanism for the improved feed utilization of rats fed clinoptilolite in the present experiment may be related to a reduction in subclinical ammonia toxicity induced by selective exchange of ammonium ions released in the lumen of the gastrointestinal tract from urea hydrolysis by microbial populations inhabiting the intestine of the rat.

The concentration of Pb in liver and kidneys tended to be linearly related to concentration in the diet (Table 2). The overall mean Pb concentration in liver was 0, 0.47, and 1.57 ppm in rats fed diets containing 0, 200, and 600 ppm of Pb, respectively; corresponding values for kidney were 0, 6.04, and 13.77 ppm. The highest tissue concentrations of Pb were found in rats fed low Ca and 600 ppm Pb (20 ppm in kidney and 2.4 ppm in liver); the presence of clinoptilolite in low or high Ca diets had no apparent effect on tissue Pb accretion.

Lead inclusion bodies have been observed in tissues of young pigs fed diets containing 1000 ppm Pb for 13 weeks (Hsu et al, 1975). Rare lead inclusion bodies were found in the femurs of rats fed Pb in the present study, but not attempt was made to quantify these findings.

Chronic ingestion of 200 or 600 ppm of Pb provided in the diet as lead acetate was not associated with decreased body weight gain, feed consumption, or efficiency of feed utilization in growing rats, but liver and kidney concentrations of Pb were increased in proportion to dietary Pb concentration. Supplemental dietary clinoptilolite had no protective effect against tissue Pb accretion, but did improve bone tissue accretion in the femur of rats fed low Ca diets. High dietary Ca offered protection against tissue accretion of Pb, in agreement with previous work (Shields and Mitchell, 1941; Six and Goyer, 1970; Hsu et al, 1975).

In summary, we conclude that the naturally occurring zeolite, clinoptilolite, although protective against tissue Pb uptake in young rapidly growing pigs fed high (1%) Ca diets (Pond et al, 1993), was not effective at the levels of clinoptilolite used (1.5 and 3.0% of the diet) in modulating tissue Pb accretion in growing rats fed 200 or 600 ppm Pb, although concentrations of Pb in liver and kidney were increased in proportion to dietary Pb level. The physiological basis for this apparent species difference in response to dietary clinoptilolite in growing animals ingesting toxic levels of Pb needs to be determined. Such parameters as erythrocyte delta-aminolevulinic acid dehydratase activity, basophilic stippling and altered learning behavior as indices of Pb toxicity are needed in future research to provide more sensitive measurements of differential effects of factors modulating dietary Pb ingestion in animals. The observed improvement in bone tissue accretion in the femurs of growing animals

fed a low Ca diet in the presence of dietary clinoptilolite suggests improved absorption of Ca; this effect has important implications for food animal production and environmental quality with respect to Ca and P utilization. The apparent stability of clinoptilolite in the gastrointestinal tract and its safety as a feed constituent has been documented (Pond et al, 1989) by measurements of tissue mineral element content in swine fed this naturally occurring zeolite throughout the growing period.

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