The Effects of Chronic Vitamin A Excess on Bone Remodeling in Aged Rats (42894)

XIAO FENG LI, BESS DAWSON-HUGHES,[1] ROBERT HOPKINS, ROBERT M. RUSSELL, WEBSTER S. S. JEE,* DANIEL BANKSON, AND XIAO JIAN LI*

The USDA Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts 02111 and Division of Radiobiology, University of Utah School of Medicine, Salt Lake City, Utah 84112*

Abstract. **This study was conducted to assess the effects of long-term ingestion of moderate excesses of vitamin A on trabecular bone remodeling in the fifth lumbar vertebral body of aged rats. Eighteen-month-old rats were fed diets with vitamin A content equal to the daily requirement (DR), 2-fold, and 5-fold the DR along with calcium content of either the DR or 0.3-fold the DR, for 14 months each. As expected, serum concentrations of 1,25-dihydroxyvitamin D were higher in the reduced than in the normal calcium intake groups (65.1 ± 2.4 SEM vs 47.8 ± 2.1 pg/ml, $P < 0.001$). Calcium balance was more positive at the higher than the lower calcium intake (5.7 vs 0.9 mg, $P < 0.001$) but was unaffected by vitamin A intake. Histomorphometric analysis of the fifth lumbar vertebral body revealed that the 2-fold but not the 5-fold excess in vitamin A intake resulted in a 15% increase in percentage of trabecular bone ($P < 0.02$). The low calcium diet depressed bone growth (total bone tissue) but did not affect percentage of trabecular bone. Several effects of the vitamin A excess and low calcium diet were noted along the trabecular surface including increased mineral apposition rate and resorption surfaces and decreased formation surfaces. The net effect of vitamin A on trabecular bone of the rat varies as intake begins to exceed the DR. At a 2-fold excess, a modest favorable effect on percentage of trabecular bone was observed.**

[P.S.E.B.M. 1989, Vol 191]

Vitamin A toxicity causes a variety of bone abnormalities in growing rats including rapid bone resorption (1, 2), destruction of bone matrix (3), and increased bone mineral turnover (4–6). Effects of excess vitamin A in man vary with the dose. Acute toxicity causes hypercalcemia (7–9) whereas use of synthetic retinoids in intermediate doses (ranging from 0.8 to 4 mg kg/day) has been associated with skeletal hyperostosis and calcification of tendons and ligaments (10, 11). No information is currently available in rats or man on whether modest excesses of vitamin A affect the skeleton or more specifically, bone remodeling. The skeletal effects of higher intakes of vitamin A raise the possibility that bone loss may be aggravated by chronic ingestion of modest excesses of vitamin A. A recent nutritional status survey of the elderly revealed that approximately 24% of the population surveyed took a supplement containing up to 10,000 IU of vitamin A daily (twice the DR) and that many took larger supplements of vitamin A (12). Other surveys have revealed that calcium intake in the elderly is often low (13). Both low calcium intake (14) and aging (15) have been associated with loss of bone mineral from the spine in the elderly.

This study was conducted to determine whether chronic ingestion of a modest excess in vitamin A, with normal or low calcium intake, has any effect on bone remodeling in aged rats. A lumbar vertebral body was selected as the measurement site since it contains reactive trabecular bone (16).

Materials and Methods

Fifty-four male Sprague-Dawley rats over 18 months of age and weighing approximately 500 g each were housed in individual cages and fed 15 g of a test diet daily over the 14-month study period. The diets were supplemented with sucrose, as needed (rarely), to maintain constant body weight. Animals were fed standard diets (17) with the exceptions that protein was reduced moderately from 17.4% to 12% and vitamin A (as stabilized retinyl acetate; Dyets, Bethleham, PA)

[1] To whom requests for reprints should be addressed at Calcium and Bone Metabolism Laboratory, USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington St., Boston, Massachusetts 02111.

Received June 29, 1988. [P.S.E.B.M. 1989, Vol 191]
Accepted January 24, 1989.

0037-9727/89/1912-0103$2.00/0

and calcium (as $CaHPO_4$) intakes were adjusted. Group 1 was fed the DR (18) of 0.5% calcium and 60 units of vitamin A (or 18 retinol equivalents, 19); Group 2 was fed 0.5% calcium and 120 units of vitamin A; Group 3 was fed 0.5% calcium and 300 units of vitamin A; Group 4 was fed 0.13% calcium and 60 units of vitamin A; Group 5 was fed 0.13% calcium and 120 units of vitamin A; and Group 6 was fed 0.13% calcium and 300 units of vitamin A daily.

Calcium Balance and Ashing Procedures. After 12 months on the test diets, each animal was placed in a metabolic balance cage for 3 days of acclimatization, followed by a 9-day balance period. Samples of each diet (in triplicate) and daily 24-hr food and fecal specimens were dissolved in hot nitric acid, diluted, and analyzed for calcium in the model SpectraSpan VI direct current plasma emission spectrophotometer (Beckman Instruments, Palo Alto, CA). Calcium balance was calculated as the daily intake minus daily loss in urine and feces.

To determine femoral and vertebral (last thoracic and first lumbar) calcium content, the bones were ashed at 450°C in a muffle furnace for 7 days. The ash was dissolved in 3 ml of concentrated HCl, diluted, and analyzed by atomic emission spectroscopy.

Laboratory Methods. Serum calcium concentration was measured according to the colorimetric method of Kessler and Wolfman (20), serum $1,25(OH)_2D$ according to the competitive protein binding method of Reinhardt *et al.* (21), and retinyl ester and retinol according to the method of Bankson and Russell (22).

Histomorphometry. Three to six rats in each group were injected subcutaneously with 10 mg/kg of calcein (Sigma Chemical Co.) on Days 26 and 27 before being killed and 25 mg/kg of tetracycline on Days 4 and 5 before being killed. The fifth lumbar vertebral bodies were dissected, trimmed, and fixed in 10% phosphate-buffered formalin for 24 hr, then stained in Villanueva Osteochrome bone stain (Polysciences, Inc., Warrington, PA) for 4 days. The specimens were then run through sequential changes of ethanol. acetone, and methyl methacrylate (Eastman Organic Chemicals, Rochester, NY). After serial transverse sections of the fifth lumbar vertebral body were sawed with a low-speed metallurgical saw (Isomet; Buehler, Ltd., Lake Bluff, Il), sections at the level which approximates 350 μm distal to the proximal growth plate were selected and ground to 20 μm with a grinder (Maruto Instrument Co., Ltd. Tokyo, Japan) for histomorphometric analysis.

Static and dynamic trabecular bone histomorphometric parameters (16, 23–25) were measured and calculated. Briefly, the perimeter or surface of the following structures were traced: total bone tissue (the trabecular bone and marrow within the endocortical envelope), trabecular bone surface, bone formation surface (surface covered with osteoid), bone resorption surface (eroded bone surface), single- and double-labeled surfaces, and the interlabeling distance between tetracycline and calcein labels. These measurements allowed a custom semiautomated Image Analysis System (coupled to a Hewlett-Packard 9816 computer) to calculate the following: total bone tissue area; trabecular bone area; percentage of trabecular bone, expressed as trabecular bone area/total bone tissue area; fractional bone formation surface, expressed as osteoid surface/trabecular bone surface; and fractional bone resorption surface, expressed as eroded bone surface/trabecular bone surface. The rate of mineral apposition was calculated as the mean distance between the calcein and tetracycline labels divided by the interdose duration (22 days). Corrected mineral apposition rate (the rate of bone formation averaged over the osteoid surface) was obtained by multiplying the bone mineral apposition rate by the ratio of labeling (i.e., mineralizing) surface to osteoid surface. The bone formation rate or bone turnover rate was calculated by the following formula: bone formation rate (total bone tissue based) = (labeling surface × mineral apposition rate/total bone tissue area) × 365 × 100 = %/year.

Student's t test was used to assess differences between group means.

Results

Measured parameters are given in Table I. Body weight did not change significantly in any group over the 14-month study. Calcium balance was positive by a mean of 0.9 mg/day in rats on reduced calcium intake (Groups 4–6) and slightly but significantly more positive (mean 5.7 mg, $P < 0.001$) in those on normal (0.5%) calcium diets (Groups 1–3). The mean serum concentration of $1,25(OH)_2D$ was higher in rats with reduced than in those with normal calcium intake (65.1 ± 2.4 SEM v 47.8 ± 2.1 pg/ml, $P < 0.001$). Liver content of both retinol and retinyl ester increased with increasing vitamin A intake. Those on the 5-fold excess (Groups 3 and 6) had significantly higher content of these compounds than the other groups, $P < 0.05$. Serum calcium, retinol, or retinyl ester concentrations were normal and did not vary among the groups (Table I). Similarly, no differences were observed in vertebral (T12 plus L1) or femoral ash calcium content among the groups (data not shown).

The net effects of 14 months of diet intervention on size and percentage of trabecular bone of the body of the fifth lumbar vertebra, in a subset of animals, are given in Table II. Total bone tissue area tended to be lower in animals on the low compared with normal calcium diet (i.e., 32% decrease in Group 4 vs Group 1, $P < 0.01$). A similar effect of the low calcium diet on trabecular bone area was observed (23% decrease in Group 4 vs Group 1, $P < 0.05$). Percentage of trabecular bone however was unaffected by the low calcium diet.

Table I. Effects of Different Diets for 14 Months on Measured Parameters[a]

Measurement	Group[2] 1	2	3	4	5	6
No.	5	7	8	8	8	7
Body weight (g)	506.9 ± 12.1	498.0 ± 12.0	481.3 ± 12.2	488.7 ± 8.5	487.8 ± 21.7	480.0 ± 28.2
Calcium balance (mg/day)	3.8 ± 8.0	8.3 ± 4.4	4.7 ± 1.9	0.6 ± 2.1	0.7 ± 1.9	1.7 ± 1.8
Plasma values						
$1,25(OH)_2D$ (pg/ml)	47 ± 12	52 ± 4	47 ± 7	64 ± 4	63 ± 12	69 ± 16
Calcium (mg/dl)	9.8 ± 1.3	10.2 ± 0.6	10.8 ± 0.8	10.2 ± 0.8	10.1 ± 0.5	9.4 ± 1.6
Retinol ester[c] (μg/dl)	0	0	1.7 ± 2.9	0.5 ± 1	1.0 ± 2.4	5.0 ± 3.5
Retinol[c] (μg/dl)	37 ± 13	59 ± 6	45 ± 4	54 ± 7	54 ± 11	49 ± 8
Liver content						
Retinyl ester[c] (μg/g)	916 ± 543	1,195 ± 242	1,871 ± 214	465 ± 139	951 ± 230	2,326 ± 503
Retinol[c] (μg/g)	54 ± 25	38 ± 19	112 ± 13	47 ± 8	69 ± 37	112 ± 46

[a] All results are mean ± SD.
[b] Groups 1–3 ingested 0.5% calcium and the DR, 2-fold DR, and 5-fold DR of vitamin A; Groups 4–6 ingested 0.13% calcium and DR, 2-fold DR, and 5-fold DR of vitamin A.
[c] Measured in two to three animals per group.

Table II. Long-Term Effects of Dietary Calcium and Vitamin A on Trabecular Bone Size and Density[a]

Group	No. of rats	Total bone tissue area (mm^2)	Trabecular bone area (mm^2)	% Trabecular bone (mm^2/mm^2)
1	4	5.10 ± 1.00	1.08 ± 0.19	0.21 ± 0.02
2	3	4.07 ± 0.58	0.99 ± 0.13	0.25 ± 0.01[b]
3	5	4.21 ± 0.51	0.88 ± 0.14	0.21 ± 0.03
4	6	3.48 ± 0.45[c]	0.83 ± 0.10[d]	0.24 ± 0.02
5	6	3.54 ± 0.69[c]	0.92 ± 0.19	0.26 ± 0.01[c]
6	5	3.27 ± 0.21[c]	0.74 ± 0.08[c]	0.23 ± 0.03

[a] All results are mean ± SD.
[b] Values differ from control (Group 1), $P < 0.02$.
[c] Values differ from control (Group 1), $P < 0.01$.
[d] Values differ from control (Group 1), $P < 0.05$.

There was no significant effect of vitamin A on total bone tissue or trabecular bone area at either calcium intake level. The two-fold excess of vitamin A was associated with a 15% increase in percentage of trabecular bone ($P < 0.02$, Table II) in animals on normal calcium intake whereas it had no effect in animals on the low calcium diet.

Trabecular surface parameters assessed at the 14-month time point are given in Table III. At normal calcium intakes bone formation surface was decreased significantly and similarly by both the 2- and 5-fold excesses of vitamin A (by 59 and 58%, $P < 0.02$). Lowering calcium intake also resulted in a significant decrease in fractional formation surface (Group 4 vs Group 1, 35% decrease, $P < 0.05$). Effects of vitamin A excess within the low calcium groups were not evident. The corrected mineral apposition rate was increased at both the 2-fold (72%, $P < 0.02$) and 5-fold (40%, $P < 0.05$) excesses of vitamin A at normal but not at low calcium intake. Low calcium intake was also associated with an increased corrected mineral apposition rate (45%, $P < 0.02$). The 5-fold excess of vitamin A was associated with a significant increase in fractional resorption surface in rats on the normal (Group 3 vs Group 1) but not reduced calcium intake (Table III). The low calcium diet itself also increased the fractional resorption surface (36%, $P < 0.02$). No effect of various combinations of vitamin A and calcium could be observed on bone formation rate.

Discussion

This study was conducted to determine whether a modest excess of vitamin A intake in the presence of normal or reduced calcium intake over their adult life-span affects remodeling in the lumbar vertebral body in rats. The adult rat was selected as the test animal since bone remodeling occurs in the spongiosa of the vertebrae of this animal after it reaches 200 g in weight (16). This region provides a sensitive measurement site since the spongiosa here is very reactive, has a large surface, and is well vascularized. Males were used to eliminate any variability in bone parameters that might have resulted from different breeding histories. The 14-month study period represents most of the late adult life-span of this animal. To increase the survival rate of the old rats in this long study, the animals were fed reduced protein diets (26) and were maintained at relatively constant weights of approximately 500 g (27, 28). Despite this, one fifth of the animals died of miscellaneous causes during the study.

As observed by others, lowered calcium intake was associated with less positive calcium balance (29) and increased serum concentration of 1,25-dihydroxyvitamin D (30, 31). These effects were likely mediated by increases in parathormone (not measured (32)). The lack of a significant effect either of lowered dietary calcium or of increased vitamin A intake on femur and mid-vertebral column (T12 + L1) ash calcium content

Table III. Vertebral Trabecular Bone Surface Measurements[a]

Group	No. of rats	Fractional formation surface (mm/mm)	Corrected mineral apposition[b] rate (μm/day)	Fractional resorption surface (mm/mm)	Bone formation rate-total bone tissue area based[c] (%/year)
1	4	0.34 ± 0.07	0.09 ± 0.02	0.11 ± 0.02	32.45 ± 5.16
2	3	0.14 ± 0.07[d]	0.16 ± 0.04[d]	0.09 ± 0.03	30.66 ± 8.03
3	5	0.14 ± 0.06[e]	0.13 ± 0.03[f]	0.15 ± 0.04[f]	29.93 ± 9.49
4	6	0.22 ± 0.08[f]	0.14 ± 0.03[d]	0.15 ± 0.03[d]	33.58 ± 7.67
5	6	0.18 ± 0.06[e]	0.16 ± 0.03[e]	0.13 ± 0.03	31.03 ± 6.57
6	5	0.20 ± 0.08[f]	0.14 ± 0.05	0.17 ± 0.02[e]	32.85 ± 5.38

[a] All results are mean ± SD.
[b] Defined as (double label width/interlabeling period) × (labeled surface/formation surface).
[c] Defined as (labeling surface × mineral apposition rate)/total bone area × 365 × 100.
[d] Differs from control (Group 1), $P < 0.02$.
[e] Differs from control (Group 1), $P < 0.01$.
[f] Differs from control (Group 1), $P < 0.05$.

seems surprising. However, it has been observed previously in younger rats in the setting of negative calcium balance (6, 33) and may reflect the insensitivity of the bone ash method or the limited selection of measurement sites.

Histomorphometric analysis of the fifth lumbar vertebral body revealed that animals with normal calcium intake and prolonged exposure to a 2-fold excess of vitamin A had similar amounts of bone tissue but significantly greater percentage of trabecular bone than control animals. This has not been observed previously and is somewhat surprising since the opposite effect has been reported at higher excesses of vitamin A (1–6). In this study the 5-fold excess of vitamin A had no net effect on either the size (total bone tissue) or percentage of trabecular bone of the vertebral body. As expected (33), long-term ingestion of low calcium diet resulted in less total bone tissue, indicating inhibition of bone growth. Although it was not evaluated in this study, the low calcium intake is expected to effect cortical as well as trabecular bone (33), with these effects likely mediated by parathormone.

On examination of the surface of the trabecular tissue, the functioning osteoblasts are laying down bone at an accelerated rate in animals on excesses of vitamin A as well as in those on low calcium intake. However, in these same animals (Groups 2, 3 and, to a lesser extent, Group 4), there was a decrease in formation surface, indicating a reduction in osteoblast number. The latter is consistent with decreased recruitment of osteoblasts. The combination of increased osteoblastic activity and decreased recruitment resulted in no change in bone formation rate from that of the controls. Finally, the observed increase in fractional resorptive surface at the 5-fold excess of vitamin A and the low calcium diet may be due to the increased rate of bone resorption and/or the uncoupling of bone formation from bone resorption. The latter may be true because numerous resorption surfaces without osteoclasts and decreased bone formation surfaces were observed. It should be emphasized that these trabecular bone surface findings represent one point in time and cannot be compared with the integrated effects of 14 months of the diets on total bone tissue and percentage of trabecular bone.

A 2-fold excess of vitamin A in the diet resulted in a modest increase in percentage of vertebral trabecular bone density (percentage of trabecular bone) in old rats whereas it did not occur with 5-fold excess of vitamin A. Reduced calcium intake resulted in depression of periosteal envelope enlargement but no change in percentage of trabecular bone. The effects of vitamin A were not observed in the setting of reduced calcium intake. Although alterations in osteoblast activity and recruitment were observed at one time point (14 months), this study provides no evidence of an adverse net effect (and instead a modest beneficial effect) of a chronic 2-fold excess in vitamin A intake on trabecular bone in old rats.

This work was supported by the USDA Human Nutrition Research Center on Aging at Tufts University, the Dana Foundation, and the Department of Energy Contract DE-AC02-76EV-00119. The authors are grateful to Ms. Judy King for manuscript preparation.

1. Belanger LF, Clark I. Alpharadiographic and histological observations on the skeletal effects of hypervitaminoses A and D in the rat. Anat Rec **158**:443–452, 1967.
2. Moore T, Sharman IM. Hypervitaminosis A combined with calcium deficiency in rats. Int J Vitam Nutr Res **49**:14–20, 1978.
3. Clark I, Bassett CAL. The amelioration of hypervitaminosis D in rats with vitamin A. J Exp Med **115**:147–156, 1962.
4. Mellanby D. Vitamin A and bone growth: the reversibility of vitamin A-deficiency changes. J Physiol **105**:382–399, 1947.
5. Wolbach SB. Vitamin A deficiency and excess in relation to skeletal growth. J Bone Joint Surg [Am] **29**:171–192, 1947.
6. Clark I, Smith HR. Effects of hypervitaminosis A and D on skeletal metabolism. J Biol Chem **239**:1266–1271, 1964.

7. Frame B, Jackson C, Reynolds W, Umphrey J. Hypercalcemia and skeletel effects in chronic hypervitaminosis A. Ann Intern Med **80**:44–48, 1974.
8. Ragavan VV, Smith JE, Belezikian JP. Vitamin A toxicity and hypercalcemia. Am J Med Sci **283**:161–164, 1982.
9. Farrington K, Miller P, Varghese Z, Baillod RA, Moorehead JF. Br Med J **282**:1999–2002, 1981.
10. Pittsley RA, Yoder FW. Retinoid hyperostosis. Skeletal toxicity associated with long-term administration of 13-cis-retinoic acid for refractory ichthyosis. N Engl J Med **308**:1012–1014, 1983.
11. DiGiovanna JJ, Helfgott RK, Gerber LH, Peck GL. Extrapsinal tendon and ligament calcification associated with long-term therapy with retinate. N Engl J Med **315**:1177–1182, 1986.
12. Krasinski SD, Russell RM, Bankson DD, Hartz S, Strauss JJ. The effect of vitamin A supplement use on serum retinol and retinyl ester levels in the elderly. J Am Coll Nutr **2**:301–302, 1983.
13. Abraham S, Carroll MD, Dresser CM, Johnston CL. Dietary intake findings, United States 1976–1980. Hyattsville, MD: National Center for Health Statistics, U.S. Department of Human Services. (PHS)83-1681, 1983.
14. Dawson-Hughes B, Jacques P, Shipp C. Dietary calcium intake and bone loss from the spine in healthy postmenopausal women. Am J Clin Nutr **46**:685–687, 1987.
15. Riggs BL, Wahner HW, Seeman E, Offord KP, Dunn WL, Mazess RB, Johnson KA, Melton LJ III. Changes in bone mineral density of the proximal femur and spine with aging. J Clin Invest **70**:716–723, 1982.
16. Baron R, Tross R, Vignery A. Evidence of sequential remodeling in rat trabecular bone: morphology, dynamic histomorphometry and changes during skeletal maturation. Anat Rec **208**:137–145, 1984.
17. Report of the AIN Ad Hoc Committee on Standards for Nutritional Studies. J Nutr **107**:1340–1348, 1977.
18. Roger A. The laboratory rat, biology and diseases. Academic Press, Vol. **I**: p 128, 1979.
19. Goodman DS. Vitamin A and retinoids in health and disease. N Engl J Med **310**:1023–1031, 1984.
20. Kessler G, Wolfman M. An automated procedure for the simultaneous determination of calcium and phosphorus. Clin Chem **10**:686–703, 1964.
21. Reinhardt TA, Horst RL, Orf JW, Hollis BW. A microassay for 1,25-dihydroxyvitamin D not requiring high performance liquid chromatography: application to clinical studies. J Clin Endocrinol Metab **58**:91–98, 1984.
22. Bankson D, Russell R. Determination of retinyl esters and retinol in serum or plasma by normal-phase liquid chromatography: Method and applications. Clin Chem **32**:35–40, 1986.
23. Frost HM. Tetracycline-based histological analysis of bone remodeling. Calcif Tissue Res **3**:211–237, 1969.
24. Merz WA, Schenk RK. Quantitative structural analysis of human cancellous bone. Acta Anat (Basel) **76**:1–15, 1970.
25. Jee SS, Haba T, Inuoe J, Ueno K, Jee KW. Stomorphometric analysis of the rat skeleton in VIIIth Conference on Calcium Regulating Hormones. Amsterdam: Elsevier, 1984.
26. Barrows CH, Kokkunen GC. Protein synthesis, development, growth, and life span. Growth **39**:525–533, 1975.
27. McCay CM, Crowell MT. Prolonging the lifespan. Sci Monthly **39**:405–414, 1934.
28. Weintraub R, Walford RL, Fligiel S, Guthrie D. Retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. J Nutr **116**:641–657, 1986.
29. Cohn SH, Teree TM, Gusmano EA. Effect of varying calcium intake on the parameters of calcium metabolism in the rat. J Nutr **94**:261–267, 1968.
30. Armbrecht HJ, Forte LR, Halloran BP. Effect of age and dietary calcium on renal 25(OH)D metabolism, serum $1,25(OH)_2D$, and PTH. Am J Physiol **246**:E266–E270, 1984.
31. Thomas ML, Ibarra MJ, Solcher B, Wetzel S, Simmons DJ. Bone Miner **4**:73–82, 1988.
32. Nerurkar MK, Sahasrabudhe MB. Metabolism of calcium, phosphorus and nitrogen in hypervitaminosis A in young rats. Biochem J **63**:344–349, 1956.
33. Ellinger GM, Duckworth J, Dalgarno AC, Quenouille MH. Skeletal changes during pregnancy and lactation in the rat: effect of different levels of dietary calcium. Br J Nutr **6**:235–253, 1952.