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Effect of low or high dietary calcium on the morphology of the rat femur

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Summary. The present study compared the effect of a calcium deficit or surfeit on femurs. Young female rats were fed with the normal (1.18%), low (0.05%), or high (2.00%) calcium diet for 3, 7, 15 or 30 days. Two groups received the low calcium diet for the first 15 days and then were followed by the normal (L-N) or high calcium diets (L-H) for the sequential 15 days. The morphology of the femur was studied together with serum calcium, parathyroid hormone (PTH), calcitonin and bone mineral density (BMD). We did not find any significant changes in the serum PTH level and bone morphology in the high calcium group. In the low calcium group, the serum PTH level increased, BMD of the whole body, the femoral weight and the femoral trabecular bone decreased as compared with the normal calcium group. There was a greater proportion of resorbing surface, less resting surface and larger vascular canal openings in the femoral endosteal surfaces in the low calcium group. In the L-N or L-H group, the femoral trabecular bone increased and the femoral resorbing surface decreased as compared with those of the low calcium group. These findings suggest that high calcium intakes do not affect the bone mass, and low calcium intakes have a deleterious effect on bone status, which may be related to vascular alternations of the bone. Reversing the low income calcium intake by a higher calcium diet can partially improve the bone alternations induced by low calcium intake.

Key words: Low calcium diet, Bone, Rat, SEM, Morphometry

Introduction

Calcium is one of the nutrients required for normal skeletal growth and mineralization, and which plays an important role in regulating bone remodeling and bone mass (Matkovic and Heaney, 1992). Calcium is stored when bone is deposited and liberated when it is resorbed. An adequate calcium intake is critical for preserving the integrity of the skeleton in humans and animals. Low calcium diet has been accepted as a method of increasing bone resorption and as one of the experimental models to reduce bone mass (Salomon, 1972; Sissons et al., 1984). Low calcium intake certainly increases the risk of osteoporosis both in animals and humans, presumably as the result of exaggerated bone resorption and decreased bone formation. The process of accelerated bone resorption induced by the low calcium diet was known to be mediated by compensatory secondary hyperparathyroidism (Stauffer et al., 1973; Persson et al., 1993; Chen et al., 2001a), but the process of bone resorption and the mechanisms leading to bone loss have not yet been elucidated. The relationship between calcium intake, circulating calcium level, calciotropic hormones, and bone status is not fully understood. The skeletal effect of the high calcium intake is less clear. There are no controlled prospective studies to show whether an increase in calcium intake promotes peak bone mass independently of energy intake. Neither are there any to show whether an increased calcium intake after longitudinal growth has ceased to have any effect on skeletal consolidation and the subsequent rate of bone loss or fracture, or both (Kanis and Passmore, 1989). The clinical usefulness of calcium supplementation to promote bone formation and prevent bone loss is controversial (Nordin and Heaney, 1990). The purpose of the present study is to evaluate the morphological changes in bone status induced by the low or high calcium intake and to clarify whether normal or higher calcium intake can improve the bone loss induced by the low calcium diet.

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Materials and methods

Four-week-old female Wistar rats with an average body weight of 79±8.6 g were divided into 14 groups of seven animals each. The normal (1.18%), low (0.05%), and high (2.00%) calcium diets (CLEA Japan Inc.) were used in this study. All animals were fed with one of the above three nutritionally complete, but calcium-altered

diets for 3, 7, 15, and 30 days. Two groups received the low calcium diet for the first 15 days and then the normal (L-N) or high calcium diet (L-H) for the sequential 15 days. The compositions of the three different diets are shown in table 1. All diets contained 0.85% phosphorus. Animals were kept in steel cages with free access to food and water. The daily food intake of a rat averaged 13.5 g on the normal and low calcium groups, while that of the high calcium group it was 11.0 g. After the experiment, the femurs of both sides were removed under ether anesthesia. The left femur was dissected and cleared. The length of the femur was measured from the tip of the greater trochanter to the distal surface of the condyle. The dry weight of the femur was measured by a precision balance.

The right femur was processed for scanning electron microscopy (SEM). The distal parts of the femurs were trimmed in the sagittal plane to expose the epiphyseal and metaphyseal trabecular bone and treated for about 4 hours with 5% sodium hypochlorite. The bones were then dehydrated in acetone and critical-point dried, mounted on stubs and coated with gold/palladium using an ion sputter. The bones were examined with a Hitachi S-3500 N SEM. Trabecular measurements of the distal femur were done from a 5 mm^2 area in the central metaphysis 1.0-2.0 mm proximal from the growth plate-epiphyseal junction. The trabecular bone volume per tissue volume (BV/TV), trabecular number (Tb. N) and trabecular thickness (Tb. Th) were measured using an image measuring system (Finetec), according to the standard nomenclature for bone histomorphometry (Parfitt et al., 1987).

The femoral diaphyses of a 5 mm-long were taken from each shaft just below the third trochanter. The endosteal surfaces were processed for SEM observation. Ten micrographs at final magnifications of 500 were taken from each animal. The mean diameter and percent area of vascular canal openings were measured. The percent area of forming, resting and resorbing surfaces were estimated using an image measuring system (Finetec). Types of bone surfaces have been described extensively (Boyde and Hobdell, 1969; Wink, 1982). In the present study, the following types were analyzed. (1) Forming surfaces composed of mineral nodules, indicative of bone-forming surfaces in various stages of mineralization. (2) Resting surfaces composed of mineralized collagen fibers, indicative of completely mineralized resting bone surfaces. (3) Resorbing surfaces showing depressions or pits with bright scalloped edges, indicative of osteoclastic resorptions.

The serum calcium level was determined by standard colorimetric method as described previously (Chen et al., 2001b). The serum PTH and calcitonin levels were determined using rat PTH and calcitonin immunoradiometric assay kits (Immutopics, Inc., San Clemente, CA, USA). The bone mineral content (BMC) and the bone mineral density (BMD) of the whole body were measured by Dual Energy X-ray Absorptiometry (DXA) using a Toyo Medic QDR type 2000.

All data are presented as mean±SEM. Statistical analysis was done using StatView J-4.5 (Abacus Concepts). Significance of the results was determined by one-way analysis of variance (ANOVA) and Fisher's PLSD test. A p value < 0.05 was considered statistically significant.

Table 1. Diet composition (g/100 g).

INGREDIENT	NORMAL CALCIUM DIET	LOW CALCIUM DIET	HIGH CALCIUM DIET
Casein	24.5	24.5	24.5
Corn oil	6.0	6.0	6.0
Cornstarch	37.5	39.87	35.0
Potatostarch	1.0	1.0	1.0
Sucrose	15.5	15.5	15.5
Cellulose	5.0	5.0	5.0
Vitamin mix[a]	1.0	1.0	1.0
Mineral mix[b]	7.0	7.0	7.0
$CaCO_3$	2.5	0.13	5.0

[a]: vitamin mix contains (per 100 g) VA 1517 IU, VD_3 250 IU, VE 7.0 mg, VB_1 1.7 mg, VB_2 1.3 mg, VB_6 1.2 mg, VB_{12} 3.4 mg, VC 19 mg, pantothenic acid 3.7 mg, niacin 16.7 mg, folic acid 0.2 mg, choline 195 mg, biotin 48.4 mg, nicotinic acid 549 mg. [b]: mineral mix contains (mg/100 g) KH_2PO_4 1730, NaCl 600, $FeC_6H_5O_7 \cdot 5H_2O$ 190, $5ZnO \cdot 2CO_2 \cdot 4H_2O$ 6, $CuSO_4 \cdot 5H_2O$ 1.26, $CoCl_2 \cdot 6H_2O$ 0.4, $Ca(IO_3)_2$ 1.54, $MnSO_4 \cdot 4H_2O$ 15.4, $MgSO_4 \cdot 7H_2O$ 800, Na_2HPO_4 1240.

Results

The serum calcium, PTH and calcitonin levels, BMD of the whole body and the femoral morphometric parameters in the high calcium group resembled those in the normal calcium group (Tables 2, 3).

During the experimental period, body weight, serum calcitonin level and the femoral length in the low calcium group were not significantly different compared with those of the normal or high calcium group (Tables 2, 3). As compared with the normal or high calcium group, the serum calcium level significantly decreased from day 3 in the low calcium group. The serum PTH level tended to elevate from day 3 and this rise was significant from day 7 in the low calcium group. The serum PTH level of the low calcium group at 15 and 30 days was 3 to 4 times higher than that of the normal or high calcium group (Table 2). The BMD of the whole body and the dry weight of the femur were significantly decreased from day 7 in the low calcium group (Tables 2, 3).

The SEM images of the trabecular bone of the femurs were similar in the normal, high, and low calcium groups at day 3 after feeding the different calcium diets (Fig. 1). As compared to the normal or high calcium group, the trabecula in the low calcium group were gradually reduced from day 7 to 30 (Figs. 2, 3).

In the SEM images of the endosteal surfaces of the femoral diaphyses, we did not find any morphological

changes in the low calcium group at day 3. There was less amount of the resting surface, more resorbing surface, larger and more vascular canal openings in the femoral endosteal surfaces from day 7 to 30 in the low calcium group (Figs. 4, 5).

The morphometric analysis of the femur showed that the trabecular bone volume in the low calcium group was significantly decreased compared with that of the normal or high calcium group during day 7 to 30 period (Table 3). The trabecular thickness significantly decreased by days 15 and 30 in the low calcium group. The trabecular number was significantly decreased from day 7. Thus, the reduction in bone mass in trabecular bone coincided with the decrease in the number and thickness of the trabecular bone.

Table 4 summarizes results of the morphometric analysis in the femoral endosteal surfaces. As compared to the normal or high calcium group, the percent area of the resting surface was significantly decreased, while the resorbing surface, the diameter and the percent area of the vascular canal openings were significantly increased from day 7 in the low calcium group. There was no significant difference with regard to the forming surface among the normal, high, and low calcium groups (Table 4).

During the 30-day experiment, the L-N and L-H groups showed similar morphological features. The BMD of the whole body, the femoral dry weight and the

Table 2. Whole body BMD, serum calcium, PTH and calcitonin levels.

DAY	DIET	BMD (mg/cm^2)	Ca (mg/100 ml)	PTH (pg/ml)	CALCITONIN (pg/ml)
	N	91.5±10.3	10.88±0.06	10.1±2.6	81.3±9.4
3	L	90.7±14.5	10.20±0.07[a]	16.5±5.2	76.5±5.1
	H	92.2±10.9	10.87±0.12	9.2±1.8	71.5±5.2
	N	92.3±11.7	11.07±0.10	12.8±3.4	89.3±5.1
7	L	88.2±12.2[a]	8.98±0.19[a]	26.8±5.8[a]	81.5±6.2
	H	93.1±12.8	10.83±0.18	12.2±3.7	77.5±8.1
	N	106.7±15.4	10.87±0.04	9.2±2.7	81.3±9.5
15	L	75.9±8.8[a]	9.86±0.13[a]	32.2±8.6[a]	76.5±5.1
	H	107.6±14.0	10.94±0.06	7.6±1.4	71.5±5.2
	N	115.1±13.6	11.06±0.09	11.4±3.7	72.3±6.2
	L	83.4±9.7[a]	9.04±0.17[a]	46.4±12.4[a]	77.0±7.7
30	H	118.7±14.6	10.97±0.16	16.0±5.2	68.9±11.3
	L-N	95.2±12.2[a,b]	10.79±0.18[b]	17.6±3.5[b]	71.5±12.4
	L-H	94.6±13.8[a,b]	10.83±0.22[b]	18.5±2.8[b]	79.5±13.7

Values are shown in mean±SEM. N: normal diet; L: low calcium diet; H: high calcium diet. [a]: $p<0.05$ vs normal calcium group; [b]: $p<0.05$ vs low calcium group.

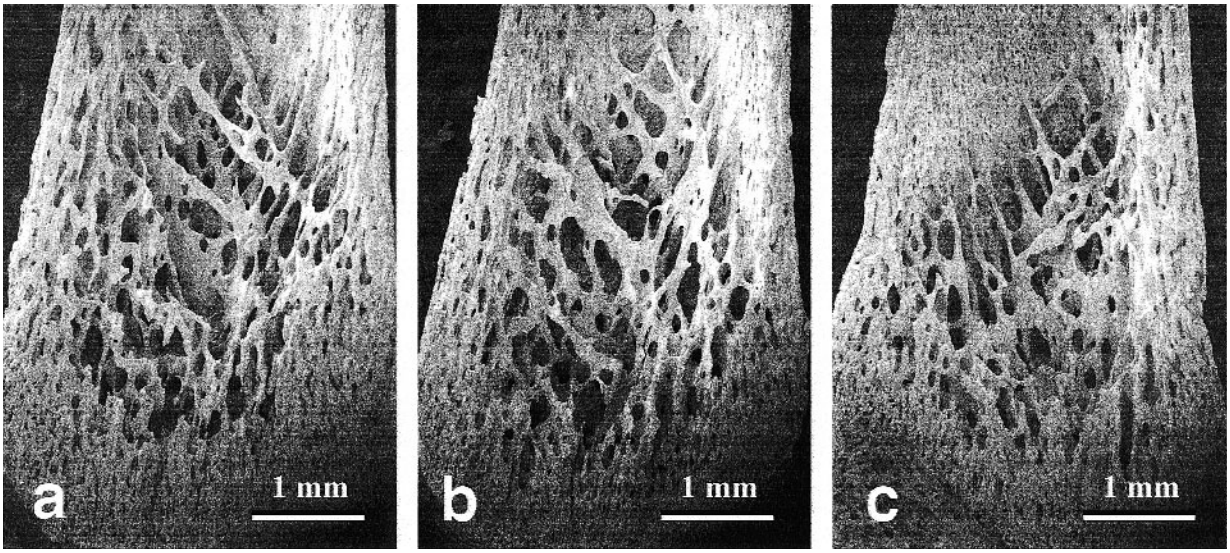

Fig. 1. The femoral distal metaphyses of the rats fed with the normal **(a),** low **(b)** and high **(c)** calcium diets for 3 days. The trabecular bones show similar features among the three groups.

femoral trabecular volume in the L-N and L-H groups were much lower than those of the normal calcium group (Tables 2, 3). As compared with the low calcium group, the serum calcium level was increased and the PTH level was significantly decreased, the BMD of the whole body, the femoral weight and the femoral trabecular volume were increased in the L-N and L-H groups (Tables 2, 3). The percent area occupied by the resting surface was increased, while the resorbing surface was significantly decreased in the femoral endosteal surface (Table 4).

Discussion

Previous studies have shown that the activity states of the bone surfaces can be diagnosed by direct examination with SEM (Boyde and Hobdell, 1969; Wink, 1982; Chen et al., 2001a). In the present study, we applied SEM and morphometric approaches to examine the effects of the low or high calcium diet on the bones in young growing rats. We did not find any significant changes in serum calcium, PTH, calcitonin levels and bone morphology in the animals fed with the high calcium diet. Kanis and Passmore (1989) stated that it was not clear whether high calcium intake increased bone mass. Some authors reported that the net calcium absorption from the small intestine seemed to be proportional to the calcium intake. A high calcium diet raised the blood calcium level and the serum calcitonin level (Persson, et al., 1993). Whereas Creedon and

Table 3. Femoral weight, length, trabecular thickness, number, and volume

DAY	DIET	FEMUR WEIGHT (mg)	FEMUR LENGHT (mm)	BV/TB (%)	Tb. Th (mm)	Tb. N (per mm)
3	N	85.6±2.4	20.15±0.13	24.8±3.6	31.3±3.9	3.2±0.4
	L	81.3±2.8	20.02±0.15	24.2±4.1	30.5±4.2	3.1±0.4
	H	84.4±2.4	20.10±0.11	23.9±3.9	32.6±4.6	3.0±0.5
7	N	109.9±0.8	21.28±0.10	25.7±3.2	33.7±4.0	3.2±0.4
	L	74.7±1.5[a]	20.60±0.25	15.3±2.1[a]	26.6±3.1[a]	2.7±0.3[a]
	H	111.3±1.5	21.05±0.08	24.8±4.1	32.9±3.7	3.3±0.5
15	N	162.2±2.8	24.67±0.24	22.0±2.8	35.2±3.8	3.4±0.4
	L	87.4±1.3[a]	23.77±0.06	11.7±1.9[a]	27.1±3.3[a]	2.4±0.3[a]
	H	161.5±4.7	23.73±0.08	23.3±2.4	36.3±4.2	3.5±0.5
30	N	286.8±4.2	30.83±0.35	21.8±2.5	30.5±3.6	3.6±0.5
	L	161.7±3.9[a]	30.47±0.24	8.5±1.4[a]	20.4±2.5[a]	1.9±0.3[a]
	H	290.1±5.7	31.09±0.46	22.5±3.9	31.1±4.4	3.4±0.7
	L-N	189.7±2.8[a,b]	30.52±0.27	10.7±1.2[a,b]	23.8±3.2[a]	2.4±0.4[a,b]
	L-H	191.2±3.2[a,b]	30.13±0.30	11.2±1.5[a,b]	22.6±2.9[a]	2.5±0.3[a,b]

Values are shown in mean±SEM. N: normal diet; L: low calcium diet; H: high calcium diet. [a]: $p<0.05$ vs normal calcium group; [b]: $p<0.05$ vs low calcium group.

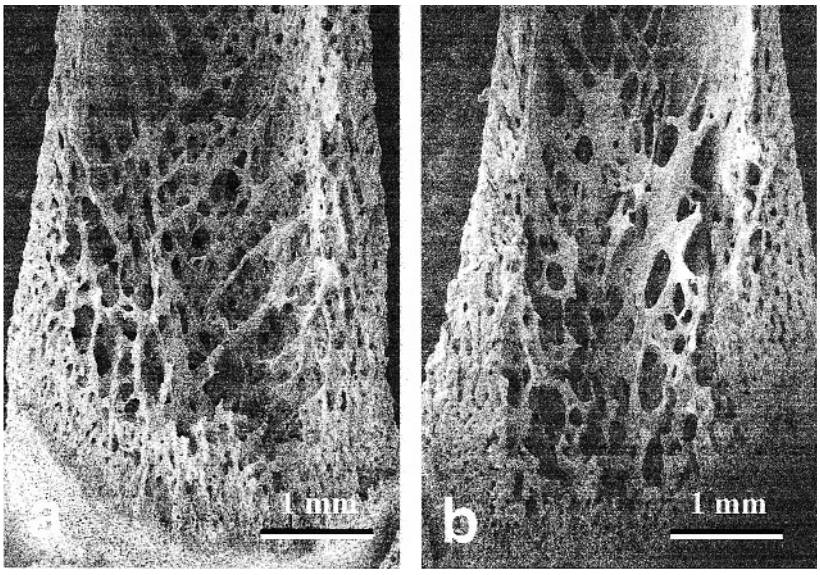

Fig. 2. The femoral distal metaphyses of the rats fed with the normal **(a)** and low **(b)** calcium diets for 7 days. The amount of the trabecular bone is reduced in the rat fed with the low calcium diet.

Cashman (2001) suggested that increasing dietary calcium intake had no effect on bone mineral composition or bone resorption in young growing female rats. In the present study, we found that the food taken by animals in the high calcium group was less than that of the normal or low calcium group. We did not measure the absorption rate of the calcium from small intestine. We speculate that the unchanged serum calcium level after intake of high calcium diet may be related to calcium absorption.

In the present study, we found that the BMD of the whole body, the dry weight of femur and the trabecular bone volume were significantly decreased in the low calcium group. These findings are consistent with the results of the previous studies indicating that a low calcium intake induces a progressive bone loss associated with a fall in serum calcium (Stauffer et al., 1973; Peterson et al., 1995). However, the body weight, bone longitudinal growth, and serum calcitonin level were not greatly affected. It was reported that the cows fed with the low calcium diet showed a significant increase in thyroid calcitonin activity (Yarrington et al., 1977). Persson et al. (1993) reported that the serum calcitonin level was unaffected in young rats fed with the

Table 4. Bone surface types and vascular canal openings on endosteal surface.

DAY	DIET	BONE SURFACE TYPE (%)			VASCULAR CANAL OPENINGS	
		Forming	Resting	Resorbing	diameter (mm)	area (%)
3	N	21.6±3.4	53.5±8.1	24.9±3.9	12.3±3.6	7.3±1.9
	L	20.2±3.7	53.3±8.8	26.5±4.2	11.5±3.2	7.6±1.8
	H	19.3±2.8	55.1±9.5	25.6±4.6	12.2±2.5	8.2±1.4
7	N	18.9±2.9	59.4±8.3	21.7±4.0	13.6±3.5	8.5±1.2
	L	22.6±4.4	44.3±9.2[a]	33.1±3.1[a]	25.1±4.6[a]	13.4±2.5[a]
	H	19.2±4.2	58.2±7.9	22.6±3.7	14.3±3.0	7.9±1.5
15	N	16.1±3.6	65.7±7.2	18.2±3.8	13.7±3.7	9.3±1.5
	L	23.3±3.2[a]	41.6±9.0[a]	35.1±3.3[a]	30.4±5.2[a]	16.2±3.7[a]
	H	17.5±4.3	65.2±8.1	17.3±4.2	14.6±2.9	9.8±1.8
30	N	13.2±3.4	72.0±9.8	14.8±2.9	16.2±2.8	10.9±1.6
	L	18.7±3.8[a]	42.9±7.7[a]	38.4±2.5[a]	31.8±5.9[a]	29.4±2.5[a]
	H	12.9±4.3	73.2±11.4	13.9±2.6	15.7±3.4	11.6±2.9
	L-N	15.4±2.7	56.8±7.2[a,b]	27.8±3.7[a,b]	28.5±6.1[a]	21.0±3.6[a,b]
	L-H	16.2±3.2	55.2±8.3[a,b]	28.6±4.2[a,b]	29.3±6.7[a]	20.7±3.5[a,b]

Values are shown in mean±SEM. N: normal diet; L: low calcium diet; H: high calcium diet. [a]: $p<0.05$ vs normal calcium group; [b]: $p<0.05$ vs low calcium group.

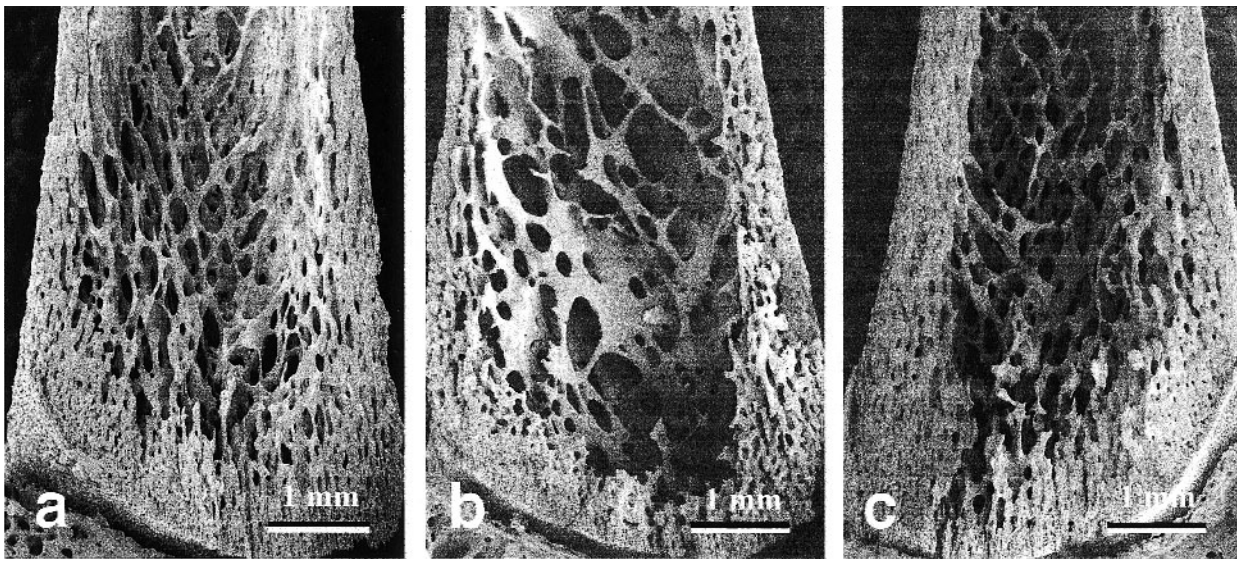

Fig. 3. The femoral distal metaphyses of the rats fed with the normal **(a)**, low **(b)** and low-normal **(c)** calcium diets for 30 days. The rat fed with the low calcium diet had less trabecular bone, while the rat fed with the low and normal calcium diets had little more trabecular bone than that of the low calcium diet.

low calcium diet. Recently, we found that there was no marked morphological change in the thyroid C cells of the rats fed with the low calcium diet (Chen et al., 2001b).

In this study, we found that the serum calcium level was continuously decreased and the serum PTH level was progressively increased in the rats fed with the low calcium diet. A study carried out on pigs showed that the bone loss in low calcium intake resulted from both stimulated bone resorption and decreased mineralization rate, without any impairment of the capacity of the osteoblasts, which were essentially due to compensatory secondary hyperparathyroidism (Eklou-Kalonji, et al., 1999). Parathyroid chief cells possess calcium-sensing receptors on their membranes (Brown et al., 1993). These cells secrete PTH strictly according to the serum calcium level. Previous reports have shown that low calcium intake significantly increased the serum PTH level and that the consequent secondary hyperparathyroidism contributes to the subsequent bone loss (Salomon, 1972; Stauffer et al., 1973; Persson et al., 1993, Naveh-Many et al., 1995, Chen et al., 2001b). Recently, we found morphologically that the rapid bone loss in young rats induced by the low calcium diet is essentially due to hyperactivity of the parathyroid gland. The stimulated gland may be a result of hypertrophy at the early stage and a combination of hypertrophy and hyperplasia at the later stage of calcium deficiency (Chen et al., 2001b). The expression of PTH gene in the parathyroid tissues and parathyroid cell proliferation

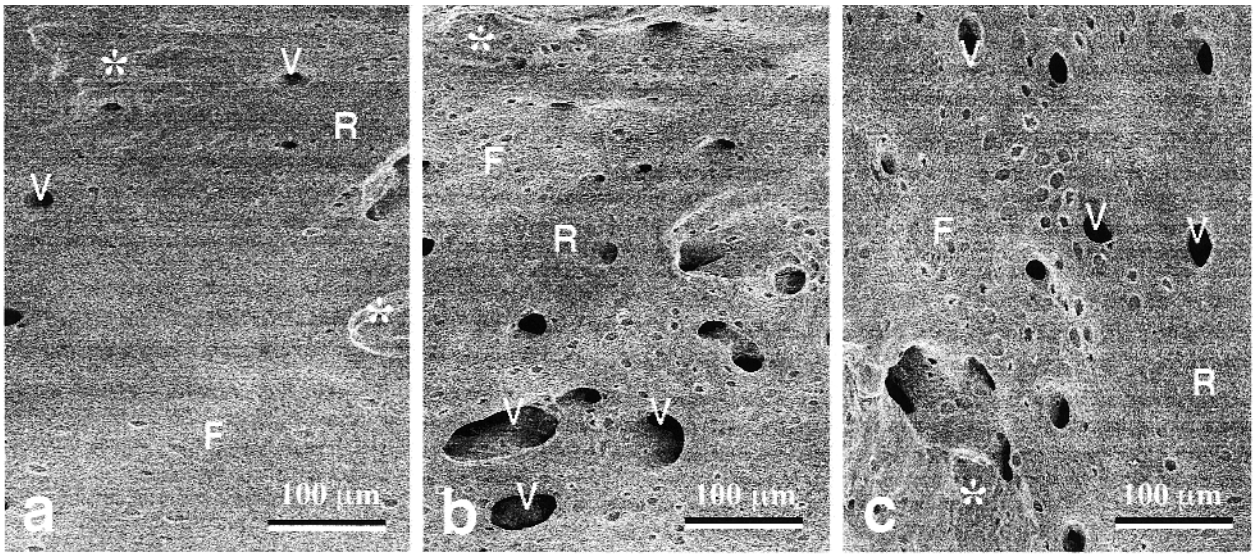

Fig. 4. The endosteal surfaces of the femoral diaphyses in the rats fed with the normal **(a)**, low **(b)** and low-normal **(c)** calcium diets for 30 days. Note numerous large vascular canal openings (V) in the rats fed with the low and low-normal calcium diets. F: forming surface; R: resting surface; Asterisk: resorbing surface.

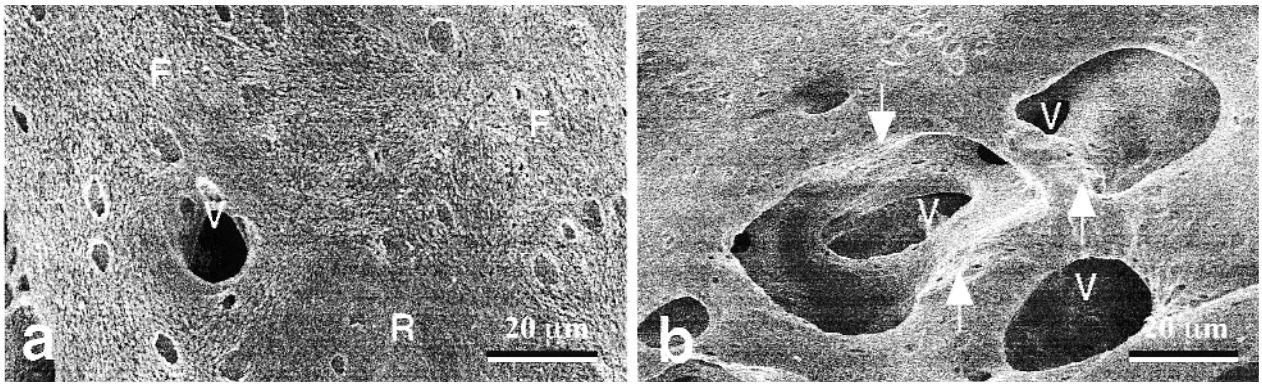

Fig. 5. The endosteal surfaces of the femoral diaphyses in the rats fed with the normal **(a)** and low **(b)** calcium diets for 30 days. The vascular canal opening (V) is small and has smooth walls in the rat fed with the normal calcium diet. Large vascular canal openings (V) with bone resorption (arrows) are found in the rat fed with the low calcium diet. F: forming surface; R: resting surface.

were also increased after feeding the low calcium diet (Naveh-Many et al., 1995). It is likely that the increase in serum PTH levels initiate the first response of bone to the reduction in serum calcium after feeding the low calcium diet (Seto et al., 1999; Mocetti et al., 2000).

In the present study, we also found that the percent area occupied by the resorbing surface in the femur increased and the resting surface decreased in the rats fed with the low calcium diet from day 7 when compared with the animals fed with the normal or high calcium diet. The percent area of the resorbing surface was larger than that of the forming surface. The vascular canal openings were larger and numerous, reflecting the porous nature of the endosteal bone in the rats after feeding the low calcium diet. These findings indicate that the bone turnover is accelerated and the bone resorption exceeds the bone formation, resulting in decreased bone mass. After feeding the low calcium diet, the blood vessels in bones were increased which might be associated with the exchange of calcium between the bone and vessel (Kayanja et al., 1969). In the endosteal surface of the femoral diaphysis from castrated rat, which showed the characteristic osteoporotic changes, the vascular canal openings were larger, the percent area of the resorbing surface increased (Wink, 1982). We believe that the femoral osteopenia in young rat after low calcium intake results from accelerated bone resorption along the blood vessel.

During the 30-day experiment, we found that normal or high calcium intake partially prevented the bone loss induced by low calcium intake. Calcium had a consistent prevention effect on the rate of bone loss in postmenopausal women. This effect was greatest when the baseline calcium was low, confirming the hypothesis of a calcium threshold beyond which the effect of calcium is reduced (Cumming et al., 1990). The variations in calcium intake throughout the early phases of the rat life cycle would affect bone mass status in later life-cycle phases (Peterson et al., 1995). Low calcium intake through adolescence had a nonreversible and deleterious effect on peak bone mass, whereas higher intake promoted greater peak bone mass and provided potential protection from age-related bone loss (Nordin and Heaney, 1990; Peterson et al., 1995). The data suggest that sufficient calcium intake is beneficial in improving the bone change induced by the low calcium intake in young growing rats.

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