PEDIATRIC DENTAL JOURNAL 14(1): 5–15, 2004

Effect of the combination of ipriflavone and 1α-OH-D_3 on debilitant bone in growing rats —Ultrastructural study of endochondral ossification—

Kazushige Ueda, Ikuko Nishida, Bin Xia, Iwan Tofani, Jianguo Wang, Yasuhiro Nishikawa and Mitsutaka Kimura

Department of Pediatric Dentistry, Kyushu Dental College
2-6-1 Manazuru, Kokurakita-ku, Kitakyushu City, Fukuoka 803-8580, JAPAN

Abstract Five-week-old male Wistar rats were used to study the effect of dietary therapy with ipriflavone combined with 1α-OH-D_3. Ultrastructural alterations in the metaphysis of debilitated tibia were observed in the growing rats.

I. Light microscopy findings
In the low-calcium diet • standard diet with supplementary ipriflavone and 1α-OH-D_3 group, calcification of the chondral matrix and ossification were active, and the tibia grew normally as in the control group.

II. Scanning electron microscopy findings
In the low-calcium diet • standard diet with supplementary ipriflavone and 1α-OH-D_3 group, dense calcospherites, distinct chondral lacunae, regularly running collagen fibers, and distinct border lines were noted.

III. Transmission electron microscopy findings
In the low-calcium diet • standard diet with supplementary ipriflavone and 1α-OH-D_3 group we found that the osteoblasts were active, the ruffled border of osteoclast was decrease, indicated this osteoclast is inactive.

In conclusion, insufficient calcium intake during the developmental period resulted in debilitated(3etaphysis tibia, whereas dietary therapy using combined ipriflavone and 1α-OH-D_3 promoted recovery.

Key words
1α-OH-D_3,
Growing rats,
Ipriflavone,
Metaphysis tibia

Introduction

Recently dietary therapies, physiologic active substances, exercise, and/or bone resorption inhibitors have been used to treat this disease[1]. Further, it has been shown that estrogen, which is known as an inhibitor of bone resorption after menopause, has a remarkable effect on osteoporosis, though it is not used routinely because of its potential carcinogenicity[2]. Other substances such as vitamin D_3, vitamin K and calcitonin have also been used, though they each have differing effect on the inhibition of bone resorption and activation of bone formation. As calcitonin, is limited to be given clinically because patients become refractory after several days of administration, which is known as the calcitonin escape phenomenon, therefore, it is not considered a suitable therapy for osteoporosis[3]. Recently, research about the effect of ipriflavone (IF), which comes from beans and is an inducer of isoflavone, on bone has been focused on. Its structure is similar to estrogen, and it has been used as an inhibitor of bone resorption, though it is considered a non-hormonal drug for osteoporosis. It also has been demonstrated to have antiallergic, anti-inflammatory, and analgesic effects[2]. Further, IF has also been reported to inhibit the activity of osteoclasts and promote that of osteoblasts[2,4,5]. While other studies have focused on the effect of a combination therapy with IF and other substances[6,7].

Bone mass reaches its peak in humans at about

Received on February 7, 2003
Accepted on September 1, 2003

Table 1 Composition of experimental diets (%)

Ingredients	Standard diet	Low-calcium diet (Ca 144 mg/100 g)
β-corn starch	38.00	37.64
Vitamin-free casein	25.00	25.00
α-potato starch	10.00	10.00
Cellulose powder	8.00	8.00
Soy bean oil	6.00	6.00
Mineral mixture	6.00	6.00
Granulated sugar	5.00	5.00
Vitamin mixture	2.00	2.00
$CaCO_3$		0.36

Table 2 The origin of element content from the mineral mixture of the diet (mg/100 g)

	Standard diet	Low-calcium diet
Ca	480	144
P	650	612
Mg	87	87
Na	220	293
K	440	746
Fe	32	32
Cu	0.46	0.5
Zn	3.4	3.0
Mn	1.6	2.6
I	0.46	0.3
Cl	170	174

the age of 20 years old, and then begins to decrease with aging. Thus, an important prophylactic treatment for osteoporosis is to improve peak bone mass and promote of osteoblasts. Vitamin D has been shown to have an effect on promoting calcium absorption, as well as normalizing bone metabolism by affecting osteoblasts[8–11]. To date, there are no reports of the combination of IF and 1α-OH-D_3 effect on endochondral ossification. Therefore, we investigated the effect of the combination of IF and 1α-OH-D_3 therapy on endochondral ossification by histopathological and ultrastructural methods.

Materials and methods

Twenty 5-week-old male Wistar rats, each weighing approximately 40 g, were randomly divided into 4 groups of 5 each. They were housed individually in small cages under conditions of 22 ± 2°C with humidity of 50 ± 5% and a 12 hour light-dark cycle. The study protocol was approved by the committee for the use of laboratory animals of Kyushu Dental College.

Table 3 Composition of experimental diets (%)

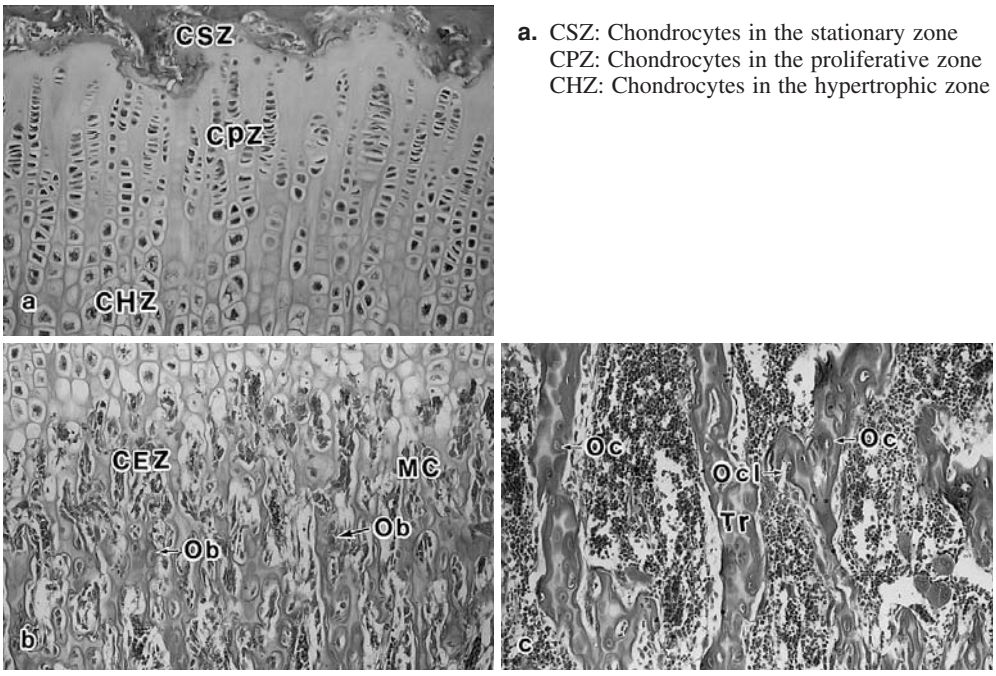
Standard diet with supplementary IF	
Standard diet	91.5
Ipriflavone	8.5

In the control group, rats were fed a standard diet and given tap water freely for 6 weeks. They were orally administered olive oil at 2 m*l*/kg of body weight 3 times each week. In the low-calcium diet group, rats were given a low-calcium diet (30% calcium of the standard diet) and distilled water freely for 6 weeks, as well as olive oil according to the protocol used in the control group. In the low-calcium diet • standard diet group, rats were fed a low-calcium diet and given distilled water freely for 3 weeks then switched to a standard diet and tap water for the next 3 weeks, as well as olive oil as in the control group. In the low-calcium diet • standard diet with supplementary ipriflavone and 1α-OH-D_3 group, rats were fed a low-calcium diet and given distilled water freely for 3 weeks, then switched to an IF (Seakuyoshitomi Co., Ltd., Japan) supplemented standard diet with tap water, and orally administered a 1α-OH-D_3 solution at 2 m*l*/kg of body weight 3 times each week for the next 3 weeks. To prepare the 1α-OH-D_3 solution, we dissolved 0.05μg/kg of 1α-OH-D_3 (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan).

All the diets were made by Oriental Yeast, Tokyo, Japan, and the components are presented in Tables 1–3.

Sample preparation

After 6 weeks of experiment, all rats were killed under deep anesthesia, and the tibia bones removed. Metaphysis tibia bone samples for examinations using light microscope, scanning electron microscope (SEM; S-3300N. Hitachi, Ltd., Japan) and transmission electron microscope (TEM; JEM-1200EX, Japanese Electric Co., Ltd.) were prepared similar to those described previously[12,13].

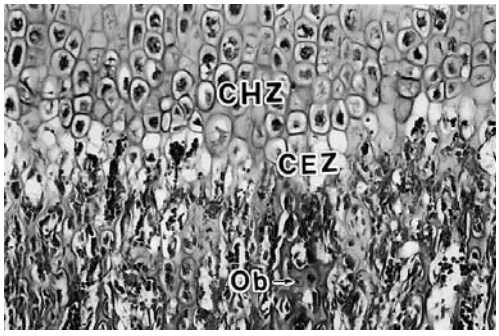


a. CSZ: Chondrocytes in the stationary zone
CPZ: Chondrocytes in the proliferative zone
CHZ: Chondrocytes in the hypertrophic zone

b. CEZ: Chondrocytes in the erosion zone
MC: Multinuclear cell Ob: Osteoblast

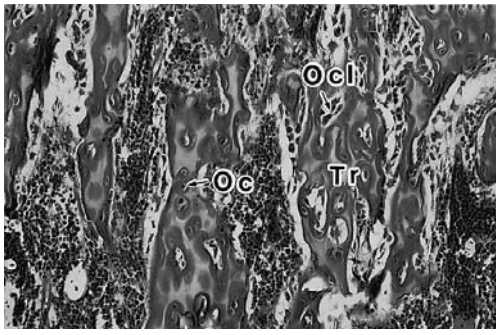
c. Oc: Osteocyte Ocl: Osteoclast
Tr: Trabecula

Fig. 1 Light microscopic findings: Metaphysis specimen from control group H•E (×100)



CHZ: Chondrocytes in the hypertrophic zone
CEZ: Chondrocytes in erosion zone Ob: Osteoblast

Fig. 2 Metaphysis specimen from low-calcium diet group H•E (×100)



Oc: Osteocyte Ocl: Osteoclast Tr: Trabecula

Fig. 3 Metaphysis specimen from IF and 1α-OH-D_3 group H•E (×100)

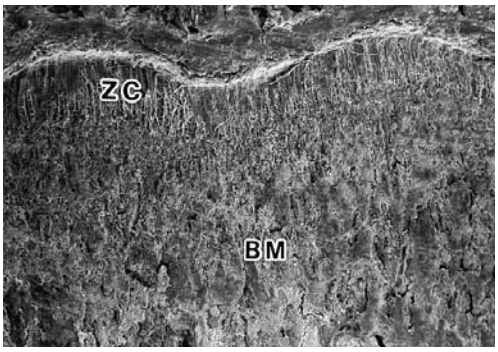
Results

Light microscopy findings

In the control group, whole images of the metaphysis tibia were shown in Fig. 1a–1c. In the low-calcium diet group, the calcification matrix surrounding hypertrophic chondrocytes, as well as the amount of primary spongy bone were decreased. Further, osteoblasts were scarce, development of the tibia was inhibited, and the trabeculae were thin (Fig. 2).

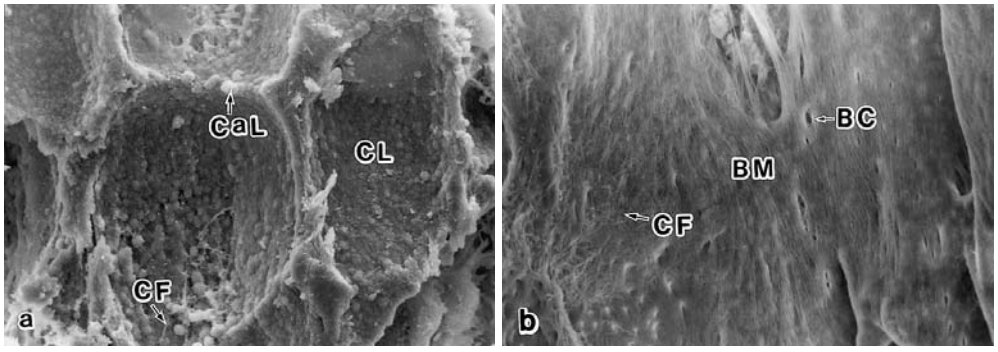
Calcification was activated in the low-calcium diet•standard diet group, as compared to the low-calcium diet group, and the number of chondroclasts was increased. Osteoblasts in the erosion zone were often noted, and bone formation was active.

In the low-calcium diet•standard diet with supplementary ipriflavone and 1α-OH-D_3 group, non-differentiated chondrocytes in the stationary zone showed abundant fibers lining the joint surfaces. Adjacent to the stationary zone was proliferative



ZC: Zone of cartilage BM: Bone matrix

Fig. 4 SEM image of tibia metaphysis specimen from the control group (×35)



a. CL: Chondral lacuna CaL: Calcospherite CF: Collagen fibrils

b. BM: Bone matrix BC: Bone canaliculus CF: Collagen fibrils

Fig. 5 SEM image of control group specimen (×2,000)

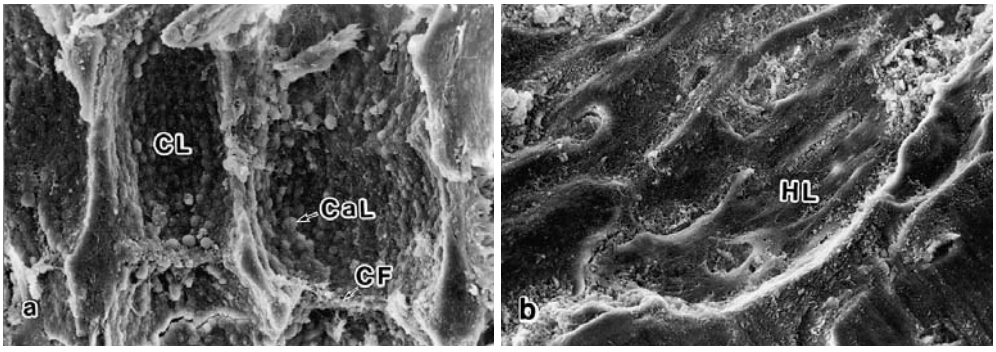
zone, in which chondrocytes had become organized into distinct columns, and demonstrated a low nucleus-to-cytoplasm ratio. Next was the hypertrophic zone, in which multinuclear chondroclasts were noted and the matrix had become mineralized. Further, some matrix vesicles were found, which suggested that calcification was taking place. Beyond that area, indication of the chondrocytes disappearance were often seen, osteoblasts and newly formed capillaries which penetrate to the bone matrix were found. These findings were similar to the control group, which showed that the displacement from cartilage to bone was normal (Fig. 3).

SEM findings

Using an SEM, we were able to observe the ultrastructure of hypertrophic chondrocytes on the cut surface of the metaphysis tibia. With low magnification, the entire image was shown in Fig. 4.

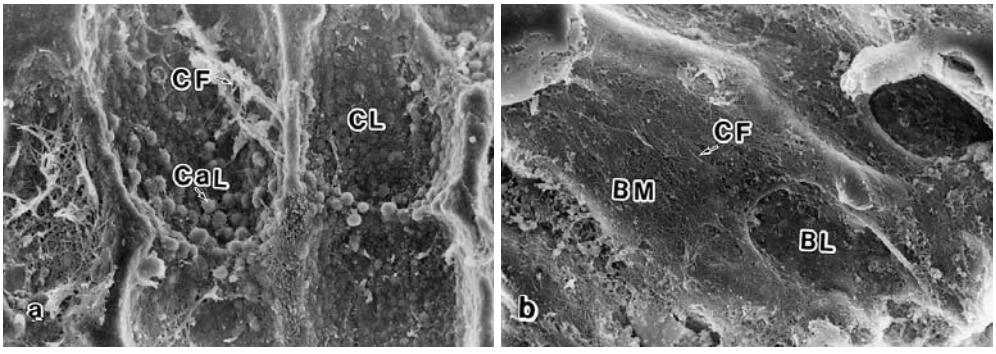
In the control group, the chondral lacunae, approximately 20–30 microns in length, showed a distinct border with an ovoid appearance and were mostly associated with regularly arranged collagen fibrils. Multiple distinct calcospherites, 1–3 microns in length, were dissolved on the walls of the chondral lacunae. The surface collagen fibers had a networked appearance, and sparse collagen fibers were found among the calcospherites (Fig. 5a). In the bone matrix, osteocyte lacunae were noted, most of which were distinct, and there were many bone canaliculi opened on the walls of the lacunae, which were rounded with regular collagen fibrils. On the bone surface, collagen fibers showed bifurcation and anastomosis (Fig. 5b). Further, Howship's lacunae, most of which were shallow with indistinct borders, were occasionally seen.

When compared with the control group, the shapes of most of the chondral lacunae in the low-



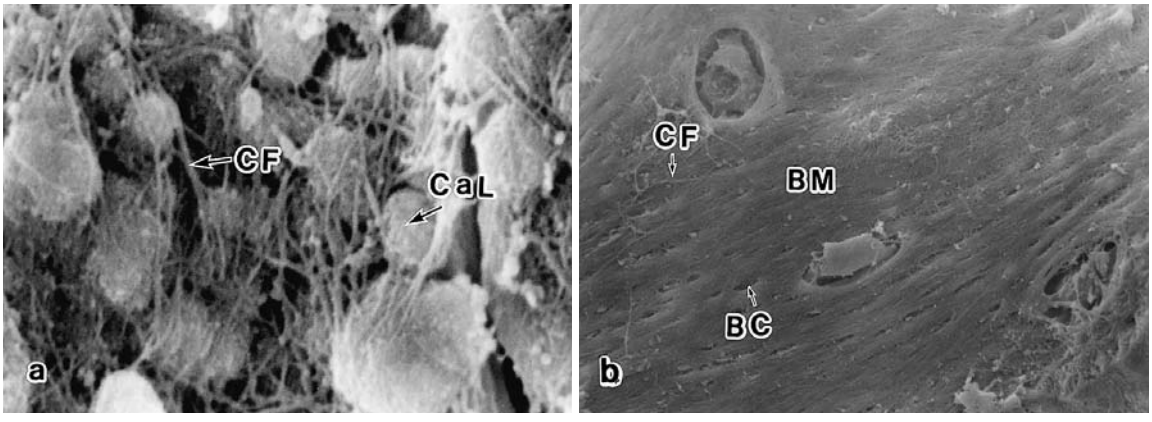
a. CL: Chondral lacuna CaL: Calcospherite CF: Collagen fibrils **b.** HL: Howship's lacuna

Fig. 6 SEM image of low-calcium diet group specimen (**a**: ×2,000, **b**: ×1,000)



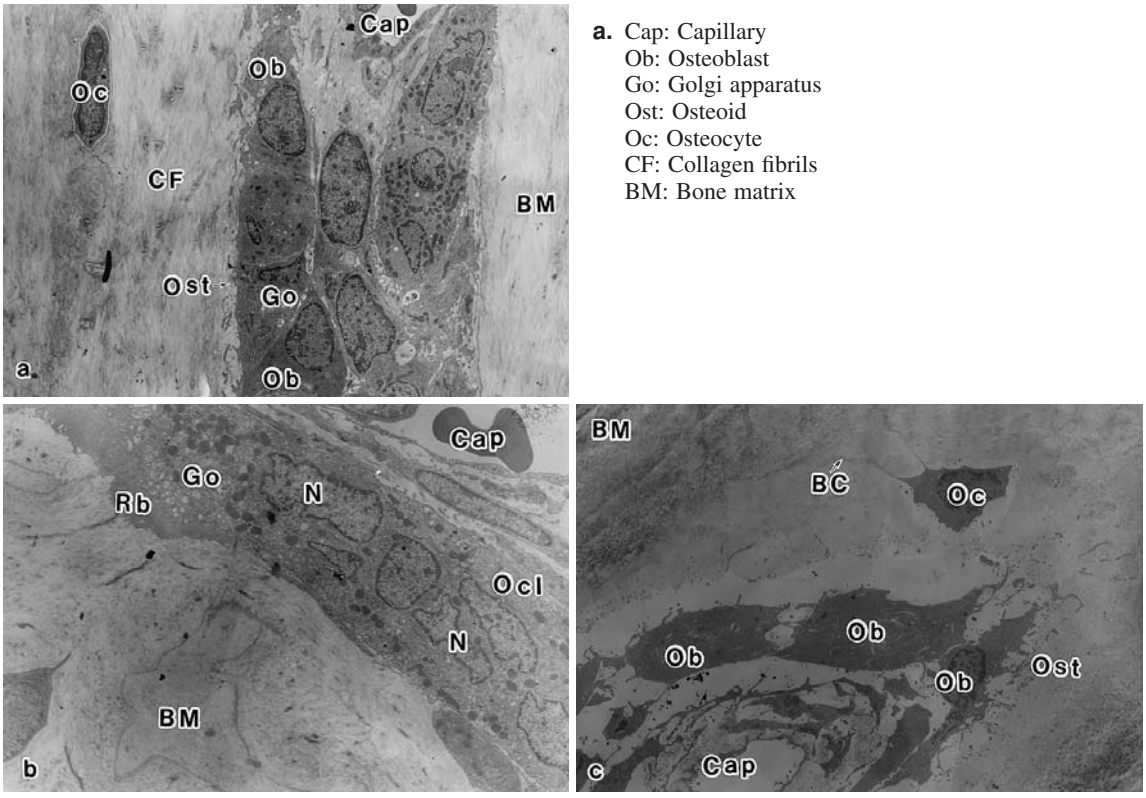
a. CL: Chondral lacuna CaL: Calcospherite CF: Collagen fibrils **b.** BM: Bone matrix BL: Bone lacuna CF: Collagen fibrils

Fig. 7 SEM image of low-calcium and standard diet group specimen (×2,000)



a. CaL: Calcospherite CF: Collagen fibrils **b.** BM: Bone matrix BC: Bone canaliculus CF: Collagen fibrils

Fig. 8 SEM image of IF and 1α-OH-D_3 group specimen (**a**: ×15,000, **b**: ×2,000)



a. Cap: Capillary
Ob: Osteoblast
Go: Golgi apparatus
Ost: Osteoid
Oc: Osteocyte
CF: Collagen fibrils
BM: Bone matrix

b. Cap: Capillary Ocl: Osteoclast
Go: Golgi apparatus Rb: Ruffled border
N: Nucleus BM: Bone matrix

c. Cap: Capillary Ob: Osteoblast
Ost: Osteoid Oc: Osteocyte
BC: Bone canaliculus BM: Bone matrix

Fig. 9 TEM image of control group specimen (**a**: ×2,000, **b**: ×2,500, **c**: ×2,500)

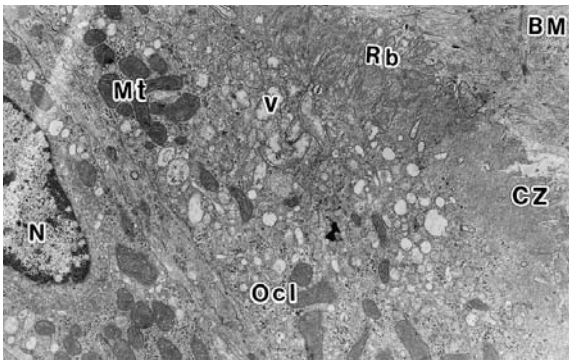
calcium diet group were irregular, the calcospherites were sparse and incomplete dissolved. In some areas, collagen fibers connecting the calcospherites were found, while in other areas only calcospherites were noted (Fig. 6a). Areas of bone resorption were seen more often than those of bone formation, which was a characteristic of this group. Most resorption lacunae were shallow with indistinct borders. Bone matrixes were observed in the networks of collagen fiber and microfibril noted on the surfaces (Fig. 6b).

In the low-calcium diet • standard diet group, the cartilage zone was distinct, and the number of calcospherite and collagen fibers in the chondral lacunae were increased, as compared with the low-calcium diet group. However, when compared with the control group, the calcospherites were not equal in size, and their dissolving incomplete. Further, the collagen fibers were indistinct (Fig. 7a). The area of bone formation was increased greatly as compared to that of the low-calcium diet group, however, the border between the matrix and bone formation was indistinct. Collagen fibers were seen irregular with their bifurcation and anastomosis (Fig. 7b).

In the low-calcium diet • standard diet with supplementary ipriflavone and 1α-OH-D_3 group, the number of calcospherites was greater and their dissolving more obvious, as compared to the low-calcium diet • standard diet group. Further, the collagen fibers were distinct and regular. Tibia growth was similar to that seen in the control group (Fig. 8a). In the bone matrix, areas of bone formation were often seen, whereas bone resorption areas were seldom found. Recovery of bone formation was also noted. The borders of the osteocyte lacunae were distinct, with an abundance of canaliculi (Fig. 8b).

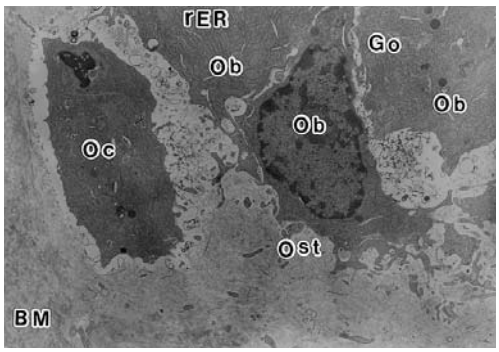
TEM findings

In the control group, osteoblasts were recognized by their mononuclear cell and cuboidal or polygonal shape. They were aggregated into a single layer of cells lying in apposition to the forming bone, with cell processes inserted into adjacent osteoid containing



Ocl: Osteoclast Mt: Mitochondria V: Vacuole
Rb: Ruffled border N: Nucleus BM: Bone matrix
Cz: Clear zone

Fig. 10 TEM image of low-calcium diet group specimen (×7,500)



Ob: Osteoblast Go: Golgi apparatus Ost: Osteoid
Oc: Osteocyte rER: rough endoplasmic reticulum
BM: Bone matrix

Fig. 11 TEM image of low-calcium/standard diet group specimen (×5,000)

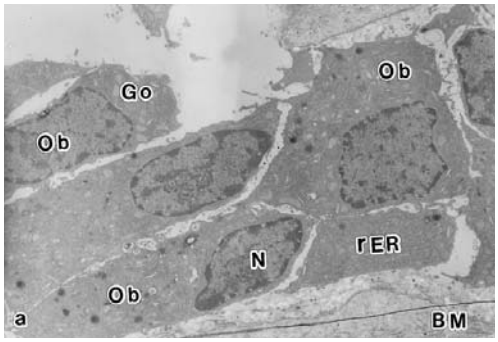
an abundance of collagen fibers. Osteoblasts are known to secrete both collagen and a ground substance that constitutes the initial non-mineralized bone or osteoid. Desmosome junctions between osteoblasts were also noted. Most of the osteoblasts in this group showed well developed mitochondria. Further, active osteoblasts were often seen, with abundant Golgi apparatus and mitochondria found in the adjacent nuclear cytoplasm (Fig. 9a).

Osteoclasts were seen as large multinucleated cells, in various shapes. In areas of resorption, the plasma membrane could be divided into 2 parts, a central region containing numerous plasma membrane infoldings forming microvilli type structures, termed the ruffled border, and a lesser ring-like perimeter of cytoplasm called the clear zone, which more or less demarcated the limits of the bone area being absorbed. Well developed rough endoplasmic reticulum, mitochondria and Golgi apparatus were noted in the cytoplasm, with various vacuoles and dense body-like lysosomes also found (Fig. 9b). The osteocytes had been enclosed by bone matrix that was previously laid down as osteoblasts. These were small and had a high nucleus-to-cytoplasm ratio, though the organelles were poorly developed. Their cytoplasmic processes extended through the canaliculi in the matrix to contact neighboring cells and the lamina limitans, while the borders between the osteoid layer and calcified matrix was distinct. Further, young osteocytes, which appeared as small osteoblasts, had well developed rough endoplasmic reticulum and Golgi apparatus (Fig. 9c).

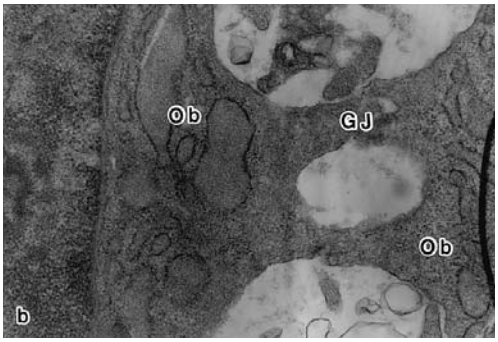
In the low-calcium diet group, the number of osteoblasts as well as the proportion of cuboidal or polygonal osteoblasts, were reduced, while most of the organelles were poorly developed. Only a few chromatins adjacent to the nuclear areas were noted. The main characteristic of this group was abundant active osteoclasts. Further, multinucleated large osteoclasts showed well developed ruffled border and abundant chromatin in the nuclear areas, with abundant vacuoles and mitochondria in the cytoplasm (Fig. 10).

In the low-calcium diet•standard diet group, young osteocytes in the osteoid area with an osteoblast appearance as well as well developed organelles, were often seen. Compared with the low-calcium diet group, the number of osteoclasts was reduced and the proportion of active osteoclasts was decreased. However, in area near the ruffled border we found vacuoles, lysosomes and fragments of collagen fibrils (Fig. 11).

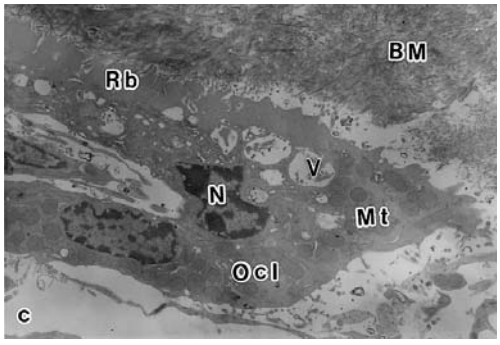
In the low-calcium diet•standard diet with supplementary ipriflavone and 1α-OH-D_3 group, osteoblasts were cubical, spherical or conical in shape and had aggregated into a single layer. The number of mitochondria in them was increased and calcification was active, while the adjacent osteoid layer was thick. Desmosome junctions between osteoblasts were often noted. Chromatins in the nucleus were abundant, and the nucleolus on one side of the nuclear area was clear. Some Golgi apparatus were noted near the nuclear area, however, they were seldom found in other areas of the cytoplasm. In the area near the bone matrix there was abundant rough endoplasmic reticulum (Fig. 12a).



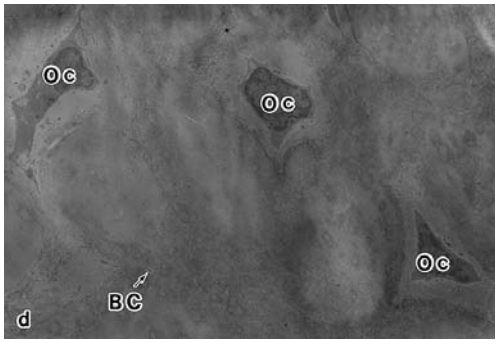
a. Ob: Osteoblast
rER: Rough endoplasmic reticulum
Go: Golgi apparatus BM: Bone matrix



b. Ob: Osteoblast GJ: Gap junction



c. BM: Bone matrix N: Nucleus
Ocl: Osteoclast V: Vacuole
Rb: Ruffled border Mt: Mitochondria



d. BC: Bone canaliculus Oc: Osteocyte

Fig. 12 TEM image of IF and 1α-OH-D_3 group specimen (**a**: ×4,000, **b**: ×30,000, **c**: ×6,000, **d**: ×2,500)

Between the osteoblast processes, gap junctions were found when viewed with high magnification (Fig. 12b). In this group, the osteoclasts were different than in the other groups, as the ruffled borders were poorly developed and there were fewer mitochondria, rough endoplasmic reticulum and lysosomes (Fig. 12c). In areas of ossification, osteocytes in newly formed bone matrix showed poorly developed organelles, which was similar to the control group, and their sizes varied. Most showed a high nucleus-to-plasma ratio and the lamina limitans were clear (Fig. 12d).

Discussion

Level of calcium intake in most Japanese is insufficient[14]. Since bone functions as a place of calcium storage and maintenance of a consistent level in serum, so insufficient calcium intake over a long period, will lead to debilitated bone[9–11]. It is known that peak bone mass in human occurs at around the age of 20 years then reduces with aging, thus it is important to maintain the mass for as long as possible. The effect of 1α-OH-D_3, a hormone that takes part in calcium regulation, is to promote calcium absorption in the intestines and nephronic loop, and it has been suggested that it also has an indirect relationship with bone formation. Further, it was reported that this hormone has an effect on osteoblasts and osteoclasts[15]. While many studies, have shown that 1α-OH-D_3 has a promoting effect on bone formation in the growth stage[8–11]. Recently, the effect of IF, a non-hormonal drug and such effects as inhibiting the activity of osteoclastic cells and improving the activity of osteoblastic cells, have been reported[16]. The positive effect of IF in combination with 1α-OH-D_3 has been expected[6,7]. Our present results demonstrated the effect of IF combined with 1α-OH-D_3 on debilitated bone in rat during the growth stage.

Light microscopy findings

In our experiments, we found that debilitated bone resulting from insufficient calcium intake could be recovered by increased calcium, as calcification was activated and the number of chondroclasts was increased in the low-calcium diet • standard diet group, as compared to the low-calcium diet group. Osteoblasts in the erosion zone were also often noted, and indicated that the bone formation was active, each of which showed the recovery of debilitated bone. These results suggest that calcium intake is very important for the recovery of debilitant bone.

It has been reported that 1α-OH-D_3 has a promoting effect on bone formation in the growing stage, though the number of osteoclasts increase and thin trabeculae have also found[8,9]. It had also been reported that high calcium intake alone could not provide effective recovery from damage caused by insufficient calcium intake[10], though another study found that a high calcium diet had beneficial effect on calcification and bone formation[17].

IF is known to inhibit bone resorption and promote bone formation[2,4,5], and it was reported that IF has other effects such as activating new bone formation, as well as osteoblasts and chondroclasts during the growth stage[18].

Notoya *et al.*[7] reported a significant effect with a combination of IF and 1α-OH-D_3 when compared to either alone, and also noted that the combination promoted bone formation and led to an increase in femoral bone mass. In the present study, newly formed capillaries were found penetrating the bone matrix, newly formed bone matrix, and trabeculae in the low-calcium diet • standard diet with supplementary ipriflavone and 1α-OH-D_3 group. These findings were similar to the control group, thus the displacement from cartilage to bone was normal.

SEM findings

We also found that the metaphysis tibia in the low-calcium diet group had an enlarged cartilage zone, irregular chondral lacunae, sparse and incomplete dissolve of calcospherites, and more resorption lacunae in trabecula, which were similar to results found in another study[17], as well as to those reported in an ultrastructural study of the mandibular condyle[19]. Calcospherites are a type of hydroxyapatite crystals[20], and it is considered that they have a close relation with calcification in endochondral ossification[21]. It has also been reported that, osteoid calcification is inactive in the erosion zone[22]. From those findings and those of the present study, we concluded that insufficient calcium intake will result in an inhibition of endochondral ossification. Moreover, a dietary therapy study in rats, it was found that an increased intake of calcium led to a reduction of bone resorption and promotion of calcification[23].

In a study using 1α-OH-D_3, it was reported that endochondral ossification was activated[24]. However, it is also known that 1α-OH-D_3 alone can not provide effective recovery for debilitated bone[10]. The effects of IF include the inhibition of bone resorption and activation of bone formation[2]. We obtained a satisfactory recovering effect from debilitated bone in the low-calcium diet • standard diet with supplementary ipriflavone and 1α-OH-D_3 group, in which the number of calcospherites was greater and their dissolving was more apparent, as compared to the low-calcium diet • standard diet group. Further, collagen fibers were distinct and regular, which showed that tibia growth was similar to that in the control group. Although similar results have been reported by Ushiroyama *et al.*[6] and Notoya *et al.*[7] they observed vertebral or unloading bone. In the present study, we used growing rats in whom debilitated bone was the result of insufficient calcium intake, and our results showed that the combination IF with 1α-OH-D_3 had satisfactory effect on the recovery of debilitated bone.

TEM findings

In bone ultrastructural studies, it was reported that insufficient calcium intake resulted in osteoblasts being inactivated and reduced, and activated osteoblasts increased, therefore, when compared with bone formation, bone resorption is dominant and bone becomes debilitated[12,25]. Further, in active osteoclast, mitochondria, vacuoles, and the ruffled border were reported to be well developed[9,12]. In studies by Baud[26] and Salmon *et al.*[27] resorption by osteoblasts and osteocytes was noted first, which they termed osteocytic bone resorption. In the present study, the characteristics of the low-calcium diet group were a reduction of active osteoblasts and an abundance of active osteoclasts, which had well developed ruffled borders and abundant chromatin in the nuclear areas, while in the cytoplasm abundant vacuoles and mitochondria were noted.

It was previously reported that when the rat diet was changed from calcium deficient diet to a standard diet, both osteoblasts and osteocytes were activated,

calcification was regulated, and an abundance of young osteocytes were noted[11]. Ozawa *et al.*[28] found that the same diet change caused the calcium in mitochondria to take a part in calcification. Kimura *et al.*[12] also noted that a change from insufficient calcium intake to normal calcium intake resulted in osteoblasts being activated with regular collagen fibers developed around them, and finally activation of bone formation. In the present study, TEM image showed the characteristics of the low-calcium diet • standard diet group to be thick osteoid formation around the osteocytes, an abundance of osteoblastic cells from pre-osteoblast to young osteocyte stage, and promoted bone formation.

In a study on the effect of 1α-OH-D_3 on bone, it was reported that the hormone had an improving effect on bone formation, though it was not so effective as calcitonin, probably because it had an improving effect on both bone formation and osteoclasts[9]. A similar result was reported by Weisbrode *et al.*[29], who found that a large amount of 1α-OH-D_3 resulted in the activation of many kinds of cells, such as chondrocytes, osteoclasts, osteoblasts and osteocytes, whereas a small amount of 1α-OH-D_3 only had an effect on osteoblasts. It is also known that formative osteocytes are similar to osteoblasts in that they have well developed organelles, which was first reported by Jande *et al.*[30]

IF is thought to combine with osteoclasts and pre-osteoclasts, and then inhibit their activity. It has been reported that IF has an inhibitory effect on osteoclast mediated bone resorption and new osteoclast formation[31] and that it improved the calcium content in osteoclasts in rats[32] and in a human pre-osteoclastic cell line, FL29[33]. IF was also shown to be able to enhance the effect of estrogen, and improve the secretion of calcitonin and resorption of bone[34]. On the other hand, IF stimulated a human osteoblastic cell line, UMR-106, to proliferate and differentiate[35]. In another study of the condition of differentiation in human and rat osteoblasts, IF had an improving effect on the activities of alkaline phosphate, collagen fiber synthesis, and osteocalcin formation, as well as the expression of some important matrix proteins and facilitation of the mineralization process[36]. In an ultrastructural report by Kimura *et al.*[12] the number of osteoclasts was reduced in the standard diet combined with IF group and most were inactive, whereas osteoblasts proliferation was active and bone formation activated.

In conclusion, although there are some studies on the skeletal effect of IF combined with 1α-OH-D_3, to our knowledge there is no report on the effect of that combination by observing histological changes of rat tibias in the growth stage. We found that IF combined with 1α-OH-D_3 promoted the recovery of debilitated bone caused by insufficient calcium intake in the growth stage.

This paper was postered at the 40th Annual Meeting of Japanese Society of Pediatric Dentistry held in Chiba, June 6th, 2002.

References

1) Yamazaki, I., Shino, A. and Tsukuda, R.: Effect of ipriflavone on osteoporosis induced by ovariectomy in rats. *J Bone Miner Metab* **3**: 205–210, 1986.
2) Agnusdei, D., Adami, S., Cervetti, R., Crepaldi, G., Di Munno, O., Fantasia, L., Isaia, G.C., Letizia, G., Ortolani, S., Passeri, M., Serni, U., Vecchiet, L. and Gennari, C.: Effects of ipriflavone on bone mass and calcium metabolism in postmenopausal osteoporosis. *Bone Miner* **19**: S43–S48, 1992.
3) Takahashi, S., Goldring, S., Karz, M., Hilsenbeck, R., Williams, R. and Roodman, G.D.: Downregulation of calcitonin receptor mRNA expression by calcitonin during human osteoclast-like cell differentiation. *J Clin Invest* **95**: 167–171, 1995.
4) Morita, I., Sakaguchi, K., Kurachi, T. and Murota, S.: Ipriflavone inhibits murine osteoclast formation *in vitro*. *Calcif Tissue Int* **51**: S7–S10, 1992.
5) Sekimoto, R.: Ipriflavone. *The Bone* **14**: 91–93, 2000.
6) Ushiroyama, T., Okamura, S. and Ikeda, S.: Efficacy of ipriflavone and 1α vitamin D therapy for the cession of vertebral bone loss. *Int J Gynecol Obstet* **48**: 283–288, 1995.
7) Notoya, K., Yoshida, K., Tsukuda, R., Taketomi, S. and Tsuda, M.: Increase in femoral bone mass by ipriflavone alone in combination with 1 alpha-hydroxyvitamin D_3 in growing rats with skeletal unloading. *Calsif Tissue Int* **58**: 88–94, 1996.
8) Matsumoto, T.: Molecular biology of bone and osteoporosis. Publisher of Medicare Co., Ltd., Tokyo, 1996, pp. 351–360. (in Japanese)
9) Beckman, M.J. and Deluca, H.F.: Modern view of vitamin D_3 and its medicinal uses. *Prog Med Chem* **35**: 1–56, 1998.
10) Rodan, G.A., Raisz, L.G. and Bilezikian, J.P.: Pathophysiology of osteoporosis in principles of bone biology. 2nd edition. Academic Press, New York, 2002, pp. 1275–1289.
11) Kinjou, T.: Radiological light and electron microscopic study on combined therapy of calcium and physiologic active substance for bone debility—especially changes in the mandibular condyle—. *J Kyushu Dent Soc* **45**: 143–174, 1991.

12) Kimura, M., Nishida, I., Xia, B., Maki, K. and Tofani, I.: Ultrastructural study on the effect of dietary therapy on bone debility in developing rats—on the enchondral ossification—. *Dentistry in Japan* **38**: 109–116, 2002.
13) Xia, B., Maki, K., Hidata, A., Nishioka, T., Kamitani, H. and Kimura, M.: The effect of zinc and exercise on the enchondral ossification of growing rats—an ultrastructural study on metaphysis of tibia—. *Ped Dent J* **12**: 53–63, 2002.
14) Department of Health and Medicine, Ministry of Health and Welfare: Nutrient survey for Japanese in 1999. Daiti Publish House, Tokyo, 2001. (in Japanese)
15) Seita, T., Ozawa, H. and Takahashi, S.: Chemistry of bone. Ishiyaku Publisher Inc., Tokyo, 1990, pp. 170–178. (in Japanese)
16) Tsuda, M., Kitazaki, T. and Itoh, T.: The effect of ipriflavone (TC-80) on bone resorption in tissue culture. *J Bone Miner Res* **1**: 207–211, 1987.
17) Yamano, W., Inoue, H., Umezu, T., Kaku, H. and Kimoto, T.: Densitometry and histopathological study on diet therapy for bone debility at the growth stage of rat—application of ipriflavone—. *Jpn J Ped Dent* **39**: 1–12, 2001. (in Japanese, English abstract)
18) Kathleen, A. and Head, N.D.: Ipriflavone: An important bone-building isoflavone. *Altern Med Rev* **4**: 10–22, 1999.
19) Fujimoto, M.: Scanning electron microscope of calcification of cartilage matrix in the mandibular condyle. *Jpn J Oral Biol* **24**: 62–71, 1982.
20) Fujimoto, M., Taniuchi, Y. and Takiguchi, R.: Energy dispersive X-ray analysis on cartilage of the mandibular condyle. *Jpn J Oral Biol* **22**: 412–417, 1980.
21) Bonnucci, E.: Fine structure of early cartilage calcification. *J Ultrast Res* **20**: 33–50, 1967.
22) Egawa, K., Niimura, A. and Takiguchi, R.: Scanning electron microscope of endochondral ossification of the mandibular condyle. *Jpn J Oral Biol* **22**: 661–665, 1980.
23) Chen, H., Hayakawa, D., Emura, S., Ozawa, Y., Okumura, Y. and Shoumura, S.: Effect of low or high dietary calcium on the morphology of the rat femur. *Histol Histopathol* **17**: 1129–1135, 2002.
24) Inoue, Y., Nishida, I., Watanabe, T. and Uchikanbori, M.: The effect of zinc combined with activated viatmin D_3 on the metaphysis of the tibia in rats at growth stage—an electron microscope study—. *Jpn J Ped Dent* **38**: 776–779, 2000. (in Japanese, English abstract)
25) Odell, W.D. and Heath, H.: Osteoporosis: pathophysiology, prevention, diagnosis, and treatment. *Dis Mon* **39**: 789–867, 1993.
26) Baud, C.A.: Submicroscopic structure and functional aspects of the osteocyte. *Clin Orthop Relate Res* **56**: 227–236, 1968.
27) Salmon, C.D. and Volpin, G.: Fine structure of bone resorption in experimental osteoporosis caused by calcium deficient diet in rats. *Calcif Tissue Res* **4**: 80–82, 1970.
28) Ozawa, H. and Yamamoto, T.: An application of energy-dispersive X-ray microanalysis for the study of biological calcification. *J Histochem Cytochem* **31**: 210–213, 1983.
29) Weisbrode, S.E., Capen, C.C. and Nagode, L.A.: Fine structural and enzymatic evaluation of bone in hypoparathyroidectomized rats receiving various levels of vitamin D. *Lab Invest* **28**: 29–37, 1973.
30) Jande, S.S. and Belanger, L.F.: Ultrastructural changes associated with osteocytic in normal trabecular bone. *Anat Res* **163**: 204, 1969.
31) Notoya, K., Yoshida, K. and Taketomi, S.: Inhibitory effect of ipriflavone on osteoclastmediated bone resorption and new osteoclast formation in long-term cultures of mouse unfractionated bone cells. *Calcif Tissue Int* **53**: 206–209, 1993.
32) Albanese, C.V. Cudd, A. and Argentino, L.: Ipriflavone directly inhabits osteoclastic activity. *Biochem Biophys Res Commun* **199**: 930–936, 1994.
33) Benvenuti, S., Petilli, M. and Frediani, U.: Binding and bioeffects of ipriflavone on human preosteoclastic cell line. *Biochem Biophys Res Commun* **201**: 1084–1089, 1994.
34) Yamazaki, I.: Effects of ipriflavone on the response of uterus and thyroid to estrogen. *Life Science* **38**: 757–764, 1986.
35) Benvenuti, S., Tanini, A. and Frediani, U.: Effects of ipriflavone and its metabolites on a clonal osteoblastic cell line. *J Bone Miner Res* **6**: 987–996, 1991.
36) Cheng, S.L., Zhang, S.F. and Nelson, T.L.: Stimulation of human osteoblast differentiation and function by ipriflavone and its metabolites. *Calcif Tissue Int* **55**: 356–362, 1994.