Effect of the combination of ipriflavone and 1α-OH-D₃ on debilitant bone in growing rats—Ultrastructural study of endochondral ossification—

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Abstract Five-week-old male Wistar rats were used to study the effect of dietary therapy with ipriflavone combined with 1α-OH-D₃. 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-D₃ 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-D₃ 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-D₃ 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 metaphysis tibia, whereas dietary therapy using combined ipriflavone and 1α-OH-D₃ promoted recovery.

Key words 1α-OH-D₃, Growing rats, Ipriflavone, Metaphysis tibia

Introduction
Recently dietary therapies, physiologic active substances, exercise, and / or bone resorption inhibitors have been used to treat this disease¹. 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 carcinogenicity². Other substances such as vitamin D₃, 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 osteoporosis⁵. 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 effects⁶. Further, IF has also been reported to inhibit the activity of osteoclasts and promote that of osteoblasts⁷,⁸. While other studies have focused on the effect of a combination therapy with IF and other substances⁹,¹⁰.

Bone mass reaches its peak in humans at about
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With aging, the age of 20 years old, and then begins to decrease. 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 \(1\alpha\)-OH-D\(_3\) effect on endochondral ossification. Therefore, we investigated the effect of the combination of IF and \(1\alpha\)-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 \(22\pm2^\circ\)C with humidity of \(50\pm5\%\) 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.

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 \(2\) ml/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 \(1\alpha\)-OH-D\(_3\) 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 \(1\alpha\)-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 \(1\alpha\)-OH-D\(_3\) solution at \(2\) ml/kg of body weight 3 times each week for the next 3 weeks. To prepare the \(1\alpha\)-OH-D\(_3\) solution, we dissolved 0.05 g/kg of \(1\alpha\)-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\(^12\)\(^,\)\(^13\).

<table>
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<th>Table 1 Composition of experimental diets (%)</th>
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<tr>
<td>Ingredients</td>
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<td>(\beta)-corn starch</td>
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<td>Vitamin-free casein</td>
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<tr>
<td>(\alpha)-potato starch</td>
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<td>Cellulose powder</td>
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<td>Soy bean oil</td>
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<td>Granulated sugar</td>
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<th>Table 2 The origin of element content from the mineral mixture of the diet (mg/100 g)</th>
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<th>Table 3 Composition of experimental diets (%)</th>
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<td>Standard diet with supplementary IF</td>
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<tr>
<td>Standard diet</td>
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<td>Ipriflavone</td>
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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-D₃ group, non-differentiated chondrocytes in the stationary zone showed abundant fibers lining the joint surfaces. Adjacent to the stationary zone was proliferative
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-
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Fig. 6 SEM image of low-calcium diet group specimen  
(a: ×2,000, b: ×1,000)

- CL: Chondral lacuna  
- CaL: Calcospherite  
- CF: Collagen fibrils  
- HL: Howship’s lacuna

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

- CL: Chondral lacuna  
- CaL: Calcospherite  
- BM: Bone matrix  
- BL: Bone lacuna  
- CF: Collagen fibrils

Fig. 8 SEM image of IF and 1α-OH-D₃ group specimen  
(a: ×15,000, b: ×2,000)

- CaL: Calcospherite  
- CF: Collagen fibrils  
- BM: Bone matrix  
- BC: Bone canaliculus  
- CF: Collagen fibrils
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-D₃ 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
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 chromat 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-D3 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).
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\(^4\). 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.
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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 debilitated bone.

It has been reported that 1α-OH-D₃ has a promoting effect on bone formation in the growing stage, though the number of osteoclasts increase and thin trabeculae have also found8,9). It had also been reported that high calcium intake alone could not provide effective recovery from damage caused by insufficient calcium intake10, though another study found that a high calcium diet had beneficial effect on calcification and bone formation17).

IF is known to inhibit bone resorption and promote bone formation2,4,5), and it was reported that IF has other effects such as activating new bone formation, as well as osteoblasts and chondroclasts during the growth stage6).

Notoya et al.7) reported a significant effect with a combination of IF and 1α-OH-D₃ 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-D₃ 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 study17), as well as to those reported in an ultrastructural study of the mandibular condyle6).

Calcospherites are a type of hydroxyapatite crystals20 and it is considered that they have a close relation with calcification in endochondral ossification21). It has also been reported that, osteoid calcification is inactive in the erosion zone22). 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 calcification23).

In a study using 1α-OH-D₃, it was reported that endochondral ossification was activated24). However, it is also known that 1α-OH-D₃ alone cannot provide effective recovery for debilitated bone10).

The effects of IF include the inhibition of bone resorption and activation of bone formation25). We obtained a satisfactory recovering effect from debilitated bone in the low-calcium diet • standard diet with supplementary ipriflavone and 1α-OH-D₃ 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-D₃ 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 debilitated12,25). Further, in active osteoclast, mitochondria, vacuoles, and the ruffled border were reported to be well developed9,12).

In studies by Baud26) 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\(^1\). 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. In 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\(^11\) and that it improved the calcium content in osteoclasts in rats\(^12\) and in a human pre-osteoclastic cell line, FL29\(^13\). IF was also shown to be able to enhance the effect of estrogen, and improve the secretion of calcitonin and resorption of bone\(^14\). On the other hand, IF stimulated a human osteoblastic cell line, UMR-106, to proliferate and differentiate\(^15\). 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\(^16\). 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.

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References