An Ultrastructural Study on the Effect of Ipriflavone and Calcitonin on Debilitated Mandibular Condyle

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Abstract

In this study, 5-week-old rats, corresponding to the childhood in human, were used. The aim of this study was to observe the effect of Ipriflavone (IF) and Eel Calcitonin (ECT) on debilitated mandibular condyle. The ultrastructural results were as follows.

1. Light microscope finding
The finding of ECT-IF group were similar to those of the control group, in that the number of trabeculae increase, the osteoblasts lined on the bone surface markedly, and the growth of mandibular condyle showed accelerated.

2. Scanning electron microscope findings
In ECT-IF group, the territorial matrix, agglutinations of calcospherite and distinct chondral lacunae were found, as seen in the control group. The collagen fiber around calcospherite arranged regularly. In bone matrix, the areas of bone formation were often seen, and distinct borders of osteocyte lacunae and canaliculi were found.

3. Transmission electron microscopy findings
The characteristic of ECT-IF group was the osteoclasts were inactive and the ruffled borders were poorly developed. The osteoblasts were active. The whole finding of the group was similar to the control group.

In conclusion, debilitated mandibular condyle resulting from insufficient calcium intake in developmental period could be recovered by dietary therapy using 1α-OH-D₃ and ipriflavone supplementary standard diet.

Key Words: Ipriflavone/Calcitonin/Mandibular Condyle/Rats
Introduction

The Eel Calcitonin (ECT) work as one of calcium regulating hormones just as vitamin D₃, parathyroid hormone (PTH). It is known that the ECT is secreted by the thyroid gland, and the target organs are bone and kidney, and it has effect on inhibiting osteoclasts, keeps content of calcium in bone and regulating the calcium content in blood plasma with PTH. Some of researches suggest that the ECT has relation with parathyroid cancer, ectopic ECT syndrome, hypercalcemia and hypocalcemia. In Japan, the calcitonin is taken from ten sorts of animals such as eel, fish, and pig, and it is used clinically. The ipriflavone (IF), which is gotten from plants and is a sort of flavonoids, was reported having an inhibiting effect on bone resorption. Moreover, it has been known that the estrogen having a proving effect on the secretion of calcitonin, so it has lots of effects such as inhibiting the bone resorption and bone mass decrease. In our knowledge, there was no report on the long effect of IF and ECT, so in this study we made an observation of the effect of ECT and IF on mandibular condyle of growing rats.

Materials and Methods

Twenty 5-week-old male Wistar rats were divided into four groups (five in each group) randomly. Rats were housed in small cages individually under similar conditions at 22±2°C, humidity 50±5% on a 12 h/12 h light–dark cycle.

In the control group, rats were fed on standard diet and given tap water freely for six weeks. The physiological saline was intramuscularly injected every day, 2 ml/kg body weight.

In low calcium diet group, rats were fed on low calcium diet (30% calcium of the standard diet) and given distilled water freely for six weeks. The physiological saline was intramuscularly injected every day, 2 ml/kg body weight.

In the low calcium and standard diet group, rats were fed on low calcium diet (30% calcium of the standard diet) and given distilled water freely for three weeks. Then the rats were fed on standard diet and tap water, physiological saline was intramuscularly injected every day, 2 ml/kg body weight, for next three weeks.

In the low calcium diet and ECT–IF group, rats were fed on low calcium diet (30% calcium of the standard diet) and given distilled water freely for three weeks. Then rats were fed on IF supplemented standard diet and tap water, the ECT was intramuscularly injected every day in a dose of 0.2 U/kg body weight for next three weeks.

All the rats' diet was made by Oriental Yeast, Tokyo, Japan. The components of the diets are presented in Table 1~3, which was the same with the study of Yamáno et al.

After 6 weeks of experiment, the rats were sacrificed under deep anesthesia (pentobarbital sodium, 250 mg/kg, Nippon Pharmacy). The mandibular condyles were removed.

1. The samples for light microscopy
The samples were fixed in 10% neutral buffered formalin for one month, decalcified in
Table 1 Composition of experimental diets (%)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Standard diet</th>
<th>Low-calcium diet (Ca144 mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-corn starch</td>
<td>38.00</td>
<td>37.64</td>
</tr>
<tr>
<td>Vitamin-free casein</td>
<td>25.00</td>
<td>25.00</td>
</tr>
<tr>
<td>α-potato starch</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>8.00</td>
<td>8.00</td>
</tr>
<tr>
<td>Soy bean oil</td>
<td>6.00</td>
<td>6.00</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>6.00</td>
<td>6.00</td>
</tr>
<tr>
<td>Granulated sugar</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Vitamin mixture</td>
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</tr>
<tr>
<td>CaCO₃</td>
<td>0.36</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Element</th>
<th>Standard diet</th>
<th>Low calcium diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>480</td>
<td>144</td>
</tr>
<tr>
<td>P</td>
<td>650</td>
<td>612</td>
</tr>
<tr>
<td>Mg</td>
<td>87</td>
<td>87</td>
</tr>
<tr>
<td>Na</td>
<td>220</td>
<td>293</td>
</tr>
<tr>
<td>K</td>
<td>440</td>
<td>746</td>
</tr>
<tr>
<td>Fe</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Cu</td>
<td>0.46</td>
<td>0.5</td>
</tr>
<tr>
<td>Zn</td>
<td>3.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Mn</td>
<td>1.6</td>
<td>2.6</td>
</tr>
<tr>
<td>I</td>
<td>0.46</td>
<td>0.3</td>
</tr>
<tr>
<td>Cl</td>
<td>170</td>
<td>174</td>
</tr>
</tbody>
</table>

Table 3 Composition of experimental diets (%)

<table>
<thead>
<tr>
<th></th>
<th>Standard diet with supplementary IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard diet</td>
<td>91.5</td>
</tr>
<tr>
<td>Ipriflavone</td>
<td>8.5</td>
</tr>
</tbody>
</table>

5 % nitric acid, dehydrated through a graded ethanol series and embedded in paraffin. 7 µm sections were made successively and stained with hematoxylin and eosin for light microscopy.

2. The samples for scanning electron microscope (SEM)

The samples were continually immersed in 2.5 % glutaraldehyde (pH7.2) for 12 hours under 4 °C then were cleaned with 10 % sodium chlorite solution by super sonic wave to remove adhesion. They were rinsed by buffered phosphate acid (pH7.2) before entering.
post-fixation, which was performed in 1% osmic acid buffer solution (pH7.2) under 4°C for two hours. After fixation, these samples were dehydrated through a graded ethanol series, and treated with t-butanol, then dried by freeze-drying method. The cracked surfaces were coated with a layer of aurum (JFC-1100, Japanese Electric Co, Ltd.) observed under a scanning electron microscope (S–3300N, Hitachi, Ltd., Japan).

3. The sample for transmission electron microscopy (TEM)

Samples were immersed in a fixative mixture of 1% paraformaldehyde and 3% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) under 4°C for one week, then rinsed with 0.05 M sodium cacodylate buffer solution (pH 7.2, 3 × 1 hour) before decalcification. The decalcification was performed in 5% EDTA–2Na solution for two weeks. The samples were rinsed with 0.05 M sodium cacodylate buffer solution (pH 7.2) again, and were additionally dissected into prismatic blocks of tissue. The postfixation was done with 2% osmic acid buffer solution (pH 7.2) for two hours, then dehydration through a graded ethanol series, diaphanization in propylene oxide, finally embedding in epoxy resin. Semithin sections were cut at 0.35 μm thickness, stained with toluidine blue, and examined using light microscope to chose target areas which would be observed in TEM. The ultrathin serial sections were cut at 70–90 nm thickness using a diamond knife on an ultramicrotome (LKB–4800, LKB) after trimming the blocks. The ultrathin sections were placed on the Cu/Rh grids, stained with uranyl acetate and lead citrate, according to the method described by Watson and Reynolds, later observed under a TEM (JEM–1200EX, Japanese Electric Co, Ltd.).

The study was approved by the committee for the use of laboratory animals of Kyushu Dental College, Japan.

Results

1. Light microscope

In control group, the whole image of mandibular condyle was as follows. The undifferentiated chondrocytes in stationary zone had a high nucleus–to–cytoplasm ration, showed ovoid–shaped, the long axis of them were in parallel with the surface of articular surface, with many regular fibers. Adjacent to the zone is the proliferative zone in which the chondrocytes undergo division and become organized into distinct columns, the cells which is called chondrocyte in proliferative zone, most of them were large having a low nucleus–to–cytoplasm ratio. Next is hypertrophic zone, in which the chondrocytes are greatly enlarged and the matrix become mineralized. In the cytoplasm of the chondrocytes, there are abundant matrix vacuoles. Beyond that is chondrocytes in erodent zone, among them the chondroclasts are often seen, and newly formed capillaries penetrate into bone matrix and osteoblasts were found. The process of endochondral ossification can be seen here. Then trabeculae were formed (Figs.1a, b).

The characteristic of low calcium diet group was that the thickness of hypertrophic zone increased and the proliferative zone decreased. The calcification matrix around hypertrophic
Ipriflavone and Calcitonin on Mandibular Condyle (Wang et al.)

Fig. 1 Mandibular condyle of the control group.
a. H-E stain (×20), b. H-E stain (×100)
  CSZ: Chondrocytes in stationary zone
  CPZ: Chondrocytes in proliferative zone
  CHZ: Chondrocytes in hypertrophic zone
  CEZ: Chondrocytes in erodont zone
  MC: Multinuclear cell
  Ob: Osteoblast
  NFT: New formed trabecula
  Tr: Trabecula

Fig. 2 SEM image of the control group.
a. (×1,000), b. (×1,000)
  CL: Chondral lacuna
  CaL: Calcospherite
  TM: Territorial matrix
  BL: Bone lacuna
  BC: Bone canaliculus
  BM: Bone matrix
  CF: Collagen fibrils

Chondrocytes and the number of primary spongy bone decrease. The chondroclast were seldom found, the endochondral ossification was inactive.

In low calcium diet and standard diet group, compared with low calcium diet group, the osteoblasts in the erodont zone were often noted, and the bone formations were active. The first spongy trabeculae were often found.

In ECT-IF group, the calcification of chondral matrix and the number of chondroclasts were similar to control group, the endochondral ossification was recovered form debilitated state and the growth of mandibular condyle was found also.

2. SEM findings
   In SEM observation, we mainly observed the ultrastructure of hypertrophic chondrocytes in mandibular condyle of the cut surface.
   In control group, the chondral lacunae showed distinct border, and multiple distinct calcospherites were noticed. The collagen fibers in the wall of chondrocytic lacunae were regular and dense, the territorial matrixes were clear, and in most chondral lacunae the
calciospherites were dense (Fig. 2a). In bone matrix, the osteocyte lacunae were noted, most of them were distinct, and there were many bone canaliculi opened their mouth, which was rounded with regular collagen fibrils, at its wall. On the bone surface, the collagen fibers were regular or irregular (Fig. 2b).

Compared with control group, the shapes of most chondral lacunae in low calcium diet group were irregular, and the calciospherites were sparse, the conglutination of them were incomplete. In some areas the collagen fiber connecting with calciospherites were found, while in other areas only the calciospherites were noted (Fig. 3a). The ratio of the area of resorption to formation is higher than that of control group, which was the characteristic of this group. Most borders of resorption bays were indistinct, in this area the osteocyte lacunae were seen and the majority of resorption bays were shallow, the mouth of canaliculi and collagen fiber were seen on the wall of osteocyte lacunae (Fig. 3b).

In low calcium diet and standard diet group, compared with low calcium diet group, the number of calciospherites and collagen fibers in chondral lacunae increased, but compared with control group, the size of calciospherites were inequable, and the agglutinations of them were incomplete and the borders of osteocyte were indistinct. Most territorial matrixes among chondral lacunae were fragmentary. The bone formation area increased greatly than that of low calcium diet group. The characteristic of this group was the areas of bone formation were often found than that of bone resorption and the thickness of newly formed trabeculae were thin (Fig. 4).

In ECT–IF group, the number of calciospherite was greater and the agglutinations of them
were more obvious than that of low calcium diet and standard diet group. Moreover, the territorial matrix and distinct chondral lacunae were easily found. Although some fragmentations of some chondral lacunae were found, the growths of mandibular condyle were similar with that of control group (Fig. 5a). In bone matrix, the areas of bone formation were often seen, while the areas of bone resorption were seldom found, the recover of bone formation was found. But some of collagen fibers run irregularly, and some indistinct borders of osteocyte lacunae were found in the group (Fig. 5b).

3. TEM findings

In control group, the osteoblasts were recognized by mononuclear cell and by their cuboidal or polygonal shape, their aggregation as a single layer or cells lying in apposition to the forming bone and the cell process of them inserting into adjacent osteoid. The osteoblasts secrete both collagen and ground substance that constitutes the initial unmineralized bone or osteoid. Most osteoblasts in this group had well developed mitochondria. The active osteoblasts were often seen, in the cytoplasm adjacent to nuclear abundant Golgi apparatus and mitochondria were found, the desmosomes between osteoblasts were also found. The osteocyte was enclosed by bone matrix that had previously laid down as an osteoblast. They were small and had a high nucleus–cytoplasm ratio, the organelles were poorly developed. They extend their cytoplasmic processes through canaliculi in the matrix to contact neighboring cells and the Lamina limitans were distinct. The young osteocyte, which seemed as small osteoblast, had well developed rough endoplasmic reticulum and Golgi apparatus. The osteoprogenitor cells, which showed spindle shape and aggregation as a single layer, were noted (Fig. 6a, b).

In the low calcium diet group, the proportion of cuboidal or polygonal osteoblasts in this group reduced, the organelles of most of them were poorly developed. The characteristic of this group was that lots of active osteoclast were noted. In some place, the osteoclasts intruded in the line of osteoblasts were found, the osteoclasts were multinucleated large cell, the Golgi apparatus and mitochondria of them were well developed, and there were
abundant chromatin in nuclear. The ruffled borders had well developed microvilli, and the clear zone, which more or less demarcated the limits of the bone area being absorbed, in this area contained abundant vacuole, lysosome and mitochondria (Fig. 7).

In low calcium diet and standard diet group group, the active osteoblasts were often seen, and between them the gap junctions were noted. Most osteoblasts had well developed free ribosome, Golgi apparatus and mitochondria and in the nuclear abundant chromatin were noted. The characteristic of this group was the number of osteoblast increased and the gap junctions between osteoblasts, osteocytes and young osteocytes were often found. Compared with low calcium diet group, the number of osteoclast reduced, and the proportion of active osteoclast decreased. But in the part near to Ruffled borders the vacuoles, lysosome and fragment of collagen fibrils were found. The osteocytes in bone matrix, connected each
other with gap junction, were noted, and some of them had a few rough endoplasmic reticulum and mitochondria (Fig. 8).

Compared with control group, the clusters of osteoblast and osteoclasts closing to osteoblasts and them parting from bone surface were often observed in ECT–IF group. The characteristic of this group was the osteoclasts were different with other groups, which was the Ruffled border of them were poorly developed, and there were few mitochondria and rough endoplasmic reticulum but there were lots of vacuoles and lysosome in cytoplasm. Some material in the vacuoles was noted in the cytoplasm the Golgi apparatus and mitochondria were well–developed, which meant that its function of bone absorption was prevented (Fig. 9a). The osteoblasts showed cubical, spherical or conical shape and aggregating as a single layer, the number of mitochondria in them increased, and the calcification was active, the thickness of osteoid was thick, and the Lamina limitans was
seldom found. The chromatins in nucleus were abundant and around them there were abundant collagen fibers. The young osteocyte, which was similar with osteoblast but was smaller than them and was in bone matrix, had well developed rough endoplasmic reticulum and mitochondria in cytoplasm. And in ossification area the osteocytes in newly formed bone matrix showed poorly developed organelles, which was similar to what was seen in control group (Fig. 9b).

Discussion

The osteoporosis is characterized by that the bone metabolism is off balance and the fragility of bone increases. The pain of waist and spine is often complained, and the fracture often takes place in aging person. After menopause the estrogen level of old women decreased obviously, which results in suffering from osteoporosis easily. Now various of drug were used for osteoporosis, such as the calcium supplement, vitamin D, vitamin K, estrogen and calcitonin. It has been thought that those material has an effect on inhibiting the decrease of bone mass. Now it has been proved that the estrogen has remarkable effect on osteoporosis but because of having carcinogenicity it was not used routinely. So other drugs which have less side effect are used. One of those drug is calcitonin, which was found in 1963, and has been proved to have effect on bone by Aliapoulis et al. (1966)\(^5\), and on osteoclast by Warshawsky et al. (1980)\(^6\). Now it is used as a drug for osteoporosis and there were lots of reports on it. In Japan, it has been reported by Orimo et al. (1996)\(^7\) and Fujita et al. (1990)\(^8\) that CT has an effect of bone mass increase. Its mechanism of it was thought that CT had inhibiting effect on pro-osteoclast and osteoclast, so the bone resorption was inhibited.

The IF has been used as an inhibitor of the bone resorption in Japan, because the structure of IF is similar to estrogen but it does not have carcinogenicity. There were lots of studies on the effect of it on menopausal osteoporosis therapy and it has been found that it was effective\(^9\).\(^{10}\) Specially, it has been reported that the IF has effect on inhibiting the bone resorption and activity of osteoclasts\(^6\), and improving the activity of alkaline phosphate\(^7\).\(^8\).

1. Light microscope

It has been known that the ossification of mandibular condyle is enchondral ossification. It was reported that the calcification of mandibular condyle was similar to other long bones till 5-day-old, and the cartilage would remain on the surface of condyle. It was also reported by Oogoshi\(^9\) that chondrocytes in mandibular condyle showed distinct zonation and differentiation in three-week-old rats, and the trabecula formation and the thickness of cartilage reduction were found in six-week-old rats. After six-week-old, the thickness of cartilage decreases resulting from the reduction of the number of chondrocyte in the zone of proliferation and hypertrophy. At the same time, the calcification was found in hypertrophic zone, the enchondral ossification took place\(^8\). In present study, the calcium deficient diet group showed the zone of mandibular condyle hypertrophied and the number
of trabeculae reduced, which was similar to our series of studies\textsuperscript{12}. In the low calcium diet and standard diet group, the recovery of bone was noted, which suggested that the bone mass decrease resulting from insufficient state of calcium could be recovered by calcium intake. Takeshima (1987)\textsuperscript{20} reported that the ECT had recovering effect on the osteoporosis, and in normal rats ECT could promote the bone formation. It has been thought that ECT has direct inhibition effect on proliferation of osteoclast, so the formation gains a dominant position. IF has been known as having inhibition effect on the proliferation of osteoclast and bone absorption. According to this, in this study the ECT–IF group showed similar light microscope finding with control group, which suggested that the ECT used with IF had satisfactory effect on the debilitated mandibular condyle recovery and growth.

2. SEM finding

There were a few reports on the calcification of cartilaginous matrix in mandibular condyle\textsuperscript{21, 22}. Fujimoto (1982)\textsuperscript{23} reported that the calcospherite existed in the growing mandibular condyle. We observed almost the same finding in control group. The debilitated state of low calcium diet group was clear, suggesting that the calcium was taken from bone in order to maintain the stability of calcium of the rats suffering from insufficient calcium intake. To mammal, maintaining the stability of calcium in serum is very important, and bone plays a role as the storage of calcium, if the level of serum calcium declines the calcium will be taken from the bone. In low calcium diet and standard diet group, bone formation was in good state, the calcospherite was dense, the calcospherite is made up from apatite the main ingredients are calcium and phosphorus, and it has been reported that the calcospherite has close relation with the primary calcification\textsuperscript{24}. But compared with control group, the condyle was still in debilitated state. In ECT–IF group, the same finding with control group was found, which meant the debilitated bone could be recovered. In the study by Takeshima (1987)\textsuperscript{20} using the ECT alone, it was reported that the recovery of bone was noted but was not as good as in control group. Similar result was reported by Monji (1991)\textsuperscript{25} who used ECT with high calcium diet. In this study, we got satisfactory results using IF with ECT.

3. TEM finding

In this study, the number of osteoblast decreased, the number of osteoclast increased and most of them were active and found in low calcium diet group, which suggested that the calcium were taken from bone. In low calcium diet and standard diet group, the number of osteoblast increased and most of them were active, which suggested that the recovery from low calcium state. In ECT–IF group, compared with low calcium and standard diet group, the activity of osteoclast was inhibited, and the differentiation and activity of osteoblast was activated. Mature osteocyte increased, and the young osteocyte showed a decreasing tendency. Compared with low calcium diet group, the active osteoclast was seldom noted, and active osteoblast were often found, which was considered as the effect of IF, so the bone mass equilibrium was turned into positive state. In the study of CT on osteoclast\textsuperscript{26}, it was reported that the ruffled border disappeared in one hour. And it was
reported by Daketomi (1993)\textsuperscript{15} that there was a possibility that IF took an inhibition effect on bone resorption alone or combined with PTH or calcitonin, whose mechanism has been known. In this study, we found that the IF had effect on activating differentiation of osteoblast and inhibiting the activity of osteoclast, so the debilitated mandibular condyle could be recovered.

Conclusion

In this study, 5–week–old rats, corresponding to the childhood in human, were used. The aim of this study was to observe the effect of IF and ECT on debilitated mandibular condyle. With the results obtained, we draw a conclusion as follow. The insufficient calcium intake in developmental period would result in mandibular condyle being debilitated, and the dietary therapy using ECT and IF supplementary standard diet had harmless effect on it and had an effect on promoting it to be recovered.

Reference


虚弱下顎頭軟骨に対するカルシトニンとイプロリフラボンの
併用療法による微細構造的
研究

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日 高 彰 子・西 川 康 博・葛 立 宏
木 村 光 孝

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ヒトの幼児期に相当する生後5週齢のWistar系雄ラットを用いて、我々はラット幼児期の虚弱下顎頭軟骨に対するホルモン製剤であるウナギカルシトニン誘導体（ECT）とイプロリフラボン（IF）の影響を微細構造的に検査し、次の結果を得た。

1. 光学顕微鏡所見

ECTとIF添加標準食を加えることにより対照群と同様の所見を呈し、軟骨層下方向に連続して著しい骨梁の増加がみられ、その表面は骨芽細胞の配列が著明で、下顎頭の成長は促進されていた。

2. 走査型電子顕微鏡所見

低カルシウム・ECT・IF添加標準食群は対照群と同様に軟骨小腔の区画が明瞭で、石灰化小球は著しく、小腔壁面は縦走基質がみられるようになり、さらに石灰化小球周辺はコラーゲン原線維が多数分布していた。骨形成面は対照群と同様に幅広らしく認められ、骨小管と周囲基質との境界は明瞭であった。

3. 透過型電子顕微鏡所見

低カルシウム・ECT・IF添加標準食群は骨吸収を遮断して抑制している破骨細胞が多くみられるのが特徴的所見であった。対照群と同様に骨芽細胞の活性は高く、ruffled borderで石灰化した骨組織に接している所もみられるが、ruffled borderは指状突起の発達が悪く、ヒダ状を呈しているものは少なく扁平化している所も多々認められた。

これらのことはECTとIFによる骨吸収作用の抑制と骨形成作用の促進が起こっているためで、骨虛弱状態からの回復が可能であることを示唆している。