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Bisphosphonate and the Eruption of Developing Teeth: Its Effects and Mechanism

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Bisphosphonates have similar chemical structures to endogenous inorganic pyrophosphate which inhibits mineral deposition in biological tissues. Even though their clinical applications have been attempted, their molecular mechanism and cellular effects on dental hard tissue development remain to be elucidated. The present study was performed to investigate their effects on the development of the tooth and their mode of action.

Alendronate, a synthetic derivative of bisphosphonates was subcutaneously daily injected in postnatal day 1 Sprague Dawley rats for successive 10 days. Animals were sacrificed at 3, 12 and 40 days after the final administration and light microscopy, RT-PCR and TUNEL were used for the analyses.

Alendronate inhibited the teeth development and retarded their eruption. In the alendronate group, the mandibular first and second molars were under-developed, compared with those in the control group at day 3. Ameloblasts in the mandibular 1st molar were discontinuous in several parts. The development of the mandibular 2nd molar was deterred by the woven bone growing into the dental papilla. The third molar tooth germ did not appear. The TUNEL positive cells were rarely seen in the normally developing hard tissue cells. But in the alendronate group, the positive cells appeared frequently in layers of ameloblasts. Furthermore, the expression of alkaline phosphatase mRNA was down-regulated in the alendronate group, suggesting that osteoblastic activity was decreased.

These results suggested that bisphosphonates may act on dental hard tissue cells, preventing tooth development and eruption.

Key words : Bisphosphonate, Tooth development, Bone

Introduction

Bisphosphonates (P-C-P in structure), also termed diphosphonates, are enzyme resistant analogues of endogenous inorganic pyrophosphate (P-O-P in structure), chelating Ca^{++} ions, selectively accumulating in hard tissue, and subsequently inhibiting mineral deposition (Fig. 1). These chemicals also inhibit osteoclasts through yet-unclear mechanisms (Fleish 1998, Fisher et al. 1999). Because of these unique properties, they have been widely studied for the clinical applications for tooth movement in orthodontics, osteoporosis, Paget disease and bone metastasis.

Fundamental actions of bisphosphonates are ambivalent: inhibition of bone resorption at low dose and inhibition of calcification at high dose. The inhibition of mineralization by these chemicals has been report-

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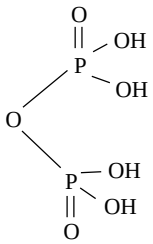
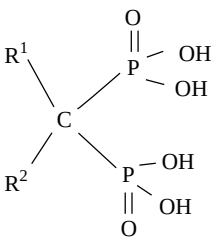


pyrophosphate (PPi)



bisphosphonate

Fig. 1. Structures of pyrophosphate and bisphosphonate.

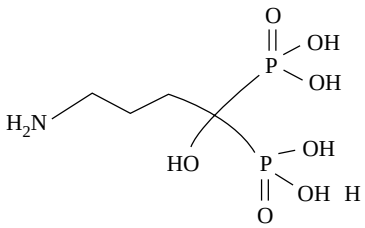


Fig. 2. Structure of alendronate used in the present study.

ed most likely due to a physicochemical mechanism to inhibit calcium phosphate formation in vitro and calcification in vivo (van Beek et al. 1994, Fleisher 1998, Ohma et al. 2000) and to delay of the dissolution of calcium phosphate crystals (Evans et al. 1980). But just these two actions are not enough to explain properties of bisphosphonates to inhibit the normal mineralization of calcified tissues including dental hard tissues. Considering the reduced chelating ability at high pH (Ebetino et al. 1998) and the active engagement of bisphosphonates on the inhibition of bone resorption, other biological mechanisms such as direct effects on osteoclasts, osteoblasts and other connective tissue cells have been suggested (Endo et al. 1996, Shipman et al. 1998). Similarly, it is postulated that bisphosphonates may act on dental hard tissue cells, affecting mineralization and tooth eruption.

Even though many clinical applications of bisphosphonates have been attempted in dental fields, their actions on other cells than osteoclasts, especially in developing dental tissues, have not been investigated yet. The developmental processes of teeth involve dynamic morphological changes from the bud stage to hard tissue apposition and eruption, which needs bone formation and resorption. The present study was focused on effects of alendronate on the development of dental hard tissue in vivo.

Materials and Methods

1. Administration of alendronate and macroscopic observation

Alendronate {(4-amino-hydroxybutylidene)bisphosphonate} (MK-217®, Merck, USA) (2.5 mg/kg in concentration) was daily subcutaneously injected for 10 days in the posterior neck region of postnatal day 1 Sprague-Dawley rats. For the control group, saline was administered at the same manner in rats of the same age. The chemical structure of alendronate is shown in Fig. 2.

Rats in the alendronate group were sacrificed by decapitation at day 12 after the final administration, when the eruption of the molar is seen and day 40 after the final administration. The maxilla and the mandible were taken out and photographed for to evaluate changes in the alveolar bone and tooth eruption.

2. Microscopic observation

Three rats were sacrificed at day 3 after the final administration. The maxilla and the mandible were taken out and immersion-fixed in 10% buffered neutral formalin. The tissues were then decalcified in EDTA solution (pH 7.4, 4˚C) and routinely processed for light microscopy.

3. TUNEL stain

TdT-mediated dUTP biotin nick end-labeling

(TUNEL) was carried out on tissue sections for apoptosis. Tissue slides were treated with 5 μg/mL proteinase K (Sigma, MI, USA) at room temperature for 15 min and then incubated in 2% H_2O_2 for 5 min to remove endogenous peroxidase. The next step was performed using Apop tag in situ detection kit (Oncor Inc. MD, USA). Briefly, being immersed in the Equilibration buffer for 15 min, sections were incubated in a mixture of terminal deoxynucleotidyl transferase and digoxygenin-dUTP mixture (TdT reagent) at 37˚C for 1 hr. The reaction was terminated by incubating them at 37˚C stop/wash solution. For the color development, sections were treated with anti-digoxigenin-peroxidase solution for 30 min, before being stained with a solution composed of 0.005% H_2O_2 and 0.002 % 3, 3′-diaminobenzidine tetrahydrochloride in Tris-HCl buffer solution. For the negative control, PBS was used in stead of TdT reagent. Tissues were finally counterstained with Meyer's hematoxylin and visualized by a light microscope.

4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was carried out to evaluate alkaline phosphatase mRNA expression from osteoblasts in the molar regions of the mandibles at day 3 after the final administration. The deep-freezed tissues were crushed and homogenized in RNase-free tubes. Total RNAs were extracted using Trizol reagent (Gibco BRL, MD, USA) according to the manufacture's instructions. All RNAs were quantitated by spectrophotometer and OD 260/280 ratios $>$ 1.8 were obtained for all samples. cDNA synthesis was carried out by incubating the RNAs in a mixture of Superscript II (Gibco BRL, MD, USA) and Oligo $(dT)_{12\text{-}18}$ (Gibco BRL, MD, USA) at 42˚C. The cDNA was then, PCR-amplified using primers for alkaline phosphatase and GAPDH. The primer sequences and product sizes were defined in Table 1. PCR cycles were performed in a Perkin-Elmer GeneAmp PCR system 2400 and the products were resolved on a 1.2% agarose gel and visualized using ethidium bromide. The intensity of the products was quantified by densitometry and the values were normalized to GAPDH signals.

Table 1. Primers to amplify alkaline phosphatase and GAPDH

Genes	Oligonucleotide sequence	Amplicon size
GAPDH	Foward 5′ CCA TGG AGA AGG CTG GGG 3′ Reverse 5′ CAA AGT TGT CAT GGA TGA CC 3′	175 bp
alkaline phosphatase	Foward 5′ TCA TGT TCC TGG GAG ATG GTA TG 3′ Reverse 5′ GCA TTA GCT GAT AGG CGA TGT CC 3′	465 bp

Results

1. Alkaline phosphatase mRNA expression

The expression of alkaline phosphatase was assessed from the portions of the mandible which contained the molar teeth. The expression measured by densitometry was approximately the half of the control group, suggesting that osteoblastic activity was decreased by the alendronate administration (Fig. 3).

2. Macroscopic findings

At day 12 after the final administration, both the maxillary and mandibular 1st and 2nd molars were partially erupted in the control group. On the contrary, all the teeth were not erupted and the alveolar ridge was thin and poorly developed in the alendronate group. At day 40 after the final administration, both all the maxillary and mandibular molars were fully erupted in the control group (Figs. 4, 5). However, the findings in the alendronate group were similar to those of day 12 except the gingival epulis in the incisal region (Figs. 6, 7, Table 2).

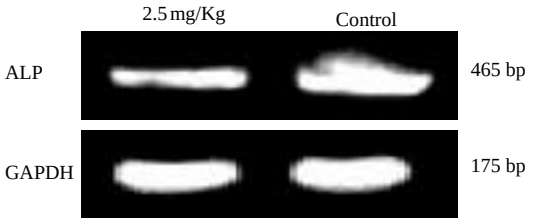


Fig. 3. The expression of alkaline phosphatase (ALP) mRNA at day 3 after the final administration in the both alendronate and control groups. ALP expression was decreased in the alendronate group.

Table 2. Eruption of the maxillary teeth at days 12 and 40 after the final administration

Days	Control				Alendronate			
	I	M_1	M_2	M_3	I	M_1	M_2	M_3
12	p	p	p	n	n	n	n	n
40	e	e	e	e	p	n	n	n

I: incisor, M_1: 1st molar, M_2: 2nd molar, M_3: 3rd molar, p: partially erupted, n: not erupted, e: erupted

3. Microscopic findings

Day 3 after the final administration: In the control group, the mandibular 1st and 2nd molars were also at the hard tissue deposition stage, forming the enamel and the dentin by ameloblasts and odontoblasts respectively. The 3rd molar was at the bell stage (Fig. 8). Osteoclasts, which were engaged in bone resorption, were observed just above the cuspal region. Fine and immature woven bone with numerous osteoblasts were observed around the tooth germs (Fig. 9). In the alendronate group, the mandibular molar tooth germs were under-developed, compared with those of the control group. Relatively small amount of dental hard tissues was formed in the mandibular 1st molar. The ameloblast layer was discontinuous in several areas, where enamel formation was defected. The surrounding woven bone matrices seemed loose and osteocytes in lacunae were sparse (Fig. 10). The woven bone grew into the dental papilla to prevent the tooth development. The third molar tooth germ did not appear (Fig 11). A bizarre proliferation of the inner and outer enamel epithelia was observed around the developing incisor ridge. The incisal ridge showed cellular entrapments in the dentin as in osteodentin (Fig. 12).

4. TUNEL staining

TUNEL positive cells were rarely seen in the normally developing tooth germs (Fig. 13). But in the alendronate group, the positive cells appeared frequently in ameloblasts, especially in abnormally proliferating ameloblasts. Enamel defects were frequently observed adjacent to the apoptotic ameloblast regions (Fig. 14).

Discussion

Being chemically similar to endogenous inorganic pyrophosphate, bisphosphonates have been widely studied for their clinical applications such as tooth movement in orthodontics and the treatment of osteoporosis, Paget disease, bone metastasis of tumor tissue and so on. The geminal carbon atom of bisphosphonates can form two additional covalent bonds, R^1 and R^2, rendering many different structures for them. These chemicals bind to bivalent metal ions such as Ca^{2+}, Mg^{2+} and Fe^{2+} by coordination of one oxygen from each phosphate group with the bivalent cation to form a three dimensional structure. The affinity for calcium and potency can be enhanced by modifying one of the side chains to form a tridentate conformation (Rogers et al. 2000). Alendronate is a second generation product of bisphosphonates which include primary amino group in the R_2 side chain (Fig. 2), having 1000 fold potency compared with etidronate in vivo (Shenk et al. 1986).

For most species, the effective daily dose of bisphosphonate was reported in the order of 5-20 mg/kg parenterally (Fleish 1998). Yaffe et al. (1997) also

suggested that alendronate 0.5 mg/kg could reduce alveolar bone resorption. Seedor et al. (1991) reported a wide of range of 0.056-7.0 mg/kg subcutaneous alendronate administration to reduce bone resorption. Ito et al. (1998) reported that alendronate had no cytotoxic effects in adult rats when it was intravenously administrated at 25 mmol/kg concentration. In the present study, 2.5 mg/kg alendronate was injected subcutaneously by the above references.

Bisphosphonates have a high affinity to bone minerals, so that they can bind to hydroxyapatite in bone. ^{3}H-labeled alendronate was found in bone resorbing surface, where hydroxyapatite crystals were exposed, rather than bone formation surface (Azuma et al. 1995, Lin 1996, Masarachia et al. 1996). Theoretically bisphosphonates can not pass cell membrane because of their high negative charge. Thus, the mechanism of bisphosphonates to inhibit normal mineralization has been largely attributed to their physicochemical properties. However, direct effects of bisphosphonate on cells in addition to their binding affinity to minerals have been extrapolated, considering the potency of a variety of bisphosphonates and effects on developing tissues (Owens et al. 1997, van Beek 1997, Rowe et al. 1999, Hiroi-Furuya 1999). In other words, cultured cells from the calvaria could take bisphosphonates by pinocytosis (Rogers 1994). Bisphosphonates could stimulate osteoblast-like cells in culture to secrete factors, inhibiting osteoclast action, detering bone resorption by osteoclasts (Sahni et al. 1993, Vitte et al. 1994). Moreover, bisphosphonates inhibited IL-6 in osteosarcoma which is engaged in osteoclast formation (Passeri et al. 1994, Manolagas and Jilka 1995).

It is generally accepted that bisphosphonates have little toxicity, which has been explained by rapid incorporation into calcified tissue and hence their short presence in the circulation. Acute toxicity is mostly due to hypocalcemia induced by the formation of complexes with calcium, leading to a decrease in ionized calcium (Fleisch 1998). Effects of bisphosphonates on the tooth, however, have been controversial. Ito et al. (1998) reported that alendronate did not have any cytotoxic effects on osteoclasts in vivo and in vitro. Similarly, Grier and Wise (1997) also suggested that it could only affect tooth eruption, not on the hard tissue formation. On the contrary, Fouda et al. (1991) reported that bisphosphonates could affect the formation of the dentin and the enamel. In the present study, a wide range of changes were observed including the impaired tooth formation, non-eruption and agenesis of the third molar. Although it is not clear what factors made a difference from the above reports, one of the reasons could be that the developing tissues used in the present study were more vulnerable than the mature tissue to alendronate. Another factor is that 50% of administered alendronate accumulates at sites of mineralization and the other half is excreted unchanged by the kidney (Rang et al. 1999). The animals used in the present study were postnatal day 1, functionally immature in the kidney function and thus might correspondingly result in more accumulation of the chemical in the body, compared with adult animals. This high accumulation of the chemical might be overburden to rat pups more than the suggested dose.

One of recently reported complications of bisphosphonates is bisphosphonate-associated osteonecrosis caused by hypovascularity and suppressed bone turnover (Najm et al. 2005, Zarychanski et al. 2006). In the present study, not a little impairs of hard tissue formation were found. These impairs can be regarded as adverse effects of alendronate. Alendronate acted on a variety of hard tissue cells, including ameloblasts and osteoblasts in addition to osteoclasts which have been widely studied with bisphosphonates. In other words, ameloblasts showed apoptotic changes and bizarre proliferation, causing defects of enamel formation.

Alkaline phosphatase has been currently used as a critical factor, which indicates secretory activity of osteoblasts because it hydrolyzes organic phosphate ester, increasing phosphate ions in local mineralizing

area and depositing calcium and phosphate ions in extracellular matrix. In the present study, the alendronate group was demonstrated reduced expression of the enzyme, which might be associated with impairs of bone formation and maturation. As a result, alendronate caused non-eruption or delay of eruption and morphological changes in the developing teeth. These findings suggested that alendronate can directly regulate cell activity and gene expression in hard tissue cells as suggested by Reinholz et al. (2000).

All together, bisphosphonate can directly act on hard tissue forming cells, besides osteoclasts, suggesting that bone deposition can be a factor for the tooth eruption.

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Legends for Figures

Fig. 4. The mandibular 1st, 2nd and 3rd molars at day 40 after the saline administration. ×10

Fig. 5. The maxillary 1st, 2nd and 3rd molars at day 40 after the saline administration. ×10

Fig. 6. The molar teeth do not erupt in the mandibular alveolar bone, which is thin and narrow in width. The incisal region shows edematous swelling at day 40 after the alendronate administration. ×10

Fig. 7. The molar and incisor teeth do not erupt in the maxillary alveolar bone, which is thin and narrow. Narrow and underdeveloped hard palate are noted at day 40 after the alendronate administration. ×10

Fig. 8. The mandibular 2nd and 3rd molars is at the stages of the crown and the bell respectively at day 3 after the saline administration in the control group. H-E, ×60

Fig. 9. Occlusal to the cuspal region of the mandibular 2nd molar, the alveolar trabecular bone appears dense and is being resorbed by osteoclasts for eruption at day 3 after the saline administration in the control group. H-E, ×150

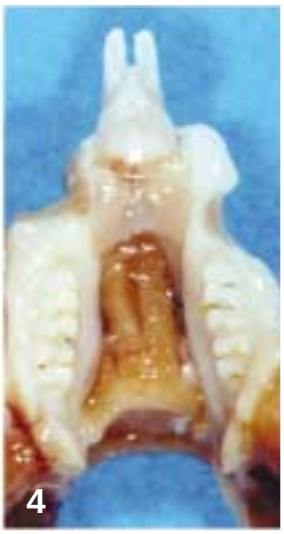
Fig. 10. The defects of enamel formation and discontinuation of ameloblast layer are noted in the mandibular molar. The trabecular bone appears loose and fills the defect area at day 3 after the last alendronate administration. H-E, ×150

Fig. 11. The surrounding alveolar bone grows to infiltrate into the mandibular 2nd molar tooth, hindering its further development at day 3 after the alendronate administration. H-E, ×100

Fig. 12. A bizarre proliferation of inner and outer enamel epithelium are covering the developing incisor ridge at 10 days after the last alendronate administration. The future incisal ridge shows cellular entrapments in the dentin as in osteodentin. H-E, ×150

Fig. 13. TUNEL positive cells are rarely seen in the normally developed molar tooth in the control group. TUNEL, ×150

Fig. 14. The ameloblastic layer in the developing mandibular 1st molar is continuous, but many ameloblasts show apoptotic figures at day 3 after the final alendronate administration, which are TUNEL positive. TUNEL, ×250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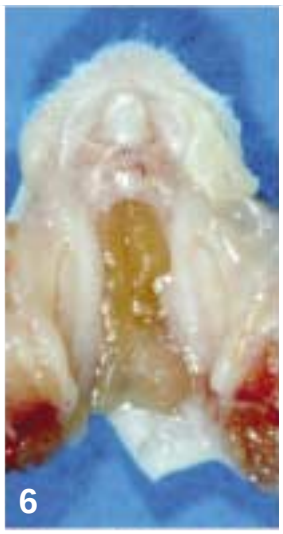
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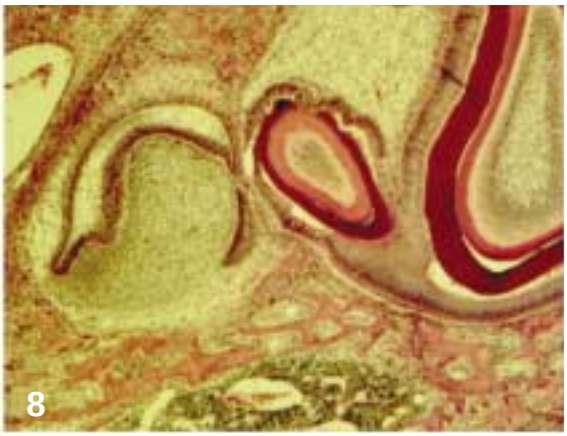
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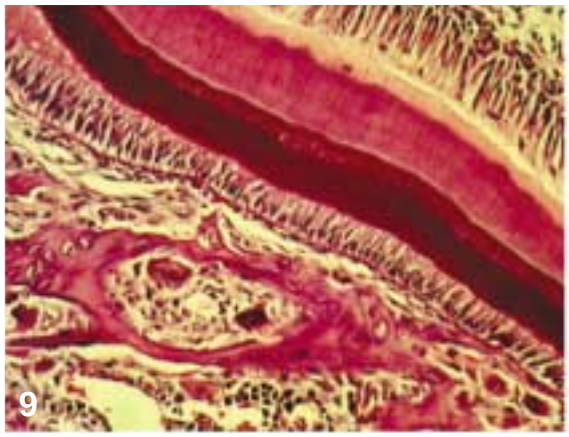
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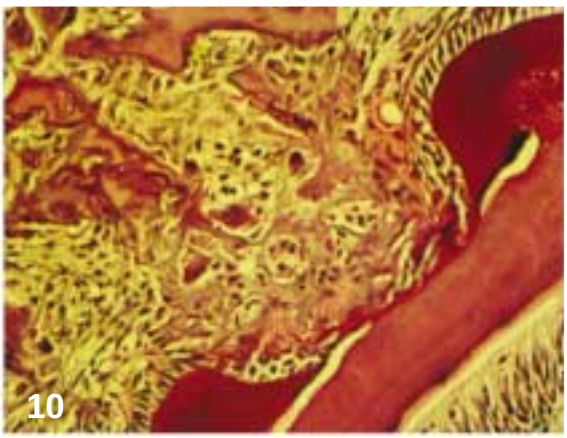
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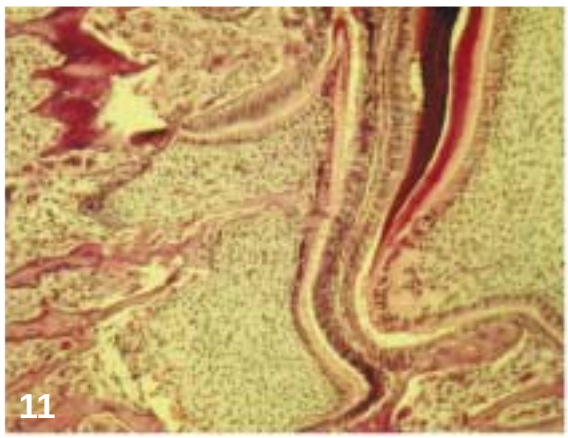
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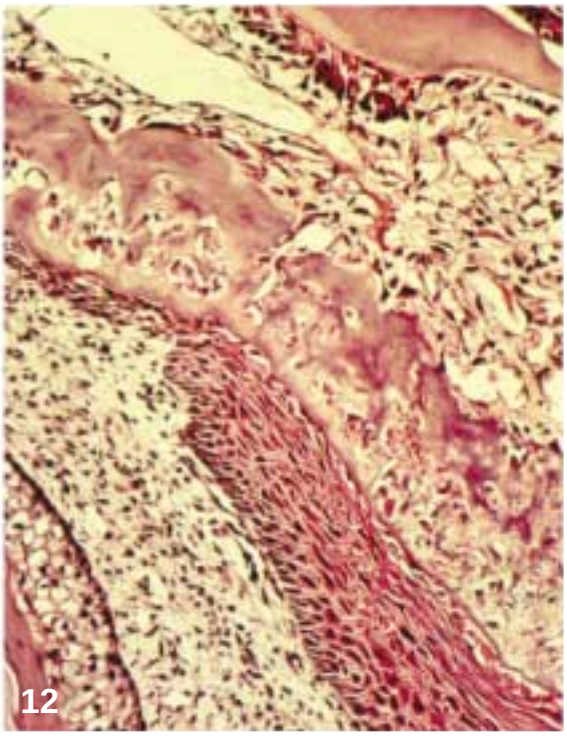
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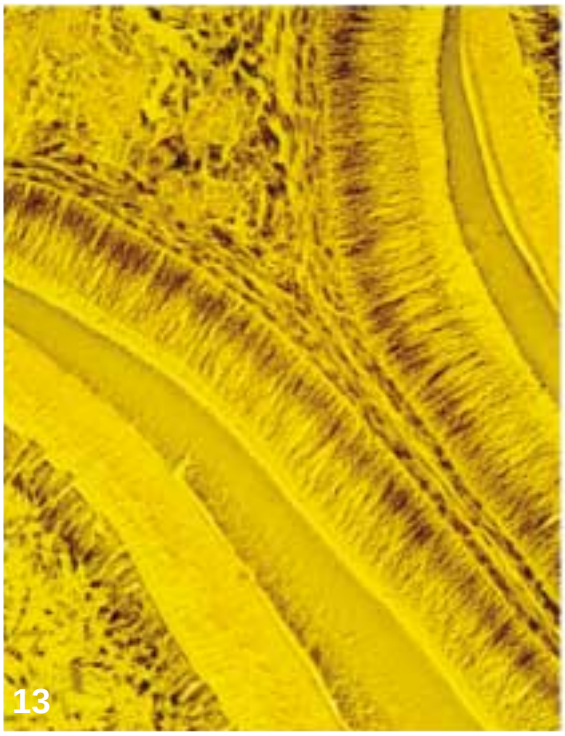
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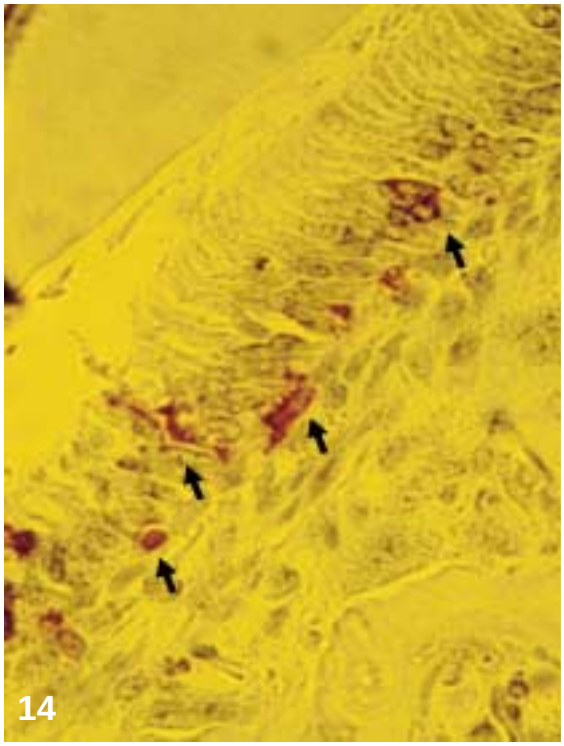
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Korean J Phys Anthropol
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Bisphosphonate가 치아 발육에 미치는 영향

김 현 진, 정 준 용, 김 선 헌
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간추림 : bisphosphonate는 수산화인회석과 결합하여 뼈파괴세포에 의한 뼈조직 흡수를 억제하는 작용을 갖는 것으로 알려져 있으나, 발생기 뼈조직, 치아조직 및 세포에 미치는 영향 및 분자적 기전은 확실하지 않다.

본 연구는 성장기 흰쥐에서 bisphosphonate계 2세대 약제인 alendronate가 치아 형성과 발육에 미치는 영향을 구명하고자 출생 1일 흰쥐에 10일간 매일 2.5 mg/kg 투여 후 3일, 12일, 40일에 희생한 후, 광학현미경적 관찰과 TUNEL 염색 및 alkaline phosphatase mRNA 발현 조사를 시행하여 다음과 같은 결과를 얻었다.

alendronate는 발생중인 치아 형성을 억제하고 맹출을 지연시키는 작용을 하였다. 투여 종료 후 3일 대조군에서 아래턱 첫째큰어금니와 둘째큰어금니는 각각 치아 단단조직 형성기에 있었으나, alendronate 투여군에서 첫째 및 둘째큰어금니의 경조직 형성은 불완전하였고 셋째큰어금니 치배는 관찰되지 않았다. 둘째큰어금니 치배 내로 뼈잔기둥이 침입하여 치배 성장이 이루어지지 않고 있었으며, 앞니의 상아질은 뼈유사상아질(osteodentin) 소견을 보이기도 하였다. TUNEL 염색에서 때로 법랑질모세포의 세포자멸사가 관찰되었다. 또한 alendronate 투여군에서 뼈모세포의 alkaline phosphatase 발현은 대조군에 비하여 현저히 저하되어 있었으며 뼈잔기둥의 방향과 성숙 정도는 대조군에 비하여 미약하였다. 투여 후 40일 경과 후에도 어금니 치배 맹출은 관찰되지 않았다.

이상의 결과는 과량의 bisphosphonate는 치아 단단조직 형성 세포에 직접 작용함으로써 성장기 치아 형성 및 발육 지연을 유발할 수 있음을 시사하였다.

찾아보기낱말 : bisphosphonate, 치아발육, 뼈형성

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