

The Effects of Alendronate on Healing of the Calvarial Defect in Rats

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I. Introduction

Periodontal disease is one of the most common diseases occurring in the oral cavity of human beings. And also, it is the primary cause of tooth loss in adults. Research for bone regeneration has been very active since implants became a modality for treatment of periodontal disease. Several proteins such as platelet derived growth factor(PDGF), insulin like growth factor(IGF), and bone morphogenetic protein(BMP) have been found to guide bone regeneration. Research for artificial materials has also made a progress.

Bisphosphonates was synthesized in Germany in 1865 for the first time. The bisphosphonates which inhibit bone resorption by osteoclast are chemical analogs of pyrophosphates. Since bisphosphonates have P-C-P bonds instead of P-O-P bonds, they are stable against heat and most other chemical reagents and completely resistant to enzymatic hydrolysis. In addition, they have a strong affinity for hydroxyapatite(1-3).

Although detailed mechanism of bisphosphonates on bone resorption has not been completely elucidated, there is a general consensus that the bisphosphonates act on osteoclasts or osteoblasts. It was reported that bisphosphonates had a direct effect on activity of the osteoclasts.¹⁷⁻¹⁸⁾ Some authors reported that bisphosphonates had an effect on osteoclast recruitment directly or indirectly through reducing the life-time of osteoclasts by programmed cell death (apoptosis)²⁰⁾. Others believe that bisphosphonates

have influences on osteoblasts as well as osteoclasts and control the differentiation or function of the osteoclasts²⁵⁾. It was reported that bisphosphonates inhibited the formation of osteoclasts and induced the osteoclast-mediated resorption inhibiting factors via osteoblasts²⁶⁾.

There are two forms of bisphosphonates; amino-bisphosphonates and non- aminobisphosphonates. In general, aminobisphosphonates is more effective than non-aminobisphosphonates. For example, Alendronate is more potent than etidronate by a fac-

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tor of 100-1000 fold⁴⁾. The effect of alendronate is believed to alter activity of osteoclast. Clinical application of alendronate is very wide and various. In particular, alendronate is being used to inhibit bone resorption of patients who have bone destruction disease such as Paget's disease, tumoral bone disease, hypercalcemia of malignancy, postmenopausal osteoporosis, osteogenesis imperfecta and so forth⁵⁻⁸⁾. However, there are some disadvantages in systematic administration of alendronate. The most common adverse effects are abdominal pain, nausea, dyspepsia, constipation and diarrhea. Sometimes it causes renal failure and pyrexia⁹⁻¹⁰⁾. Additional disadvantage is very low oral bioavailability of 0.9 to 1.8%⁸⁾. Thus local delivery overcomes the absorption problems encountered as well as potential adverse effects on other tissues.

In the application of alendronate on dentistry, the effects of suppression of alveolar bone resorption by periodontitis have been revealed¹¹⁾. Some author reported that alendronate was effective in promoting the healing process of rat's extraction socket and increasing the hardness of cortical bone³⁴⁾. Others have said that the local delivery of alendronate significantly reduced bone resorption activated by surgical separation of periosteum from bone^{12,13)}. It was also found that locally applied alendronate was influential in increasing bone formation rate and bone to implant contact in the regenerative treatment of periimplant defects¹⁴⁾. This agent and its effect on alveolar bone or its use with guided bone regeneration (GBR) have not been investigated extensively, although preliminary studies have shown a positive effect with the systematic use of the drug.

Until now, various mediators for local drug delivery, such as gelatin sponge, tricalcium phosphate, fibronectin, collagen were reported. Collagen which occupies the largest portion of protein of higher ver-

tebrate animals has high tensile strength, low antigenicity and facilitates wound healing and blood coagulation. It is also reported that collagen membrane has osteoconductive property and high liquid-absorbability due to excellent porosity¹⁶⁾.

Therefore in this study, we investigated the effects of local application of alendronate and the efficiency of collagen membrane as mediator on healing of the calvarial defect in rats, which has a good experimental design to regeneration of tissue destruction.

II. Materials and Methods

1. Animals and Drug treatment

A total of 18 Sprague-Dawley rats were used for experimental animals in this study and these animals were 2 months old. Six animals were sacrificed in the first, second and fourth week after initiation of the experiment. The defects of the right side were designated as testing groups and those of the left side were control groups.

Mixed solution of 0.82cc Rompun (Bayer Korea, Korea), 7cc Ketamine hydrochloride (Youhan Pharm, Korea) and 2cc normal saline were injected intraperitoneally for general anesthesia and the rats were maintained anesthetized condition by intermittent inhalation with ether.

The dorsal part of the cranium was shaved and prepared aseptically for surgery and 2% Lidocaine (contained epinephrine 1:80,000, Youhan Pharm, Korea) were injected for bleeding control and topical anesthesia in the mid-line of cranium. A 20 mm long incision in the scalp along the sagittal suture was made. Skin, musculature, and periosteum were reflected, and then the parietal bones were exposed. Two full-thickness bone defects with a diameter of 5mm trephined in the dorsal part of the parietal bone lateral to the sagittal suture. A 5-mm

trephine bur was used to create the defects under constant irrigation with sterile physiologic saline to prevent overheating of the bone edges. Alendronate solution was prepared by dissolving 10 mg alendronate in 1 mL saline solution. 6mm diameter collagen membrane(CollaTape[®], Sulzer Calcitek, INC.) absorbed with 20 μ l(10mg/mL, 1mg/kg) alendronate (Yu Yu Pharm, Korea) solution was inserted in the defects of the right side and 6mm diameter collagen membrane(CollaTape[®], Sulzer Calcitek, INC.) absorbed 20 μ l physiologic saline was inserted in the defects of the left side. The periosteum and scalp were sutured with 5-0 vicryl[®]. Topical ointment, Penicillin G(tardomyocel[®]-L) was applied and 0.1ml/kg Enrofloxacin(Baytril[®] 25) I.M. was administered to prevent infection.

2. Histologic evaluation (H&E)

All animals were sacrificed by heart perfusion, and specimens were taken at the site of parietal bone around the calvarial defect areas. Specimens from the test (alendronate-treated) or control sites were fixed with the mixture of 4% paraformaldehyde in 0.1M phosphate buffered saline(PBS). After demineralization with 10% EDTA, the specimens were dehydrated with a graded series of ethanol, embedded in paraffin, sectioned at 5 μ m with microtome, and stained with hematoxylin and eosin (H&E).

3. TRAP staining

To evaluate osteoclastic activity, tartrate resistant acid phosphatase (TRAP) staining was done. TRAP activity was detected by incubation with a mixture of 0.1mg/ml naphthol AS-MX phosphate (Sigma, USA), 0.5% N, N-dimethylformamide, 0.6 mg/ml fast red violet LB (Sigma, USA) in 0.1M acetate buffer solution (pH 5.0) at 37 $^{\circ}$ C for 10 min.

4. Hardness measurement

The hardnesses of specimens from test or control sites were estimated by the hardness measurement instrument (FUTURE-TECH, FM-7, Matsuzawa 10, Japan) under the condition of load 50 g, loading time 10 sec. Specimens were obtained on 1, 2 and 4 weeks after surgery and hardness was measured 6 times per specimen.

5. Statistical analysis

In order to investigate the difference in the number of TRAP(+) cells and hardness, the mean values were tested statistically with ANOVA test.

III. Results

1. Histologic findings (by H&E staining)

1) 1 week after surgery

Collagen membrane were partially shown and inflammatory cells infiltrated around the marginal bone of the defect of test and control groups. New bone formations were not shown and similar healing aspects were seen with both test and control groups(Figure 3a, 3b).

2) 2 week after surgery

Inflammatory cell infiltrations were significantly decreased and collagen membranes were resorbed but still remained on both groups. Formation of new bones was shown at the marginal bone of the defects and similar healing aspects are seen with both test and control groups(Figure 4a, 4b).

3) 4 week after surgery

Inflammatory cell infiltrations disappeared and osteogenic repairs were observed with most areas of

Table 1. The number of TRAP(+)cells for control and test groups. (1mm² area around the defect margin)

Time(wks)	Groups	
	Control	Test
T1	16,4±0,9	7,8±0,9 **
T2	4,7±0,7	4,0±0,6
T4	7,1±0,5	7,7±0,7
D1(T2-T1)	-11,7 *	-3,1
D2(T4-T1)	-9,3 *	-0,1

* : Significantly different between two groups ($p < 0,05$).

** : Significantly different between two groups ($p < 0,01$).

Each value represents mean and standard deviation.

T1, T2 and T4 imply 1 week, 2 weeks and 4 weeks specimens respectively.

D1(T2-T1) is time difference between 1 week and 2 weeks.

D2(T4-T1) is time difference between 1 week and 4 weeks.

the defects on both groups. Similar bony healing aspects were seen for both test and control groups(Figure 5a, 5b).

2. Osteoclastic activity by TRAP staining (Table1, Figure 1, Figure 6-8)

In 1 week after surgery, test groups($7,8 \pm 0,9$ cells) displayed the lower number of TRAP(+) cells than control group($16,4 \pm 0,9$ cells) ($p < 0,01$). In 2 week after surgery, statistically significant difference between control group($4,7 \pm 0,7$ cells) and test group($4,0 \pm 0,6$ cells) was not observed. In 4 week after surgery there was also no statistically significant

difference between control group($7,1 \pm 0,5$ cells) and test group($7,7 \pm 0,7$ cells).

Differences in number of TRAP(+) cells measured after 1 week and 2 weeks by time course were 11,7 cells on the control group, and 3,1 cells on the test group. These results showed statistically significant differences with the control but not with the test group($p < 0,05$). The difference between 1 week and 4 weeks by time course were 9,3 cells for control group, and 0,1 cells for test group. These results showed statistically significant difference on control group and not test group($p < 0,05$).

3. Hardness measurement (Table 2, Figure 2)

Table 2. Knoop Hardness of Specimens of control and test groups by time course.

Time(wks)	Groups	
	Control	Test
T2	16,8±0,47	24,0±4,6 *
T4	46,9±0,16	44,4±1,4
D1(T4-T2)	30,1±1,4 **	20,4±1,4 **

* : Significantly different between two groups ($p < 0,05$).

** : Significantly different between two groups ($p < 0,01$).

Each value represents mean and standard deviation.

T2, T4 imply 2 weeks, 4 weeks specimens respectively.

D1(T4-T2) is time difference between 4 weeks and 2 weeks.

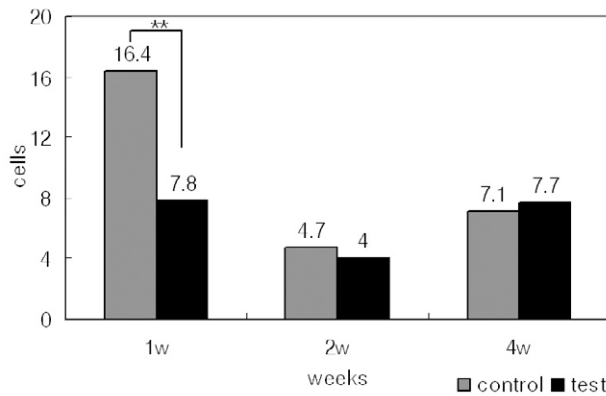


Figure 1. The number of cells of control and bisphosphonate treated groups

** : very significantly different between two groups ($p < 0.01$).

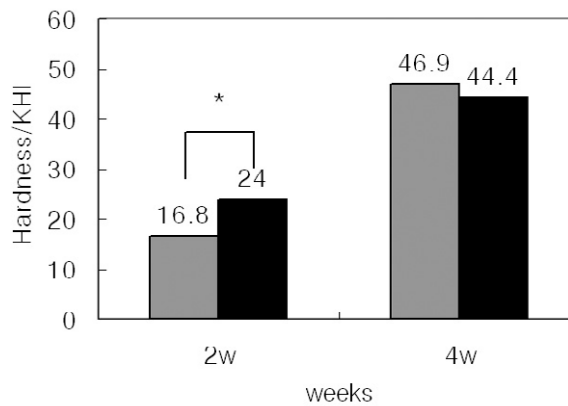


Figure 2. Comparative study of knoop hardness of control and alendronate treated groups

KHN is Knoop Hardness Number (kg/mm^2).

* : significantly different between two groups ($p < 0.05$).

For samples taken 1 week after surgery, the measurement was impossible because of sample cracking during measurement. For samples taken 2 weeks after surgery, hardness of the bone was 16.8 ± 4.7 for the control group, and 24.0 ± 4.6 for the test group. This is statistically significant difference ($p < 0.05$). For samples taken 4 weeks after surgery, hardness of the bone was 46.9 ± 1.6 for the control group, and 44.4 ± 1.4 for the test group indicating no difference in two groups. The hardness difference for 2 weeks and 4 weeks by time course were

-30.1 ± 1.4 for the control group, and -20.4 ± 1.4 for the test group. These results showed statistically significant difference for both control group and test group ($p < 0.01$).

IV. Discussion

Research for various materials that guide bone regeneration in periodontal disease and implant dentistry has been progressing recently. Bisphosphonates, inhibitors of bone resorption by

osteoclasts, were introduced by Fleisch *et al.*¹⁾ in 1969, and have been used for the treatment of bone destruction disease such as, Paget's disease, osteoporosis, tumoral bone disease, hypercalcemia of malignancy, osteogenesis imperfecta, and so on⁵⁻⁸⁾.

The action mechanism of bisphosphonates on bone resorption has not clearly been elucidated. However, Hughes¹⁸⁾, Fleisch¹⁾ and Nishikawa²⁵⁾ *et al.* reported that bisphosphonates inhibited the differentiation of osteoclasts. Murakami *et al.*²¹⁻²²⁾ said that they inhibited the bone resorption by suppressing the action of the enzyme regulating the cytoskeletal organization in osteoclasts (protein tyrosine phosphatase). Selander *et al.*^{23,24)} said that bisphosphonates reduced the life-time of osteoclasts by programmed cell death (apoptosis). In addition to the fact that bone resorption suppression of bisphosphonates is caused by the direct effect on osteoclasts, an existing theory states that bisphosphonates give an indirect effect on osteoclasts by influencing osteoblasts. Nishikawa *et al.*²⁵⁾ reported that bisphosphonates had an effect on osteoblasts by inhibiting the later stage of osteoclastogenesis. Vitte *et al.*²⁶⁾ said that bisphosphonates induced osteoblasts to secrete the factors inhibiting osteoclast-mediated resorption. There is a general agreement that the final target of bisphosphonates action is the osteoclast now. Four mechanisms appear to be involved: 1) inhibition of osteoclast recruitment; 2) inhibition of osteoclastic adhesion; 3) shortening of the life span of osteoclasts; 4) inhibition of osteoclast activity.

Etidronate was the first bisphosphonates which was used to treat a human disease. Until now, a great number of bisphosphonates, such as risedronate, pamidronate, alendronate, clodronate, zoledronate *et al.*, have been synthesized and each of them possesses its own physicochemical and biological characteristics. This implies that it is not possible to extrapolate from the results of one com-

pound to others with respect to their actions.

In our study, we used alendronate to evaluate the effect of bone regeneration. Alendronate is a sort of aminobisphosphonates, which have high potency and far greater selectivity for inhibition of bone resorption because of amino group at the end of the side chain. In animal studies, alendronate is not metabolized and is cleared from the plasma by uptake into bone and elimination via renal excretion. Although the drug distributes widely in the body soon after administration, this transient state is rapidly followed by nonsaturable redistribution to skeletal tissue. Oral bioavailability is very low about 0.9 to 1.8% and food markedly inhibits oral absorption⁴⁾. In preclinical study, alendronate has some animal toxicity, such as hypocalcemia, renal damage, and inhibition of normal materialization. In humans, the oral administration of alendronate can be accompanied by esophageal and gastrointestinal side effects such as nausea, dyspepsia, vomiting, gastric pain, and diarrhea, and sometimes even ulceration. Moreover, a small number of renal failure and pyrexia were reported.⁴⁾

So local delivery, especially in the application of dentistry, overcomes the absorption problems encountered as well as any potential adverse effects on other tissues. In order to deliver drugs locally for bone regeneration, we used collagen membrane which has excellent absorption property and rat's calvarial defect formed by intramembranous bone formation.

Critical size, the minimal size that can't get spontaneous regeneration of calvarial defect of rats, was known as 5mm¹⁵⁾. However, this research showed 5mm-diameter had complete bone regeneration in 4 weeks in all experimental animals. It is considered that the collagen membranes worked as osteoconductive materials¹⁶⁾. Our examination showed severe inflammatory reaction on 1 and 2 weeks after

surgery in all experimental animals. We thought that the applied collagen membrane caused an inflammatory reaction. Kim et al²⁷⁾ reported that the collagen membrane caused inflammatory reaction in calvarial defect but there was no difference in the healing of defect with the control group. Ripamonti et al²⁸⁾ said that low pH resulted from collagen-mediated inflammatory reaction inhibited bone regeneration because of low pH. In their study, inflammation lasted for 4 weeks, but in our study, inflammation fairly decreased in 2 weeks and disappeared in 4 weeks. Therefore, it is thought that the inflammation rarely affected healing, although the effect of the agent was somewhat reduced by this inflammatory reaction.

Some other methods for local delivery have been suggested. Adachi et al²⁹⁾ used subperiosteal injection methods and reported that topical application of risedronate may be helpful in anchoring and retaining teeth under orthodontic movement.

Denissen^{30,31)} said that HA-coated implants were soaked in 0.1mmol sterile solution of alendronate for 1 week at 37°C, which was shown to have a slow, constant release of alendronate from HA over-time. This concentration has been shown to be bioactive and not to have cytotoxic effects in vitro.

Yaffe et al^{12,13,32)} applied the gelatin sponge absorbing alendronate for local delivery. They concluded that topical delivery of alendronate at the time of surgery reduces bone loss in periodontal procedures involving mucoperiosteal flap surgery, and they said that the most effective dose is 200µg for topical delivery at the surgical site and 400µg for distant site³³⁾.

In this study, though 200µg alendronate was applied, there was similar histologic finding between test and control groups each week.

Though the 200µg alendronate is effective for reducing bone loss in alveolar bone, it is thought that the 200µg alendronate may be insufficient or the

concentration 10mg/ml is not enough to accelerate bone regeneration. Other thought is that the collagen membrane may not release alendronate slowly. So most of alendronate may be released at the early stage of healing.

Test groups have shown significantly less TRAP(+) cells than control groups only after 1 week. There were statistically significant differences on the control groups within 1, 2 and 4 weeks and not on the test groups. These results indicate that the effect of alendronate appeared only in early stage of the healing process.

Motoie et al³⁵⁾ found that bone mass and mechanical strength were increased by alendronate in rats. In this study, hardness measurement was impossible due to cracking of samples of 1 week. Test groups showed significantly higher hardness than control groups not in 4 weeks but in 2 weeks. There were significant increases of hardness on both groups as time went by. These results are different from those of histologic finding and TRAP finding. Though the agent's effect evaporates in an early stage, we thought that increasing hardness was maintained for a long period of time. Another thought is that the histologic finding was a study confined to specific area and specific surface, while the hardness study was a more broad area to estimate, so we could obtain more credible and satisfactory results.

In conclusion, local application of alendronate with the collagen membrane was somewhat effective in reducing osteoclastic activity and increasing hardness during an early stage of healing and it was necessary to research various factors related to growth and calcification of bone such as bone matrix protein and to find a way to release the agent slowly.

V. Summary

The present study aimed to examine the effects of

topical application of alendronate with a collagen membrane on the healing of the calvarial defect in rats, which has a good experimental design for the healing of tissue destruction,

To study the effect of alendronate on bone healing, the collagen membrane containing 200 μ g alendronate was inserted in the defects of the right side and collagen membrane treated with physiologic saline was inserted in the defects of the left side. After 1, 2 and 4 weeks, observation of histologic feature after H&E staining, cell counting after TRAP staining, and hardness measurement(Knoop) were performed. In histologic finding, similar features were shown for both test and control groups each week. In cell counting only the 1 week test groups showed significant reduction of TRAP(+)cells than control groups($p < 0.01$) and the control groups showed statistically significant difference for 1, 2, 4weeks($p < 0.05$). In hardness measurement, The 2 week test groups showed significant higher hardness than control groups. ($p < 0.05$) and not 4 weeks. There was significant increase of hardness for both groups as time goes by. ($p < 0.01$)

Therefore local application of alendronate with collagen membrane was somewhat effective in reducing osteoclastic activity and increasing hardness in the early stage of healing. Further investigation concerning the actual effect of alendronate for bony healing will be necessary to apply the clinical cases

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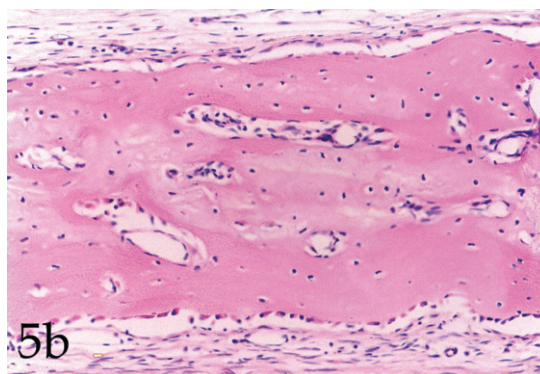
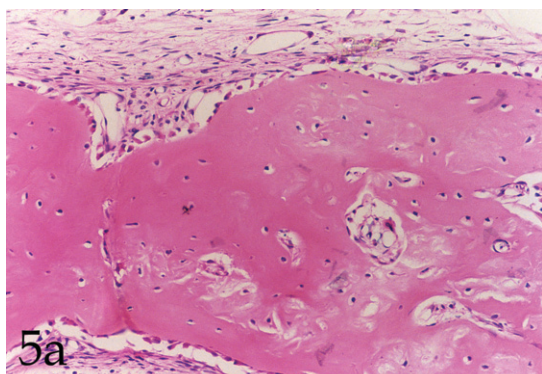
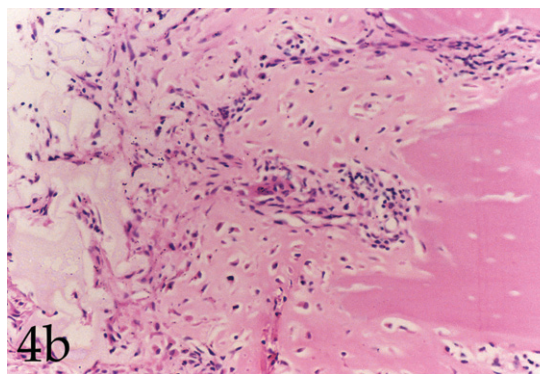
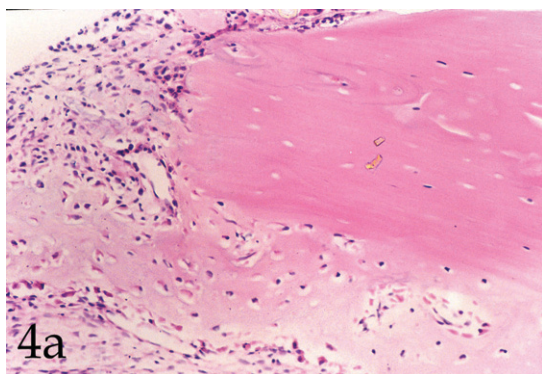
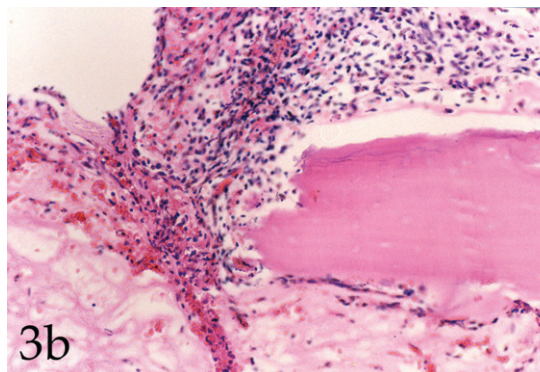
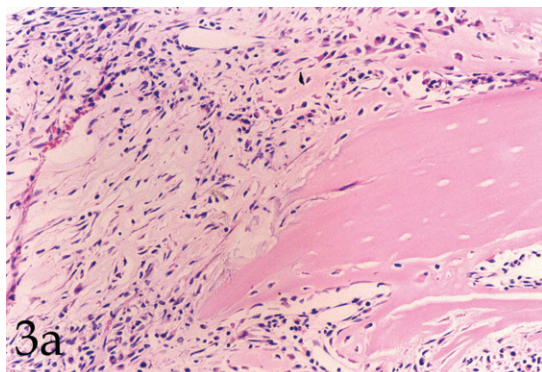
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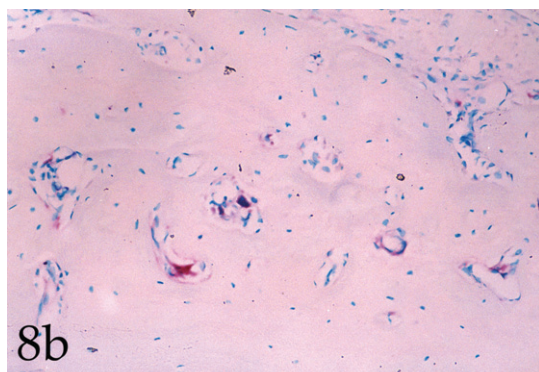
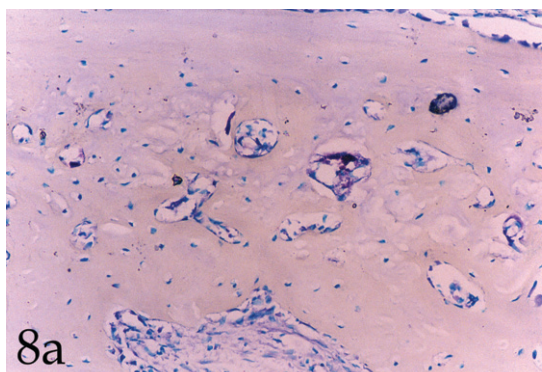
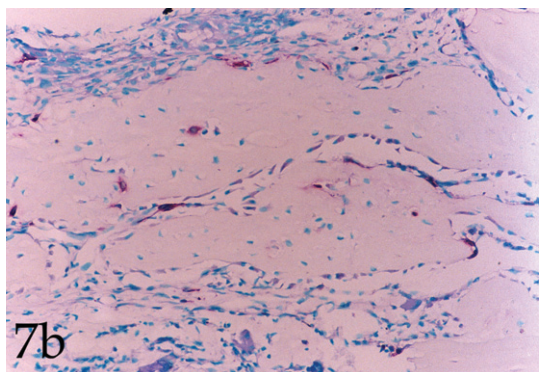
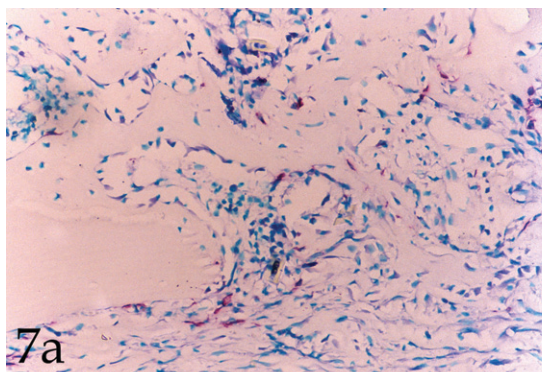
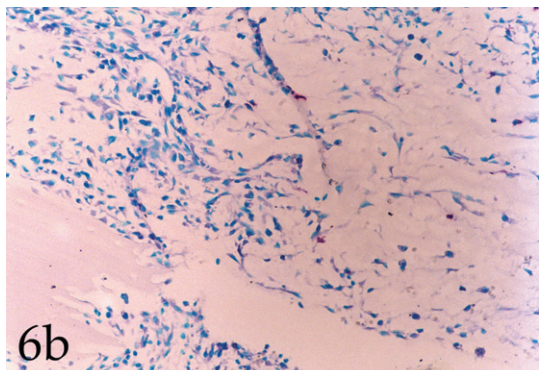
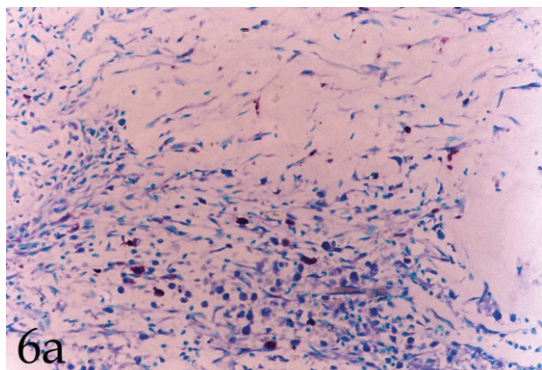
사진부도 설명

- Figure 3 (a:control, b:test)
Histologic findings of control and test groups (1 week after surgery)
Inflammatory cells infiltrated around the marginal bone of the defect on test and control groups. New bone formations were not shown and similar healing aspects were seen on both test and control groups. (H&E, $\times 200$).
- Figure 4 (a:control, b:test)
Histologic findings of control and test groups (2 weeks after surgery)
Inflammatory cell infiltrations were significantly decreased. New bone formations were shown on the marginal bone of the defects and similar healing aspects are seen on both test and control groups. (H&E, $\times 200$).
- Figure 5 (a:control, b:test)
Histologic findings of control and test groups (4 weeks after surgery)
Inflammatory cell infiltrations disappeared and osteogenic repairs were observed on most areas of the defects on both groups. Similar bony healing aspects were seen on both test and control groups. (H&E, $\times 200$).
- Figure 6 (a:control, b:test)
Histologic findings of control and test groups (1week after surgery)
Test groups showed significantly lower number of TRAP(+) cells than control group. (TRAP, $\times 200$).
- Figure 7 (a:control, b:test)
Histologic findings of control and test group (2weeks after surgery)
There were similar TRAP(+)cell numbers between the control and test groups. (TRAP, $\times 100$).
- Figure 8 (a:control, b:test)
Histologic findings of control and test group (4weeks after surgery)
There were similar TRAP(+)cell numbers between the control and test groups. (TRAP, $\times 200$).

사진부도 (I)



사진부도 (Ⅱ)



백서 두개골 결손부의 치유과정에 alendronate가 미치는 영향

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Bisphosphonates는 파골세포에 의한 골흡수를 방지하는 물질로 알려져 있으며 임상에서 널리 쓰이고 있다. 그 중 Alendronate는 Aminobisphosphonates의 한 종류로 non-aminobisphosphonates인 etidronate보다 100-1000배 더 강한 효과를 보이는 것으로 알려져 있다.

본 실험의 목적은 백서두개골 결손부의 골재생을 실험모델로 하여 alendronate의 국소투여 효과를 알아보는 것으로 액체의 흡수성과 골전도성이 우수한 것으로 알려진 collagen membrane을 사용하여 결손부 양측에 alendronate와 physiologic saline을 각각 적용하여 1주, 2주, 4주의 조직학적 치유양상, 파골세포활성도, 경도를 평가하였다. 조직학적 치유양상은 1주째 collagen membrane에 의한 염증성 침윤이 나타났으며 2주째부터 골성회복이 관찰되었고 4주째 완전한 골성회복을 보여 각주별 실험군, 대조군 공히 유사한 양상을 보였고 실험에 사용한 200 μ g의 용량은 조직학적으로 관찰할만한 골재생의 향상을 위해서는 부족한 용량으로 사료되는 바이다. TRAP(+) cell은 1주째 대조군에 비해 실험군에서 유의하게 적은 수를 보였으며($p < 0.01$) 2주와 4주째는 유의한 차이를 나타내지 않았고 경도측정에서는 2주째 대조군에 비해 실험군에서 유의하게 높은 경도를 보였으며 4주째 유의한 차이를 나타내지 않았다($p < 0.05$).

이상의 실험에서 alendronate는 골조직 치유과정의 초기에 파골세포의 활성도와 경도에 다소 영향을 미친 것으로 사료되며 향후 골조직 재생을 위한 임상적응에 응용을 위해서는 치유과정을 더욱 향상시킬 수 있는 추가적인 연구가 필요하리라 사료된다.

주요어: 골재생, 골아세포, 파골세포, bisphosphonates, Alendronate