Effects of Alendronate on Bone Remodeling around Osseointegrated Implants in Rats

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(Directed by Prof. Hong Seok Moon, D.D.S., M.S.D., Ph.D.)

A Dissertation Thesis
Submitted to the Department of Dental Science
And the Graduate School of Yonsei University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy in Dental Science

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June 2012

This certifies that the dissertation thesis
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June 2012

감사의 글

본 논문이 완성되기까지 오랜 시간 지도와 격려로 저를 이끌어주신 문홍석 지도 교수님께 진심으로 감사를 드립니다. 논문 심사과정에서 세심한 지도와 가르침을 주신 정한성 교수님, 박영범 교수님, 김성태 교수님, 김지환 교수님께 깊은 감사를 드립니다. 시편 제작, 자료 분석 및 계측에 많은 도움을 주신 박상현 기사 및 이채은 연구원에게도 감사의 마음을 전합니다. 그리고 실험을 도와주며 많은 시간 애를 써 준 보철과 후배 윤기준, 오경철에게 진심으로 감사를 전합니다. 또한 지면을 통해 일일이 언급하지는 못하지만 저에게 도움과 격려를 주신 모든 분들께도 다시 한번 진심으로 감사 드립니다.

마지막으로 항상 저를 사랑으로 지켜봐 주시는 아버지, 어머니, 장인어른, 장모님께 감사 드리며, 제가 하는 일에 언제나 전념할 수 있도록 세심하게 배려해주는 사랑스런 아내 현정과 하루가 다르게 성장해 나가면서 저에게 큰 기쁨이 되고 있는 장남 제현, 차남 성현에게도 고마움과 사랑을 전하며 이 논문을 함께 나누고자 합니다.

2012 년 06 월

황우진 드림

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Abstract

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Woojin Hwang D.D.S., M.S.D.

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Purpose: Since the first report about BRONJ (bisphosphonate-related osteonecrosis of the jaw) in 2003, many reports have been issued. Recently, bisphosphonate-associated late failures of dental implants have been reported. There are still fewer reports about peri-implant bone remodeling associated with bisphosphonates. The purpose of this study is to evaluate the chronological effects of alendronates on peri-implant bone remodeling in rats.

Materials & Methods: Thirty-six Sprague-Dawley rats (body weight 130-140g, 4 weeks old, male) were first divided into the bisphosphonate and control groups. Then, they were again divided into 4-week, 8-week, and 12-week groups according to the period of subcutaneous administration. The maxillary first molars in each rat were extracted. At 1 month after the extraction, custom-made implants were inserted bilaterally into the extraction sites. After the 4-week-long period of osseointegration, the rats started to get

subcutaneous injection of alendronate or saline for the predetermined periods of time. Then, the rats were euthanized. The specimens were prepared for histologic and micro-computed tomographic analyses. The s-CTX and s-OC levels of the 12-week groups were measured 4 times on a regular basis.

Results: The empty lacunae in the 4-week bisphosphonate group outnumbered those in the 4-week control group significantly. No significant differences were found in the TRAP-positive cell counts, BV / TV data, and BMD data among the control and bisphosphonate groups. The s-CTX levels of the bisphosphonate group declined with time without significance, and those of the control group also declined significantly. The difference of s-OC levels between the control and bisphosphonate groups at +8 weeks was significant. The median values of s-OC in the bisphosphonate group were continuously lower than those in the control group at each time of blood sampling.

Conclusion: Within the limitations of this study, there were no consistent results among the histologic, micro-computed tomographic and biochemical data regarding the peri-implant bone remodeling associated with alendronate. At some specific points of time, the reduction of osteoclastic and osteoblastic functions was observed. An s-OC measurement is seen as a more reliable test tool for bisphosphonate effects. In summary, alendronate seems to have affected the bone remodeling around osseointegrated implants.

Key words: Alendronate, Bisphosphonate, BRONJ, Implant, Empty lacuna, BMD, Bone volume, s-CTX, s-OC

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I. Introduction

Bisphosphonates are powerful compounds with known efficacy for inhibition of bone resorption. They are prescribed for patients with diseases such as osteoporosis, Paget's disease, hypercalcemia of malignancy, multiple myeloma, and bone metastases associated with breasts, prostates, and lungs.[1-4] These drugs have a high affinity for Ca^{2+} ions, due to the presence of the two phosphonate groups, and rapidly bind to bone surfaces. Although small amounts of bisphosphonates are naturally released from the bone surface, their

release is dramatically accelerated during bone resorption.[5] The released bisphosphonates are ingested by osteoclasts, altering their resorptive capacity and survival.[6]

Some adverse effects have been reported in patients on bisphosphonates for the treatment of bone-associated diseases. One of them is bisphosphonate-associated osteonecrosis of the jaw (BRONJ). BRONJ is defined as the presence of exposed necrotic bone in the maxillofacial region that does not heal within 8 weeks after clinical identification by a health care provider, in a patient who is currently receiving or previously had been exposed to a bisphosphonate and has not had radiation therapy to the craniofacial region.[7]

The mechanism of BRONJ incidence is still unclear and controversial. A number of assumptions have been suggested in the literature. Bone remodeling suppression by bisphosphonates is one of the most popular hypotheses.[8,9] It has also been hypothesized that soft tissue of the oral cavity could play a significant role in the BRONJ incidence. Reid et al[10] have proposed that bisphosphonates, which accumulate in the bone, have direct toxic effects on the oral epithelium, and inhibit normal healing of soft tissue lesions caused by either dental intervention or some other traumas, such as traumas from dentures, chewing food, and local infections. Thus, bisphosphonate toxicity to non-osteoclast cells is facilitated by the presence of osteoclasts, as it occurs at an infected bone surface due to resorption and the concomitant release of cytotoxic bisphosphonates.[10] Bisphosphonates also inhibit the proliferation and functions of macrophages, which originate from the same lineages as osteoclasts.[11,12] Osteoclasts are usually the only cells that internalize toxic amounts of bisphosphonates, but in the high concentrations of bisphosphonates, toxicity to other bone cells might occur.[10] Firth et al.[13]

reported that bisphosphonates upregulate macrophage apoptosis. Inhibition of macrophages eventually affects normal immunity, angiogenesis, and wound healing, which might lead to BRONJ.[14] Miglorati et al.[15] have suggested that BRONJ may be attributable to consecutive events: a decrease in bone resorption, decrease of activation of bone multicellular units, leading to decreased bone cellularity and reduced blood flow. All of these events would predispose the jaw bones to osteonecrosis.[15] On the contrary, there are reports that increased number of osteoclasts was found after bisphosphonate administration.[16-18]

The risk for BRONJ occurrence after dental implant therapy in patients who received bisphosphonates is not well-known and is still controversial.[19] Several case reports have reported that patients with symptoms of BRONJ around implants had been taking oral bisphosphonates. Wang et al.[20] reported a case of the patient who had been taking an oral bisphosphonate for over 10 years and had unexplained clinical signs of bone necrosis after routine implant placement. They suggested that bisphosphonate use should be considered with caution.[20] Marx et al.[21] reviewed 119 bisphosphonate-related bone exposure cases, and reported that of the 119 BRONJ cases, 3.4% were associated with dental implantation. They suggested that implant placement might be a precipitating factor for BRONJ although its incidence was low.[21] Lazarovici et al.[22] studied 27 BRONJ cases associated with dental implants. They reported that only 22.2 % of BRONJ cases were surgically related, and that 77.8% developed spontaneously and occurred as a late complication in the 23 patients whose dental implants had been placed after the initiation of their bisphosphonate treatments.[22] The remaining 4 BRONJ cases were associated with the dental implants that had been placed a few years before bisphosphonate treatment was

initiated.[22] Yuan et al.[14] documented a recent case report of severe peri-implant infection that developed after the patient had used an oral bisphosphonate for 3 years, and they suggested that patients treated with bisphosphonates who receive dental implants should be followed up carefully for long periods. On the contrary, Grant et al.[23] reviewed a total of 468 dental implants placed in the 115 patients who had received oral bisphosphonate therapy. They reported that there was no evidence of bisphosphonate-associated osteonecrosis of the jaw in any of the patients evaluated, and that their implant survival rate was similar to those of the patients who had not received bisphosphonate therapy. Madrid and Sanz[24] reported that placement of a dental implant might be considered a safe procedure in patients taking an oral bisphosphonate for less than 5 years with regard to the occurrence of BRONJ, since in their studies no BRONJ case had been reported. They[24] also reported that the intake of oral bisphosphonates did not influence short-term (1-4 years) implant survival rates. Fugazzotto et al.[25] reported that a history of oral bisphosphonate use for 3 years did not contribute to the incidence of osteonecrosis after implant placement. Jeffcoat[26] conducted a parallel-group controlled trial involving patients subjected to dental implant surgery, and he concluded that there was not a statistically significant difference in the incidence of osteonecrosis between patients taking nitrogen-containing oral bisphosphonates and the control group. Despite conflicting conclusions about effects of bisphosphonates on dental implant success, we could expect more cases of BRONJ associated with dental implants in the future than before. It is because more people have to get on bisphosphonate treatments for the treatment of some types of cancer or osteoporosis as their life expectancy is getting longer.[22] Therefore, although the incidence of bisphosphonate-related implant failure is

considered relatively low, the morbidity of BRONJ is so severe that the association between implant failure and bisphosphonates should not be underestimated.[14]

Some previous animal studies regarding the effects of bisphosphonates on dental implant osseointegration reported that bisphosphonates did not affect dental implants negatively.[27-30] Viera-Negron et al.[27] reported that osseointegration of the implants, which were placed in the maxilla of rats, was improved by bisphosphonates. Kim et al.[30] reported that bisphosphonates did not significantly affect bone-to-implant contact in bisphosphonate groups. There are some animal studies, regarding the effects of bisphosphonates on osseointegration of implants, where implants were not placed in jaw bones. Chacon et al.[28] used rabbit tibias, and Narai S. and Nagahata S.[29] used rat femurs for dental implantation. However, considering that the development and physiology of jaw bones are different from those of tibia and femur bones, it seems to be more appropriate to use jaw bones than to use skeletal bones for researches on dental implants.

The rate of bone turnover, characterized by the degradation of old bone and the formation of new bone, can be assessed by measuring bone matrix components released into blood circulation during bone remodeling. Biochemical markers of bones have been used to monitor the efficacy of anti-resorptive therapy in osteoporosis patients. C-terminal telopeptide of type I collagen (CTX), as a bone resorption marker and a useful clinical assessment tool for regular osteoporosis follow-up, is also used to help assess risk for BRONJ in patients taking oral or intravenous bisphosphonates,[31] although controversy and debate continue regarding the use of a CTX test. There is still a paucity of evidence-based, peer-viewed clinical studies demonstrating that a CTX test is a reliable predictor of BRONJ incidence after dental surgery.[32,33] It is one of several known serum and urine

tests that measure breakdown products of bone resorption. CTX tests specially measure a specific cross-link peptide of type I collagen, which comprises 98% of the total protein in bones. Its level in serum is therefore proportional to the amount of osteoclastic resorption occurring when the blood is drawn. Lower values of CTX measurements represent degrees of suppression of bone remodeling. Some bone turnover markers are also linked to bone formation. Measurements of bone-specific alkaline phosphatase and serum-osteocalcin are used for the evaluation of bone formation. Osteocalcin (OC) is non-collagenous proteins released into blood circulation by osteoblasts during their activity of bone matrix synthesis.[34] OC is likely involved in influencing osteoid mineralization and in providing negative feedback during the bone remodeling process. The serum level of osteocalcin is well correlated with bone formation rate which is assessed by bone histomorphometry.[35] Newly synthesized osteocalcin is largely incorporated in the extracellular bone matrix, and a small fraction of it is released into the blood circulation providing a marker of bone formation.[34] Kwon et al.[36] reported that the values of both CTX and OC were decreased in the BRONJ patients. Therefore, they suggested that the simultaneous considerations of CTX and OC values might be a set of risk markers which assess risk prediction for BRONJ before invasive dental surgery. However, many pre-analytical factors influence bone marker measurements, including: food intake, growth, sex, circadian variation, age, disease, *etc*.[37,38] Therefore, the sample (blood or urine) should be taken under controlled conditions like consistency in the time of blood sampling and fasting. Because fasting reduces the amplitude of diurnal variation of s-CTX levels, a sample taken in the fasting state will increase the sensitivity of the test.[39] Both s-CTX and s-OC levels have a circadian rhythm, so much care is needed for

consistency in the time of their samplings, but an s-OC level is not influenced significantly by food intake, so fasting is not always needed for measurements of s-OC levels.[37]

In our study, we focused on the effects of alendronate on peri-implant bone remodeling of already-osseointegrated implants and the concomitant variation of bone marker values (s-CTX and s-OC) in a rat model. A hindrance to our understanding of the pathophysiology of BRONJ is an inability to conduct prospective well-controlled clinical studies on human patients. Thus, animal models are needed to find BRONJ causes and to settle the controversies about the role of bisphosphonates in the BRONJ pathogenesis.[16,30] As over 190 million oral bisphosphonate prescriptions have been dispensed worldwide,[22] the risk of BRONJ occurrence and late failure of implant osseointegration in patients, who already have dental implants in their mouths and start to take bisphosphonates afterward, needs to be studied. Serial measurements of bone markers are very easy and useful clinical tools for the assessment of bone-related diseases, for monitoring treatment efficacy, and for evaluation of compliance of long-term therapy for chronic diseases. In a clinical situation, laboratory tests through blood sampling may be much easier than tissue manipulations such as tissue biopsy for the prediction of BRONJ. Thus, it seems to be meaningful to study correlation between levels of two bone markers (s-CTX and s-OC) and the phenomenon around dental implant surfaces at the microscopic level.

Most of the studies regarding dental implants associated with bisphosphonates are about case reports and initial establishment of osseointegration. There are fewer reports about peri-implant bone remodeling associated with bisphosphonates post-osseointegration. Considering the reports about suppression of bone turnover and

remodeling due to bisphosphonate administration, it is probable that bisphosphonates will affect long-term prognoses of implants.

The purpose of this study was to evaluate the chronological effects of alendronates, which were administered after establishment of implant osseointegration, on peri-implant bone remodeling in the maxilla of rats, and to investigate the utility of biochemical markers, such as s-CTX and s-OC, as risk assessment tools for BRONJ.

Our study's null hypothesis is as follows;

Alendronate did not affect bone remodeling around implants negatively.

II. Materials and Methods

1. Experimental animals

Thirty-six Sprague-Dawley rats (body weight 130-140g, 4 weeks old, male) were first divided into the bisphosphonate group and control group. Then, they were again divided into 4-week, 8-week, and 12-week groups according to the period of subcutaneous bisphosphonate or saline administration (Table I).

Alendronate was injected subcutaneously into each rat belonging to the bisphosphonate groups, and likewise the same amount of saline was injected subcutaneously into each rat belonging to the control groups. All rats under the study were given free access to food pellets and tap water, housed and taken care of at the animal experimental laboratory of Yonsei University, College of Dentistry, Seoul, South Korea. All experimental procedures were performed in accordance with the guidelines for animal experiments of Yonsei University, College of Dentistry.

Table I. The experimental animals

Injection Period		4 wk	8 wk	12 wk
Control groups	Rats (4 wk, male)	6	6	6
Bisphosphonate groups	Rats (4 wk, male)	6	6	6

2. Experimental procedures

The overall experimental protocol is presented in Fig. 1. The detailed implantation protocol followed the one described in the previous report.[30] In summary, all needed surgical procedures were conducted under general anesthesia through the intramuscular injection of an anesthetic mixture composed of Rompun® (xylazine, 20 ㎎ / ㎖, 0.5 ㎖ / ㎏ body weight; Bayer, Leverkusen, Germany) and Zoletil® (tiletamine and zolazepam, 100 ㎎ / ㎖, 0.5 ㎖ / ㎏ body weight; Virbac Lab., Carros, France).[40] Then, maxillary first molars on both sides in each rat were carefully extracted with dental forceps and dental explorers. The extraction sockets were allowed to be healed for one month before implant placements. At 1 month after tooth extraction, a small, full thickness flap was elevated at each recipient site for dental implantation under general anesthesia in the same manner as mentioned before. 1.5 mm-deep implantation osteotomy was prepared with a low-speed ∅1.0 mm round bur and subsequently a Ø1.3 mm fissure bur. Profuse saline irrigation was maintained throughout the osteotomy process. Then, custom-made sterile implants (Ø1.5 mm X 1.5 mm) made of grade IV titanium were inserted bilaterally into the drilled cavities by tapping them with a mallet so that their tops were situated just at the cortical bone surface or roughly 0.5 mm below the bone (Fig. 2 and Fig. 3). Then the flaps were repositioned carefully without suturing.[30] After the surgery, the rats were housed with free access to food pellets and tap water. The implants were allowed to be osseointegrated for 4 weeks.

After the 4-week-long period of implant osseointegration, rats in the bisphosphonate groups started to get subcutaneous injection of Alendronate Sodium Salt (Merial Inc., Parramatta, AUS) as follows: the dose of 1.0 mg / kg, 2 times per week for the predetermined period of time: 4, 8, 12 weeks for each bisphosphonate group, respectively. Likewise, rats in the control groups started to get subcutaneous injection of saline by the same procedure as in the bisphosphonate groups. The rats were allowed to survive for 4, 8, and 12 weeks, respectively after the start-ups of alendronate or saline administrations, and then they were euthanized at the end of survival period. After the rats were anesthetized generally enough as described above on the predetermined day, they were perfused transcardially with 4% paraformaldehyde for euthanization.[41] Then their maxillae, including implants, were removed *en bloc,* immersed in the same fixative for an additional 24 hours.

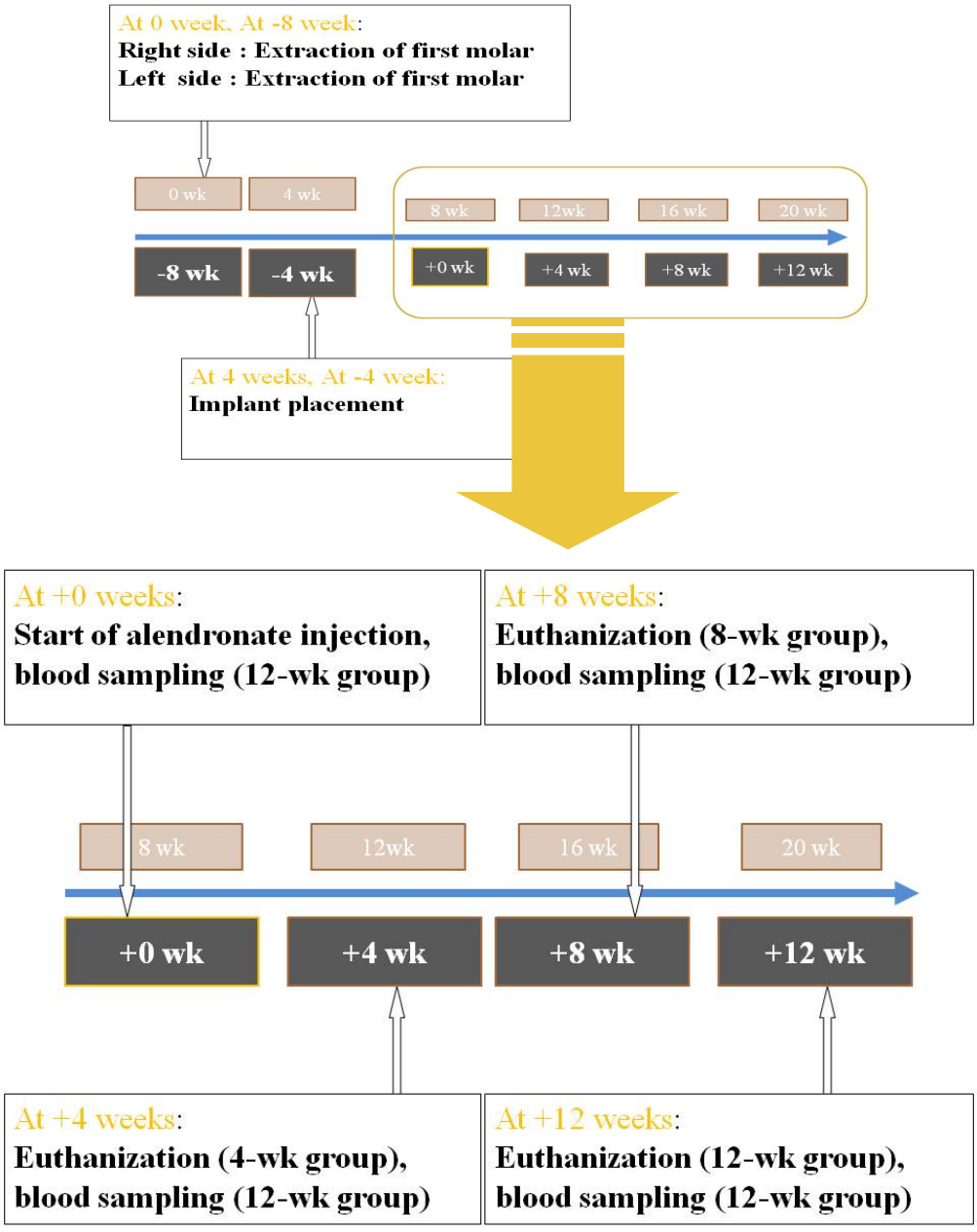

Fig. 1. Experimental design protocol.

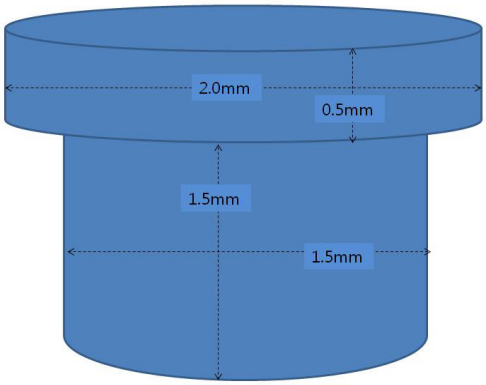

Fig. 2. Dimension of custom-made mini implant with smooth surface.

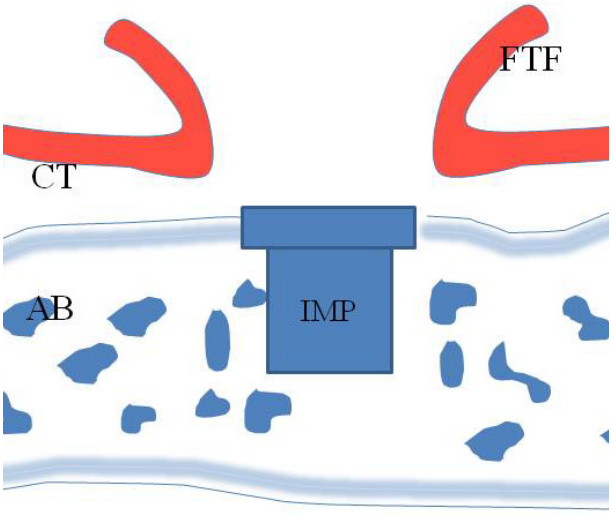

Fig. 3. Surgical procedures of extraction of maxillary first molar and implantation.
* AB: alveolar bone, IMP: implant, CT: connective tissue, FTF: full-thickness flap

3. Histologic analyses

After micro-CT taking, all specimens were decalcified with 10% EDTA at 4 ℃ for one month. The decalcified specimens were first embedded in paraffin wax using standard protocol, and then a series of 7-㎛ thick sections were prepared. The specimens were stained with Hematoxylin-Eosin (H&E) stain using acid phosphatase leukocyte kit (Sigma, St. Louis, MO, USA) on the base of the procedures of the previous study.[30] The stained specimens were observed with a light microscope (Leica DM2500, Leica Microsystems, Germany).

TRAP-positive cells and empty lacunae were counted in the ROI (region of interest) for the quantification of necrotic bone by using IMT i-Solution Lite ver. 8.1 (IMT i-Solution Inc., Korea) (Fig. 4). According to Futami et al.[41], injured pre-existing bone during implantation is usually located within 100 ㎛ from cavity surface. Kenzora et al. [42] suggested a possibility that the injured areas might be located 500 ㎛ beyond the bone cavity margin. In our study, the width of peri-implant ROI was set as 300 ㎛ from the surface of an implant.

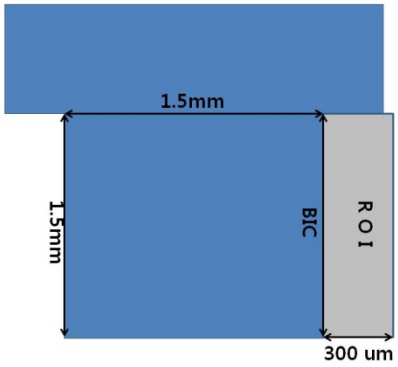

Fig. 4. The analyzed area in the proximity of an implant.
Empty lacunae and TRAP-positive cells were counted in the ROI.
* ROI: region of interest, BIC: bone to implant contact.

4. Micro-computed tomographic analyses

Before decalcification of the specimens, a 3-dimensional micro-CT image was taken for each rat using a micro-CT scanner (Skyscan 1076, Aartselaar, Belgium) at 50 kV and 30 μA. The image was reconstructed to obtain volumetric information and BMD (bone mineral density) about bone remodeling pattern in the proximity of an implant defined by the VOI (volume of interest) (Fig. 5). The density difference between bone and an implant can allow separation of the image of bone compartment from the image of an implant compartment, which enables 3-dimensional evaluation of the peri-implant area. The threshold value for the separation was determined by analyzing the gray-level distribution and picking up the intermediate gray-level value between the two peaks of the materials to be distinguished. BMD, 3-dimensional TV (tissue volume: total volume),

3-dimensional BV (bone volume), and BV / TV % (percentage of bone volume) were computed for the 3-dimensional VOI of each implant site.[43]

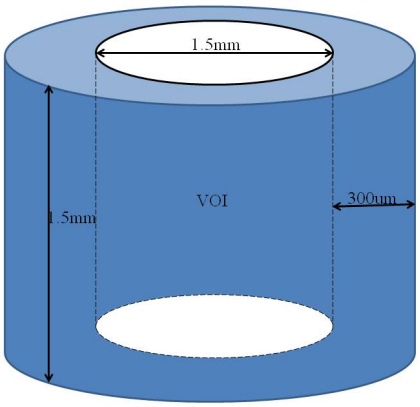

Fig. 5. Volume of interest in the micro-CT image.
* VOI: volume of interest.

5. Biochemical analyses: s-CTX and s-OC

A blood sample was taken from each rat of the 12-week groups, scheduled to be administered with saline or alendronate for 12 weeks, at 0 week, 4 weeks, 8 weeks, and 12 weeks after the start of saline or alendronate infusion, for a total of 4 times. To reduce the variability of measurement, the sampling procedure was done at a regular time under fasting state. The serum samples were obtained by centrifugation and then stored at -200℃ until laboratory assay. The levels of serum-CTX and serum-OC were measured by Elecsys 2010 using β-CrossLaps/Serum (Roche Diagnostics Corp.,

Indianapolis, USA) kit and N-MID® Osteocalcin (Roche Diagnostics Corp., Indianapolis, USA) kit, respectively.

6. Statistical analyses

Median, maximal, minimal, 75% percentile, and 25% percentile values were determined for each group. Mann Whitney U test and Kruskal Wallis test were used to evaluate the histological, micro-computed tomographic, and biochemical data. Dunn's test was used for post hoc multiple tests. All calculations were performed using a specific statistical program (SPSS Ver. 18.0, IBM Co., Somers, NY, USA).

III. Results

Of 36 rats, 5 were dead unintentionally during the study: 1 from the 8-week control group, 1 from the 8-week bisphosphonate group, 1 from the 12-week control group, and 2 from the 12-week bisphosphonate group. A total of 9 implants were lost among the remaining 31 rats: 3 from the 4-week control group, 2 from the 4-week bisphosphonate group, 1 from the 8-week control group, 2 from the 12-week control group, and 1 from the 12-week bisphosphonate group.

1. Micro-computed tomographic findings

3-dimensional micro-CT images were taken to obtain BV / TV percentage (bone volume / tissue volume percentage) and BMD (bone mineral density) data in the VOI (volume of interest) depicted in Fig. 5. There were no significant differences in BV / TV and BMD data among the control and bisphosphonate groups. A representative 3-D image for each group is shown in Fig. 6. The implants are shown to be partially surrounded by trabecular bones. The processed data are presented in Fig .7 and Fig. 8.

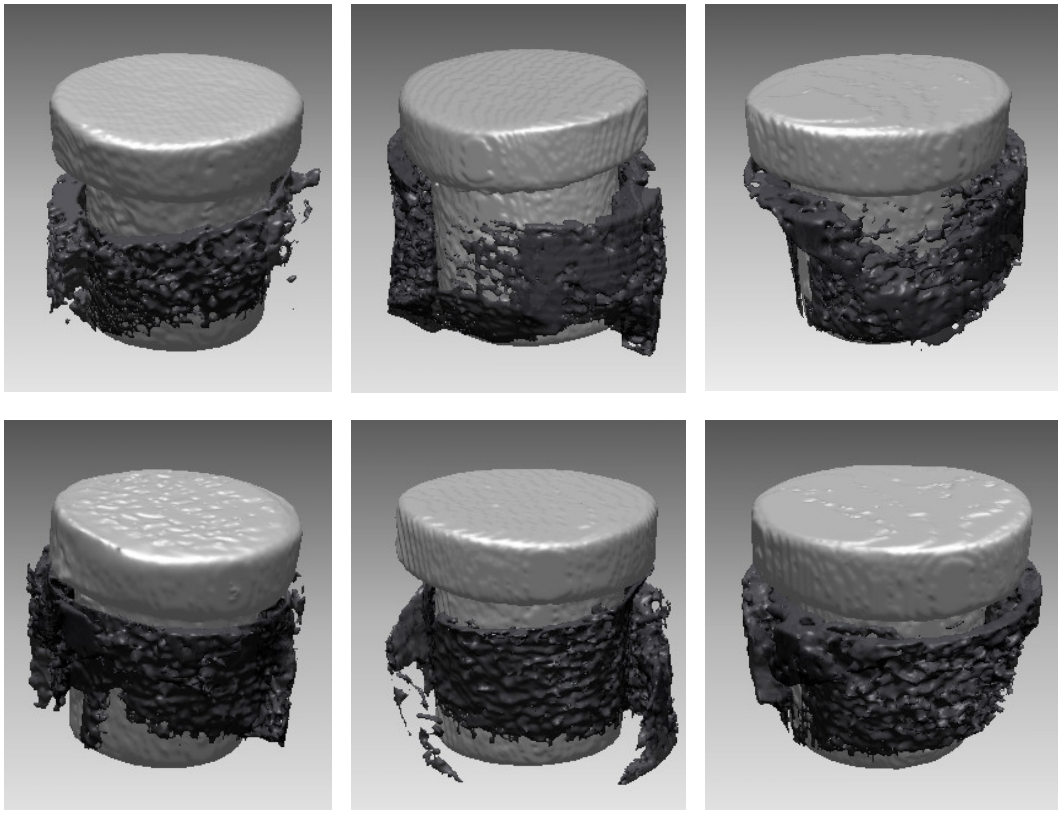

Fig. 6. 3-dimensional reconstructed images of peri-implant sites.
Upper left: the bisphosphonate group at 4 weeks after alendronate infusion.
Lower left: the control group at 4 weeks after saline infusion.
Upper center: the bisphosphonate group at 8 weeks after alendronate infusion.
Lower center: the control group at 8 weeks after saline infusion.
Upper right: the bisphosphonate group at 12 weeks after alendronate infusion.
Lower right: the control group at 12 weeks after saline infusion.

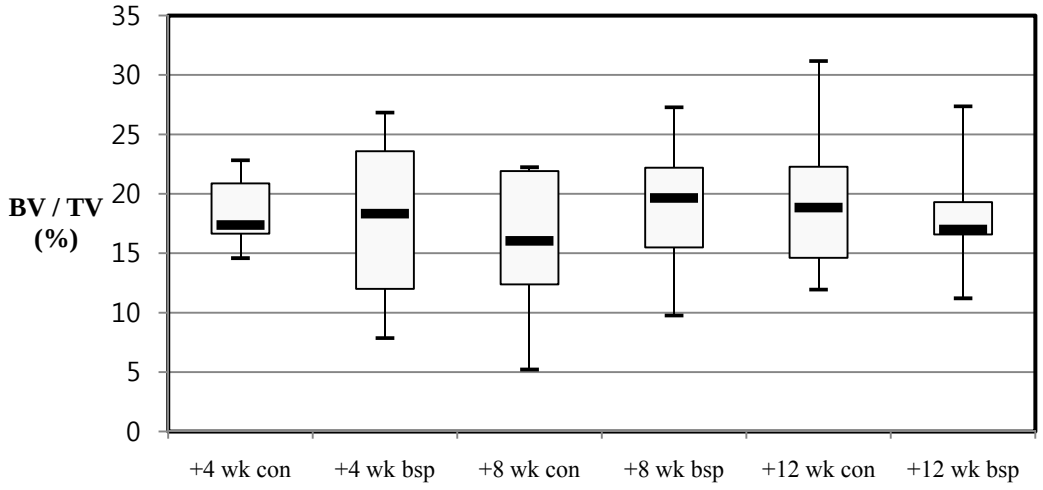

Fig. 7. Bone volume / tissue volume percentage in the VOI (volume of interest). Data are presented as median (*thick horizontal line*), interquartile range (*enclosed boxes*), range between highest and lowest values (*thin horizontal line*).
* wk: week, con: control group, bsp: bisphosphonate group.

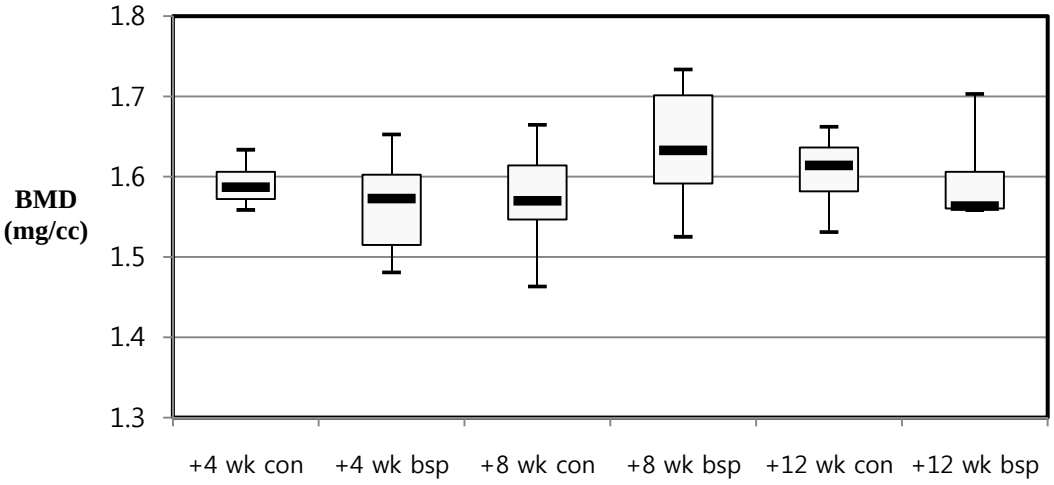

Fig. 8. Bone mineral density in the VOI (volume of interest). Data are presented as median (*thick horizontal line*), interquartile range (*enclosed boxes*), range between highest and lowest values (*thin horizontal line*).
* wk: week, con: control group, bsp: bisphosphonate group.

2. Histologic findings

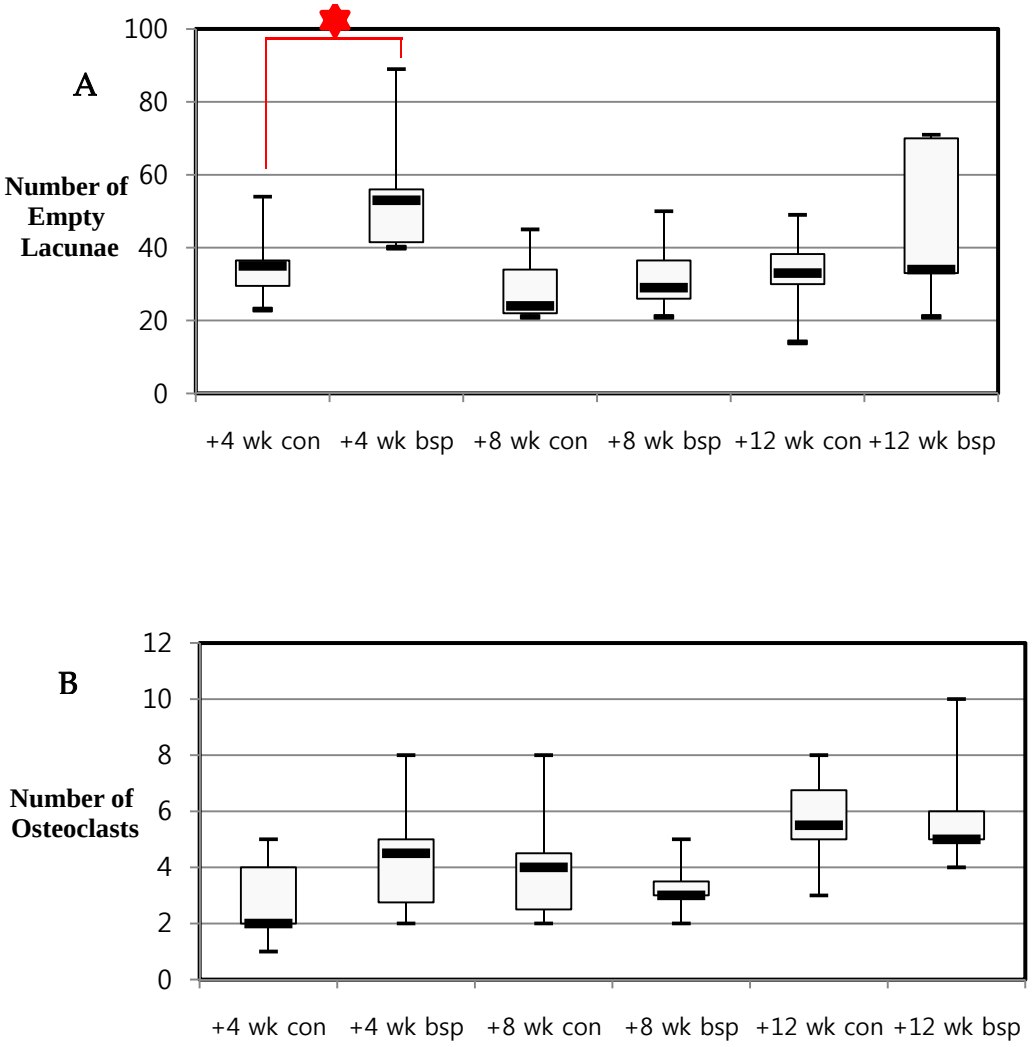

Fig. 9. Histomorphometric results in implant sites. Data are presented as median (*thick horizontal line*), interquartile range (*enclosed boxes*), range between highest and lowest values (*thin horizontal line*).
A: The number of empty lacuna in the ROI.
B: The number of TRAP-positive osteoclasts in the ROI.
* Red stars indicate that there was significant difference ($p<0.05$).
* ROI: region of interest.
* wk: week, con: control group, bsp: bisphosphonate group.

To investigate the effects of alendronate on peri-implant bone remodeling at the histomorphometric level, we obtained H&E-stained and TRAP-stained images containing the ROI (region of interest) at two magnification powers (12.5x and 100x), at three points of time (at 4, 8, and 12 weeks after the start of drug infusion) (Fig. 10 - 13). The empty lacunae and TRAP-positive osteoclasts were counted. The empty lacunae in the 4-week bisphosphonate group outnumbered those in the 4-week control group significantly, but there were no significant differences for the 8-week and 12-week groups (Fig. 9A). No significant differences were found in the TRAP-positive cell counts among the 4-week, 8-week, and 12-week groups (Fig. 9B).

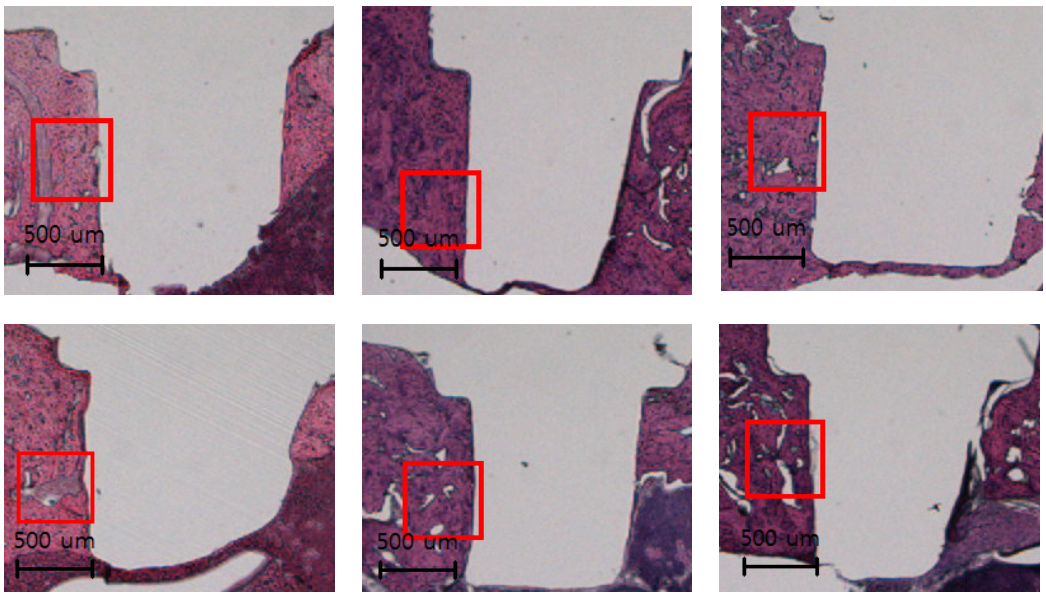

Fig. 10. H&E stained images of the implant sites at 4, 8, and 12 weeks after start of drug infusion. Scale bar = 500㎛ (H&E stained image 12.5x).
Upper left: the bisphosphonate group at 4 weeks after alendronate infusion.
Lower left: the control group at 4 weeks after saline infusion.
Upper center: the bisphosphonate group at 8 weeks after alendronate infusion.
Lower center: the control group at 8 weeks after saline infusion.
Upper right: the bisphosphonate group at 12 weeks after alendronate infusion.
Lower right: the control group at 12 weeks after saline infusion.

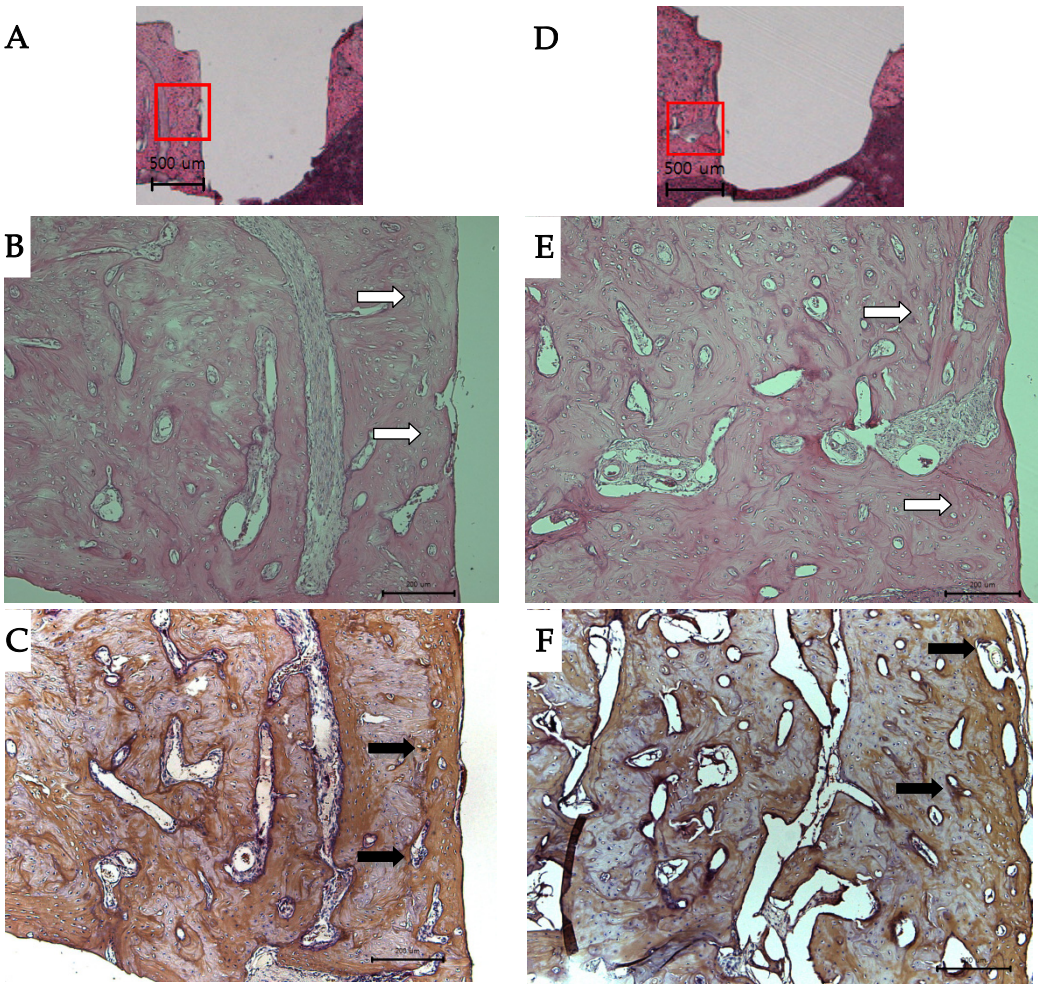

Fig. 11. Histologic images of implant sites at 4 weeks after the start of drug infusion.
A,B,C: for bisphosphonate group. D,E,F: for control group.
A & D: H & E stained images at lower magnification (12.5x). Scale bar = 500 ㎛.
B & E: H & E stained images of the red boxes in A & D (100x). Scale bar = 200 ㎛.
C & F: TRAP stained images of the red boxes in A & D (100x). Scale bar = 200 ㎛.
* White arrow: empty lacuna.
* Black arrow: TRAP-positive osteoclast.

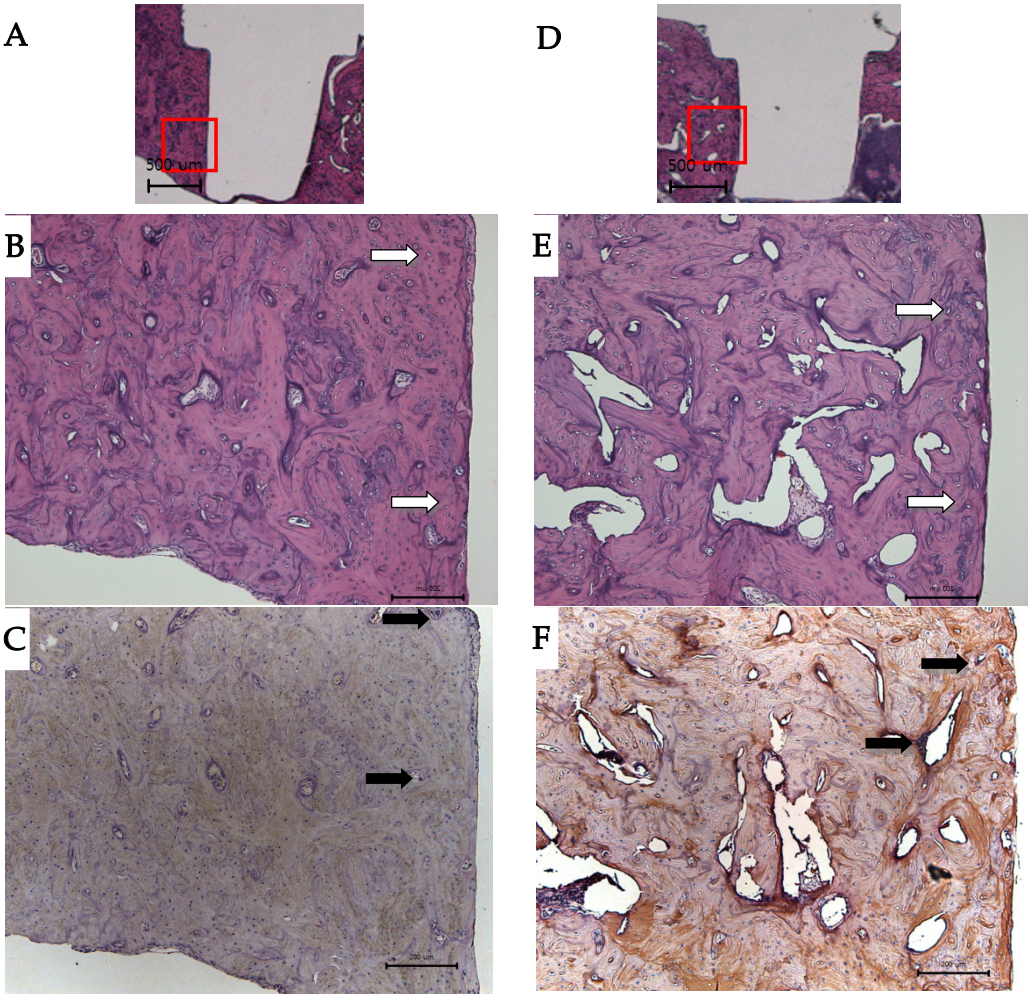

Fig. 12. Histologic images of implant sites at 8 weeks after the start of drug infusion.
A,B,C: for bisphosphonate group. D,E,F: for control group.
A & D: H & E stained images at lower magnification (12.5x). Scale bar = 500 ㎛.
B & E: H & E stained images of the red boxes in A & D (100x). Scale bar = 200 ㎛.
C & F: TRAP stained images of the red boxes in A & D (100x). Scale bar = 200 ㎛.
* White arrow: empty lacuna.
* Black arrow: TRAP-positive osteoclast.

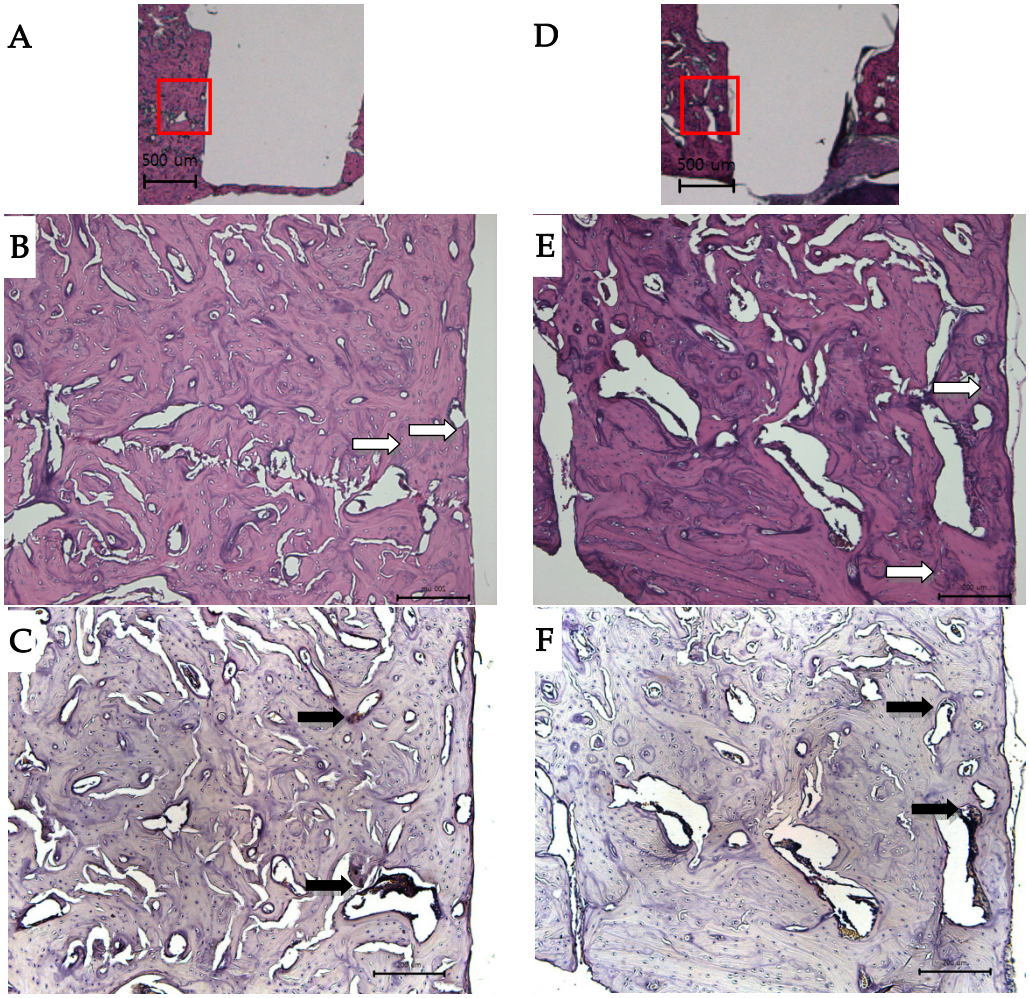

Fig. 13. Histologic images of implant sites at 12 weeks after the start of drug infusion.
A,B,C: for bisphosphonate group. D,E,F: for control group.
A & D: H & E stained images at lower magnification (12.5x). Scale bar = 500 ㎛.
B & E: H & E stained images of the red boxes in A & D (100x). Scale bar = 200 ㎛.
C & F: TRAP stained images of the red boxes in A & D (100x). Scale bar = 200 ㎛.
* White arrow: empty lacuna.
* Black arrow: TRAP-positive osteoclast.

3. Biochemical findings: s-CTX and s-OC values

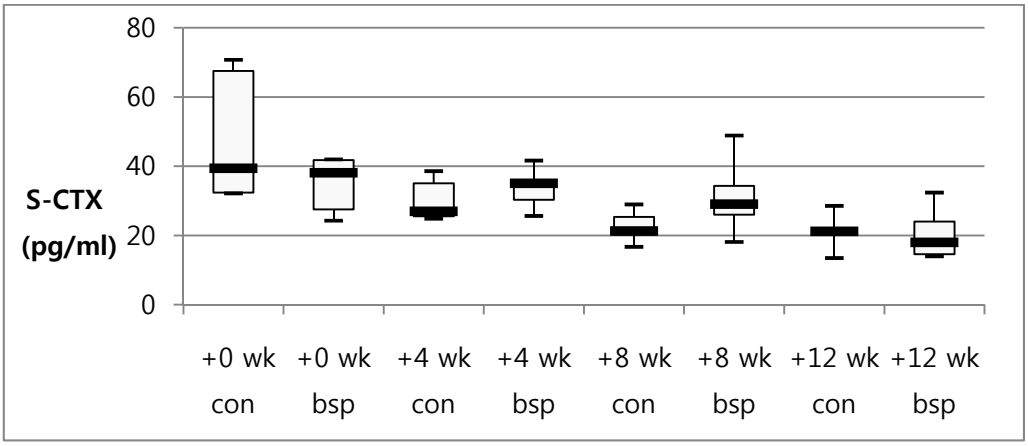

Fig. 14. S-CTX levels by timing of blooding sampling. Data are presented as median (*thick horizontal line*), interquartile range (*enclosed boxes*), range between highest and lowest values (*thin horizontal line*).
* Red stars indicate that there was significant difference ($p<0.05$).
* wk: week, con: control group, bsp: bisphosphonate group.

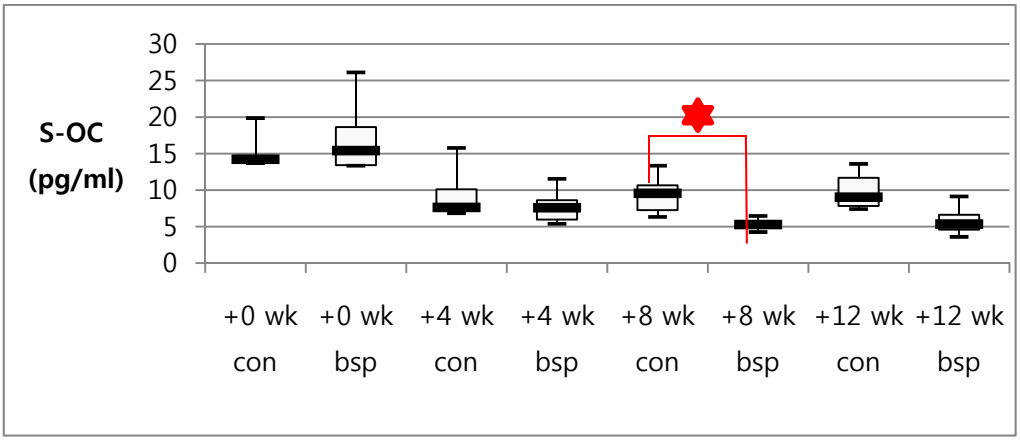

Fig. 15. S-OC levels by timing of blooding sampling. Data are presented as median (*thick horizontal line*), interquartile range (*enclosed boxes*), range between highest and lowest values (*thin horizontal line*).
* Red stars indicate that there was significant difference ($p<0.05$).
* wk: week, con: control group, bsp: bisphosphonate group.

The s-CTX and s-OC levels of the 12-week groups were measured to evaluate the biochemical effects of alendronate on bone metabolism from the start of drug infusion to the time of euthanization, at intervals of 4 weeks, a total of 4 times. There were no significant differences in the s-CTX levels between the 12-week control and 12-week bisphosphonate groups (Fig. 14). While alendronate is known to naturally decrease s-CTX levels, the s-CTX levels of the 12-week bisphosphonate group declined with time without significance, and those of the 12-week control group also declined significantly. The difference of s-OC levels between the 12-week control group and the 12-week bisphosphonate group at +8 weeks was significant. The median values of s-OC in the 12-week bisphosphonate group were lower than those in the 12-week control group at +4 weeks, +8 weeks, and +12 weeks.

IV. Discussion

In this study, an animal experiment using rats was performed to investigate the effects of bisphosphonates on bone remodeling around a dental implant. Alendronate, one type of bisphosphonates, was used for this study. Since its first availability in 1995, alendronate has been used for the treatment of osteoporosis. The risk of BRONJ for patients receiving intravenous bisphosphonates is known to be significantly greater than the risk of BRONJ for patients receiving oral bisphosphonates such as alendronate.[7,22,31,44] Although its incidence in patients taking oral bisphosphonates is much less than in patients receiving intravenous bisphosphonates, there are rising concerns that osteoporotic patients will encounter BRONJ more frequently as they are treated for longer periods of time.

There are reports about BRONJ occurrences associated with dental implants.[14,20-22,24-26,31,45,46] Most of them are retrospective studies or case reports, but there still is a lack of well-controlled prospective studies. Among case reports reporting the incidence of BRONJ associated with dental implants, some reported patients suffering from BRONJ despite its relatively low incidence,[20-22,45] but others reported that bisphosphonate administered orally did not cause any harm to dental implantation.[24-26] Despite many case reports about the failure of dental implantation associated with bisphosphonates, its mechanism and cause have not been thoroughly understood and remain controversial. In our study, we focused on possibilities of late failure associated with dental implants, and

designed an animal experimental model to investigate effects of alendronate on bone remodeling around osseointegrated implants.

Some previous studies reported about the initial healing after implantation associated with bisphosphonates. Kurth et al.[47] reported the improved initial osseointegration of implants in rats. Chacon et al.[28] and Narai et al.[29] conducted animal studies using femurs or tibias for studying effects of bisphosphonates on osseointegration of implants, and they reported improved removal torque of implants. But these kinds of long bones are different biologically from alveolar bones where dental implants are placed clinically, and bisphosphonates will affect in different ways depending on the types of bones. Therefore, experimental animal models using alveolar bones for a dental implant study are considered appropriate. According to previous studies using a rat model, the new bone formation was found at 5 days post-implantation, and the osseointegration was established at 28 days post-implantation.[48,49] There are still fewer studies about peri-implant bone remodeling after the implant placement associated with bisphosphonates although there are many studies about the initial bone healing. Peri-implant bone reinforcement will occur through bone remodeling after implant osseointegration. In our study, bisphosphonate administration started at 4 weeks after implant placement when reportedly osseointegration had been established.

Previous animal experiments applied a variety of bisphosphonate doses, and administration methods. Viera-Negron et al.[27] did subcutaneous injection at a dosage of 5 ㎎ / ㎏ bw. Hikita et al.[50] did local administration at a dosage of 1 mg / kg bw. Aguirre et al.[51] did subcutaneous injection at two levels of doses which were 15 ㎍ / ㎏

bw and 150 μg / kg bw. In our study, we applied the dosage of Hikita et al.'s study and administered alendronate twice a week like in Aguirre et al.'s study. The doses, adopted by Negron et al.'s, Hikita et al.'s and by our study, are much higher than those for osteoporotic human patients. However, the determination of an optimal alendronate dosage for experimental rat models for a BRONJ study is considered not yet to be standardized but it is worthy for a research topic.

To investigate the effects of bisphosphonates on bone remodeling around osseointegrated implants, histological, micro-computed tomographic and biochemical changes were observed in this study. The reduced bone resorption possibly leads to remaining of dead bones caused by injury during implantation. According to previous studies, empty lacunae would appear after dental trauma such as dental extraction or implantation, and their number will reduce as healing processes continue.[41,52] Recent studies reported that the number of empty lacunae did not decrease because of bone remodeling depressed by bisphosphonates.[16,53] In our study, more empty lacunae were observed significantly in the 4-week bisphosphonate group than in the 4-week control group. The 8- and 12-week bisphosphonate groups had higher median values than the 8- and 12-week control groups, but with no significant difference. Therefore, the bone resorption rate seems to have been reduced to some extent due to bisphosphonates. But it is still controversial whether direct toxic effects of bisphosphonates on osteocytes cause osteocyte death or suppressed bone remodeling fails to renew bone areas naturally under cell death.[54] Similarly, the bisphosphonate administration was reported to have increased the dead bone formation in the mice in a previous study where zoledronate and an

immuno-suppressive drug were infused, and a BRONJ-like disease was induced as a result.[16] However, a distinct BRONJ-like disease was not located in our study. The reason for this seems to be that different types of bisphosphonates and ways to administrate them were used in that study.

In our study, TRAP (tartrate-resistant acid phosphatase) staining was conducted to count TRAP-positive osteoclasts in order to investigate bisphosphonates' direct effects on osteoclasts. It is known that bisphosphonates will block bone resorption by way of the inhibition of geranyl pyrophosphate synthase or farnesyl pyrophosphate synthase inside osteoclasts, and subsequently interfere with osteoclasts' membrane specializations required for bone resorption.[55] Hughes et al.[56] reported that bisphosphonates promoted apoptosis leading to decreasing the number and activity of osteoclasts. Hikita et al.[50] reported that bisphosphonates diminished the number of TRAP-positive cells. However, Weinstein et al.[17] reported that long-term alendronate treatment was associated with an increase in the number of osteoclasts, which included distinctive giant, hypernucleated, detached osteoclasts that were undergoing protracted apoptosis. Bi et al.[16] also reported an increased number of TRAP-positive cells in mice after long-term bisphosphonate administration. These two studies[16,17] also mentioned that the function of osteoclasts was reduced, although their number increased. It was also proposed that bisphosphonates may have increased the lifespan of osteoclasts.[17,18] In our study, there was no significant difference in the number of TRAP-positive cells between the bisphosphonate group and the control group, and considering this result together with the increased number of empty lacunae, the activity of osteoclastic resorption seems to have been lowered.

To easily identify and treat people who are osteoporotic or at risk of osteoporosis, many biochemical bone markers have been developed and used for the evaluation of bone formation and resorption. Biochemical bone markers show the effect of drugs on bone metabolism and help establish the lowest or optimal dose inducing the largest change in the marker level because the treatment-related responses of such markers are more rapid compared with radiographic methods such as BMD. Biochemical bone markers have analytical and pre-analytical variability of the measurement.[57] Some of them have a broad range of values among individuals and dependency on patients' age,[58] even on rats' age.[59,60] Marx et al.[31] proposed that the risk of BRONJ could be assessed by using s-CTX tests. However, the reliability of the s-CTX test, when evaluating the risk of BRONJ incidence, is still under controversy. Kwon et al.[36] proposed that simultaneous consideration of s-CTX and s-OC might be a set of risk markers assessing risk prediction for BRONJ incidence. Compared with studies about osteoclasts, there are still not so many studies about the effects of bisphosphonates on osteoblasts. In this study, both s-CTX and s-OC levels of the 12-week groups were measured 4 times throughout the period of drug infusion to biochemically investigate bone remodeling. In this study, the levels of s-CTX had no significant difference between the 12-week bisphosphonate and 12-week control groups at each time of blood sampling. However, the levels of s-CTX in the 12-week control group decreased over time significantly and those in the 12-week bisphosphonate group also decreased without significance. Interestingly, s-CTX and s-OC values were reported to decline naturally with age during adolescence in male rats.[59,60] Our study was carried out while the rats grew from the age of 4 weeks to the age of 20 weeks, and it seems to be no wonder that the levels of s-CTX and s-OC in this study

declined with time both in the bisphosphonate and even control groups. Therefore, regarding s-CTX, it was hard to conclude definitely whether alendronate had affected bone resorption activity in our study, although it is known that s-CTX is a bone resorption marker and its decreased level is expected easily from a bisphosphonate treatment. Regarding s-OC in our study, while there was a significant difference in the s-OC level at 8 weeks post-administration, the s-OC levels of the 12-week bisphosphonate group kept lower than those of the 12-week control group at 4, 8, and 12 weeks after drug administration. Therefore, it can be assumed that alendronate affected the bone formation activity to some extent. Fink et al.[61] reported that s-OC values decreased slightly during bisphosphonate therapy. Baim and Miller[62] reported that bone formation can be rather reduced by inhibited bone resorption during the bone-remodeling process. In our study, although the activity and number of osteoblasts were not investigated on a microscopic level, the continuously lower levels of s-OC in the 12-week bisphosphonate group, together with the increased number of empty lacunae, could reflect the impairment of bone remodeling process and sequentially signify the increase of bone disease risk.

Micro-computed tomographic analysis of peri-implant bone was conducted for the evaluation of the bone remodeling in our study. Compared with histomorphometry, micro-computed tomography is a fast and non-destructive method that allows three-dimensional evaluation of bone morphometry parameters such as bone volume, bone surface, trabecular thickness, and trabecular separation. On the other hand, histomorphometry process allows high resolution and image contrast, but has traditionally been assessed in two-dimensional sections, not allowing 3-dimensional analysis of the bone structure.[43] Micro-CT evaluation is an alternative method to quantify bone in three

dimensions. In our study, a rat bone biopsy containing a titanium mini implant was analyzed using a micro-CT scanner before the histologic slice preparation. There were neither significant differences nor tendencies found in the micro-computed tomographic results regarding bone volume / tissue volume % and bone mineral density. It could reflect that alendronate didn't affect the peri-implant bone structure significantly. Otherwise, it was likely to be due to micro-CT morphometry's limitations of measuring only mineralized tissues over a certain threshold of mineralization, while traditional histomorphometry can measure minimally mineralized and even non-mineralized bone tissue.[43] From a different point of view, there is a possibility that the morphometric changes of peri-implant bones had occurred practically but the micro-CT process could not detect them numerically. Therefore, another study with longer period of research is assumed to be necessary. As the evaluation of correlation between histomorphometry and micro-computed tomographic morphometry is beyond the scope of our study, it was not conducted. Despite many technical advancements of radiology, it still seems that small changes in bone structure can only be evaluated by conventional histology in spite of its tedious procedures. However, as significant correlations between conventional histomorphometry and micro-computed tomographic morphometry have been reported in some reports,[63,64] the researcher will possibly be exempt from conventional histology processes, which are tedious and time-consuming, with the advent of a micro-CT scanner with more precision in the future.

In our study, the implants were kept submerged without functional loading. Practically, peri-implant bone remodeling will be affected by a variety of parameters in oral cavities under the functional loading state. Reportedly, long-term administration of a

bisphosphonate will depress bone remodeling severely and accumulate post-injury micro-damage, eventually leading to decrease of bone toughness without reduction of bone strength.[65,66] In a clinical situation, overlapped with poor dental prostheses, infections, traumas, poor oral hygiene, and systemic conditions, long-term administration of bisphosphonates possibly could result in localized bone necrosis in the situation demanding dynamic osseous repair.

Our study also had several limitations. First, this study was ended with a small number of samples in each group. Second, rats at juvenile age, of which biochemical bone markers are quite variable, were used. Third, the placed implants remained submerged throughout the study period, and had no chance of functioning time in oral cavities. Fourth, osteoblasts were not studied in the histomorphometric analysis.

In summary, it was found in this study that the osteoclastic and osteoblastic functions may have been suppressed by alendronate from histologic and biochemical points of view. From a 3-dimensional radiographic point of view, it was hard to make a definite conclusion about the alendronate effects on peri-implant bone remodeling. A further study is necessary considering the limitations shown above.

V. Conclusion

The purpose of this study was to evaluate the effects of alendronate on the bone remodeling around osseointegrated implants. Within the limitations of this study, the following conclusions can be drawn:

1. There was no consistent result among the histologic, micro-computed tomographic, and biochemical data regarding the peri-implant bone remodeling associated with alendronate. However, at some specific points of time, the reduction of osteoclastic and osteoblastic functions was observed.
2. The pre-analytical variation parameters of s-CTX placed limitations on the conclusive analysis of the s-CTX results. The s-OC median values of the bisphosphonate group remained consistently lower than those of the control group from +4 weeks to +12 weeks post-administration. An s-OC measurement is seen as a reliable test tool for bisphosphonate effects.
3. In summary, alendronate seems to have affected the bone remodeling around the osseointegrated implants.

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국문요약

실험용 쥐에서 골유착된 임플란트 주위 골개조에 알렌드로네이트가 미치는 영향

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목적: 비스포스포네이트와 연관된 악골 괴사가 2003 년에 처음으로 보고된 이후 최근엔 비스포스포네이트와 관련된 임플란트 late failure 가 보고되고 있다. 비스포스포네이트가 임플란트 주위 골개조에 미치는 영향에 대해 체계적으로 진행된 연구는 별로 없다. 본 연구의 목적은 골유착된 임플란트 주위 골개조에 알렌드로네이트가 미치는 영향을 알아보는 것이다.

방법: 36 마리의 실험용 쥐를 실험군 (알렌드로네이트 주입)과 대조군 (식염수 주입)으로 나누고 다시 약물투여 기간에 따라 4 주, 8 주, 12 주군으로 나누어 총 6 개 군으로 실험을 진행하였다. 모든 쥐에서 상악 제 1 대구치를 발치 후 한 달간의 치유기간을 가졌고 그 후 맞춤 제작된 미니

임플란트가 식립되었다. 그러고 한 달간의 골유착 과정을 거쳤다. 골유착 후 군별로 4, 8, 12 주 동안 약물이 정해진 용량으로 투여되었으며 그 후 희생되어 조직학적 분석, 방사선학적 분석 과정에 들어갔다. 12 주군을 대상으로 약물이 투여된 12 주 동안 s-CTX, s-OC 측정을 4 회 시행했다.

결과: 투약 4 주차에서 empty lacunae 의 수는 대조군과 실험군 사이에 유의차가 있었다. TRAP-positive 세포 수, BV/TV 측정치, BMD 측정치는 대조군과 실험군간에 유의차는 없었다. S-CTX 결과치는 투약 시간이 경과함에 따라 대조, 실험군에서 감소하는 경향을 보였는데 대조군에서 유의차가 있었다. S-OC 결과치는 투약시간 경과에 따라 대조군과 실험군에서 유의성 있게 감소했고 실험군의 중간값이 대조군의 것보다 계속 낮게 유지 되었다.

결론: 알렌드로네이트가 임플란트 주위 골개조에 미치는 영향에 대한 조직학적, 마이크로시티, 생화학적 결과들 사이에 동일한 경향을 보이지 않았다. 비스포스포네이트의 효과를 평가하는데 있어서 s-OC 는 신뢰할만한 검사 방법이라고 여겨진다. 본 연구의 한계 내에선 골유착이 완성된 임플란트 주위 골개조에 알렌드로네이트가 영향을 미친 것으로 보인다.

핵심되는 말: 알렌드로네이트, 비스포스포네이트, BRONJ, 임플란트, 상실된 골소강, 골밀도, 골부피, s-CTX, s-OC