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ORIGINAL ARTICLE

Effect of alendronate on healing of extraction sockets and healing around implants

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OBJECTIVES: The purpose of this study was to evaluate the effect of alendronates on healing of extraction sockets and healing around implants in the maxilla of rats.
MATERIALS AND METHODS: Twenty-four Sprague–Dawley rats were used. The rats in bisphosphonate group were subcutaneously injected with alendronate (5.0 mg kg^{-1}) three times a week for 4 weeks. Both sides of the maxillary first molars were extracted, and customized titanium implants (Ø1.5 × 2.0 mm) were placed immediately into one side. Rats were killed at 3, 7, 14, or 28 days following surgery.
RESULTS: New bone formation in extraction sockets, bone area around the implant site, and bone–implant contact were not delayed in the bisphosphonate group. The tartrate-resistant acid phosphatase positive cell count did not differ between bisphosphonate and control groups; however, empty lacunae were observed significantly more in bisphosphonate group. The differences in empty lacunae were shown at different time points between the implant sites and extraction sites: at 7 days after extraction, and at 14 and 28 days after implantation.
CONCLUSIONS: Alendronates seemed to decrease bone resorption but not to decrease bone formation. Empty lacunae were observed significantly more at later time points in implant sites compared to extraction sockets.
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Keywords: alendronate; bisphosphonate-related osteonecrosis of the jaw; implant; extraction; empty lacuna; bisphosphonate; osteoporosis

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Introduction

Bisphosphonates have been used routinely as bone-resorption inhibitors to treat bone metabolic abnormalities such as osteoporosis and Paget's disease. The clinical efficacy of oral bisphosphonates for the treatment of osteopenia/osteoporosis is well established. They are also used to manage cancer-associated bone disease (Drake *et al*, 2008). Bisphosphonates have a high affinity for bone mineral and inhibit hydroxyapatite breakdown, thereby effectively suppressing bone resorption. Several studies on the use of bisphosphonate in dentistry, such as reduction of bone resorption after dental tooth extraction (Graziani *et al*, 2008) or periodontal surgery, (Binderman *et al*, 2000) have been reported. However, osteonecrosis of the jaw, atrial fibrillation, and hypocalcemia have been reported as complications associated with bisphosphonate therapy (Drake *et al*, 2008).

In 2003, Marx first reported observing osteonecrosis in 36 patients who received bisphosphonates intravenously following dental treatment (Marx, 2003). In 2004, Ruggiero *et al* (2004) reported that oral bisphosphonate could also be a contributing factor for BRONJ (bisphosphonate-related osteonecrosis of the jaw). Since these reports, many others on BRONJ have emerged; however, the mechanisms of BRONJ have not yet been elucidated. According to Allen and Burr's review of the various hypotheses underlying BRONJ, suppression of remodeling, impairment of angiogenesis, and infection have been proposed as causative factors (Allen and Burr, 2009).

Invasive dental treatment may be a risk factor for BRONJ. According to the position paper on BRONJ by the American Association of Oral and Maxillofacial Surgeons, the following dental treatments have been suggested as local risk factors for BRONJ: extraction, dental implant placement, and periodontal surgery involving osseous injury (Ruggiero *et al*, 2009). Because extraction is one of the most frequently reported risk factors for BRONJ, many researchers have investigated

the effect of bisphosphonates on the healing of extraction sites.

The healing process in the tooth extraction socket differs from that in the osseointegration of the implant (Kojima *et al*, 2008). Therefore, the effect of bisphosphonate on healing around implants may differ from bisphosphonate's effect on the healing of extraction sockets. The risk for BRONJ following implant therapy in patients who have received bisphosphonates is unknown and controversial. Madrid and Sanz reported that implant therapy is contraindicated in patients who received bisphosphonates intravenously; however, it is not contraindicated in patients who took bisphosphonates orally (Madrid and Sanz, 2009). Grant *et al* (2008) also reported no evidence of BRONJ in 115 patients who received oral bisphosphonate therapy, and implant survival rate was similar to the patients who had not received bisphosphonates. However, symptoms of BRONJ around implants in patients who had been taking oral bisphosphonates have been reported (Martin *et al*, 2010; Park *et al*, 2010). According to animal studies on the effect of bisphosphonates on dental implants (Chacon *et al*, 2006; Viera-Negron *et al*, 2008), bisphosphonates did not affect dental implants negatively. Most animal studies, however, did not use the jaw bone (Chacon *et al*, 2006) and the effect of bisphosphonate on bone healing needs to be investigated in jaw bones because the development and physiology of jaw bone differ from those of other bones such as tibia or femur. (Futami *et al*, 2000). The overall rate of turnover of alveolar bone is 10 times greater than that of the long bones (Dixon *et al*, 1997). Furthermore, Ruggiero and Drew have reported the elective involvement of bisphosphonates in the jaw bones compared to other bone sites because bisphosphonates are preferentially deposited in bones with high turnover rates such as the jaws (Ruggiero and Drew, 2007). The purpose of this study was to evaluate the effect of alendronates on the healing of the extraction socket and healing around implants in the maxilla of rats.

Materials and methods

Experimental animals and procedures

Twenty-four male Sprague–Dawley rats (body weight, 130–140 g; age, 4 weeks) were divided into the bisphosphonate and control groups. Animals were given free access to food pellets and tap water and housed at the animal experimental laboratory at Yonsei University, College of Dentistry, Seoul, Korea. All experiments were performed in accordance with the guidelines for animal experiments of Yonsei University, College of Dentistry. Alendronate sodium salt (Merial Inc., Parramatta, NSW, Australia) was injected subcutaneously at a dose of 5.0 mg kg^{-1}, three times a week for 4 weeks before surgical intervention until the time animals were killed (Viera-Negron *et al*, 2008) in the bisphosphonate group, and the same amount of saline was injected subcutaneously following the same protocol in the control group.

Surgical interventions were conducted under general anesthesia by intramuscular injection of an anesthetic cocktail composed of Rompun (xylazine, 20 mg ml^{-1}, 0.5 ml kg^{-1} body mass; Bayer, Leverkusen, Germany) and Zoletil (tiletamine and zolazepam, 100 mg ml^{-1} 0.5 ml kg^{-1} body mass; Virbac, Carros, France) (Del Signore *et al*, 2006; Pereira *et al*, 2007). Then, maxillary first molars were extracted from both sides. In the extraction sockets of the mesial roots (the largest root) of the maxillary right first molars, osteotomy was performed with a 1.3-mm diameter bur. Then, the customized mini-implants (Ø1.5 × 2.0 mm) with machined surface made of grade IV titanium were inserted into the osteotomy sites until the top of the mini-implant reached the peripheral bone by pressing. Rats were killed at 3, 7, 14, or 28 days after surgical intervention. They were deeply anesthetized as described above, then perfused transcardially with 4% paraformaldehyde (Figure 1) (Futami *et al*, 2000).

Histologic analyses

All specimens were decalcified with 10% EDTA at 4°C for 2 months. The decalcified samples were first embedded in paraffin wax using a standard protocol, then a series of 7-μm sections were prepared. The specimens were stained with hematoxylin–eosin (H&E), Trichrome (Masson) using a kit (Sigma, St. Louis, MO, USA), and TRAP (tartrate-resistant acid phosphatase) stain using an acid phosphatase leukocyte kit (Sigma). Immunohistochemistry was conducted with collagen type I (Col I) (ab6308; Abcam, Cambridge, UK) which was osteoblastic marker. The samples were observed with a light microscope (Leica DM 2500; Leica Microsystems, Wetzlar, Germany) after the various staining treatments. The bone area was measured in the extraction socket of the mesial root using Image Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD, USA). The socket wall could be clearly identified by regular contours where there was no new bone and by reversal lines (Iizuka *et al*, 1992). The new bone area was confirmed by Trichrome staining. TRAP-positive cells and empty lacunae were counted in four regions of interest (Figure 2).

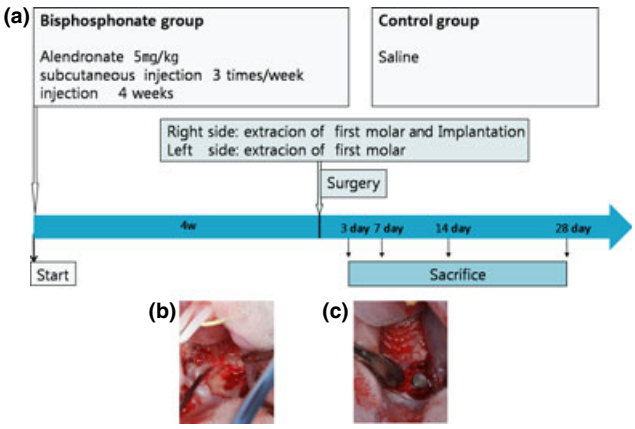

Figure 1 Experimental design. (**a**) Experimental design. (**b**) Extraction sockets of the right maxillary first molar of the rat. (**c**) Implant placed in the socket

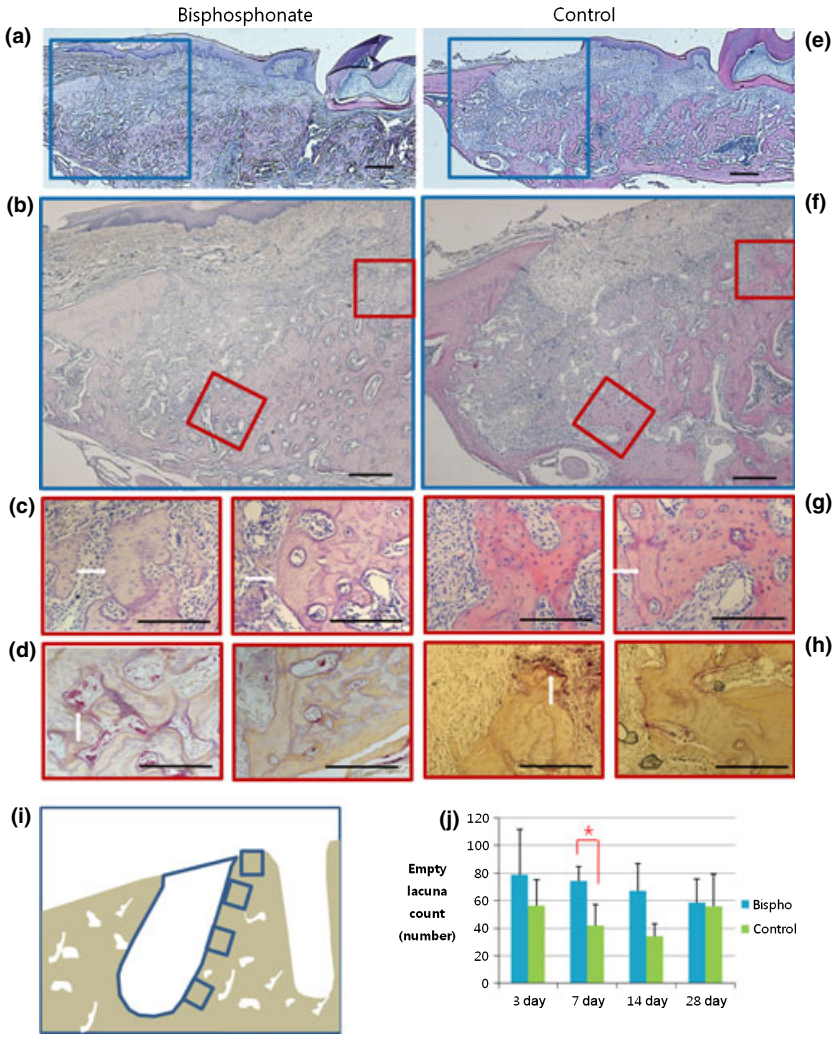

Figure 2 Histologic images of extraction sockets at 7 days after extraction. (**a**–**d**) Bisphosphonate group. (**e**–**h**) Control group. (**a**, **e**) H&E-stained images of extraction sockets of right maxillary first molar of the rat at lower magnification (×12.5). Blue boxes indicate the extraction sockets of mesial roots. Scale bar = 500 μm. (**b**, **f**) H&E-stained images of the blue boxes in A&E (×50). Scale bar = 200 μm. (**c**, **g**) H&E-stained images of the red boxes in B and F (×100). Scale bar = 200 μm. White arrows indicate empty lacunae. (**d**, **h**) Tartrate-resistant acid phosphatase (TRAP)-stained images of the red boxes in B and F (×100). Scale bar = 200 μm. White arrows indicate TRAP-positive cells. (**i**) The selected areas for measurement in the extraction socket. Each blue square, 300 × 300 μm. (**j**) Empty lacunae count in extraction sockets. *Indicates significant difference between bisphosphonate and control groups ($P < 0.05$)

In the implant site, bone implant contact was measured at the distal surface of the implant. TRAP-positive cells and empty lacunae were counted within the blue box indicated in Figure 3 in the distal side of the implant space. Bone area was measured in the same box (Figure 3).

Statistical analyses

Mean values and standard deviations were calculated. The mean differences were verified with repeated measures analysis of variance and independent t tests with a significance level of 5% ($P < 0.05$). All calculations were performed using SPSS ver. 18.0 (IBM, Somers, NY, USA).

Results

Among the total 24 implants, one implant was lost in the control group, while all other implants were well fixed and healed. The healing of the extraction sockets appeared normal without any complications.

Histologic findings in extraction sockets

We identified no significant difference between the bisphosphonate and control groups in new bone formation in the extraction socket. Bony projections were found from the inferior extraction socket in both groups at 3 days after extraction (Figure 4). The osteoblast marker Col I was expressed in the extraction socket in both groups at 7 days after extraction (Figure 4), indicating that the function of the osteoblasts was not delayed in bisphosphonate groups. The number of TRAP-positive cells did not differ between the bisphosphonate and control groups. Well-stained osteoclasts were observed in the bisphosphonate group at 28 days after extraction (Figure 4); however, empty lacunae were observed significantly more in the bisphosphonate group compared to control group at 7 days after extraction (Figure 2). Thus, the number and shape of osteoclasts in the bisphosphonate group did not differ from the control group, but the function of osteoclasts was decreased in the bisphosphonate group (Table 1).

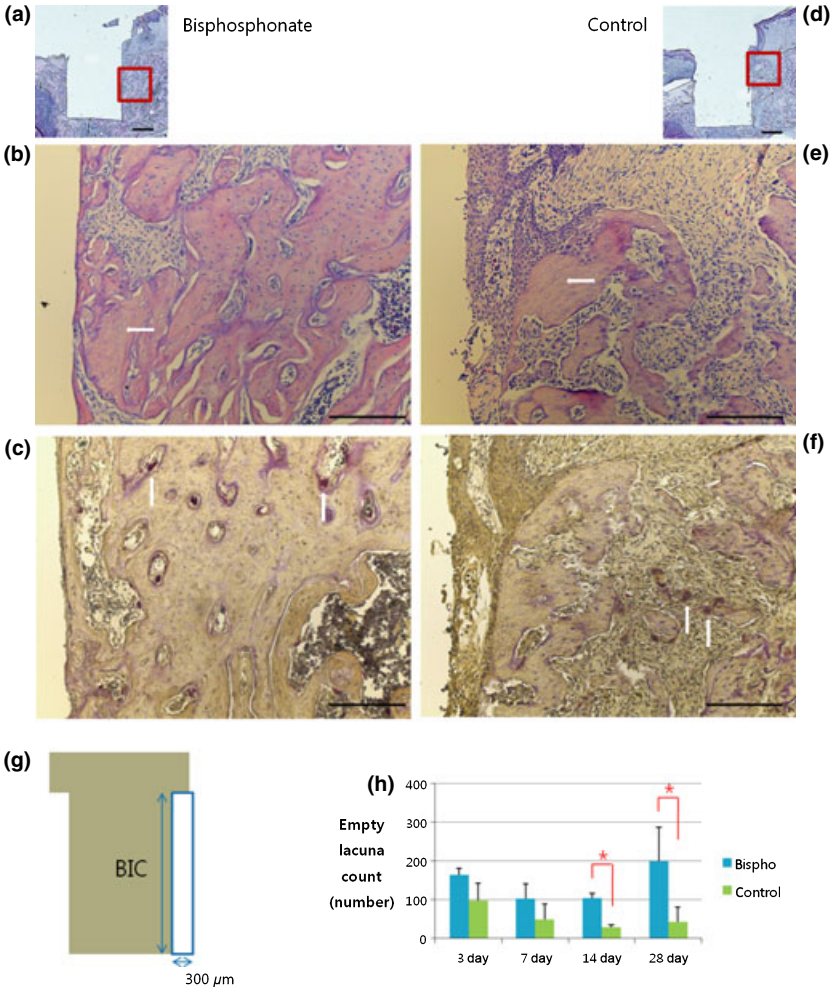

Figure 3 Histologic images of implant sites at 28 days after implantation. (**a**–**c**) Bisphosphonate group. (**d**–**f**) Control group. (**a**, **d**) H&E-stained images at lower magnification (×12.5). Scale bar = 500 μm. (**b**, **e**) H&E-stained images of the red boxes in **a** and **d** (×100). Scale bar = 200 μm. White arrows indicate empty lacunae. (**c**, **f**) Tartrate-resistant acid phosphatase (TRAP)-stained images of the red boxes in A and D (×100). Scale bar = 200 - μm. White arrows indicate TRAP-positive cells. (**g**) The selected areas for measurement in the implant site. BIC (bone implant contact) was measured at the distal side of the implant surface, and the bone area was measured in the blue square. The empty lacunae and TRAP-positive cell count were also measured in the same square. (**h**) The numbers of empty lacunae in implant sites. *Indicates significant difference between bisphosphonate and control groups ($P < 0.05$)

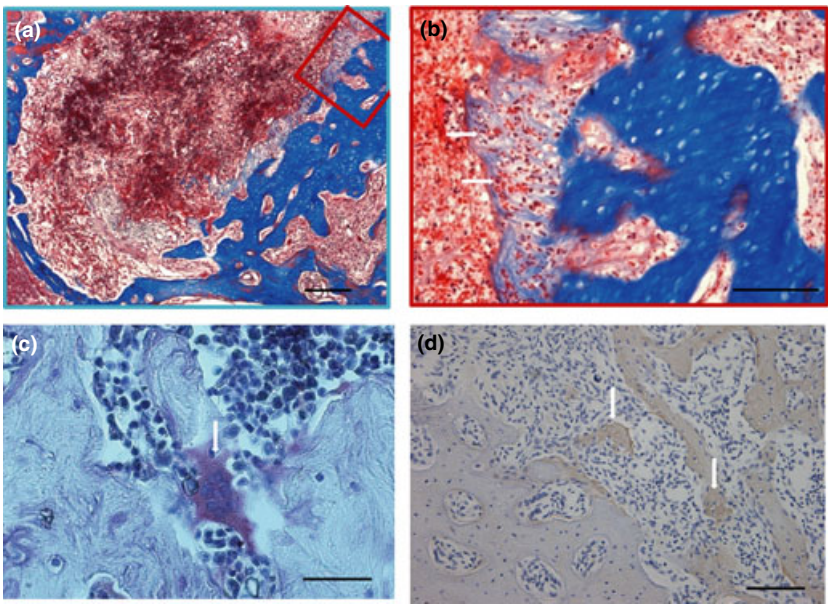

Figure 4 Histologic findings in extraction sockets. (**a**) Trichrome-stained image of bisphosphonate group at 3 days after extraction (×50). Scale bar = 200 μm. (**b**) Trichrome-stained image of the red box in A (×200). White arrows indicate the bone matrix. Scale bar = 100 μm. (**c**) Tartrate-resistant acid phosphatase (TRAP)-stained image of bisphosphonate group at 28 days after extraction (×400). White arrow indicates the TRAP-positive cells, which have several nuclei. Scale bar = 50 μm. (**d**) Col I–stained image of the bisphosphonate group at 7 days after extraction (×200). White arrows indicate brown staining of collagen type I. Scale bar = 100 μm

Histologic findings in implant sites

Bone–implant contact did not significantly differ between the control and bisphosphonate groups. The bone area was observed to be significantly larger in the bisphosphonate group at 14 days after implantation, however. The TRAP-positive cell counts did not significantly differ

Table 1 Histometric results in extraction sockets (mean ± s.d.)

	Group	*3 day*	*7 day*	*14 day*	*28 day*
Bone area (%)	Bisphosphonate	2.40 ± 0.93	14.73 ± 6.23	50.27 ± 5.09	61.33 ± 2.67
	Control	5.35 ± 1.28	14.91 ± 3.85	50.55 ± 8.61	64.07 ± 1.64
Trap-positive cell (number)	Bisphosphonate	17.67 ± 1.53	30.33 ± 15.37	24.00 ± 7.21	35.33 ± 13.80
	Control	22.00 ± 8.66	29.33 ± 8.74	24.00 ± 7.55	17.67 ± 9.24
Empty lacuna (number)	Bisphosphonate	79.00 ± 32.51	74.33 ± 10.50*	67.00 ± 20.07	58.67 ± 17.01
	Control	56.33 ± 18.93	41.67 ± 15.50*	34.00 ± 9.07	55.67 ± 23.67

Trap, tartrate-resistant acid phosphatase.
*Means significant differences ($P < 0.05$) (Number of samples for each group; $n = 3$).

Table 2 Histometric results in implant sites (mean ± s.d.)

	Group	*3 day*	*7 day*	*14 day*	*28 day*
Bone area (%)	Bisphosphonate	39.31 ± 8.23	44.26 ± 8.77	56.57 ± 8.41*	46.85 ± 16.54
	Control	37.39 ± 1.39	18.39 ± 19.26	31.16 ± 4.53*	33.38 ± 19.33
Trap-positive cell (number)	Bisphosphonate	23.67 ± 5.13	37.00 ± 9.17	19.33 ± 3.79	11.00 ± 7.55
	Control	22.00 ± 0	26.00 ± 8.89	17.00 ± 4.24	12.00 ± 2.00
Bone implant contact (%)	Bisphosphonate	10.15 ± 4.80	9.55 ± 2.31	27.77 ± 9.12	55.22 ± 15.24
	Control	17.01 ± 5.45	9.23 ± 4.37	29.15 ± 22.26	41.20 ± 20.50
Empty lacuna (number)	Bisphosphonate	163.00 ± 18.08	102.33 ± 38.37	103.67 ± 12.22*	199.33 ± 87.36*
	Control	97.33 ± 45.08	49.00 ± 39.03	28.50 ± 6.36*	42.67 ± 37.75*

Trap, tartrate-resistant acid phosphatase.
*Mean significant differences ($P < 0.05$) (Number of samples for each group; $n = 3$).

between the control and bisphosphonate groups, but empty lacunae were observed more in the bisphosphonate group than in controls at 14 days after implantation and 28 days after implantation (Figure 3) (Table 2).

Discussion

Experimental animal models for investigating BRONJ have been recently reported, but most of them have been focused on the effect of bisphosphonates on the healing of extraction sockets (Hikita *et al*, 2009; Aguirre *et al*, 2010; Bi *et al*, 2010; Kikuiri *et al*, 2010). Recently, many case reports have reported the late failure of implantation in relation to bisphosphonates (Lazarovici *et al*, 2010). There were a few studies concerning the effect of bisphosphonates on implants but the mechanism has not been thoroughly investigated. In our study, we used normal male rat maxilla model to evaluate the pure effects of alendronate on the early healing of extracted sockets. We wanted to evaluate the effect of bisphosphonate *per se*. Among the previous animal studies, such as Hikita *et al* (2009) used male rat. In addition, we focused on osseointegration of implants and compared the difference of healing pattern between extracted sockets and implantation in this animal model. The effect of bisphosphonates can vary according to the type and method of administration and the concentration (Drake *et al*, 2008). We used alendronate in this experiment because implants are placed mainly in patients who receive oral bisphosphonates therapy rather than intravenous bisphosphonates therapy. Previous animal experiments with alendronate have used various concentrations and administration methods (Viera-Negron *et al*, 2008; Hikita *et al*, 2009; Aguirre *et al*, 2010) (e.g., 5 mg kg^{-1}, 1 mg kg^{-1}, 15 μg kg^{-1}, and 150 μg kg^{-1}). In our study, we followed the method of Viera-Negron *et al* (2008), who used subcutaneous injection with the highest concentration among all the previous studies. Determination of the appropriate concentration for experimental models may be a potential research topic. To select the method of bisphosphonate administration, we reviewed previous animal studies. Although alendronate is commonly administered orally in human, it is very challenging to feed rats with constant concentration of alendronate orally. Therefore, we injected alendronate subcutaneously. Most animal studies used subcutaneous (Viera-Negron *et al*, 2008, Aguirre *et al*, 2010) or intraperitoneal (Bi *et al*, 2010) injection methods. We wanted to know the effect of bisphosphonate *per se*. Therefore, we used male rat without ovariectomy. Previous animal study by Hikita *et al* (2009) also used male rat.

In our results, bisphosphonate did not affect the function of osteoblasts. Similar to the findings of Iizuka *et al* (1992), in our study, bone projections were found from the inferior extraction socket in both groups at 3 days after extraction. The bone area around the implants was observed significantly more at 14 days after implantation in the bisphosphonate group, similarly to results of Miettinen *et al* (2009). Previous reports noted that bisphosphonate prevented apoptosis of osteoblasts and enhanced osteoblast function (Bellido and Plotkin, 2010); however, the specific mechanisms remained unclear. In contrast, Hikita *et al* reported that bisphosphonates delayed the initial bone formation in the extraction socket. They suggested that bisphosphonate diminished the number of TRAP-positive cells and that inhibition of osteoclast formation just after extraction is related to delayed initial healing. Hughes *et al*

(1995) also reported that bisphosphonates promoted apoptosis of murine osteoclasts; however, in our study, the number of TRAP-positive cells did not differ between bisphosphonate and control groups. Weinstein *et al* (2009) and Bi *et al* (2010) recently reported that the long-term administration of bisphosphonate increased the number of normal shaped osteoclasts. These studies suggested that osteoclast function was reduced although the number was increased.

According to previous studies, empty lacunae owing to trauma can appear in the extraction socket or around the implant and are reduced as healing progresses (Shimizu *et al*, 1998; Futami *et al*, 2000; Chen *et al*, 2010). In the septum adjacent to the extraction socket and the nearby region in our study, a lacuna lacking osteocytes (an empty lacuna) formed at the early stage of extraction and then gradually disappeared during the healing process. This progression indicates that damage and necrosis arose in the surrounding bones after extraction and then resolved through the normal healing process (Bi *et al*, 2010). In our study, such empty lacunae were found both in the experimental group and the control group, but significant differences were observed between the two groups at some time points. These differences may be understood as the result of suppressed bone remodeling by bisphosphonate and are similar to the results of other studies showing increased dead bone formation following bisphosphonate administration (Bi *et al*, 2010; Kikuiri *et al*, 2010). Burr and Allen (2009) reported that it is not clear whether osteocyte death occurs because of the direct toxic effects of bisphosphonates on osteocytes or because of suppressed remodeling. There is no direct evidence of the *in vivo* effects of bisphosphonates on osteocytes. Therefore, the empty lacunae could be a result of suppressed bone remodeling which cause failure to renew areas that naturally undergo cell death (Allen and Burr, 2009).

Empty lacunae in the extraction socket were observed significantly more in the bisphosphonate group at 7 days after extraction, while they were observed significantly more in the bisphosphonate group at 14 and 28 days after implantation. Thus, the effect of suppressed resorption arises in the early stage in the extraction socket but occurs later in the implants. In our results, the effect of bisphosphonate on extraction socket was transient, but the effect of bisphosphonate on healing around implant lasted for a long period of time. There may be differences in the healing patterns between the extraction and implantation (Kojima *et al*, 2008), because of the difference in the defect needed for bone formation and the presence of the implant. The bone stock may have been greater in the bisphosphonate group because of the suppressed resorption, and it could be helpful for the initial stability of implant (Miettinen *et al*, 2009). A previous study reported that remodeling was reduced by bisphosphonate administration and that microcracks were increased in bones by the accumulation of older bones (Brennan *et al*, 2010). Because microcracks form in regions of attenuation of the lacunae–canalicular network, there is the risk of increased microcracks if the empty lacunae remain for a long period of time (Brennan *et al*, 2010). Therefore, remaining of empty lacunae without resolving around implant may be related to the late failure of implant which was recently reported by many researchers (Lazarovici *et al*, 2010; Martin *et al*, 2010).

In conclusion, although the small number of samples is a limitation of this study, we found that our animal experiment model was useful for evaluating the effect of bisphosphonate on healing around implants. Alendronate administration decreased osteoclasts function, but did not decrease osteoblasts function. Empty lacunae by decreased osteoclasts function were more observed at a later time of healing in implants compared to the healing of extraction sockets. Thus, long-term follow-up may be needed to evaluate the effect of alendronate on the osseointegration of implants in further studies.

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Author contributions

J.H. Kim: research design, drafting the paper main author. Y.B. Park: research design. Z. Li: interpretation of data. M.K Chung: research design. H.S. Jung: interpretation of data. H.S. Moon: revising paper. J.S. Shim: revising paper.

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