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The effect of two locally administered anti-resorptive agents on bone regeneration in a rat fibula model: Alendronate and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2

Jung Woo Nam [a], Jin Il Kwon [b], Joo Young Hong [b], Hyung Jun Kim [b, *]

[a] Department of Oral and Maxillofacial Surgery, Wonkwang University Sanbon Hospital, 321, Sanbon-ro, Gunpo-si, Gyeonggi-do 15865, Republic of Korea
[b] Department of Oral and Maxillofacial Surgery, College of Dentistry, Yonsei University, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea

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ABSTRACT

Bisphosphonates are well-known drugs as inhibitors of bone resorption acting on inducing programmed cell death of osteoclasts. However, many *in vitro* studies report that optimal concentration of the bisphosphonate affects not only osteoclasts but also osteoblasts, that is, it induces the anabolic effects of osteoblasts. Recently reported 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is an endogenous ligand of peroxisome proliferator-activated receptor-gamma, with an inhibitory activity on bone loss. Researchers have also suggested that 15d-PGJ_2 has the ability to reduce bone destruction and as the possibility of regeneration of bone.

The purpose of this study is to demonstrate the anabolic effect of two anti-resorptive materials, alendronate and 15d-PGJ_2, in a critical sized segmental defect model of rat fibula. The regenerated bone on the operative site was assessed through gross, radiographic (plain X-ray, and micro-computed tomography), histomorphologic evaluation, and statistical analysis. Consequently, the locally applied alendronate prevented resorption of grafted materials, and had a positive effect on bone regeneration with positive micro-architectural modification of the surrounding bone, although this study did not verify a significant capacity of bone regeneration of 15d-PGJ_2 and instead only shed a light on its possibility.

1. Introduction

For the last several decades, as the demand for bone graft has increased for reconstruction of various bone defects in the dental and orthopedic fields, many efforts have been made continuously to develop new bone graft materials, such as bone morphogenetic proteins (BMPs), substituted for conventional bone graft materials such as autogenous bone, allogeneic bone, and xenogeneic bone, all of which have their own inherent limitations. Under these circumstances, many researchers have attempted to discover some materials with a focus on aspects different from their own native properties and uses, and bisphosphonates (BPs) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) are among them. Both materials are known as anti-resorptive agents and are used for osteoporosis and bone metastasis, but the possibility of an anabolic effect has been reported recently as well (Im et al., 2004; von Knoch et al., 2005; Kim et al., 2015). BPs are well known for acting mainly on inducing programmed cell death of osteoclasts and inhibiting bone resorption and reducing bone remodeling, thereby, causing positive bone balance (Sato et al., 1991; Murakami et al., 1995; Luckman et al., 1998; Fisher et al., 1999). However, several studies *in vitro* and *in vivo* showed that BPs affected not only osteoclasts but also osteoblasts, causing an anabolic effect (Im et al., 2004; von Knoch et al., 2005). 15d-PGJ_2, one of the terminal products of the cyclooxygenase-mediated arachidonic acid pathway, is an endogenous ligand of peroxisome proliferator-activated receptor-gamma (PPARγ) which has anti-inflammatory and anti-tumor activity at micro-molar concentrations (Diez-Dacal and Perez-Sala, 2010; Kim et al., 2015). In 2012, Kim et al. reported that 15d-PGJ_2 prevented and treated the destruction of bone associated with breast cancer bone metastasis, and it also suppressed estrogen deficiency-induced bone loss (Kim et al., 2015). Therefore, in view of these

* Corresponding author. Fax: +82 2 2227 7825.
E-mail address: kimoms@yuhs.ac (H.J. Kim).

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aspects of the two anti-resorptive agents, the researchers planned two animal experiments with both materials in the rat fibula models. The rat fibula, which has a critical sized segmental defect (CSD), is one of the first choices of animal models for experimentation to evaluate the bone regeneration capacity. CSD refers to a minimum size of bone defect that does not heal spontaneously (Hollinger and Kleinschmidt, 1990). The aim of this study is to demonstrate the anabolic effect of two anti-resorptive materials, BPs (Alendronate, ALN) and 15d-PGJ_2, in the CSD model of rat fibula.

2. Materials and methods

2.1. Study design

Two experiments, Experiment 1 (alendronate with biphasic calcium phosphate, ALN-BCP group) and Experiment 2 (15d-PGJ_2 with absorbable collagen sponge, 15d-PGJ_2-ACS group), were designed using CSD models of fibulae in 16 rats, respectively. The result of both experiments was assessed through gross, radiographic, histomorphologic, and statistical analysis at the fourth and eighth weeks after operation (Fig. 1).

2.2. Animals

In Experiments 1 and 2, sixteen and seventeen 8-week-old male Sprague–Dawley (SD) rats weighing 250–300 g were used, respectively. Basically, 16 rats were planned for the 15d-PGJ_2-ACS group, but two more rats were added to the experiment since a rat died after operation. The rats were maintained in plastic cages in a 12-h day/12-h night cycle room at a temperature of 21 °C. They were provided with water and standard feed pellets *ad libitum*. The animal selection, management, surgical protocols and preparations were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of the Department of Laboratory Animal Medicine, Medical Research Center, Yonsei University College of Medicine (approval nos. 2010-0381 and 2013-0051).

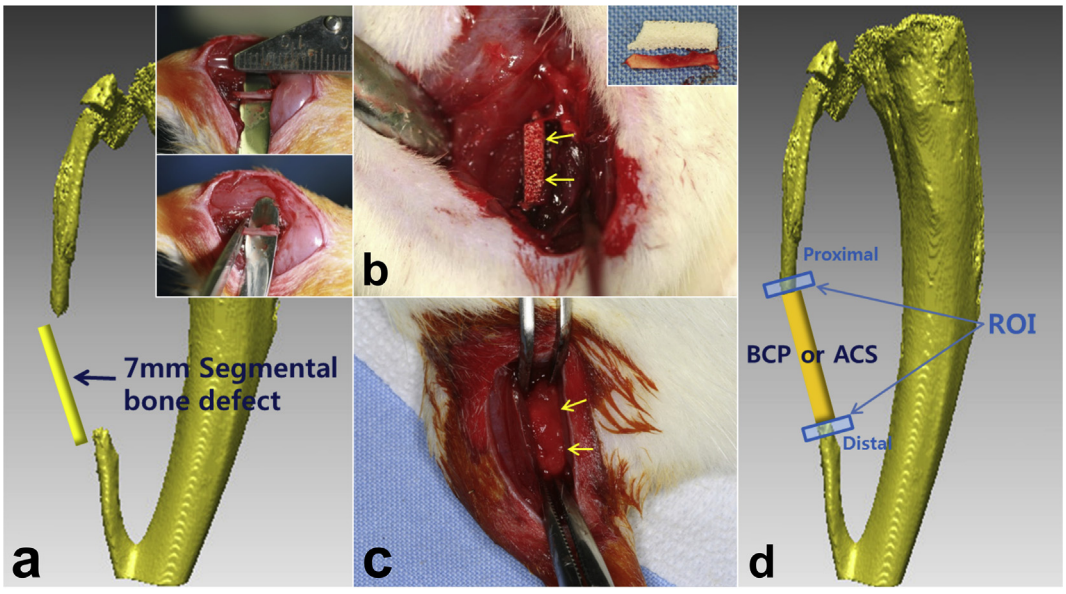
2.3. Agents and biomaterials

In Experiment 1, BP solution (ALN, Fosamax; MSD, Malmo, Sweden) and biphasic calcium phosphate (BCP) block (Osspol; HA 60%, β-TCP 40%, Dongsung, Seoul, South Korea) as a drug carrier were used. The BP solution was produced by dissolving ALN in normal saline solution under sonication and removing the impurities by 0.2-μm millipore filter. The BCP blocks were prefabricated in 2 × 2 × 8 mm size. In Experiment 2, 15d-PGJ_2 (Cayman Chemicals, Ann Arbor, MI, USA) and absorbable collagen sponge (ACS, CollaHeal, Bioland, Korea) as a drug carrier were used.

2.4. Surgical procedure and experimental design

In both experiments, the rats were sedated by intramuscular injection with Zoletil (Virbac, Carros, France, 30 mg/kg) and Rumpun (Bayer Korea, Korea, 10 mg/kg) on their rumps. Preparation for surgery such as shaving, scrubbing with 10% povidone–iodine solution, and local anesthesia with lidocaine including 1:100,000 epinephrine was carried out. After skin incisions, the fibula bones were exposed, and the complete bony defects of 7 mm (Hollinger and Kleinschmidt, 1990), more than a critical size, were formed in bilateral fibula bones of all the rats (Fig. 1a).

Then, in the ALN-BCP group, for the experimental group, the BCP block, dipped in 2.0 mg/ml ALN for 1 h, was inserted into the right fibula defect, and for the control group, the BCP block, dipped in



	Agents	Animal Model		Group	Number (sites)	f/u period (weeks)
Experiment 1	ALN (2.0mg/ml)	Rat fibula	[1]Exp.	ALN + BCP block	16	4, 8
			[2]Con.	NS + BCP block	16	
Experiment 2	15d-PGJ_2 (200μg)		Exp.	15d-PGJ_2 + ACS	17	
			Con.	NS + ACS	17	

[1]*Exp. ; Experimental group ; Right side of rat fibulae* [2]*Con. ; Control group ; Left side of rat fibulae*

Fig. 1. Rat fibula experimental model. (a) A segmental bone defect of 7 mm was formed in a rat fibula model. (b) Experiment 1: A BCP block (with/without ALN) was positioned in the defect. (c) Experiment 2: ACS (with/without 15d-PGJ_2) was positioned in the defect. (d) ROI was defined on both ends of the grafted materials.

normal saline for an hour, was inserted into the left one (Fig. 1b). In 15d-PGJ_2-ACS group, for the experimental group, the ACS, dipped in 200 μg (1 mg/500 μl, 100 μl) of 15d-PGJ_2, was inserted on the right fibula defect, and for the control group, the ACS, dipped in 100 μl normal saline solution, was inserted on the left one (Fig. 1c).

2.5. Evaluation methods and histology procedure

In the ALN-BCP group, eight rats were sacrificed in a CO_2 asphyxiation chamber at the fourth and eighth weeks after the operation, respectively. In the 15d-PGJ_2-ACS group, nine rats were sacrificed at the fourth week and eight rats were sacrificed at the eighth week.

The regenerated bone on the operative site was assessed through gross, radiographic (plain X-ray, micro-computed tomography [micro-CT] imaging), histomorphologic evaluation, and statistical analysis. The intact legs of rats including fibula, tibia bone and soft tissue underwent a plain X-ray as follows: voltage (kV) = 60, current (mA) = 70, exposure (s) = 0.08 and micro-CT (Microtomography, SkyScan 1076, Belgium; resolution = 18 μm, voltage (kV) = 100, current (μA) = 100, filter = 0.5 mm aluminum, exposure (ms) = 1180, rotation step (degrees) = 0.500/360 rotation) in both experiments. Three-dimensional (3D) reconstructed images of regenerated bone were acquired through the NRecon (Reconstruction software, SkyScan). Statistical analysis of the measured microstructural trabecular bone parameters, such as bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), trabecular number (Tb.N, mm^{-1}), and structure model index (SMI) (15d-PGJ_2-ACS group only), of region of interest (ROI) on micro-CT was conducted with a two-sample t-test using IBM SPSS 19.0 software (IBM Corp., Armonk, NY, USA). The ROI was defined as the region of 18 μm from both cutting ends (Fig. 1d).

The experimental sites (right fibulae) were fixed at 4 °C in 4% paraformaldehyde/0.01 M phosphate-buffered saline solution (PBS, pH 7.4) for 24 h. They were decalcified using formic acid–sodium formate at room temperature and then embedded in paraffin. Serial, 7-μm-thick sections were cut and then stained using hematoxylin and eosin (H–E) stain and Masson's trichrome stain (ALN-BCP group only), and examined by optical and polarized microscopy.

3. Results

3.1. Anabolic effects and bone regeneration

3.1.1. Experiment 1 (ALN-BCP group)

Gross and radiographic findings: The control groups showed no bony union between BCP block and fibula bone at the fourth and eighth weeks. Grafted BCP blocks completely disappeared in five rats, and only three BCP blocks remained attaching to fibula bone with fibrous unions in the 4-week control group (Fig. 2a). Unlike the 4-week group, most of the 8-week group (seven rats) had the enlarged defect (more than 7 mm) as they absorbed the end of remaining fibula with loss of BCP block except for one rat, which showed a fibrous union between BCP and fibula (Fig. 2c). On the other hand, most of the experimental group showed bony unions between BCP block and fibula bone with no loss of BCP blocks at the fourth and eighth weeks (Fig. 2b and d). Some rats that had BCP blocks away from their original position showed ectopic bone formation from the fibula end to BCP block (Fig. 2e). There was little difference between the 4- and 8-week experimental groups in regard to bone regeneration.

Micro-CT findings: The longitudinal section of micro-CT images showed the majority of non-unions and some fibrous unions on fibula defect sites in the 4- and 8-week control groups. Bone density of the cutting ends of fibula was similar to that of cortical bone, and loss of bone marrow opening was observed through bone mineral density color window mode in all rats of the 4-week control group. The results of the 8-week control group were not different from those of the 4-week group, but most of the defects were larger than those of the 4-week group due to the absorption of the ends of fibulae bone (Fig. 3a and b). In contrast, most of rats in the 4- and 8-week experimental groups showed various amounts of newly formed bone around the grafted materials. The ectopic bone formation between the fibula end and BCP block was also observed in some rats that had BCP blocks away from their original position. Unlike the control groups, bone marrow opening and bone regeneration with newly formed bone with a similar degree of mineralization to medullary bone were observed in all experimental groups. The 8-week experimental group is deemed to have undergone more bone remodeling to look like the intact fibula than the 4-week group (Fig. 3c and d).

Statistically, all four measured parameters, BV/TV, Tb.Th, Tb.N, and Tb.Sp, were significantly different under 95% confidence levels between the control and the experimental groups at the fourth and eighth week except the variable of Tb.Sp of the 4-week group ($p = 0.106$) (Table 1).

Histomorphologic findings: In the 4-week control group, the BCP blocks were surrounded by soft tissue, and there was no evidence of new bone formation. The number of new blood vessels of the control group was much less than that of the experimental group. In contrast, newly formed bone with angiogenesis, osteoblasts, and osteocytes were observed in grafted BCP pore in the 4-week experimental group. The 8-week control group showed results similar to those of the 4-week control group; there was no incorporation between BCP block and recipient fibula bone, but interposition of soft tissue. Scattered type I collagen without new bone formation was observed in bone defects by Masson's trichrome stain (Fig. 4a and b). However, the 8-week experimental group

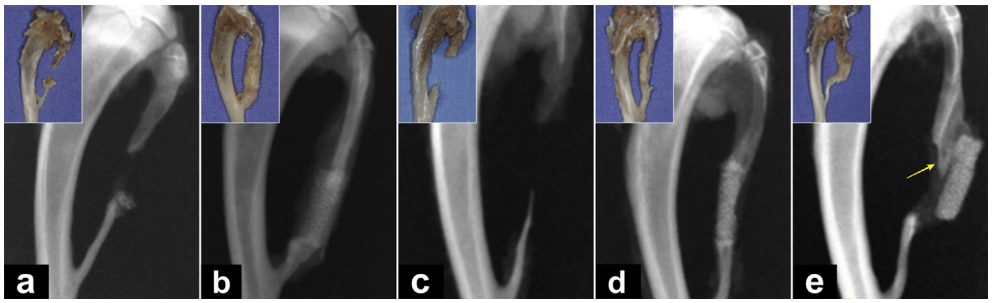


Fig. 2. Gross and radiographic findings in ALN-BCP group. (a) Four-week control group. (b) Four-week experimental group. (c) Eight-week control group. (d, e) Eight-week experimental group (yellow arrow; ectopic bone formation).

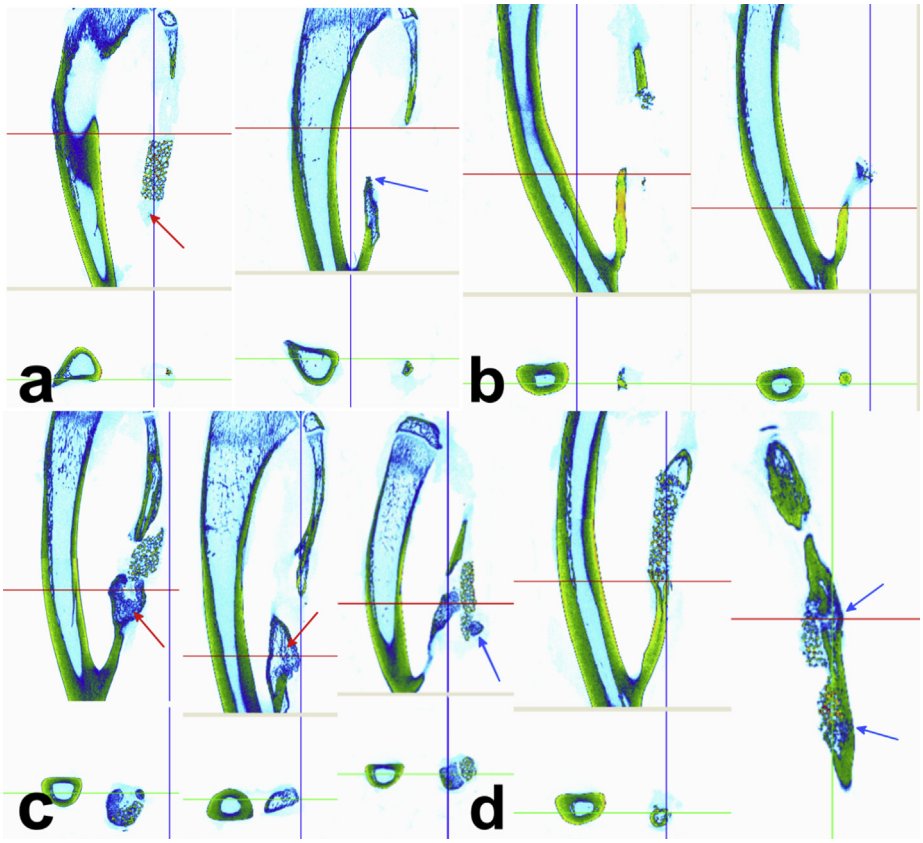


Fig. 3. Micro-CT findings in ALN-BCP group. (a) Four-week control group: Fibrous union around grafted BCP (red arrow) and sharply resorbed cutting surface of fibula (blue arrow) were observed. (b) Eight-week control group: Non-unionized fibula cutting ends were absorbed. (c) Four-week experimental group: Bone marrow opening and micro-architecture modification of the marrow (red arrows), as well as heterotopic bone formation (blue arrow), which was evidence of osteostimulation, were observed. (d) Eight-week experimental group: Opening of bone marrow, enhanced volume of trabecular bone and micro-architectural modification were revealed. Complete bone regeneration was observed in all sections on micro-CT.

Table 1
Statistical analysis of bone morphometric indices of the control and experimental groups in the ALN-BCP group.

	Group	BV/TV (%)	Tb.Th (μm)	Tb.Sp (μm)	Tb.N (/mm)
4 Weeks	Control	4.37(1.43)	179.45(52.91)	542.42(11.49)	0.25(0.04)
	Experimental	8.43(2.69)	240.82(75.57)	530.48(25.83)	0.38(0.13)
	p	0.000*	0.012*	0.106	0.003*
8 Weeks	Control	3.25(3.61)	111.54(102.31)	313.67(285.77)	0.11(0.12)
	Experimental	8.95(2.60)	233.96(44.20)	533.37(16.70)	0.45(0.16)
	p	0.000*	0.000*	0.008*	0.000*

Data are mean with standard deviation in parentheses.
*$p < 0.05$.

showed bony union of bone defects with regenerated bone, which was continuous and had even diameter and inner bone marrow structure through remodeling process. Newly formed bone and ingrowth of it into remaining BCP pores were definitely observed in the fibula bone defects by Masson's trichrome stain (Fig. 4c and d).

3.1.2. Experiment 2 (15d-PGJ$_2$-ACS group)

Gross, radiographic and micro-CT findings: All of the 4- and 8-week control groups showed non-union of fibula defects. Some rats in the 4-week control group showed bony bulging of cutting ends of fibula, but sharpened cutting ends and/or increased defect size due to absorption of fibula cutting ends were observed in the 8-week control group (Fig. 5a and b). In the 4-week experimental group, three rats had evidence of bone regeneration of fibula defects with continuous newly formed bone from one side of fibula cutting ends, but two rats showed increased defects in this experimental group. The result of the 8-week experimental group was not much different from that of the 4-week experiment group. There were also some rats with evidence of newly formed bone that had the shape of cortical bone and marrow cavity in the 8-week experimental group (two rats), but not significant existence of bony union or regeneration compared to the 4-week experimental group (Fig. 5c and d). There was no sign of inflammation and infection on surrounding soft tissue in all groups.

Among the five measured parameters, BV/TV, Tb.Th, Tb.N, Tb.Sp, and SMI, there were statistically significant differences in the variables of BV/TV and Tb.N in the 4-week group and BV/TV, Tb.N, Tb.Sp, and SMI in the 8-week group from those in the control groups with a 95% confidence level (Table 2).

Histomorphologic findings: In the 4-week control group, any new bone formation did not occur around the fibula cutting ends that were surrounded by the fibroblasts and adipocytes (Fig. 6a). The 8-

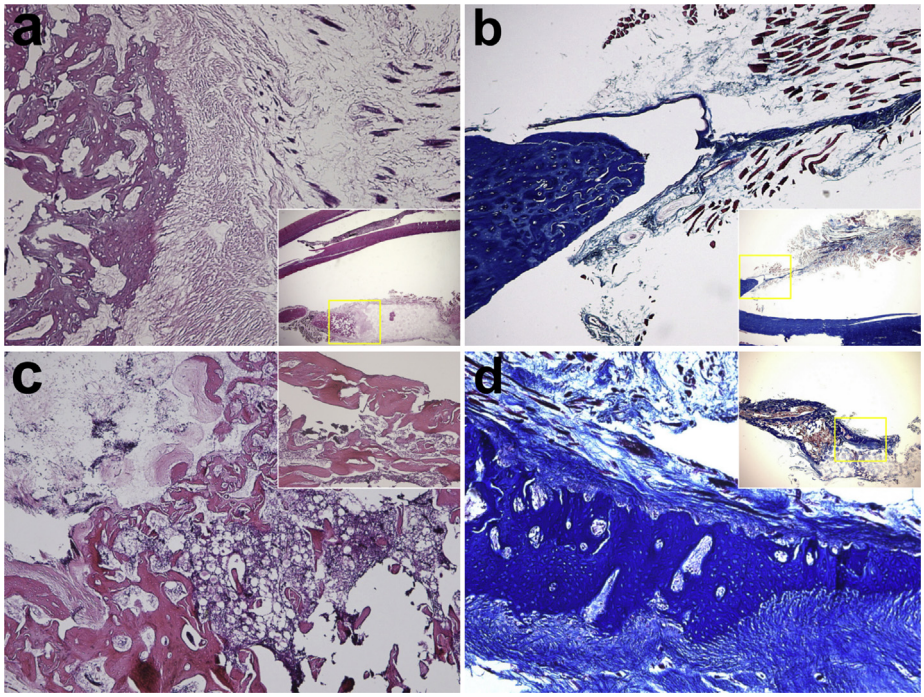


Fig. 4. Histomorphologic findings of the 8-week groups in ALN-BCP group (hematoxylin and eosin and Masson's trichrome stain). (a, b) Control group: Interpositional soft tissue between BCP block and fibula was observed. BCP block was encapsulated with soft tissue and there was no bone ingrowth to micro-pores. (c, d) Experimental group: A continuous connecting cortical bone was noticeable between the remaining fibula and newly formed neo-fibula bone. The inner part of the new bone was gradually replaced by bone marrow. Magnification: a: ×12.5, ×100; b: ×12.5, ×50; c: ×50, ×200; d: ×12.5, ×100.

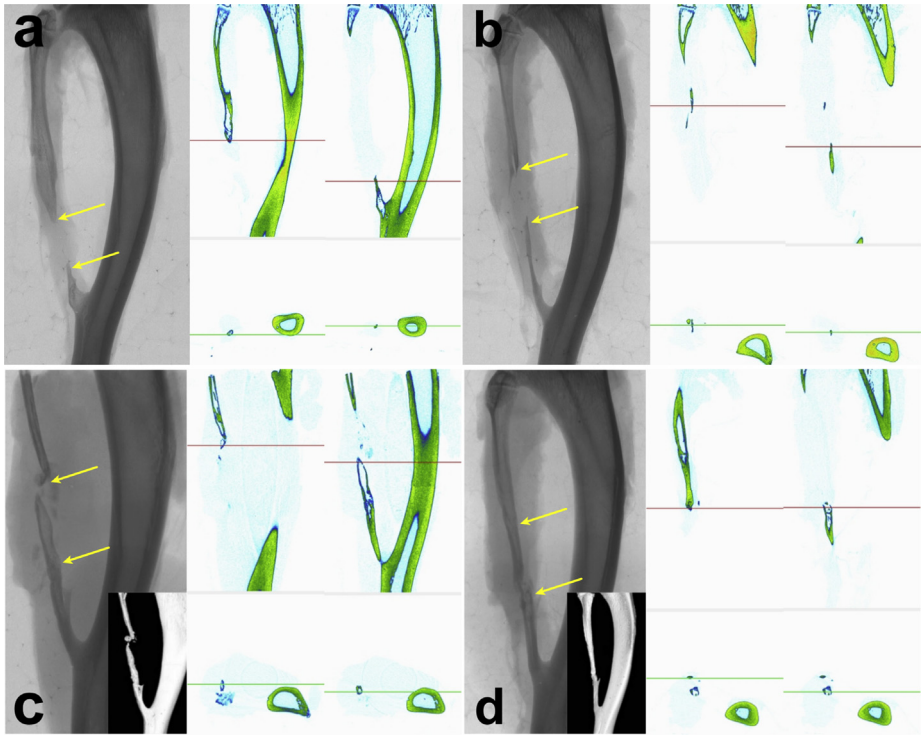


Fig. 5. Micro-CT findings in 15d-PGJ$_2$-ACS group. Yellow arrows indicate what were thought to be the fibula cutting ends. (a, b) The 4- and 8-week control groups: Bone defects remained, and non-unionized fibula cutting ends were absorbed. (c, d) The 4- and 8-week experimental groups: In some rats, newly formed bones, continuous with fibula cutting ends, were observed.

Table 2
Statistical analysis of bone morphometric indices of the control and experimental groups in 15d-PGJ_2-ACS group.

	Group	BV/TV (%)	Tb.Th (μm)	Tb.Sp (μm)	Tb.N (/mm)	SMI
4 Weeks	Control	13.1(7.32)	117.58(12.89)	137.51(3.13)	1.13(0.65)	2.34 (0.32)
	Experimental	24.5(14.76)	122.71(14.78)	134.44(8.13)	1.99(1.17)	2.20 (0.32)
	p	0.007*	0.275	0.144	0.011*	0.206
8 Weeks	Control	9.71(7.87)	124.68(17.2)	140.13(0.81)	0.66(0.44)	2.48 (0.28)
	Experimental	19.39(10.16)	131.69(7.24)	138.73(1.87)	1.4737(0.77)	2.04 (0.37)
	p	0.005*	0.143	0.012*	0.001*	0.001*

Data are mean with standard deviation in parentheses.
*$p < 0.05$.

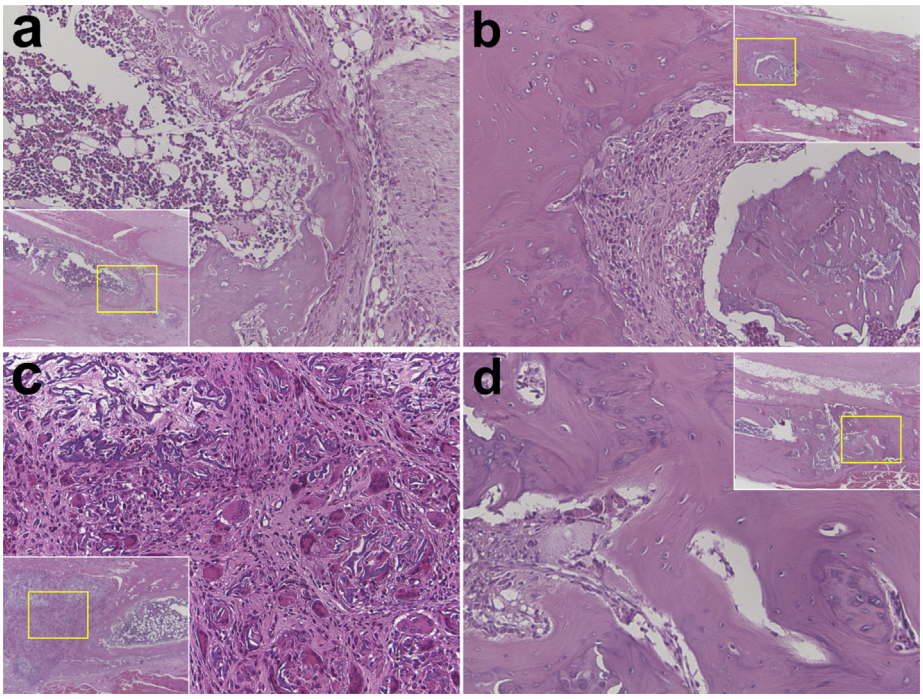


Fig. 6. Histomorphologic findings in 15d-PGJ_2-ACS group (hematoxylin and eosin stain). (a) Four-week control group: The fibula cutting ends were surrounded by fibroblasts and adipocytes. (b) Eight-week control group: Resorption of fibula bone and interpositional soft tissue were observed. (c) Four-week experimental group: An abundance of osteocytes and blood vessels were observed around the fibula cutting ends. (d) Eight-week experimental group: Some rats showed continuously regenerated cortical bone from fibula cutting ends. Magnification ×50 (inner box), ×200.

week control group showed significant resorption of fibula ends and interposition of soft tissue in most respects (Fig. 6b). Some of the 4-week experimental group showed new bone formation with an abundance of osteocytes and blood vessels. Medullary cavity was observed at the new bone area, and an abundance of fibroblasts, vessels, and bone matrix mixture were seen around the fibula cutting ends that failed to achieve bony union (Fig. 6c). Some rats, whose newly formed bone were observed, in the 8-week experimental group showed continuously regenerated cortical bone from the fibula cutting ends with more mature mineralized matrix (Fig. 6d). However, there were also some rats showing no additional bone formation in the experimental groups.

4. Discussion

This study, composed of two experiments, was designed to determine the anabolic effect of the two drugs ALN and 15d-PGJ_2, which are known for preventing bone resorption acting on osteoclasts. The rat fibula was selected as the animal model for evaluating bone regeneration capacity of the bio-materials, and the reasons were as follows: first, the rat was easy to use and it required a simple surgical procedure; second, the rat fibula was known for the fact that its spontaneous healing was very difficult in bone defects more than 6 mm, so the definite effect of osteoinductivity of experimental materials could be estimated in this model; and third, the graft materials could be retained in their original position without any fixation because it is surrounded by muscle cuff and the fibula did not load (Hollinger and Kleinschmidt, 1990; Lazard et al., 2011).

Because of pharmacological mechanism of the BPs that induces apoptosis in osteoclasts (Sato et al., 1991; Murakami et al., 1995; Luckman et al., 1998; van beek et al., 1999; Rodan and Martin, 2000; Reszka and Rodan, 2003), medical doctors have widely used them via systemic administration for the maintenance of positive bone balance of the skeletal system (Bone et al., 2004). However, several complications from systemic administration of the BPs, such as problems with the digestive system (Bone et al., 2000) and osteonecrosis of the jaw (Allen and Burr, 2009), have raised the need for their local application in the clinic. Since the early 2000s, some orthopedists have conducted several experiments to identify the effect of local application of the BPs in animal experimental models, and they found that the local application of before grafting of bone substitutes prevented resorption of the grafted materials, and the degree of its effectiveness depends on

the concentration of ALN (Aspenberg and Astrand, 2002; Agholme and Aspenberg, 2009). These *in vivo* studies were basically focused on the positive bone balance by inhibiting osteoclasts, but recently, the anabolic effect of the BPs, such as proliferating osteoblasts and inducing differentiation of mesenchymal stem cells to osteoblasts, has become gradually established (Im et al., 2004; von Knoch et al., 2005; Wang et al., 2010). Although the direct mechanism of the anabolic effect of the BPs to the osteoblasts is not completely understood (Allen and Burr, 2009), a hypothesis that the mechanism is related to inhibition of the enzyme in the mevalonate pathway during the synthesis of cholesterol in osteoclasts has been proposed (Giuliani et al., 1998; Mathov et al., 2001; Im et al., 2004). Two pieces of theoretical evidence for the hypothesis were suggested. The first one was the inference that the N-containing BPs, similar to the statins that were clinically widely used as lipid-lowering medications, interfered with the synthesis of cholesterol by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co-A) reductase in mevalonate pathways during the synthesis of cholesterol in the liver, and increased expression of the gene of BMP-2, so this accelerated differentiation and induction of osteoblasts (Mundy et al., 1999). The second piece of evidence was based on the research finding that specific concentrations of ALN suppressed the expression of BMP/Wnt antagonist (Follistatin/Dan) in cells and accelerated their differentiation to osteoblasts in an *in vitro* study (Hayashi et al., 2009).

Therefore, in ALN-BCP group, the researchers put forward a hypothesis that ALN directly worked on the osteoblasts, as well as the osteoclasts, and had an anabolic effect. As briefly mentioned above, a concentration-dependent phenomenon of the BPs was observed, in that ALN, at a specific concentration (10^{-8} M), had osteoinductive capacity, but cell proliferation and maturation were inhibited at high concentrations of ALN *in vitro* and *in vivo* (Im et al., 2004; Bobyn et al., 2009; Jakobsen et al., 2010). In this study, the researchers determined 2.0 mg/ml (0.00615 M) as the proper concentration of ALN for local application referring to previous animal studies of other researchers. A drug delivery system should be maintained for a sufficient length of time without disruption in the early stage, and effective localization and slow release of the drugs are possible by chemical bonding of the BPs and calcium phosphates. Therefore, BCP was selected as the drug carrier of this experiment for the local application of the BPs *in vivo* (Ranade, 1991; Yaffe et al., 1997).

The bone metabolism is controlled by the interaction between receptor activator of nuclear factor-κB ligand (RANKL) and RANK (receptor activator of nuclear factor-κB) from osteoclast, causing a hard tissue resorption, and inhibition of RANKL via osteoprotegerin (OPG), also known as osteoclastogenesis inhibitory factor, which is responsible for avoiding excessive hard tissue resorption by binding to RANKL (Lacey et al., 1998; Yasuda et al., 1998; Khosla, 2001). Both responses to inhibition of RANK, a central activator of NF-κB, by coupling OPG to RANKL, and inhibition of RANKL–RANK system due to suppressed NF-κB by 15d-PGJ_2, seem to have similar influences of inhibition of osteoclast activity (Baud'huin et al., 2007). 15d-PGJ_2 is a high-affinity ligand for PPARγ, and the activated PPARγ controlled various physiological reactions and induced bone loss by reducing osteoblast differentiation and activating osteoclast differentiation (Straus et al., 2000; Wan, 2010; Kim et al., 2015). In addition, 15d-PGJ_2 blocks osteoclastogenesis by decreasing tartrate-resistant acid phosphatase (TRAP)-positive cells, which are increased by RANKL, and inhibits the formation of resorption pits by decreasing the activities of cathepsin K and matrix metalloproteinases (MMP), which are secreted by mature osteoclasts (Kim et al., 2015). The researchers put forward a hypothesis for the 15d-PGJ_2-ACS group that 15d-PGJ_2 acts on not only on NF-κB to inhibit bone destruction by suppression of RANK and decreasing differentiation of mature osteoclasts, but also osteoblasts have capacities of differentiation and formation of bone like the BPs.

Two pilot studies were performed first to determine proper concentration of 15d-PGJ_2. Appropriate regulation of its concentration is important, because it is a cytokine that acts directly on nuclear receptor and has various effects. In the first pilot study, three different concentration (0.5 mg/500 μl, 2 mg/500 μl, 5 mg/500 μl) of 15d-PGJ_2 100 μl were applied to 12 sites of 6 rats (4 sites of 2 rats, respectively) by the same method as in this study (Experiment 2), and the rats were sacrificed after 4 weeks. Comparison between different concentration groups was performed without a control group through gross and radiographic findings. In the second pilot study, two different concentration (0.5 mg/500 μl, 2 mg/500 μl) of 15d-PGJ_2 100 μl were applied to 16 sites of 8 rats (8 sites of 4 rats, respectively) by the same method as above without a control group as well. Four weeks later, the rats were sacrificed and the effect of the agent was evaluated through gross and radiographic findings, and 1 mg/500 μl of 15d-PGJ_2 100 μl was finally decided to be used in this study.

4.1. Anabolic effects and bone regeneration

4.1.1. ALN-BCP group

Grafted BCPs of experimental groups were less absorbed and well-maintained than those of control groups in both 4- and 8-week groups. The reason was thought to be that the well-known mechanism of action of the BPs to inhibit osteoclasts could prevent absorption and collapse of graft materials. Grafted materials of bone defects should be maintained without absorption to play a role as a scaffold for bone regeneration until new bone formation *in vivo* (Lichte et al., 2011). It is thought that the local application of the proper concentration of BPs to prevent early absorption of bone graft materials may have a favorable outcome during reconstruction of bone defects using autogenous or artificial bone grafts. In gross, radiographic, and micro-CT findings, it was confirmed that bone regeneration of the experimental group was much better than that of the control group. Especially, in micro-CT findings, evidence of bone regeneration, such as open bone marrow cavity of the cutting side, the enlarged diameter of the regenerated fibula bone, and the ingrowth of newly formed bone on the bone defect sites were observed in the experimental group unlike the control group. The mechanism of new bone formation after graft of bone substitutes is not completely understood, but based on several studies, it was thought that endochondral ossification could mainly be observed during new bone formation by osteoinduction of graft materials, and intramembranous ossification could be seen during new bone formation by osteoconduction of graft materials (Yuan et al., 2002; Habibovic et al., 2008; Barradas et al., 2011). Therefore, several findings in ALN-BCP group, such as endochondral ossification around bone graft, and the occurrence of bone regeneration mainly just around bone graft materials without direct bone deposition into the pore of graft materials, could indirectly prove the osteoinduction capacity of ALN-loaded BCP. Some of rats in the experimental group showed a tendency toward bone regeneration in response to BCP even without bony contact due to axial deviation of grafted materials away from recipient sites, and it meant that ALN-loaded BCP had an osteoinduction capacity (Lazard et al., 2011). Furthermore, the results of statistical analysis using microstructural trabecular bone parameters showed positive micro-architectural modification on medullary bone around the defect, although the values of Tb.Sp were not consistent with the results of the other parameters. On the basis of previous studies such as a study by Verron et al., which reported improvement of bony micro-architecture as evident by scanning electron microscopy after the local application of BP to the osteoporotic femurs of

ewes (Verron et al., 2010), the result of Experiment 1 in our study could also be presumed to indicate that positive micro-architectural modification was caused by an increase in osteoblast differentiation due to locally released bisphosphonates. However, verification via immunohistochemistry will be required to identify the exact cause.

4.1.2. 15d-PGJ_2-ACS group

There was no significant difference in gross and radiographic findings between the experimental and control groups, but in micro-CT findings, BV/TV and Tb.N of 4-week group and BV/TV, Tb.N, Tb.Sp, and SMI of 8-week group were statistically significant difference between the control and experimental groups. Histomorphologically, active deposition of calcified matrix, woven bone formation, and an increase in the amount of trabecular bone were also observed in some specimens in the experimental group. If new bone formation did not occur in the CSD model, the size of defect would be enlarged with sharpened and absorbed cutting edges, and open bone marrow would not be seen (Lazard et al., 2011). However, some rats in the experimental group in this study showed open bone marrow cavities on the cutting ends, so this could be regarded as evidence of bone regeneration. Previous studies have reported that prostaglandin is a powerful factor in bone formation (Hakeda et al., 1985), and PGD_2, a precursor of 15d-PGJ_2, stimulates calcification of osteoblasts (Koshihara and Kawamura, 1989). If the capacity and the mechanism of bone formation of 15d-PGJ_2 are validated, it will provide a clinically effective bone substitute.

5. Conclusion

The researchers concluded that the locally applied ALN prevented resorption of grafted BCP and had a positive effect on bone regeneration, with positive micro-architectural modification of the surrounding bone through the ALN-BCP group, whereas the 15d-PGJ_2-ACS group did not verify the significant capacity of bone regeneration of 15d-PGJ_2, and only shed a light on its possibility.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

HJK designed the study and prepared the first draft of the paper. He is guarantor. JWN, JIK and JYH contributed to the experimental work, and they were responsible for statistical analysis of the data. All authors revised the paper critically for intellectual content and approved the final version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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