Effect of doxycycline doped bone substitute on vertical bone augmentation on rat calvaria

Peng ZHANG¹, Lin DING¹,² and Shohei KASUGAI¹

¹Department of Oral Implantology and Regenerative Dental Medicine, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan
²Foshan Stomatology Hospital, School of Stomatology and Medicine, Foshan University, 5th Hebin Road, Chancheng district, Foshan 528000, China

Corresponding author, Lin DING; E-mail: dinglin032001@gmail.com

INTRODUCTION

Bone augmentation is frequently required in orthopedic and dental fields. Although autologous bone graft is still the golden standard for bone augmentation, the limitation of the harvestable bone volume and the inflammation of the donor site are the problems with this classical modality. Instead, various bone substitutes have been clinically used, among which, Bio-Oss (BO: Geistlich Pharma, Wolhusen, Switzerland), deproteinized bovine bone xenograft, is the most widely used in dental field. The inorganic bone substitute is comprised of a hydroxyapatite (HA) skeleton that chemically and physically resembles human spongiosa bone in terms of trabecular architecture. Its porosity and inner surface area act well as a biocompatible and osteoconductive scaffold for osteoblasts recruited from the adjacent preexisting bone, resulting in new bone formation and ingrowth of lamellar bone.

Clinically, BO is applied with guided bone regeneration (GBR) technique, in which a barrier membrane covers the bone regenerative site to block the soft tissue invasion. Although the combination with BO and GBR is clinically effective, vertical bone augmentation is still challenging because the augmented site is not surrounded by the bony walls resulting in insufficient recruitment of osteogenic cells. Furthermore, due to the insufficient amount of soft tissues to cover the augmented site, the infection following membrane exposure often occurs, which is the most serious complication of this modality.

Doxycycline (DOX), a semi-synthetic broad-spectrum antibiotic, is a structural isomeride of tetracycline. It has been widely studied regarding its well-known antibacterial effects by inhibition of microbial protein synthesis, anti-inflammatory properties by suppression of the polymorphonuclear leukocytes, and anticollagenase feature by altered regulation of cytoplasmic calcium as mediated by osteoclasts, thereupon further inhibiting bone resorption through osteoclastic collagenase. The recent studies have shown that DOX inhibits osteoclast lineage differentiation, which makes DOX beneficial to bone regeneration. Furthermore, our previous study and the study of Walter et al. both revealed the advantage of DOX as an osteogenic agent on the surface of implants coated with HA or consisted of titanium zirconium. Since it demonstrates high affinity to HA of bones and dental hard tissues, DOX is widely used in dental treatment for periodontitis and peri-implantitis. On the other hand, BO is capable as a delivery carrier of DOX, as it retains vast surface area. Thus, it is likely that combination of DOX with BO would not only inhibit infection but also stimulate bone formation in vertical bone augmentation. The aim of the present study was to investigate the effects of BO+DOX via GBR on vertical bone augmentation and to explore the mechanism of bone formation affected by BO+DOX.

MATERIALS AND METHODS

BO+DOX sample preparation

For BO+DOX group, 1 mg DOX (Doxycycline hyclate, SIGMA-ALDRICH, St. Louis, MO, USA) supplied in powder form were dissolved in 1 mL pure ethanol, i.e. 1 mg/mL DOX ethanol solution. Five hundred milligram (Approximately 1 cm³) BO granules ranging from 3-1.5 mm were soaked in 1 mL of DOX ethanol solution for 24 hours at room temperature. Then, it was stirred at 100 rpm for 15 minutes and centrifuged at 5000 rpm for 5 minutes to collect the BO+DOX particles. The hydroxyapatite (HA) content in the BO+DOX particles was measured by X-Ray Diffraction (XRD) analysis and the weight ratio of HA to BO was calculated to be 0.58.

Dental Materials Journal 2019; 38(2): 211–217
0.25 to 1 mm in diameter were directly immersed into aforementioned 1 mg/mL DOX ethanol solution 1 mL for 30 min, i.e. 2 mg DOX/1 g BO. Then the compounds were air-dried in the fume hood.

**Animal care and surgical procedures**

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University, Tokyo, Japan (Approval No. A2017-205A). Forty 12-week-old male albino Sprague-Dawley rats (Sankyo Labo Service, Tokyo, Japan) weighing from 360–390 g, were randomized into the following two groups: (1) BO; (2) BO+DOX. Animals were allowed to acclimatize for 1 week and housed per pair in a standard cage in an experimental animal room with standardized room temperature, humidity, light/dark cycle and diet.

Animals were anesthetized preoperatively with an intraperitoneal injection of ketamine (100 mg/kg, DAIICHI SANKYO, Tokyo, Japan) and xylazine (5 mg/kg, BAYER, Tokyo, Japan). All the surgical procedures were conducted under standard aseptic condition. The foreheads of the animals were shaved and disinfected with 10% povidone-iodine.

A cutaneous flap was created by making a U-shape incision above the calvaria and was raised laterally, and the subcutaneous connective tissue was mobilized carefully. The periosteum was exposed and incised from the midline and detached and lifted by a raspatory to confirm the surgical field of the calvaria surface.

A large circular groove was drilled on the midline using a trephine bur (inner/outer diameter 4/5 mm), under profuse saline irrigation. At the experimental site, four holes (diameter 0.5 mm) were drilled inside the central circle avoiding perforation, to induce bleeding from the marrow space so that graft material was infused with blood.

The Teflon rings (TFR) (4 mm in diameter and 2 mm in height) were fixed in the circular groove on the calvaria and tightly adapted to the denuded bone. BO granules with or without DOX were filled into the TFR using a curet. A piece of collagen membrane (Bio-Gide, Geistlich Pharma) (BG) were carefully placed over the TFR completely and fixed by suturing and repositioning the mucoperiosteal flaps, followed by skin suture (Fig. 1).

At 4 and 8 weeks after the surgery, the animals of both groups were euthanized averagely by suffocation with CO₂. After fixation, specimens were scanned with a high resolution microcomputed tomography (μCT) imaging system (InsepXio, SMX-100 CT, Shimadzu Science East, Tokyo, Japan) with the tube voltage of 70 kV, tube current of 30 μA and voxel size of 0.03 mm/voxel. The scanned images were reconstructed, and volumetric data was analyzed with three-dimensional image analysis software program (TRI/3D-BON, RATOC System Engineering, Tokyo, Japan).

The region of interest (ROI) was defined as a cylinder with 4 mm in diameter and 1 mm in height that could cover the entire thickness of the graft. By using the volume correction function of the software, artifacts were extracted from the raw data. Inside the wall of bone, trabecular bone was defined as binarized contrast number of 32,000–41,000 and was extracted directly from 3D image, whereas the residual graft was defined as above 41,000. Due to the well-calcification of the wall of new bone, its contrast was sometimes above 41,000, so we manually selected the region of wall of new bone and define it as above 36,000. All these binarized numbers were set based on the tissue of original bone and soft lining tissue.

**Histological evaluation**

The fixed samples were subsequently decalcified in 10% ethylene diamine tetraacetic acid (EDTA) for 4 weeks. After decalcification, all samples were dehydrated in ascending grades of ethanol and embedded in paraffin. Five micrometer-thick sagittal sections of the grafted area were prepared and collected on SuperFrost-plus slides to be stained with hematoxylin and eosin (H&E.
RNA extraction and RT-PCR analysis
Total RNA was isolated using Trizol lysis reagent (Invitrogen, Waltham, MA, USA). The purity and concentration of RNA was determined with a Nanodrop spectrophotometer (Nano-Drop ND 2000, Nanodrop Technologies, Wilmington, DE, USA). cDNA was synthesized from total RNA using SuperScript III First-Strand Synthesis Super-Mix for RT-PCR (Invitrogen). The RT-PCR analysis was carried out with the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and the Power SYBR Green PCR Master Mix (Life Technologies, Warrington, UK). The following genes were analyzed: Alkaline Phosphatase (ALP), Bone Morphogenetic protein 2 (BMP2), Collagen type I (ColI), Osteocalcin (OCN), and Transforming growth factor beta 1 (TGFβ1) and β-Catenin. Moreover, the custom-made primers of genes synthesized by Thermo Fisher Scientific (Yokohama, Japan) were listed in Table 1. The expression level of mRNA was normalized with the housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and calculated by the 2-∆∆Ct method.

Statistical analysis
Results were presented as mean±standard deviation (SD) of the experiments. Statistical analysis was carried out with statistical software IBM SPSS Statistics version 19.0 for Windows (IBM, Armonk, NY, USA). A one-way analysis of variance (ANOVA) with paired t-test were used to quantify differences. p Values<0.05 were considered to be statistically significant. Error bars in the figures represent the SD.

RESULTS
Visual observation after the surgery
Postoperatively, all the rats tolerated the surgical procedures and recuperated uneventfully without any symbol of wound dehiscence, graft migration, visible infection, or obvious complication. All the samples underwent radiological, histological and RT-PCR analyses.

Radiographic evaluation
In order to investigate peri-graft bone dimensions inside the TFR, radiographic examinations were performed by μCT measurement. The content inside the TFR was outlined on the reconstrucive CT-scan image (Figs. 2A, B). We also observed that well-decalcified new bone climbed up along the outer surface of TFR under the cover of collagen membrane (Fig. 2A).

At 4 weeks in both BO and BO+DOX groups, the new bone climbed along the inner wall of TFR and grew toward the membrane covering the substitute, and the newly formed bone showed high-density radio-opacity which is only slightly weaker than the substitute indicating remarkable mineralization. It demonstrated thicker wall of new bone in BO+DOX group in comparison with BO group. None of the 4-week samples built a complete ring around the content that reached the top. The immature new bone with low-density radio-opacity grew around the substitute in both groups and more new bone appeared in BO+DOX group than BO group (Figs. 2C, D).

At 8 weeks, the wall of new bone became thicker and reached higher as compared to that at 4 weeks and the radio-opacity was also stronger than the one at 4 weeks. The wall of new bone reached the top of TFR and formed a complete ring. New bone volume around the substitute also increased from 4 weeks (Figs. 2E, F).

The quantitative parameters of the bone morphometric data obtained from μCT images were presented in Table 2, which demonstrated more increments of bone volume in BO+DOX group at 4 and 8 weeks than the ones in BO group at both time points. Furthermore, in both groups the new bone volume at 8 weeks was higher compared to the one at 4 weeks. However, no statistical significance was found between the two groups (Table 2, Fig. 2G). The alterations were also shown by significantly larger trabecular number (Tb. N) and lower trabecular separation (Tb. Sp) in BO+DOX compared to the ones in BO at 4 weeks.
Fig. 2 Radiological analysis of the ratio of BV.
(A) A representative 3D image of the surgery by μCT. (B) the complex inside the TFR. Representative reconstructive 3D image of μCT of each group.
(C) BO at 4 weeks. (D) BO+DOX at 4 weeks. (E) BO at 8 weeks. (F) BO + DOX at 8 weeks. C–F, green regions: BO; red regions: MNB. (G) quantified mean percentage of BV/TV. BV: bone volume; TV: total volume.

Table 2 Quantitative bone morphometric data using μCT for trabecular bone in rat calvarias

|                  | 4 weeks          | 8 weeks          |
|------------------|------------------|------------------|
|                  | BO               | BO+DOX           | BO               | BO+DOX           |
| Bone Volume fraction (BV/TV) % | 5.93 (±1.21) | 9.23 (±3.89) | 27.34 (±13.14) | 31.68 (±17.24) |
| Trabecular Thickness (Tb.Th) μm | 51.88 (±13.24) | 43.80 (±8.70) | 90.74 (±23.93) | 93.83 (±80.13) |
| Trabecular Number (Tb.N) mm⁻¹ | 0.82 (±0.22)  | 2.15 (±0.55)* | 2.04 (±0.65)  | 3.48 (±2.94) |
| Trabecular Separation (Tb.Sp) μm | 279.76 (±94.83) | 152.27 (±57.97)* | 120.20 (±37.55) | 122.47 (±59.22) |

* p<0.05 vs. BO group at 4 weeks.

Table 3 The mean proportions of NGT, MNB, NCT and BRG, taking into account the volume variation of the bone augmentations

|                  | 4 weeks          | 8 weeks          |
|------------------|------------------|------------------|
|                  | BO               | BO+DOX           | BO               | BO+DOX           |
| NGT              | 57.07 (±4.67)   | 65.36 (±3.46)*  | 59.32 (±4.80)   | 70.98 (±4.71)*  |
| MNB              | 22.08 (±1.72)   | 29.42 (±3.84)*  | 30.24 (±5.38)   | 33.53 (±4.44)   |
| NCT              | 36.68 (±4.86)   | 36.00 (±1.29)   | 28.04 (±7.08)   | 32.98 (±3.45)   |
| BRG              | 41.23 (±2.70)   | 34.58 (±6.66)   | 41.72 (±5.31)   | 33.49 (±7.85)   |

NGT: newly generated tissue; MNB: mineralized new bone; NCT: non-mineralized connective tissue; BRG: BO residual graft. NGT is composed of MNB and NCT. Mean and SD were presented. * p<0.05 vs. BO group respectively.
Histological and Histomorphometrical analyses

At 4 and 8 weeks, inside the TFR, there were BO residual graft material (BRG) and newly generated tissue (NGT). NGT was composed of mineralized new bone (MNB) and non-mineralized connective tissue (NCT). The areal proportions of these components at 4 and 8 weeks were presented in Table 3. At both time points, compared to BO group, in BO+DOX group, NGT was larger whereas BRG was smaller.

In specific, BO particles of various sizes and configurations were distributed within the TFRs. Although some of BO particles were lost and only left blank artifact due to the H&E staining procedures\textsuperscript{13}, the surrounded tissues were distinguished mainly as MNB stained as pink, and occasionally as NCT stained as dark purple. The MNB near the basal calvarial bone was more mineralized than that of upper MNB. Along the inside wall of the TFR, the newly formed bone was evident on both sides of the sagittal section if only there was space between TFR and substitutes. The new bone was much more mature compared to inside the MNB as indicated by the light pink staining close to the basal calvarial bone. Moreover, small lacunars and bone marrows could be identified on the wall of new bone. The character of wall of new bone is consistent with the μCT observation. On the top part of TFR, the space between the BO particles and the membrane were fulfilled by a layer of NCT (Figs. 3A, C).

Particularly at 8 weeks in BO+DOX group, on the wall of new bone, mature bone with well-arranged laminar structure and abundant bone marrow cavity were witnessed. The substitutes were preserved better through H&E staining procedure than other

\textbf{Fig. 3} Histological appearance and analysis of the whole sagittal graft area.

Representative histological appearance of the specimens of each group. (A) BO at 4 weeks. (B) BO at 8 weeks. (C) BO+DOX at 4 weeks. (D) BO+DOX at 8 weeks. (E) quantified mean percentage of NGT/TA. *p<0.05 vs. BO group respectively. NGT: newly generated tissue; TA: total area.

\textbf{Fig. 4} \textit{In vivo} mRNA expression of bone formation marker TGF-β, BMP2, β-catenin, ALP, OCN and Coll levels presented as fold changes.

Data are shown as the mean±SD (n=5). *p<0.05 vs. BO group at 4 and 8 weeks respectively, #p<0.01 vs. BO+DOX group.
groups encapsulated by MNB, which indicates better osseointegration around the interface between substitutes and surrounded tissues (Figs. 3B, D).

**Real-time PCR analysis**

In order to investigate more details of cellular and molecular responses to the bone substitute with or without DOX, gene expressions of osteogenic molecules including ALP, BMP2, TGFβ1, OCN, ColI and β-catenin were examined with RT-PCR.

TGFβ1 expression was up-regulated significantly both at 4 and 8 weeks (p<0.05) in BO+DOX group compared to BO group (Fig. 4). Meanwhile, in BO+DOX group, BMP2 and β-catenin were more expressed at 4 weeks; however, there were no difference at 8 weeks. Moreover, in comparison with BO group, in BO+DOX group, CollI at 4 and 8 weeks as well as ALP and OCN at 4 weeks showed an upward tendency. Nevertheless, at 8 weeks, there was not difference of the expression level of BMP2, β-catenin, ALP and OCN between the two groups with or without DOX.

**DISCUSSION**

A new concept of sub-antimicrobial dose DOX (SDD) or low-dose DOX (LDD) as adjuvant therapy for periodontal diseases has been approved by U.S. Food and Drug Administration and other national regulatory agencies in Europe and Canada. DOX used as gel form (10% DOX gel) or electrochemically bound implant surface (1 mg/mL) or attached on the HA-coated titanium surface (5 mg/mL with wash), were proved to be able to promote bone regeneration in vivo and/or in vitro. DOX was also used as a combination with BO, in a rabbit sinus lift study, where the bone quantities and densities and bone to material contact were not statistically different between BO with or without DOX, probably due to the low concentration of DOX (0.1 mg/mL).

Another in vitro research focusing on the antimicrobial properties revealed that the extent of adsorption release of DOX was concentration and time dependent, and also positive correlation with BO amount. Additionally, the adsorption kinetics of the antibiotics onto bone graft is also affected by graft porosity and surface area. Based on these previous studies, we speculated that continuous release of DOX to the surrounding environment may promote the bone formation.

In our preliminary experiment, we analyzed DOX release behavior from the BO granules in PBS for 14 days (data not shown) and found out that in the first 12 h DOX was rapidly released and that the peak of the DOX release was observed at 48 h. The release pattern was similar to the previous report of Dashti et al.

We confirmed the continuous DOX release from BO granules up to 14 days although the amount of released DOX decreased with time.

In the present study, histological observation clearly validated new bone formation around the BO/BO+DOX granules surface, reconfirming promising osteoconductivity and osseointegration of BO/BO+DOX granules by GBR. Histologically, BO+DOX particles appeared to be more integrated with the newly formed bone at 4 and 8 weeks than BO particles. This was based on the fact of less blank space, which was more frequently observed between BO particles and MNB in BO at each time point, in comparison with the corresponding time point in BO+DOX. This phenomenon coincided with the findings of a previous sinus lift study in human at two time points for comparison.

NGT area is mainly consisted of two parts, i.e. the unfilled space and the space resorbed by substitute, and the unfilled space in both groups was almost similar to each other. According to histomorphometric analysis, NGT of BO+DOX at both time points showed significant increase as compared to BO. Moreover, the BRG of BO+DOX also presented a decreasing trend. Based on these results, we speculate that the bone formation and remodeling in BO+DOX group were more active along the BO+DOX granule surface and led to more resorption of the substitute (Table 3).

In addition to radiographical and histological analyses, mRNA expression levels of several genes were investigated. BMP proteins, as multifunctional paracrine growth factors belonging to the TGFβ super family, take crucial part in the osteogenesis. Among BMPs, BMP2 is at the center of interest for research and clinical application. Moreover, signaling cross-talk between TGFβ/BMP pathways controls a number of events, including cell proliferation and differentiation.

It has been demonstrated that BMP2 acts synergistically with β-catenin to promote chondrocyte and osteoblast differentiation. In vitro, the present results of the gene expressions in vivo indicate that the upregulation tendency of ALP and OCN in BO+DOX at 4 weeks might mainly be mediated via synergistical functional cross talk of BMP and Wnt/β-catenin pathways as compared to BO.

**CONCLUSION**

The results of the present study indicated that the treatment with DOX to BO promotes bone apposition around the graft and demonstrated that the combination...
of BO and DOX enhances vertical bone augmentation in rodent model. Furthermore, it is likely that BMP and Wnt/β-catenin pathways involve in this DOX accelerating effect.

REFERENCES

1) Lambert F, Lecloux G, Léonard A, Sourice S, Layrolle P, Rompen E. Bone regeneration using porous titanium particles versus bovine hydroxyapatite: a sinus lift study in rabbits. Clin Implant Dent Relat Res 2013; 15: 412-426.
2) Wang X, Zakaria O, Madi M, Hao J, Chou J, Kasugai S. Vertical bone augmentation induced by ultrathin hydroxyapatite sputtered coated mini titanium implants in a rabbit calvaria model. J Biomed Mater Res B Appl Biomater 2015; 103: 1700-1708.
3) de Lange GL, Overman JR, Farré-Guasch E, Korstjens CM, Hartman B, Langenbach GE, Van Duin MA, Klein-Nulend J. A histomorphometric and micro-computed tomography study of bone regeneration in the maxillary sinus comparing biphasic calcium phosphate and deproteinized cancellous bovine bone in a human split-mouth model. Oral Surg Oral Med Oral Pathol Oral Radiol 2014; 117: 8-22.
4) Dashti A, Ready D, Salih V, Knowles JC, Barralet JE, Wilson M, Donos N, Nazhat SN. In vitro antibacterial efficacy of tetracycline hydrochloride adsorbed onto Bio-Oss bone graft. J Biomed Mater Res B Appl Biomater 2010; 93: 394-400.
5) Sheikh Z, Sima C, Glogauer M. Bone replacement materials and techniques used for achieving vertical alveolar bone augmentation. Materials 2015; 8: 2953-2993.
6) Roccuzzo M, Savoini M, Dalmasso P, Ramieri G. Long-term outcomes of implants placed after vertical alveolar ridge augmentation in partially edentulous patients: a 10-year prospective clinical study. Clin Oral Implants Res 2017; 28: 1204-1210.
7) Silva AC, Oliveira MR, Amaral LF, Ferreira S, Garcia-Jr IR, Mariano RC. Effect of doxycycline in gel form on bone regeneration: histomorphometric and tomographic study in rat calvaria. J Periodontol 2016; 87: 74-82.
8) Golub LM, McNamara TF, Ryan ME, Kohut B, Blieden T, Payonk G, Sipos T, Baron HJ. Adjunctive treatment with subantimicrobial doses of doxycycline: effects on gingival fluid collagenase activity and attachment loss in adult periodontitis. J Clin Periodontol 2001; 28: 146-156.
9) Kinugawa S, Koide M, Kobayashi Y, Mizoguchi T, Ninomiya T, Muto A, Kawahara I, Nakamura M, Yasuda H, Takahashi N, Udagawa N. Tetracyclines convert the osteoclastic-differentiation pathway of progenitor cells to produce dendritic cell-like cells. J Immunol 2012; 188: 1772-1781.
10) Ding L, Zhang P, Wang X, Hao J, Aoki K, Kuroda S, Kasugai S. Effect of doxycycline-treated hydroxyapatite surface on bone apposition: A histomorphometric study in murine maxillae. Dent Mater J 2018; 37: 130-138.
11) Walter MS, Frank MJ, Satué M, Monjo M, Remold HJ, Lyngstadaas SP, Haugen HJ. Bioactive implant surface with electrochemically bound doxycycline promotes bone formation markers in vitro and in vivo. Dent Mater 2014; 30: 200-214.
12) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 2001; 25: 402-408.
13) Berghund T, Lindhe J. Healing around implants placed in bone defects treated with Bio-Oss. An experimental study in the dog. Clin Oral Implants Res 1997; 8: 117-124.
14) Saito T, Takeuchi R, Hirakawa K, Nagata N, Yoshida T, Koshino T, Okuda K, Takema M, Hori T. Slow releasing potential of vancomycin-loaded porous hydroxyapatites blocks implanted into MRSA osteomyelitis. J Biomed Mater Res 2002; 63: 245-251.
15) Queiroz AC, Santos JD, Monterio FJ, Gibson IR, Knowles Jc. Adsorption and release studies of sodium ampicillin from hydroxyapatites composites. Biomaterials 2001; 22: 1393-1400.
16) Kitayama S, Wong LO, Ma L, Hao J, Kasugai S, Lang NP, Mattheos N. Regeneration of rabbit calvarial defects using biphasic calcium phosphate and a strontium hydroxyapatite-containing collagen membrane. Clin Oral Implants Res 2016; 27: e206-e214.
17) Valentini P, Abensur D, Wenz B, Peetz M, Schenk R. Sinus grafting with porous bone mineral (Bio-Oss) for implant placement: a 5-year study on 15 patients. Int J Periodontics Restorative Dent 2000; 20: 245-253.
18) Grimaud E, Heymann D, Rédini F. Recent advances in TGF-β effects on chondrocyte metabolism: potential therapeutic roles of TGF-β in cartilage disorders. Cytokine Growth Factor Rev 2002; 13: 241-257.
19) Schmidt-Bleek K, Willisio BM, Schwabe P, Seemann P, Duda GN. BMPs in bone regeneration: less is more effective, a paradigm-shift. Cytokine Growth Factor Rev 2016; 27: 141-148.
20) Guo X, Wang XF. Signaling cross-talk between TGF-β/BMP and other pathways. Cell 2009; 108: 896-905.
21) Rawadi G, Vayssiere B, Dunn F, Baron R, Roman-Roman S. BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. J Bone Miner Res 2003; 18: 1842-1853.
22) Mbulavie G, Sheikh S, Stains JP, Salazar VS, Cheng SL, Chen D, Civitelli R, Smolen in vivo and in vitro. Gartner RJ, Knowles JC. Adsorption and release studies of sodium ampicillin from hydroxyapatites composites. Biomaterials 2001; 22: 1393-1400.
23) Zhang M, Yan Y, Lim YB, Tang D, Xie R, Chen A, Tai P, Harris SE, Xing L, Qin YX, Chen D. BMP-2 modulates beta-catenin signaling through stimulation of Lrp5 expression and inhibition of beta-TrCP expression in osteoblasts. J Cell Biochem 2009; 108: 896-905.