Yasunori Ayukawa, DDS, PhD

Submitted for publication August 6, 2018.
Accepted in revised form September 17, 2018.

*Correspondence: Section of Implant and Rehabilitative Dentistry, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
(e-mail: ayukawa@dent.kyushu-u.ac.jp).

Effect of a Single Injection of Benidipine-Impregnated Biodegradable Microcarriers on Bone and Gingival Healing at the Tooth Extraction Socket

Mikio Imai,¹ Yasunori Ayukawa,^{1,*} Noriyuki Yasunami,¹
Akihiro Furuhashi,¹ Yoko Takemura,¹ Naomi Adachi,¹
Jiangqi Hu,¹ Xudiyang Zhou,¹ Yasuko Moriyama,¹
Ikiru Atsuta,¹ Kosaku Kurata,² and Kiyoshi Koyano¹

¹Section of Implant and Rehabilitative Dentistry, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, Fukuoka, Japan.

²Department of Mechanical Engineering, Faculty of Engineering Kyushu University, Fukuoka, Japan.

Objective: A dihydropyridine-type calcium channel blocker, benidipine (BD), is extensively used in hypertension therapy. *In vitro* study reported BD promoting bone metabolism. We evaluated the effect of sustained release of BD-loaded poly(lactic-co-glycolic acid) (PLGA) microcarriers on the promotion of bone and gingival healing at an extraction socket *in vivo*. In addition, the effect of BD on osteoblasts, osteocytes, fibroblasts, and epithelial cells was evaluated *in vitro*.

Approach: The maxillary first molar of rats was extracted. Next, PLGA microcarriers containing BD were directly injected into the gingivobuccal fold as a single dose. After injection, bone and soft-tissue healing was histologically evaluated. Effect of BD on proliferation, migration, and gene expression of gingival and bone cell was also examined *in vitro*.

Results: After tooth extraction, BD significantly augmented bone volume and density, and also epithelial wound healing. During *in vitro* studies, BD promoted significant proliferation and migration of fibroblasts and epithelial cells. Real-time RT-PCR revealed that BD upregulated messenger RNA expression of *Ahsg* (alpha 2-HS glycoprotein) and *Csf2* (colony-stimulating factor 2) in osteoblasts.

Innovation: The prevention of bone and soft-tissue reduction associated with tooth extraction has been eagerly anticipated in the field of dentistry. This study first reported the effect of BD on extraction socket healing.

Conclusion: A single dose of topically administered BD-loaded PLGA microcarriers promoted bone and soft-tissue healing at the extraction site of tooth.

Keywords: benidipine, osteocyte, tooth extraction socket, PLGA microcarriers, wound healing

INTRODUCTION

A DIHYDROPYRIDINE-TYPE CALCIUM channel blocker, benidipine (BD), has been widely used as an antihypertensive and anticardiac arrest drug,^{1–3} and the anabolic effect of BD on bone

in vitro was recently reported. Several studies have suggested that BD increases the alkaline phosphatase activity of osteoblasts and stimulates mineral matrix deposition.^{4–6} In addition, BD has been reported to

decrease RANKL (receptor activator of nuclear factor kappa-B ligand) expression in human osteoblasts, indicating the suppression of osteoclast differentiation.⁷ Furthermore, BD promoted cell proliferation and osteogenic differentiation at concentrations from 1×10^{-6} to 1×10^{-9} M by upregulating gene expression levels of runt-related transcription factor-2 (RUNX2), bone morphogenetic protein (BMP)-2, and osteocalcin (OCN).⁶ This increased the differentiation of bone marrow stromal cells (BMSCs) toward osteoblasts.⁸ However, the detailed mechanism remains unclear, and no studies have examined the effects of this antihypertensive drug on bone function in animal models.

In this study, we focused on these properties of BD and explored the possibility of accelerating the healing of the extraction socket soft and hard tissues. Accordingly, we introduced newly developed sustained-releasable BD-loaded microcarriers and evaluated the effect of single transmucosal injection of BD-loaded microcarriers.

CLINICAL PROBLEM ADDRESSED

A sufficient volume of alveolar bone is one of the most important factors that determine favorable results in the fields of prosthetic dentistry and dental implantology. In addition, an appropriate volume of soft tissue is essential for dental esthetics.^{9–11} However, alveolar bone volume always decreases to a certain extent after tooth extraction. The consequences of these physical soft- and hard-tissue changes greatly affect clinical outcomes.

A variety of materials, such as autografts, allografts, xenografts, and alloplastic materials, have been applied to extraction sockets to avoid a decrease of bone volume.^{12–17} However, these materials have several disadvantages, and there are currently no gold standard treatment modalities to preserve bone volume.

Platelet-rich plasma or similar blood concentrate has also been used for this purpose without any risk of infection because it can be harvested from the patient's own blood. This concentrate is believed to include several types of growth factors, but it is still insufficient to preserve bone at the extraction socket.^{18–21} Consequently, to eliminate the possibility of extraction socket volume reduction, the development of a novel method to enhance bone regeneration is anticipated.

When using certain drugs and growth factors, an appropriate drug-delivery system (DDS) is important to obtain a clinically successful outcome. For

bone formation, a DDS must feature a long-lasting and steady release of the drug.

MATERIALS AND METHODS

In vivo study

Materials and animals. A total of 100 five-week-old male rats (130–150 g; 80 rats for histomorphometry, 20 for micro-computed tomography [CT]) were used for two experimental (15 or 45 μ g/kg of BD) and two control (no administration or poly(lactic-co-glycolic acid) [PLGA] without BD) groups. Each group comprised four subgroups based on the experimental period. Consequently, each subgroup comprised five animals for histomorphometry. Throughout this study, we complied with the ARRIVE guidelines and experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals, and the rats were treated according to the guidelines for animal care established by Kyushu University under the approval of the institution's ethical committee (approval no.: A26-223-0).

The microcarriers were prepared using an oil phase consisting of BD (Wako Pure Chemical Industries, Osaka, Japan) in a mixture of PLGA (PLGA-7510; Wako Pure Chemical Industries) dissolved in dichloromethane. The oil phase was cooled and subsequently poured into an aqueous solution of 0.25% polyvinyl alcohol. The oil-in-water emulsion was stirred to aid dichloromethane evaporation. The hardened microcarriers were then filtered using sieves with apertures of 70 μ m, rinsed with water, and lyophilized into powder, which was dried under reduced pressure. As a result, 1 mg microcarrier contained 15 μ g BD. To observe the microcarriers, they were coated with gold (JEC-550 Twin Coater; JEOL, Tokyo, Japan) and observed with a scanning electron microscope (S-3100; Hitachi High-Technologies, Tokyo, Japan) at 15 kV (Fig. 1a). To confirm sustained-release characteristic, the microcarriers (1 mg) were suspended in 2 mL of simulated body fluid (SBF)²² at 37°C. An aliquot (70 mL) of SBF supernatant was sampled every 2 days and analyzed by spectrophotometry at 237 nm (Fig. 1b).

Tooth extraction and injection of microcarriers. The maxillary right first molars of the rats were extracted under a combination anesthetic (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol). The animals were randomly categorized into the abovementioned four groups. After extraction, 0.1 mL of 150 or 450 μ g/mL

Figure 1. (a) Scanning electron microscope image of the microcarriers. (b) Cumulative amounts of BD in SBF. (c) Connective tissue area (blue) was defined as the area between the gingivobuccal fold and the palatal end of the alveolar ridge. (d) Epithelial wound healing was defined as the distance between both edges of epithelium (between arrowheads). (e) Scheme for calculation of bone density. The percentage of bone density was defined as bone volume (A)/tissue volume (B) × 100. (f) Vertical bone height was defined as the bone height at the center of the socket (arrow). (g) Bone volume was defined as the volume of new bone formed at the site where maxillary right first molar was previously present (from the mesial end of the mesial root socket to the distal end of the distal root socket of the maxillary right first molar). BD, benidipine; SBF, simulated body fluid. Color images are available online.

microcarrier-suspended saline (the final dose of BD was 15 or 45 $\mu\text{g/kg}$, respectively) was immediately injected into the gingivobuccal fold near the extraction socket. The concentration of BD was defined as follows: according to interview form of Coniel[®] tablet (bland name of BD), peroral administration of 8 mg of BD (maximum dose of BD for hypertension) resulted in the concentration of a maximum of 4 ng/mL in blood. One milligram of microcarrier released ~ 120 ng/mL SBF per month (Fig. 1b) and it was equivalent to 4 ng/mL SBF per day. Thus we decided to use this concentration. In addition, threefold concentration was also used for the test purpose.

On 1, 3, 7, and 28 days after the treatment, the animals were killed by an overdose of pentobarbital sodium and perfusion fixed through the ascending aorta with a 0.9% saline and 4% paraformaldehyde solution (pH 7.4).

Micro-CT. The maxillae were further immersed in the same fixative for 24 h. Next, bone biopsies were examined using a micro-CT scanner (SkyScan 1076; SkyScan, Aartselaar, Belgium). Scanning was performed with an in-lane pixel size and slice thickness of 9 μm . Reconstruction was conducted using built-in SkyScan NRecon program.

Tissue preparation. Specimens were decalcified in 20% tetrasodium ethylenediaminetetraacetate (pH 7.4), dehydrated, and embedded in paraffin. Sectioning was performed parallel to the coronal plane and the tissues were stained with hematoxylin and eosin.

Analysis of epithelial wound healing, connective tissue area, and bone density. Sections representing the central portion of each extraction socket were selected, and images were obtained using a microscope (BZ-9000; Keyence, Osaka, Japan). Epithelial wound healing was evaluated by measuring the distance between the both epithelial edges on days 1, 3, and 7. The connective tissue area and bone density were evaluated on day 28 after tooth extraction (Fig. 1c–e).

Vertical bone height and bone volume. Using micro-CT images, vertical bone height was measured. Among frontal plane slices, a slice that included mesiodistally median slice of corresponding tooth of opposite site was used for the measurement of bone height. Bone height was measured at the buccolingually median position of alveolus. Bone volume was measured at the extraction socket using a built-in numerical analysis software (CTAn; SkyScan) (Fig. 1f, g).

In vitro study

Cell culture. Mouse osteoblast cell line MC3T3-E1 cells (RIKEN BRC Tsukuba, Ibaraki, Japan) were cultured in α -minimum essential medium (α -MEM) containing 100 U/mL penicillin, 100 U/mL streptomycin, and 10% fetal bovine serum (FBS) in a humidified incubator at 37°C and 5% carbon dioxide (CO_2). Mouse osteocyte cell line MLO-Y4 cells were cultured on type I collagen-coated plates (0.15 mg/mL, rat tail collagen type I; BD Biosciences, Bedford, MA) with α -MEM (Gibco BRL, Paisley, United Kingdom) supplemented with 2.5% FBS (Gibco BRL), 2.5% iron-supplemented calf serum (HyClone Laboratories, Logan, UT), and antibiotics (100 IU/mL penicillin and 100 g/mL streptomycin; Gibco BRL).²³ The cells were subcultured every 3 days in the presence of 0.25% trypsin.

Mouse fibroblast cell line NIH3T3 cells (RIKEN BRC Tsukuba) were cultured in α -MEM containing 10% FBS. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Mouse oral epithelium cell line GE1 cells (RIKEN BRC Tsukuba) were cultured in serum-free medium (SFM-101; Nissui, Tokyo, Japan) supplemented with 1% FBS and epidermal growth factor. The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 33°C. The cells were subcultured every 3 days in the presence of 0.25% trypsin.

Cell proliferation assay. MC3T3-E1, MLO-Y4, NIH3T3, and GE1 cells were seeded in 96-well plates (5,000 cells per well) and incubated overnight. BD was dissolved in 0.1% dimethyl sulfoxide (DMSO) at different concentrations (final concentrations of 0.1–10,000 nM) and added to the cell cultures. Cells without BD but with DMSO were used as negative control, and wells without cells were used as blanks. Further incubation for 12 h (NIH3T3 and GE1) or 24 h (MC3T3-E1 and MLO-Y4) was performed. Next, 10 μL of WST-8 reagent (Cell Count Reagent SF; Nacalai Tesque, Kyoto, Japan) was added, and the cells were further incubated for 2 h. Subsequently, the optical density at 450 nm was measured on a microplate spectrophotometer (Infinite M200; Tecan, Salzburg, Austria). The cell proliferation rate was calculated according to the following formula: $(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}})/(\text{OD}_{\text{control}} - \text{OD}_{\text{blank}})$.

Cell migration assay. Cell migration was measured using a scratch assay. NIH3T3 and GE1 cells were grown to a semiconfluent monolayer in a 35 mm dish. Next, the surface of the dish was scratched using a pipette tip (~ 300 μm in diameter

Figure 2. (a) Histological images of extraction socket on day 3. (A) Tooth extraction only (control). (B) Injection of PLGA without BD (active control). (C) Injection of PL-BD at 1.0 mg/kg. (D) Injection of PL-BD at 3.0 mg/kg. In the extraction socket, blood clotting and granulation tissue were observed. In the PL-BD groups, a recovery of epithelial continuity was observed. (b) Histological images of extraction socket on day 28. (A) Tooth extraction only (control). (B) Injection of PLGA (active control). (C) Injection of PL-BD at 1.0 mg/kg. (D) Injection of PL-BD at 3.0 mg/kg. At day 3, the distances between edges of epithelia in PL-BD groups (C, D) were significantly shorter than that of the control group (A). Hematoxylin and eosin staining. Bar = 1,000 μm ($n=5$). PL-BD, poly(lactic-co-glycolic acid) with benidipine; PLGA, poly(lactic-co-glycolic acid). Color images are available online.

of apex). Thus, a cell-free band ~ 300 μm in width was created. The cells were incubated and images were captured every 1 h for 12 h using a time-lapsed microscope (BioStation IM; Nikon, Tokyo, Japan) in the presence of BD at doses of 0 and 100 nM. The number of cells migrating into the cell-free band and the distance from the edge were measured.

Real-time PCR assay. MC3T3-E1 cells were seeded at a concentration of 2.5×10^4 cells/mL in a 35 mm dish containing α -MEM, 10% FBS, and osteogenic induction supplement containing 10 mmol/L β -glycerophosphate disodium and 0.15 mmol/L ascorbic acid (Sigma, St. Louis, MO). A series of dilutions of BD (final concentration, 100 nM) were added to the culture medium in the 35 mm dish for 7 days. MC3T3-E1

cells treated solely with osteogenic induction supplement were used as a control group.

Quantitative Reverse transcriptase PCR assays were performed using the RT²Profiler™ PCR array: mouse osteogenesis (catalogue no.: PAMM-026; Super-Array, Frederick, MD), according to the manufacturer's instructions. The array contained primer sets for 84 osteogenesis-related genes and five housekeeping genes. The starting amount of RNA was 1 μg. PCR was performed using a Light-Cycler® 96 System (Roche Molecular Systems, Pleasanton, CA) using the following parameters: 1 cycle for 10 min at 95°C, 45 cycles for 15 s at 95°C, and 1 min at 60°C. Data were analyzed using the PCR Array Data Analysis Web Portal.

All quality control parameters, that is, genomic DNA control, reverse transcription control, and

positive PCR control, were within the manufacturer's recommended limits for each assay. Results for each assay were normalized to the average of all five housekeeping genes. The average thresholds were compared, and a gene expression change was reported if statistically significant difference between control and BD groups was present.

Statistical analysis

The statistical significance of differences with respect to migration assay, cell proliferation assay, and other data were determined by a one-tailed Student's *t*-test, one-way analysis of variance with *post hoc* Dunnett or Tukey test, respectively. Differences were considered significant if *p*-values were <0.05.

RESULTS

Epithelial wound healing

There were no significant differences in epithelial wound healing among the groups on day 1 (data not given). On day 3, wound closure significantly progressed in the PLGA with BD (PL-BD) 1.0 and 3.0 groups (each administered 1 or 3 mg/kg microcarriers that contained 15 or 45 µg/mL, respectively) compared with the control group

(Fig. 2a). On day 7, complete wound closure of the epithelium was observed in all groups (data not given).

Connective tissue area

No significant differences between the groups were observed on day 28 (Fig. 3a).

Bone density

On day 28, bone density was significantly larger in both the PL-BD groups than in the control and PLGA groups (Figs. 2b and 3b).

Vertical bone height

On day 28, the vertical bone height value at the extraction socket was significantly larger in the PL-BD 1.0 and 3.0 groups than in the control and PLGA groups (Fig. 3c).

Bone volume

On day 28, bone volume was significantly higher in the PL-BD 1.0 and 3.0 groups than in the control and PLGA groups (Fig. 3d).

Cell proliferation assay

After incubation for 24 h, the proliferation of MC3T3-E1 was significantly promoted by BD at

Figure 3. (a) Connective tissue area on day 28. No significant differences were observed between the groups. (b) Bone density on day 28. Bone density values were significantly larger in the PL-BD 1.0 and 3.0 groups than in the control and PLGA groups. (c) Vertical bone height on day 28. Vertical bone height values were significantly higher in the PL-BD 1.0 and 3.0 groups than in the control and PLGA groups. (d) Bone volume on day 28. Bone volumes were significantly higher in the PL-BD 1.0 and 3.0 groups than in the control and PLGA groups (*n*=5). **p*<0.05; ***p*<0.01.

Figure 4. Cell proliferation ratios after incubation for 24 h (MC3T3-E1 and MLO-Y4) or 12 h (NIH3T3 and GE1). **(a)** Cell proliferation of MC3T3-E1 was significantly promoted by BD at doses of 10, 100, and 1,000 nM. **(b)** Cell proliferation of MLO-Y4 was significantly promoted by BD at doses of 0.1, 1, 10, 100, 1,000, and 10,000 nM. **(c)** Cell proliferation of NIH3T3 was significantly promoted by BD at doses of 1, 10, 100, and 1,000 nM. **(d)** Cell proliferation of GE1 was significantly promoted by BD at dosages of 1, 10, 100, 1,000, and 10,000 nM ($n=5$). * $p<0.05$; ** $p<0.01$.

doses of 10, 100, and 1,000 nM. The proliferation of MLO-Y4 was significantly promoted by BD at doses of 0.1, 1, 10, 100, 1,000, and 10,000 nM. After incubation for 12 h, the proliferation of NIH3T3 was significantly promoted by BD at doses of 1, 10, 100, and 1,000 nM. The proliferation of GE1 was significantly promoted by BD at doses of 1, 10, 100, 1,000, and 10,000 nM (Fig. 4a–d).

Cell migration assay

The number of NIH3T3 and GE1 cells that migrated into scratch was increased by BD at a dose of 100 nM (Fig. 5a, b).

Real-time PCR

Relative to control, six genes in MC3T3-E1 were significantly upregulated by BD (Table 1).

DISCUSSION

The antihypertensive mechanism of BD involves blocking of three kinds of calcium channels. Particularly, blocking of the T-type calcium channel

reportedly inhibits the activity of Rho kinase.²⁴ This study demonstrates that the local administration of BD facilitates epithelial wound healing as early as 3 days after tooth extraction. These findings are persistent with our *in vitro* findings that the migration and proliferation of NIH3T3 and GE1 cells are accelerated by BD. Cell migration is promoted by inhibiting the Rho-ROCK (rho-associated kinase)-myosin signaling.²⁵ These findings prove that BD affects the activation of epithelial proliferation and migration and subsequent wound healing after tooth extraction.

In view of calcium channels other than T-type, the blocking of N-type calcium channels, which is also conducted by BD, is known to inhibit adrenergic receptors.^{26,27} Both osteoblasts and osteoclasts possess adrenergic receptors, and adrenergic stimulation from sympathetic nerves enhances bone resorption.^{28–30}

The region of connective tissue around the extraction socket showed no significant differences between the groups on day 28. Some calcium

Figure 5. Scratch assay after incubation for 12 h. Both NIH3T3 (**a**) and GE1 (**b**) showed migration promoted by BD at a dosage of 100 nM. Bar = 100 μ m ($n=5$). * $p<0.05$; ** $p<0.01$.

channel blockers are known to have a side effect of gingival overgrowth by fibroblastic collagen overproduction; however, this finding was not observed in this study. This is consistent with a previous study reporting that BD does not have the side effect of gingival overgrowth.³¹

This study demonstrated that 1.0 or 3.0 mg/kg BD-impregnated PLGA microcarriers promote bone density, bone volume, and vertical bone height of the extraction socket. As mentioned previously, BD inhibits Rho-ROCK-myosin signaling. Another ROCK inhibitor, Y-27632, reportedly increases cell movement into bone formation sites, resulting in enhanced osteogenesis.²⁵ Furthermore, Y-27632 enhances BMP-4 production.³² With regard to gene expression of MC3T3-E1, six

genes were statistically significantly upregulated. Especially, the expression changes of two genes were greater than fourfold. Fetuin, a gene product of *Ahsg* that is enhanced by BD expression in this study reportedly combines with calcium phosphate to form calciprotein particles and promotes bone mineralization.³³ Similarly, BD upregulates colony-stimulating factor 2 (CSF-2) expression. CSF-2 is known to inhibit differentiation of osteoclast precursors into osteoclasts.³⁴

Cell proliferation is a key attribute of the bone healing process. In this study, the promotional effect of BD on MC3T3-E1 proliferation is observed. A previous study also demonstrated that BD promoted MC3T3-E1 proliferation at concentrations from 1×10^{-6} to 1×10^{-9} M.⁶ The results in this study are consistent with this finding. Similarly, BD promoted MLO-Y4 proliferation. This finding has not been previously reported. Osteoblasts are known to possess L-type calcium channels, and T-type calcium channels are expressed on osteocytes but not osteoblasts.²⁸ However, other studies reported that T-type calcium channels were expressed in osteoblasts during skeletal development.^{29,30} Our results indicate that BD may also act on osteocytes that are abundant in bone tissue.

In this study, a novel drug-delivery microcarrier for sustained release of BD has been developed

Table 1. Expressions of 84 osteogenesis-associated genes

Gene	Fold Change	p
<i>Csf2</i>	7.37	0.037
<i>Ahsg</i>	4.42	0.015
<i>Nog</i>	2.49	0.043
<i>Sost</i>	1.53	0.003
<i>Bmp5</i>	1.50	0.037
<i>Mmp10</i>	1.29	0.002

The gene expressions of benidipine-administered MC3T3-E1 were compared with those of the control. Genes whose expression statistically significantly changed are shown. No downregulated genes were indicated ($n=3$).

using PLGA. This is expected to facilitate a long-lasting effect, even with the minimal dose (*i.e.*, single transmucosal injection) at the socket. In our previous studies, an injectable fluvastatin-loaded PLGA microsphere suitable for local injection was developed, which had a long-term effect on bone formation. This DDS has been shown to release fluvastatin at a constant rate for >1 month.^{35,36} The method of fabricating microcarriers in this study is referenced in these preceding studies with a modification in the impregnation procedure of drugs into PLGA. This is because fluvastatin is water soluble, but BD is water insoluble. BD in this DDS demonstrates release at an invariable rate for >1 month. Consequently, despite the single injection of BD-impregnated PLGA microcarriers near the extraction socket, accelerated epithelial healing and considerable improvements in bone volume, bone density, and vertical bone height are observed on day 28 in this study.

The limitation of this study is that the results are not necessarily extrapolatable to humans. In addition, some cell lines used for culture study are nongingival or alveolar bone origin that has a possibility to bring misinterpretation. Moreover, BD is usually prescribed for oral dosage. Thus, local injection of BD with a long-lasting effect may give rise to unexpected side effects.

At present, a variety of methods have been used to promote socket healing; however, these methods present problems such as infection, difficulty in surgical skill, or insufficient clinical outcome. Thus, the PLGA and BD microsphere is expected to be a novel potent modality to improve the outcome of socket preservation with its concurrent enhancement characteristics of soft- and hard-tissue healing at the extraction site.

INNOVATION

Alveolar bone volume always decreased after the teeth extraction. The consequences of these physical soft- and hard-tissue changes greatly affect the clinical outcomes. This animal study demonstrates that a single transmucosal application of PLGA-BD microcarriers promotes healing of both bone and gingiva at the extraction socket. Culture study demonstrates that BD promotes proliferation and migration of bone tissue and oral soft-tissue cells through the enhancement of expression of some genes in osteoblasts that have metabolic effects on bone.

KEY FINDINGS

- BD promoted bone healing of extraction socket *in vivo*.
- BD upregulated messenger RNA expression of *Ahsg* and *Csf2* in osteoblasts.
- BD promoted gingival healing at the extraction socket *in vivo*.
- BD promoted the migration of epithelial cell and fibroblast.

ACKNOWLEDGMENTS AND FUNDING SOURCES

The authors thank Dr. Lynda Bonewald and Dr. Yoichi Kato for the kind gift of MLO-Y4 cells. This work was supported by a KAKENHI Grant (No. JP24249089) to K.K. from the Japan Society for the Promotion of Science.

AUTHOR DISCLOSURE AND GHOSTWRITING

All authors declare no competing financial interests exist. The authors listed expressly wrote the content of this article. No ghostwriters were used to write this article.

ABOUT THE AUTHORS

Mikio Imai, DDS, Naomi Adachi, DDS, Jiangqi Hu, DDS, and Xudiyang Zhou, DDS, are students of Graduate School of Dental Science, Kyushu University. **Yasunori Ayukawa, DDS, PhD**, is an associate professor; **Noriyuki Yasunami, DDS, PhD, Akihiro Furuhashi, DDS, PhD, Yasuko Moriyama, DDS, PhD**, are assistant professors; **Yoko Takemura DDS, PhD**, is a resident; **Ikiru Atsuta, DDS, PhD**, is a lecturer; and **Kiyoshi Koyano, DDS, PhD**, is a professor and chairperson of Section of Implant and Rehabilitative Dentistry, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University. They focus on the regeneration of soft and hard tissue of orofacial region. **Kosaku Kurata, PhD**, is an associate professor of Department of Mechanical Engineering, Faculty of Engineering, Kyushu University. His field of specialization is bioengineering and biothermal engineering. In this study he played a crucial role in cell culture, especially in culture of MLO-Y4.

REFERENCES

- Ogihara T, Matsuzaki M, Matsuo H, et al. The combination therapy of hypertension to prevent cardiovascular events (COPE) trial: rationale and design. *Hypertens Res* 2005;28:331–338.
- Nishizawa T, Cheng XW, Jin Z, et al. Ca(2+) channel blocker benidipine promotes coronary angiogenesis and reduces both left-ventricular diastolic stiffness and mortality in hypertensive rats. *J Hypertens* 2010;28:1515–1526.
- Matsuzaki M, Ogihara T, Umemoto S, et al. Prevention of cardiovascular events with calcium channel blocker-based combination therapies in patients with hypertension: a randomized controlled trial. *J Hypertens* 2011;29:1649–1659.
- Nishiya Y, Sugimoto S. Effects of various antihypertensive drugs on the function of osteoblast. *Biol Pharm Bull* 2001;24:628–633.
- Nishiya Y, Kosaka N, Uchii M, Sugimoto S. A potent 1,4-dihydropyridine L-type calcium channel blocker, benidipine, promotes osteoblast differentiation. *Calcif Tissue Int* 2002;70:30–39.
- Wang B, Bi M, Zhu Z, Wu L, Wang J. Effects of the antihypertensive drug benidipine on osteoblast function in vitro. *Exp Ther Med* 2014;7:649–653.
- Shimizu H, Nakagami H, Yasumasa N, et al. Links between hypertension and osteoporosis: benidipine ameliorates osteoporosis in ovariectomized hypertensive rats through promotion of osteoblast proliferation and inhibition of osteoclast differentiation. *Curr Cardiovasc Risk Rep* 2012;6:274–280.
- Ma ZP, Liao JC, Zhao C, Cai DZ. Effects of the 1, 4-dihydropyridine L-type calcium channel blocker benidipine on bone marrow stromal cells. *Cell Tissue Res* 2015;361:467–476.
- Nozawa T, Enomoto H, Tsurumaki S, Ito K. Biologic height-width ratio of the buccal supra-implant mucosa. *Eur J Esthet Dent* 2006;1:208–214.
- Schropp L, Wenzel A, Kostopoulos L, Karring T. Bone healing and soft tissue contour changes following single-tooth extraction: a clinical and radiographic 12-month prospective study. *Int J Periodontics Restorative Dent* 2003;23:313–323.
- Araujo MG, Lindhe J. Dimensional ridge alterations following tooth extraction. An experimental study in the dog. *J Clin Periodontol* 2005;32:212–218.
- Araujo MG, Lindhe J. Socket grafting with the use of autologous bone: an experimental study in the dog. *Clin Oral Implants Res* 2011;22:9–13.
- Zubillaga G, Von Hagen S, Simon BI, Deasy MJ. Changes in alveolar bone height and width following post-extraction ridge augmentation using a fixed bioabsorbable membrane and demineralized freeze-dried bone osteoinductive graft. *J Periodontol* 2003;74:965–975.
- Carmagnola D, Adriaens P, Berglundh T. Healing of human extraction sockets filled with Bio-Oss. *Clin Oral Implants Res* 2003;14:137–143.
- Araujo MG, Lindhe J. Ridge preservation with the use of Bio-Oss collagen: a 6-month study in the dog. *Clin Oral Implants Res* 2009;20:433–440.
- Sandor GK, Kainulainen VT, Queiroz JO, Carmichael RP, Oikarinen KS. Preservation of ridge dimensions following grafting with coral granules of 48 post-traumatic and post-extraction dento-alveolar defects. *Dent Traumatol* 2003;19:221–227.
- Lindhe J, Araujo MG, Buler M, Liljenberg B. Biphasic alloplastic graft used to preserve the dimension of the edentulous ridge: an experimental study in the dog. *Clin Oral Implants Res* 2013;24:1158–1163.
- Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). *Trends Biotechnol* 2009;27:158–167.
- Albanese A, Licata ME, Polizzi B, Campisi G. Platelet-rich plasma (PRP) in dental and oral surgery: from the wound healing to bone regeneration. *Immun Ageing* 2013;10:23.
- Ogino Y, Ayukawa Y, Tsukiyama Y, Koyano K. The effect of platelet-rich plasma on the cellular response of rat bone marrow cells in vitro. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2005;100:302–307.
- Ogino Y, Ayukawa Y, Kukita T, Koyano K. The contribution of platelet-derived growth factor, transforming growth factor-beta1, and insulin-like growth factor-I in platelet-rich plasma to the proliferation of osteoblast-like cells. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006;101:724–729.
- Cho SB, Nakanishi K, Kokubo T, Soga N, Ohtsuki C, Nakamura T. Apatite formation on silica gel in simulated body fluid: its dependence on structures of silica gels prepared in different media. *J Biomed Mater Res* 1996;33:145–151.
- Kurata K, Heino TJ, Higaki H, Vaananen HK. Bone marrow cell differentiation induced by mechanically damaged osteocytes in 3D gel-embedded culture. *J Bone Miner Res* 2006;21:616–625.
- Sugano N, Wakino S, Kanda T, et al. T-type calcium channel blockade as a therapeutic strategy against renal injury in rats with subtotal nephrectomy. *Kidney Int* 2008;73:826–834.
- Ichida M, Yui Y, Yoshioka K, et al. Changes in cell migration of mesenchymal cells during osteogenic differentiation. *FEBS Lett* 2011;585:4018–4024.
- Hayashi K, Wakino S, Sugano N, Ozawa Y, Homma K, Saruta T. Ca2+ channel subtypes and pharmacology in the kidney. *Circ Res* 2007;100:342–353.
- Shimizu H, Nakagami H, Yasumasa N, et al. Cilnidipine, but not amlodipine, ameliorates osteoporosis in ovariectomized hypertensive rats through inhibition of the N-type calcium channel. *Hypertens Res* 2012;35:77–81.
- Thompson WR, Majid AS, Czymmek KJ, et al. Association of the alpha(2)delta(1) subunit with Ca(v)3.2 enhances membrane expression and regulates mechanically induced ATP release in MLO-Y4 osteocytes. *J Bone Miner Res* 2011;26:2125–2139.
- Shao Y, Alicknavitch M, Farach-Carson MC. Expression of voltage sensitive calcium channel (VSCC) L-type Cav1.2 (alpha1C) and T-type Cav3.2 (alpha1H) subunits during mouse bone development. *Dev Dyn* 2005;234:54–62.
- Bergh JJ, Shao Y, Puente E, Duncan RL, Farach-Carson MC. Osteoblast Ca(2+) permeability and voltage-sensitive Ca(2+) channel expression is temporally regulated by 1,25-dihydroxyvitamin D(3). *Am J Physiol Cell Physiol* 2006;290:C822–831.
- Ono M. Incidence of gingival overgrowth caused by calcium channel blockers. *Jpn Soc Oral Ther Pharmacol* 2008;27:79–85.
- Yoshikawa H, Yoshioka K, Nakase T, Itoh K. Stimulation of ectopic bone formation in response to BMP-2 by Rho kinase inhibitor: a pilot study. *Clin Orthop Relat Res* 2009;467:3087–3095.
- Brylka L, Jahnen-Dechent W. The role of fetuin-A in physiological and pathological mineralization. *Calcif Tissue Int* 2013;93:355–364.
- Udagawa N, Horwood NJ, Elliott J, et al. Interleukin-18 (interferon-gamma-inducing factor) is produced by osteoblasts and acts via granulocyte/macrophage colony-stimulating factor and not via interferon-gamma to inhibit osteoclast formation. *J Exp Med* 1997;185:1005–1012.
- Yasunami N, Ayukawa Y, Furuhashi A, et al. Acceleration of hard and soft tissue healing in the oral cavity by a single transmucosal injection of fluvastatin-impregnated poly (lactic-co-glycolic acid) microspheres. An in vitro and rodent in vivo study. *Biomed Mater* 2015;11:015001.
- Masuzaki T, Ayukawa Y, Moriyama Y, et al. The effect of a single remote injection of statin-impregnated poly (lactic-co-glycolic acid) microspheres on osteogenesis around titanium implants in rat tibia. *Biomaterials* 2010;31:3327–3334.

Abbreviations and Acronyms

Ahsg	= alpha 2-HS glycoprotein
BD	= benidipine
BMP	= bone morphogenetic protein
BMSCs	= bone marrow stromal cells
CO ₂	= carbon dioxide
Csf2	= colony-stimulating factor 2
CT	= computed tomography
DDS	= drug-delivery system
DMSO	= dimethyl sulfoxide
FBS	= fetal bovine serum
MEM	= minimum essential medium
OCN	= osteocalcin
PL-BD	= poly(lactic-co-glycolic acid) with benidipine
PLGA	= poly(lactic-co-glycolic acid)
RANKL	= receptor activator of nuclear factor kappa-B ligand
ROCK	= rho-associated kinase
RUNX2	= runt-related transcription factor-2
SBF	= simulated body fluid