



LOCALLY DELIVERED ETHYL-2,5-DIHYDROXYBENZOATE USING 3D PRINTED BONE IMPLANT FOR PROMOTION OF BONE REGENERATION IN A OSTEOPOROTIC ANIMAL MODEL

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Abstract

Osteoporosis is a disease characterized by low bone mass, most commonly caused by an increase in bone resorption that is not matched by sufficient bone formation. The most common complications of postmenopausal osteoporosis are bone-related defects and fractures. Fracture healing is a multifactorial bone regeneration process, influenced by both biological and mechanical factors related to age, osteoporosis and stability of the osteosynthesis. During the treatment of bone defects in osteoporotic conditions, imbalanced bone remodeling is the leading cause for implant failure. To overcome these problems, ethyl-2,5-dihydroxybenzoate (E-2,5-DHB), a drug that promotes bone formation and inhibits bone resorption, was used. E-2,5-DHB-incorporating titanium (Ti) implants using poly(lactic-co-glycolic acid) (PLGA) coating for local delivery of E-2,5-DHB were developed and the effects on bone healing of femoral defects were evaluated in an osteoporotic model. The release of E-2,5-DHB resulted in decreased bone resorption and increased bone formation around the implant. Thus, it was confirmed that, in the osteoporotic model, bone healing was increased and implant fixation was enhanced. These results suggested that E-2,5-DHB-coated Ti implants have great potential as an ultimate local drug delivery system for bone tissue scaffolds.

Keywords: Osteoporosis, bone formation, bone resorption, bone fracture healing, ethyl-2,5-dihydroxybenzoate.

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Introduction

Osteoporosis is a global emerging healthcare issue and a socio-economic problem characterized by reduced bone mass, weak bone strength and microarchitectural aggravation in the cortical and trabecular skeleton. These features lead to skeletal fragility and sensitivity to fractures. Osteoporosis is an age-related disease caused by a disproportion between osteogenic bone formation and osteoclastic bone resorption, which is often the result of postmenopausal estrogen deficiency (Pei and Tontoz, 2004; Rodan and Martin, 2000).

In recent years, fracture healing has been widely studied, but less attention has been placed on osteoporotic fractures. Since osteoporosis-related fractures generally occur at long bone sites subjected to loading stress, mechanical intervention may affect the healing process. A few studies report the

therapeutic effect of local implantation of biomimetic scaffolds during osteoporotic defect regeneration (Cheng *et al.*, 2013; Zhang *et al.*, 2012). Fractures of osteoporotic bones present diverse challenges, including a large proportion of fixation failure and impaired healing capability, which make their medical management challenging (Kyllönen *et al.*, 2015). Osteoporotic bone fracture, not implant breakage, is the primary cause of failure of internal fixation. Because bone mineral density associates with the ability to hold screws, osteoporotic bone often lacks the strength to hold plates and screws securely (Alho, 1993; Sjostedt *et al.*, 1994; Strømsøe *et al.*, 1993). The major challenges in the treatment of osteoporotic fractures are stable internal fixation, without implant loosening, and subsequent successful bone healing. However, alterations in bone turnover, bone tissue quality and delays in fracture healing are some of the well-known key issues that must be addressed

by clinicians and researchers to improve treatment outcomes for patients with osteoporotic fractures (Barrios *et al.*, 1993; Gardner, 2004).

The discovery of osteopromotive and inductive agents, with the potential to stimulate bone formation, has raised hope for the augmentation of osteoporotic fracture healing. Local release of a bone anabolic agent at the site of an osteoporotic fracture would potentially facilitate a rapid increase in bone strength and quality, as well as reduce the bone healing period and prevent the development of problematic non-union. Local delivery is expected to increase the utility and efficacy of bone anabolic or antiresorptive drugs and decrease adverse effects related to their systemic administration.

Ethyl-2,5-dihydroxybenzoate (E-2,5-DHB) is a derivative of ethyl-3,4-dihydroxybenzoate (E-3,4-DHB), which is an element of the *Rubus coreanus* extract. *Rubus coreanus* total extract is a natural bioactive substance with bone-protective effects during postmenopausal osteoporosis in ovariectomized rats (Do *et al.*, 2008). A natural bioactive substance, it has been considered as a biomedical material due to its good biocompatibility and various bio-active characteristics. E-3,4-DHB also improves osteoinductive activity by stimulating osteoblast differentiation and reduces bone resorption by inhibiting osteoclast differentiation (Kwon *et al.*, 2014). However, the effect of E-2,5-DHB on bone healing in osteoporotic conditions has yet to be clearly defined. Therefore, it was hypothesized that E-2,5-DHB, with its dual effects on osteoblasts and osteoclasts, would promote bone defect healing in an osteoporotic model.

Titanium (Ti) and its alloys are extensively used as implant materials in the orthopedic and dental fields because of their excellent biocompatibility and mechanical properties (Long *et al.*, 1998). Ti facilitates new bone formation and provides long-lasting bone-implant stability (Ravichandran *et al.*, 2012). Ti and its alloys are widely used as hard tissue replacements in artificial bones, joints and dental implants. As these types of replacements, their low elastic modulus is generally viewed as a biomechanical advantage because they can produce smaller stress shielding (Sáenz de Viteri and Fuentes, 2013). If

several interconnected pores are introduced into Ti and its alloys, their elastic modulus decreases to a level similar to that of cancellous bone (Pattanayak *et al.*, 2011). Porous bone scaffolds can be made by a variety of methods and selective laser melting (SLM) processes are used to produce porous bone substitutes (Mullen *et al.*, 2009; Stamp *et al.*, 2009; Thijs *et al.*, 2010).

Our previous study reports whether E-2,5-DHB can be an effective material and whether it plays a role in inducing osteoblast differentiation and inhibiting osteoclast differentiation (Kwon *et al.*, 2016). In the treatment of bone defects in osteoporotic conditions, imbalanced bone remodeling is the leading cause of implant failure. To overcome these problems, E-2,5-DHB, a drug that promotes bone formation and inhibits bone resorption, was used. In this study, an E-2,5-DHB-incorporating Ti implant using poly(lactic-co-glycolic acid) (PLGA) coating for local delivery of E-2,5-DHB was developed and its effects on bone healing of femoral defects in an osteoporotic rat model were evaluated.

Materials and Methods

Materials

The PLGA polymer (polylactic acid/polyglycolic acid = 50/50) was obtained from Evonik (Birmingham, AL, USA). Acetone was obtained from Duksan Pure Chemicals Co., Ltd (Kyungki Do, Korea). E-2,5-DHB was supplied by Cell Safe Ltd. (Kyungki Do, Korea). MC3T3-E1 mouse preosteoblast cells were purchased from Riken BRC cell bank (Tokyo, Japan). α -modification minimum essential medium (α -MEM) and fetal bovine serum (FBS) were obtained from WelGENE (Daegu, Korea). Bradford assay kit was purchased from Bio-Rad (Hercules, CA, USA).

Three-dimensional printed porous titanium implants containing E-2,5-DHB

Three-dimensional (3D) printed porous Ti implants were produced by a powder bed fusion (PBF) metal 3D printer (SLM system: Metasys250; Winforsys, Kyungki Do, Korea) using Ti powder (Ti-6Al-4V; LPW-Ti64Gd5; LPW Technology Ltd., Sci-Tech,

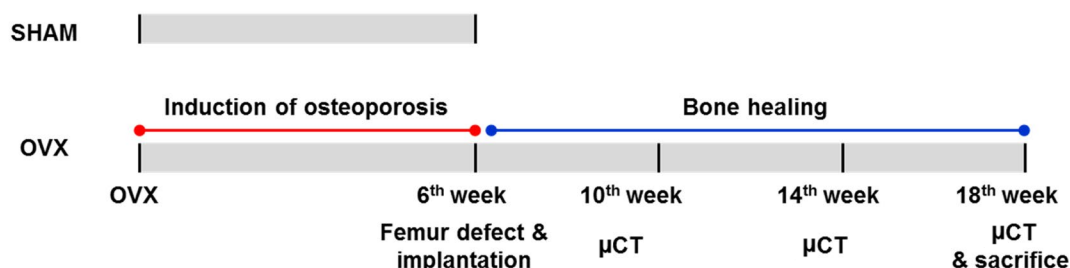


Fig. 1. Schematic diagram indicating the surgery time points for rats in the sham surgery (SHAM) and ovariectomy (OVX) groups. 6 weeks after ovariectomy, establishment of the osteoporotic model was confirmed in the OVX rats, using SHAM rats as control. Also at 6 weeks, femur defect drilling was performed in the OVX rats, followed by implantation with 3D printed Ti implants. After 4, 8 and 12 weeks, OVX rats were sacrificed and femur specimens were removed for assessment.

Table 1. Ethyl-2,5-dihydroxybenzoate (E-2,5-DHB) concentration in the E-2,5-DHB-incorporating PLGA-coated titanium implants. Data are shown as mean \pm SD.

	Initial E-2,5-DHB concentration (mg)	
	1	2
Measured concentration (mg)	1.1 \pm 0.7	1.9 \pm 2.2

Daresbury, UK). The Ti implants were manufactured using an SLM system (Metasys250; Winforsys). This system contains a Yb fiber laser beam with a nominal diameter of 70 to 150 μ m and maximum power of 150 W. Cylindrical specimens of 3 mm in diameter and height were manufactured. The porous Ti implants were solvent-cleansed by serial ultrasonic treatment in acetone, ethyl alcohol and de-ionized water. The 3D printed porous Ti implants were sterilized before coating by incubation in 70 % (v/v) ethanol for 30 min and then washed 5 times with distilled water. PLGA was dissolved in acetone to prepare 15 % PLGA-acetone solutions. To accelerate its dissolution in acetone, PLGA was stirred for 1 h. E-2,5-DHB in acetone was incorporated into the PLGA solution before coating. 3D printed porous Ti implants were immersed in the PLGA-acetone-E-2,5-DHB solution for 5 min to force the solution into the porous structure. Then, the PLGA-coated 3D Ti implants were dried for 5 d to remove the residual acetone.

Characterization of the E-2,5-DHB-incorporating PLGA-coated titanium implants

Surface morphology of the Ti implants was observed with a field emission scanning electron microscope (FE-SEM S-800; Hitachi, Tokyo, Japan) using an accelerating voltage of 20 kV, as well as by micro-computed tomography (μ CT) (NFR Polaris-G90; NanoFocus Ray Co., Jeonju, Korea).

E-2,5-DHB loading capacity was determined by measuring the concentration of coated E-2,5-DHB. The E-2,5-DHB-incorporating PLGA-coated Ti implants were immersed in DMSO. After vortexing, the solution was measured spectrophotometrically at 338 nm. To determine the release kinetics of E-2,5-DHB from the Ti implants, the E-2,5-DHB-incorporating PLGA-coated Ti implants were immersed in 1 mL of phosphate-buffered saline (PBS) solution at 37 °C. At predetermined time intervals of 1, 3 and 5 h and 1, 3, 7, 14, 21 and 28 d, the supernatant was collected and replaced with fresh PBS solution. The amount of E-2,5-DHB released in the collected solution was measured spectrophotometrically at 240 nm.

In vivo bone healing in an osteoporotic animal model using 3D printed titanium implants coated with PLGA incorporated with E-2,5-DHB

All animal experiments were performed in accordance with the Korean Food and Drug Administration guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee

of the Yonsei Laboratory Animal Research Center (Permit No. 2015-0120). All animals were kept at 20–26 °C under a 12 h light/dark cycle and allowed food and water *ad libitum*.

20 female Sprague-Dawley rats (3 months old), which underwent several procedures during the course of the experiment (Fig. 1), were acclimatized for 1 week before the experiments. The animals were anesthetized by an intramuscular injection of Zoletil (30 mg/kg; MNS Korea, Kyungki Do, Korea) and Rompun (10 mg/kg; MNS Korea). The abdominal cavity was accessed by a 20 mm midline dorsal skin incision, which allowed for blunt dissection of the connective tissue between the skin and the muscular layer of the abdominal wall. The ovary was located in a fat pad and gently pulled outside the abdominal cavity. Two ligatures were placed with absorbable 4-0 sutures. The ovaries were cut and removed and the uterine horns were placed back into the abdominal cavity. The abdominal muscles were closed with absorbable 4-0 sutures using a continuous suture technique, after which the skin was sutured with multiple single absorbable sutures. The animals were administered 1 mg/kg meloxicam and 5 mg/kg gentamicin subcutaneously for postoperative analgesia and infection prophylaxis, respectively. The SHAM-operated group underwent the same surgical procedure as the ovariectomy (OVX) group, except their ovaries were not removed. After 6 weeks, all animals were assessed for total body bone mineral density (BMD) using dual-energy X-ray absorptiometry (DXA; InAlyzer, Medikors, Seongnam, Korea) and μ CT (NFR Polaris-G90, NanoFocus Ray Co.) to confirm the usefulness of the OVX group as a model of osteoporosis. The animals were also weighed weekly.

Six weeks after the OVX or SHAM surgery, a femoral defect was created. The rats were divided into the following five groups: SHAM (sham-operation), OVX, OVX + Ti, OVX + Ti (1 mg) and OVX + Ti (2 mg). The OVX + Ti group received a Ti implant with only PLGA-coating, whereas the OVX + Ti (1 mg) and OVX + Ti (2 mg) groups received a Ti implant with PLGA-coating containing 1 mg and 2 mg of E-2,5-DHB, respectively.

The animals were anesthetized by an intramuscular injection of Zoletil and Rompun. Both hind limbs of the rats were shaved. A longitudinal incision through the skin and the muscle was made on the medial surface of the femur. After exposure of the medial side of the distal femur, a bone defect 3 mm in diameter and 3 mm in depth was created perpendicular to the bone's surface. To create the defect, a dental drill

(Marathon-N1, Saeyang Co., Daegu, Korea) was used with constant saline cooling. The defects were irrigated with normal saline to remove bone residue. Then, the drilled cavity was washed with saline and dried using a sterile cotton gauze. Defects were made bilaterally and transplants were randomized. The defect was either left empty (as the control) or the 3D printed porous Ti implants were positioned within the cavity. The subcutaneous tissue layer was closed with absorbable 4-0 sutures, after which the skin was closed. Finally, the defects were covered by returning the quadriceps to their original position and repositioning the patella.

Dual-energy x-ray absorptiometry (DXA)

Bone mineral content (BMC) and BMD of the femur were assessed by DXA. All femora were evaluated 6 weeks after the ovariectomy.

μ CT analysis

At 4, 8 and 12 weeks following transplantation of Ti implants containing E-2,5-DHB into the OVX rats, the operated animals were scanned using a high resolution μ CT imaging system. The animals were placed in a supine position onto the scan stage and sedated by inhalation of 2 % isoflurane. The following μ CT settings were used: resolution 39 μ m, tube voltage 65 kV, tube current 115 μ A and exposure time 34 ms for each of the 360° rotational steps. The reconstruction image size was 1024 × 1024 pixels and the number of slices was 512. 3D models of the trabecular bones of the femur were reconstructed using the Lucion software (Mevisys, Seoul, Korea) to evaluate bone alterations. The μ CT sagittal section through the midline of the defect was selected to measure each defect.

Histological analysis

Histology was performed on the femora of each group of animals to study the bone-Ti interface and bone morphology. After harvesting the femora and removing the surrounding soft tissues, the bones were fixed in 10 % neutral-buffered formalin solution, dehydrated in graded ethanol solutions ranging from 70 % to 100 % and then embedded in Technovit 7200 methacrylate resin (Heraeus Kulzer, Bad Sachsa, Germany). Sections of 40 ± 5 μ m were obtained using an EXAKT diamond cutting system (EXAKT 300 CP, Bamberg, Germany) and stained with Goldner's trichrome to study mineralization. The stained sections were observed using an optical microscope (BX51, Olympus, Tokyo, Japan).

Mechanism of osteoclast differentiation inhibition by E-2,5-DHB: western blot

MC3T3-E1 mouse preosteoblast cells were maintained in a α -MEM without ascorbic acid, supplemented with 10 % FBS and 1 % antibiotic and antimycotic solution. For all experiments, cells were used up to the 10th passage. They were grown at 37 °C in a 5 %

carbon dioxide atmosphere incubator. To evaluate osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B ligand (RANKL) expression, MC3T3-E1 cells were seeded onto 6-well plates at 2 × 10⁵ cells/well density and cultured in the presence or absence of E-2,5-DHB at 5 and 10 μ g/mL. After being cultured for 7 d, the cells were extracted by adding ice-cold radioimmunoprecipitation assay (RIPA) buffer. The cell lysates were centrifuged at 14,000 ×g for 15 min at 4 °C. Protein determination was performed by Bradford assay according to the manufacturer's protocol. Protein samples were separated by electrophoresis using a 12.5 % sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and then transferred onto polyvinylidene difluoride membranes. The membranes were incubated overnight at 4 °C with a rabbit polyclonal anti-OPG antibody (1 : 1000 dilution; ab183910, Abcam, Cambridge, UK), a mouse monoclonal anti-RANKL antibody (2 μ g/mL; MAB6263, R&D system, Minneapolis, MN, USA) or a rabbit polyclonal anti- β -actin antibody (1 : 1000 dilution; 4967S, Cell Signaling, Danvers, MA, USA). Finally, the membranes were incubated with a horseradish-peroxidase-conjugated secondary antibody [anti-mouse IgG (1 : 2000; 7076S, Cell Signaling) or anti-rabbit IgG (1 : 2000; 7074P2, Cell Signaling)]. Protein expression was detected by enhanced chemiluminescence (Cell Signaling). All bands were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All results are expressed as the mean ± standard deviation (SD) of at least three determinations. Statistical analysis was performed by analysis of variance (ANOVA), using SPSS software (SPSS Inc. Chicago, IL, USA). Statistically significant values were defined as $p < 0.05$.

Results

Characterization of titanium implants containing E-2,5-DHB

To determine the loading efficacy of E-2,5-DHB, the concentration of E-2,5-DHB coating the Ti implants was measured (Table 1). Ti implants containing E-2,5-DHB were loaded with different amounts of E-2,5-DHB. The loading concentrations of E-2,5-DHB for the 1 mg and 2 mg implants were 1.1 mg and 1.9 mg, respectively.

The surface morphology of the Ti implants was observed after PLGA coating (Fig. 2a). The prepared Ti implants had porous structures with open micropores ranging from 200 to 250 μ m. After being coated with PLGA, the Ti implants still maintained a large pore structure. Scanning electron microscopy (SEM) images of the Ti implants revealed that their pore walls exhibited a surface with regular micropores. After being coated by PLGA,

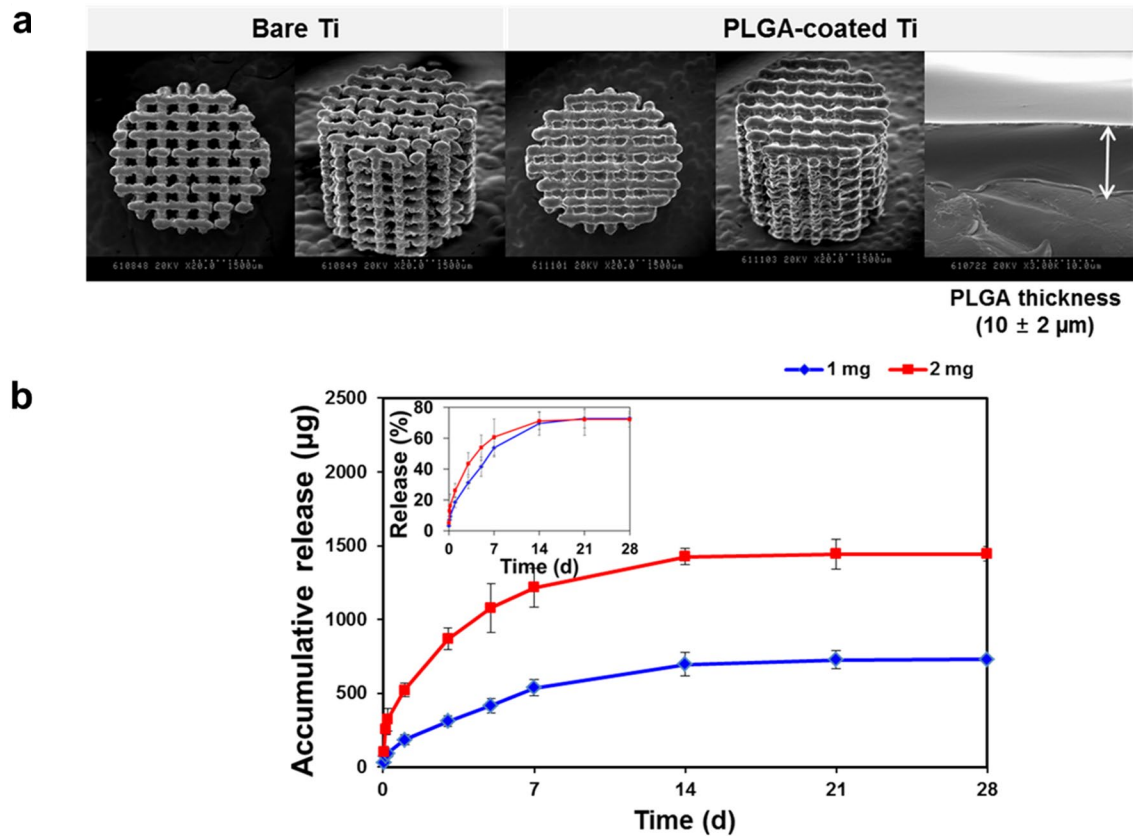


Fig. 2. Characterization of E-2,5-DHB-incorporating PLGA-coated Ti implants. **(a)** SEM images of Ti-only implants and PLGA-coated Ti implants. **(b)** E-2,5-DHB release kinetic from E-2,5-DHB-incorporating PLGA-coated Ti implants in PBS at 37 °C. The data are presented as mean \pm SD ($n = 4$).

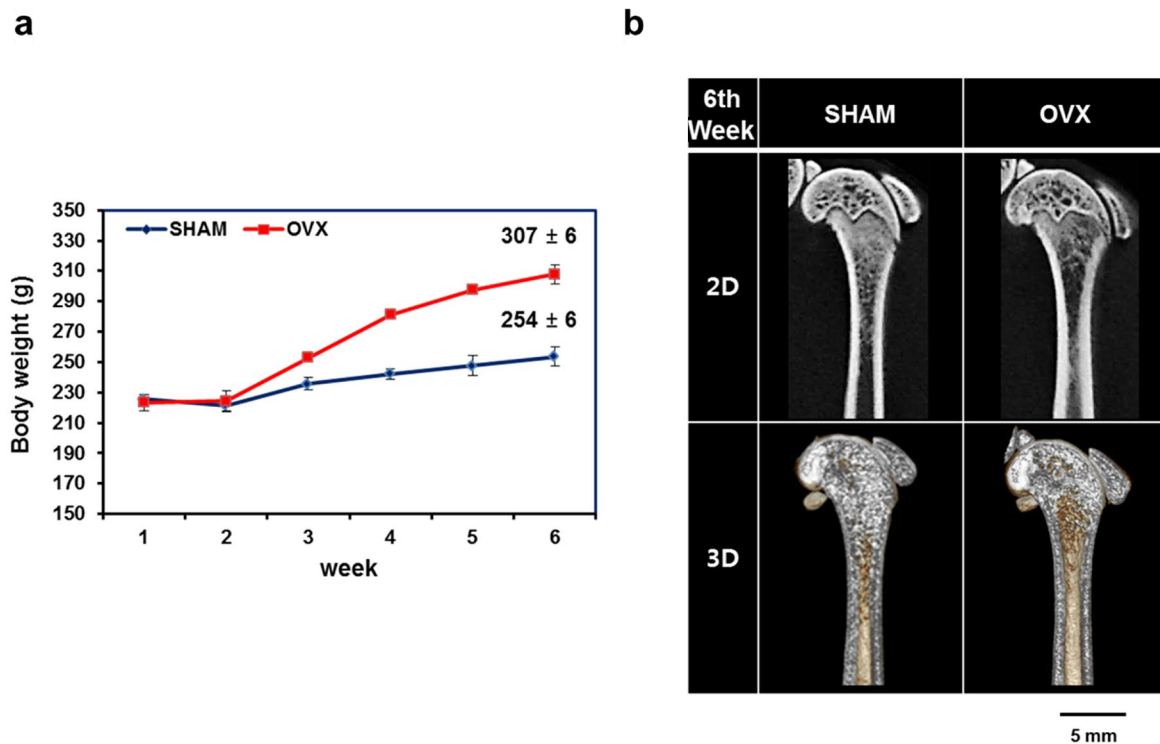


Fig. 3. Confirmation of the rat osteoporotic model. **(a)** The body weight of OVX rats was significantly larger than that of the SHAM control rats. **(b)** μCT : 2-dimensional (2D) and 3D images of distal femora from SHAM and OVX rats (scale bar = 500 μm) at 6 weeks.

Table 2. Confirmation of the rat osteoporotic model. Femur BMC and BMD were significantly lower in the OVX group compared to the SHAM group. Data are shown as mean \pm SD.

	SHAM	OVX
BMC (g)	0.107 \pm 0.008	0.101 \pm 0.017
BMD (g/cm ³)	0.374 \pm 0.025	0.347 \pm 0.015

the Ti implants still maintained this pore structure. The coated-PLGA thickness was approximately 10 ± 2 μ m. Release profiles obtained after the release of E-2,5-DHB from E-2,5-DHB-incorporating PLGA-coated Ti implants are shown in Fig. 2b. At day 7, the amount of E-2,5-DHB released from the implants containing 1 and 2 mg of E-2,5-DHB was 54 % and 61 %, respectively. E-2,5-DHB released from the E-2,5-DHB-incorporating PLGA-coated Ti implants exhibited a burst release, with most of the drug being released within the first 7 d. Over the 28 d period, 70 % of the E-2,5-DHB was released from both the 1 and 2 mg E-2,5-DHB-incorporating PLGA-coated Ti implants, resulting in a sustained release profile.

Characterization of the osteoporosis animal model

Animals in the sham-operated group showed a steady increase in body weight during the 6 weeks observation period (Fig. 3a). Following ovariectomy, OVX rats weighed significantly more than SHAM rats at the evaluated time intervals. Initial body weights (221 ± 6 g) were similar among all rats. At the end of the 6 weeks period of osteoporosis induction, the final body weight was significantly higher in OVX rats (307 ± 6 g) than in SHAM rats (254 ± 6 g) ($p < 0.01$).

6 weeks after the initial surgery, the mean femoral BMC was significantly lower in the OVX group than in the SHAM group: 0.101 ± 0.017 g and 0.107 ± 0.008 g, respectively ($p < 0.05$) (Table 2). Likewise, the mean BMD of the femur was significantly lower in the OVX group (0.347 ± 0.015 g/cm³) than in the SHAM group (0.374 ± 0.025 g/cm³) ($p < 0.05$).

μ CT analysis revealed structural differences between the OVX and SHAM groups with respect to the trabecular architecture of the femur (Fig. 3b).

Bone healing of femoral defects in ovariectomy rats

μ CT images were acquired to analyze the morphology and mineralization of new bone formation within the femoral defects. As shown in Fig. 4a (representative longitudinal μ CT images), in the E-2,5-DHB group, newly formed bone was more than in the control group. This confirmed healing of the defects in the presence of E-2,5-DHB. Much less mineralized tissue was present in the untreated defects. A minimal amount of new bone formation was found in the only-PLGA-coated Ti group at all time intervals considered. The E-2,5-DHB-coated Ti groups exhibited the largest amounts of mineralized bone.

The bone newly formed in the Ti implant area after 3 months was assessed by PACS software, which

measures bone density in Hounsfield units (HU) (Fig. 4b). Values obtained from μ CT scans taken 4, 8 and 12 weeks postoperatively were compared and revealed the type of bone formed in the vicinity of the implant. Radiological evaluations using μ CT scans showed a substantial increase in bone volume in the regions implanted with E-2,5-DHB after 12 weeks. The bone formed around the implant was more bone regeneration, with higher HU values. The enhancement of bone formation 8 and 12 weeks postoperatively in the E-2,5-DHB-coated Ti groups was superior (with higher HU values) compared to the only-PLGA-coated Ti group. HU values increased by 167 % and 144 % at 8 weeks and 12 weeks, respectively, in the E-2,5-DHB (1 mg) group and 154 % and 144 % in the E-2,5-DHB (2 mg) group. In the only-PLGA-coated group, the volume of bone formed was less compared to the successfully implanted E-2,5-DHB-coated group areas, with low HU values and signifying poor quality of formed bone. Moreover, as shown in Fig. 4c, no defect healing was observed in the defect area of osteoporotic rats during the transplantation period.

Histology analysis

Histologic findings (Goldner's trichrome staining) at the fracture sites supported the biomechanical evidence of accelerated defect healing by the released E-2,5-DHB (Fig. 5). Areas of newly formed bone, stained blue, differed considerably and only-PLGA-coated Ti implants showed less bone than E-2,5-DHB-coated Ti implants (1 mg and 2 mg). Bone formation inside the porous space was mainly located at distal sites and resulted in direct bone-Ti contact. Direct bone-Ti contact was found throughout the entire bone-Ti interface. Trabecular bone areas around the E-2,5-DHB-coated Ti implants in animals were connected and more newly formed bone was seen in direct contact with the E-2,5-DHB coated surface compared with the only-PLGA-coated control implants.

Infiltration of newly formed bone was observed inside the pores filled with newly formed bone and within the Ti implants (Fig. 6a). Image J analysis showed that the result was not statistically significant, but the area of positive staining in animals with E-2,5-DHB coated Ti implants was large. Quantification of the Goldner's trichrome staining images revealed differences between the only-PLGA-coated Ti group and the E-2,5-DHB-coated Ti groups, as the defects revealed less staining in the only-PLGA-coated control animals. The amount of bone around only-PLGA-coated Ti implants in osteoporotic rats

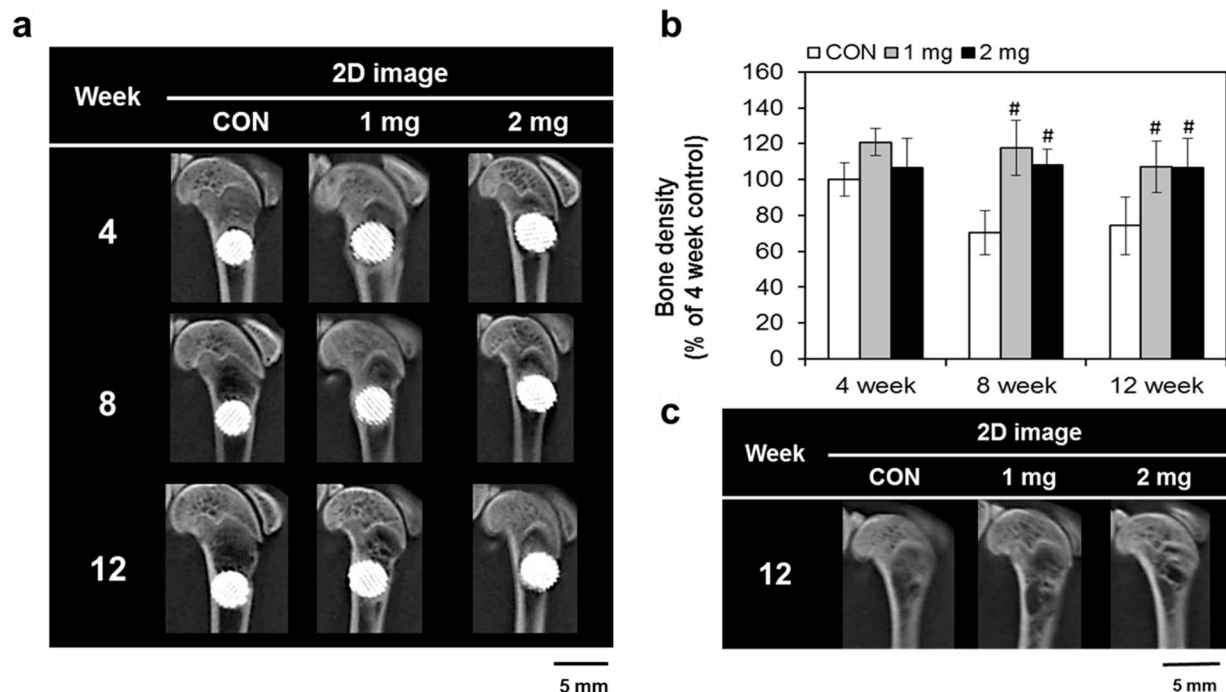


Fig. 4. Bone healing of femoral defects in OVX rats. (a) Representative μ CT images of the distal femur showing mineralized bone formation within defects in the control (CON) group, titanium (Ti) + 1 mg E-2,5-DHB group and Ti + 2 mg E-2,5-DHB group, 12 weeks after surgery. The CON group received a Ti implant coated with only PLGA. Scale bar = 5 mm. (b) Quality of bone formed in the Ti implant area 3 months postoperatively was assessed by PACS software (Piviewer, INFINITT Healthcare, Seoul, Korea), which measures bone density in Hounsfield units. Data are shown as mean \pm SD (* p < 0.05 compared with the Ti group). (c) Representative μ CT images of the distal femur showing mineralized bone formation within empty defect at 12 weeks. Scale bar = 5 mm.

was smaller with respect to the E-2,5-DHB-coated groups (Fig. 6b). Statistically, the positive staining area percentage was significantly larger in the E-2,5-DHB-coated Ti group than in the only-PLGA-coated Ti group.

Inhibition of osteoclast differentiation by E-2,5-DHB

E-2,5-DHB treatment inhibits osteoclast differentiation (Kwon *et al.*, 2016). To investigate the mechanism of inhibition of osteoclast differentiation, OPG and RANKL expression were measured. The concerted actions of OPG, receptor activator of nuclear factor- κ B (RANK), and RANKL play a central role in maintaining the delicate balance between bone resorption and formation and in preserving bone mass (Boyle *et al.*, 2013; Khosla *et al.*, 2001; Hofbauer and Schoppet, 2004). To investigate the effect of E-2,5-DHB on OPG and RANKL expression, which are synthesized by osteoblasts, MC3T3-E1 cells were treated with E-2,5-DHB and OPG and RANKL protein levels were measured by western blot analysis (Fig. 7). The results indicated that expression of RANKL at the protein level was downregulated by E-2,5-DHB, whereas OPG levels were significantly upregulated. These results suggested that E-2,5-DHB (5 and 10 μ g/mL) was more effective in stimulating the secretion of OPG as opposed to RANKL, thus exerting inhibitory effects on osteoclast differentiation.

Discussion

The aim of this study to investigate the effect of E-2,5-DHB on bone defects healing in osteoporotic conditions. The results confirmed the ability of E-2,5-DHB-incorporating Ti implants using PLGA coating for local delivery of E-2,5-DHB to enhance fracture healing in osteoporotic bones.

Using conventional fabrication techniques, it is difficult to adjust the internal pore size, geometry and distribution (Pattanayak *et al.*, 2009; Yang *et al.*, 2000). In contrast, the SLM process is an effective technique for fabricating 3D porous structures directly from metal powders. Consequently, the process is suitable for designing artificial bone substitutes with complex inner and outer geometries that will fit a patient's defective bone. In this study, 3D printed Ti implants with pore sizes of 200 to 250 μ m were prepared. These implants were coated with a PLGA surface layer. PLGA coating did not decrease the pore size of the Ti implants. The small pore size benefits cell ingrowth and enhances diffusion of nutrients into the 3D scaffolds (Barou *et al.*, 2002). Our results suggested that PLGA modification was a viable method to improve the physicochemical and drug-delivery properties of the Ti implants.

BMC and BMD at the femur site of OVX rats were shown to be decreased at week 6 post-surgery, when compared to the SHAM group. μ CT images

of the OVX rats confirmed a decrease in trabecular bone density, volume and thickness. OVX rats are commonly accepted as a suitable preclinical animal model for human postmenopausal osteoporosis (Jee and Yao, 2001; Lelovas *et al.*, 2008; Turner, 2001). Similarly to previously published studies (Jee and Yao, 2001; Kalu, 1991; Lelovas *et al.*, 2008; Turner, 2001), our results indicated that over the course of the experiment, OVX rats presented most of the characteristics observed in human postmenopausal osteoporosis. This included a significant decrease in trabecular bone thickness and mass and an increase in trabecular spacing, compared to healthy (SHAM group) animals.

Drugs can be administrated locally or systemically. Drugs delivered systemically are absorbed into the bloodstream and spread throughout the body by the circulatory system. This can lead to kidney and liver complications, lack of penetration into targeted tissues, systemic toxicity, as well as hospitalization of the patient. On the other hand, when the drug is delivered locally, the risk of side effects and overdosage, as for the systemic administration, may be limited and a high concentration of drug can effectively reach the target site (Price *et al.*, 1996; Ruszczak and Friess, 2003; Somayaji *et al.*, 1998). Local delivery is expected to increase the availability and efficacy of these bone anabolic or antiresorptive drugs and reduce the adverse effects associated with their tissue administration.

Reports of previous studies evaluate treatments to enhance new bone formation or suppress excessive bone resorption during bone regeneration, such as estrogen, parathyroid hormone (PTH), selective

estrogen receptor modulators, strontium ranelate, calcitonin, bisphosphonates, sclerostin antibodies and Dickkopf-1 antibodies (Agholme *et al.*, 2011; Cao *et al.*, 2002; Li *et al.*, 2007; Nozaka *et al.*, 2008). However, antiosteoporotic drugs (*i.e.* bisphosphonates and RANKL inhibitors) often reduce new bone formation because they disrupt the linkage between osteoclast and osteoblast activity. PTH is an anabolic drug that can directly stimulate bone formation and, currently, it is the only anabolic agent approved for osteoporosis treatment by the Food and Drug Administration (Tashjian and Gagel, 2006). However, the anabolic effects of PTH are usually related to enhanced bone resorption and withdrawal of PTH associates with loss of its therapeutic effects (Tanizawa *et al.*, 1998). Strontium ranelate is one of the agents reported to facilitate osteoporotic fracture healing, exhibiting the dual effects of promoting bone formation and inhibiting bone resorption (Bonnelye *et al.*, 2008; Goldberg *et al.*, 1985; Habermann *et al.*, 2010). Results of the current study showed that E-2,5-DHB, similar to strontium ranelate, could also be used as an effective agent to promote bone fracture healing in osteoporotic conditions.

No signs of foreign body reactions or inflammatory responses were observed in our rat model. Porous titanium implants have recently received increased attention as a new biomaterial that can act as an osteoconductive scaffold, providing direct mechanical support in large bone defects at weight-bearing sites (Murr *et al.*, 2010). However, they remain bioinert and, thereby, lack the ability to provide strong biological cues that enhance bone regeneration. This led to the hypothesis that E-2,5-DHB incorporated into porous

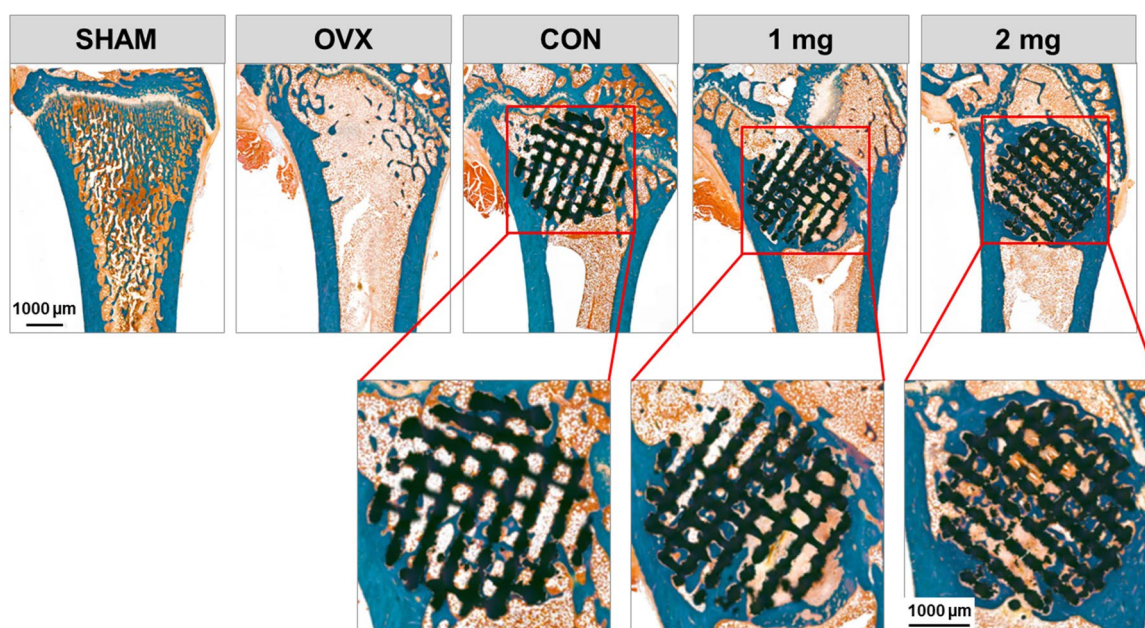


Fig. 5. Representative Goldner's trichrome staining of femur defects areas from SHAM, OVX and Ti implant groups at 12 weeks, respectively. The SHAM group underwent sham surgery; the OVX group underwent ovariectomy but received no implant; the Ti groups underwent ovariectomy and received implants containing PLGA coating only (CON group), 1 mg of E-2,5-DHB in PLGA (1 mg group) or 2 mg of E-2,5-DHB in PLGA (2 mg group). Scale bars = 1000 μ m.

Ti implants would result in a biologically active and mechanically strong composite biomaterial that would enhance bone healing of large bone defects.

Ideal orthopedic scaffolds should be biocompatible with surrounding cells to expedite satisfactory bone attachment between implants and surrounding bone structures (Borsari *et al.*, 2009). It is well known that successful implant fixation is closely related to the parallelism of the bone structure near the implant and the thickness of the bone (Brånemark *et al.*, 1997).

Additional bones formed around and near the implant, due to E-2,5-DHB elution, can be helpful in a wide range of orthopedic implant applications for increasing the reliability and accuracy of the device stability. Our results showed that local release of E-2,5-DHB directly from the implant had a positive effect on bone healing, while avoiding the disadvantages of systemic exposure.

Some herbal medicines are reported to be effective in treating various diseases. Local administration of 4-herb formula (CDNR) controls inflammation and promotes bone regeneration (Siu *et al.*, 2015). Other studies report the prevention and treatment of osteoporosis using plant-derived natural products (Caichompoo *et al.*, 2009; Jeong *et al.*, 2005; Ha *et al.*, 2003; Kim *et al.*, 2008; Li *et al.*, 2012; Suh *et al.*, 2007). Lycii radices cortex (LRC) extract may be a good natural herbal medicine candidate for the

treatment of osteoporosis (Park *et al.*, 2014) and for increasing spinal BMD (Wang *et al.*, 2013). However, these studies evaluate the efficacy of herb extract as a drug to prevent osteoporosis and the effect of healing on osteoporotic defects is not evaluated. In the current study, unlike other herbal extras, E-2, 5-DHB showed a dual action on osteoblasts and osteoclasts, demonstrating bone defect healing effect in osteoporotic conditions.

Osteoclasts originate from the monocyte/macrophage hematopoietic lineage and are responsible for bone resorption (Boyle *et al.*, 2003; Zhao *et al.*, 2007). RANKL, expressed by osteoblasts, plays a key role in the activation of osteoclasts by binding to the RANK receptor (Jimi *et al.*, 1999). OPG, produced by osteoblasts, inhibits osteoclast differentiation by binding to RANKL (Hofbauer and Heufelder, 2000). The inhibitory effects of E-2,5-DHB on osteoclast differentiation are likely regulated by various paracrine factors, including OPG and RANKL, released from osteoblasts. In this study, it was demonstrated that E-2,5-DHB mediated an increase in the levels of secreted OPG, whereas it decreased RANKL levels. This suggested that E-2,5-DHB could affect the balance of OPG/RANKL pathway and promote bone healing in osteoporotic conditions.

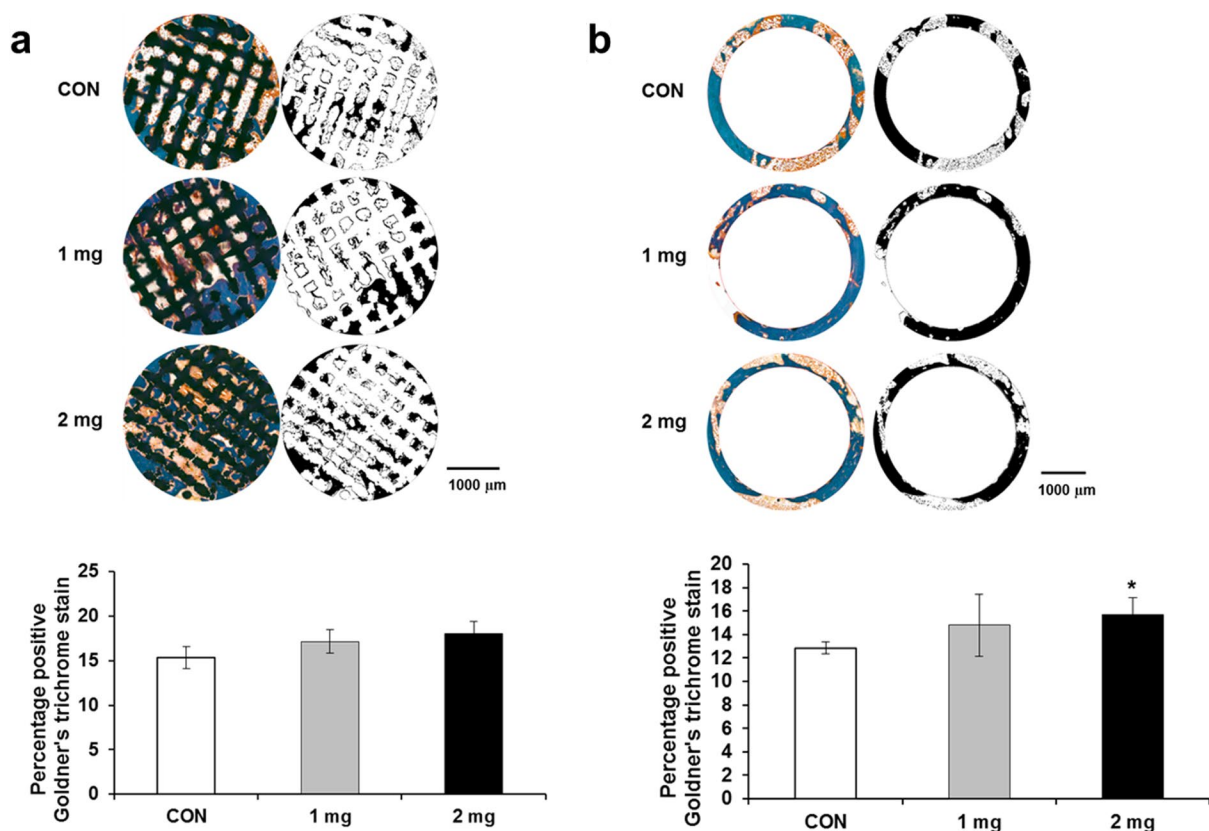


Fig. 6. Goldner's trichrome staining was analyzed by Image J software. For each sample, a circular region of the same size was chosen: (a) a circle with a diameter of 3 mm including the entire implant structure and (b) a 0.3 mm-thick ring of surrounding bone tissue. Data are shown as mean ± SD. * $p < 0.1$ compared to the Ti group in (b).

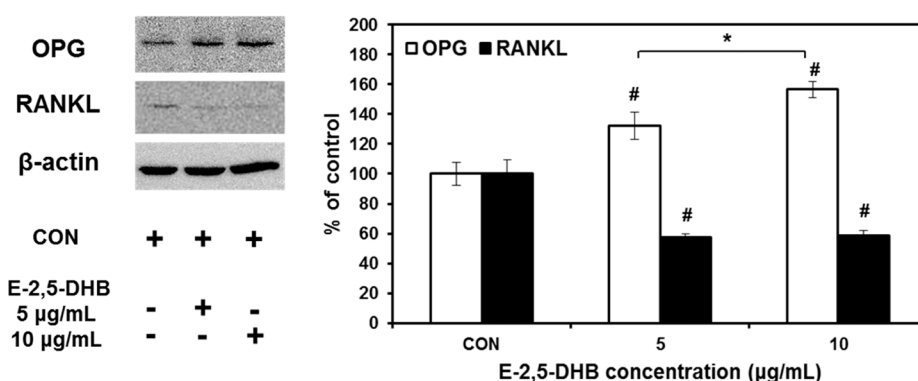


Fig. 7. Efficacy study of E-2,5-DHB evaluating osteoclast differentiation. Western blot of OPG and RANKL. Data are presented as mean \pm SD. *, # statistically significant differences (* $p < 0.05$, # $p < 0.01$). CON, control.

Conclusion

E-2,5-DHB-incorporating PLGA-coated Ti discs were prepared and whether this could influence osteoblast functions, as well as inhibit osteoclast differentiation, was evaluated. Subsequently, the local release of E-2,5-DHB from E-2,5-DHB-coated Ti implants was used to enhance bone formation around implants inserted into femoral defects. This study demonstrated that local treatment with E-2,5-DHB resulted in significantly more new bone formation in the rat femur deficient area compared to the control (PLGA-coated only Ti disc). Comparing the total new bone formation, it was revealed that both 1 and 2 mg of E-2,5-DHB induced significantly more bone formation. Therefore, it appeared that E-2,5-DHB-coated Ti implants have great potential as an ideal local drug delivery system when using bone tissue scaffolds.

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Discussion with Reviewer

Wing Hoi Cheung: What is the potential to translate E-2,5-DHB-incorporating titanium implant for the use in clinical application?

Authors: E-2,5-DHB-incorporating titanium implants showed promising clinical potential, enhancing implant performance in the osteoporotic bone condition. In recent year, there has been an increasing effort to restore bone defects in patients with osteoporosis. Also, E-2,5-DHB-incorporating titanium implants showed great potential for implant surface coating in several clinical applications, including dental, plastic and orthopedic surgery.

Wing Hoi Cheung: Is there any side effect of E-2,5-DHB?

Authors: E-2, 5-DHB is a derivative of *Rubus coreanus* extract, which does not represent a serious concern for safety since it is used as health food supplements. No serious adverse events, such as foreign body reactions or inflammatory reactions, have been observed in our rat model. Nevertheless, any side effect during clinical applications need to be confirmed.

Editor's note: The Scientific Editor responsible for this paper was Chris Evans.