Modulation of bone formation and resorption using a novel zoledronic acid loaded gelatin nanoparticles integrated porous titanium scaffold: an in vitro and in vivo study

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Abstract
Osteoporotic bone defects are a major challenge in clinics for bone regeneration. With the condition of osteoporosis, excessive bone absorption and impaired osteogenesis result in unexpectedly long healing procedures for defects. In order to simultaneously enhance bone formation and reduce bone resorption, a polydopamine-coated porous titanium scaffold was designed, to be integrated with anti-catabolic drug zoledronic acid nanoparticles (ZOL loaded gelatin NPs), which was able to achieve a local sustained release of ZOL as expected. The in vitro study demonstrated that extracts of the composite scaffolds would stimulate osteoblast differentiation; they also inhibited osteoclastogenesis at a ZOL loading concentration of 50 µmol l⁻¹. In the subsequent in vivo study, the composite scaffolds were implanted into ovariectomy-induced osteoporotic rabbits suffering from femoral condyles defects. The results indicated that the composite scaffolds without ZOL loaded gelatin NPs only induced callus formation, mainly at the interface margin between the implant and bone, whereas the composite scaffolds with ZOL loaded gelatin NPs were capable of further enhancing osteogenesis and bone growth into the scaffolds. Moreover, the research proved that the promoting effect was optimal at a ZOL loading concentration of 50 µmol l⁻¹. In summary, the present research indicated that a new type of porous titanium scaffold integrated with ZOL loaded gelatin NPs inherited a superior biocompatibility and bone regeneration capability. It would be an optimal alternative for the reconstruction of osteoporosis-related defects compared to a traditional porous titanium implant; in other words, the new type of scaffold offers a new effective and practical procedure option for patients suffering from osteoporotic bone defects.

1. Introduction

With the ongoing aging of society, osteoporosis (OP) has become a common disease, encountered particularly in postmenopausal women, with an incidence of up to 74% [1]. Patients with OP are vulnerable to OP-related fractures, such as critical size bone defects [2, 3]. Although tissue-engineered bone substitutes, including various synthetic materials, have been considered, with promising alternatives to bone graft in bone defect repairing [4, 5], the treatment of critical size bone defects, particularly under the condition of OP, still has challenging requirements in clinical practice [6, 7]. On one hand, the poor osteogenesis in bone defects locally interferes with integration between the bone and implant. Previous studies have indicated that the local bone resorption continues to be very active in osteoporotic fractures after eight weeks, while collagen fiber formation and mineralization is relatively non-active; thus, new bone formation and reconstruction are further delayed [8, 9]. On the other hand, the quality and mechanical strength
of a newly-formed callus within the defect are lower than that of non-osteoporotic bones [9].

When developing implants for promoting osteogenesis in osteoporotic defects, the biomechanical stability of the implant within the defect site is a prerequisite for lesion healing. Such stability depends mainly on the mechanical strength of the implant. Medical-grade titanium alloy is currently the main material used for metal implants, due to its excellent biocompatibility, superior corrosion resistance and high mechanical strength. Moreover, the mechanical strength of a titanium scaffold can be tailored to the pore size and density using computer-aided design layered manufacturing combined with electron beam melting (EBM) technology [10]. However, artificial bone scaffolds made of titanium alloy have no local anti-OP and osteoconduction effects, although they have satisfactory mechanical strength. Under an osteoporotic condition, this drawback leads to delayed bone fusion with the implant, increasing the risk of re-fracture, refractory healing and delayed bone fusion with the implant, increasing the risk of re-fracture and refractory healing. Under an osteoporotic condition, this drawback leads to delayed bone fusion with the implant, increasing the risk of re-fracture and refractory healing.

2. Materials and methods

2.1. Materials and sample preparation

First, gelatin NPs were prepared using a two-step coagulation method as described in previous research [22]. The NPs were then dipped into a ZOL solution of different concentrations (0 µmol l⁻¹, 1 µmol l⁻¹, 10 µmol l⁻¹, 100 µmol l⁻¹, 500 µmol l⁻¹). Subsequently, the mixtures were incubated in a refrigerator, with a constant temperature of 4 °C, vibrating for 12 h, to make sure that the gelatin nanospheres and ZOL were fully integrated.

The model of the Ti6Al4V bionic porous scaffold (pore size 520 ± 35 µm, porosity 57 ± 4.2%) was designed by Magics software with a diameter of 5 mm and a height of 6 mm; it was fabricated with an Arcam’s EBM machine (EBM A1; Arcam AB, Sweden) (refer to [23] for further details). Then, a layer of pDA coating was prepared on the surface of the porous titanium scaffold, as described in previous research [17]. Accordingly, dopamine (2 mg ml⁻¹) was dissolved in 10 mM Tris-HCl (pH 8.5), with the scaffolds dipped into the solution for 12 h, to form the polydopamine coatings. When they were ready, the pDA coated scaffolds were dipped into the ZOL loaded gelatin NPs solution and incubated with rocking for 24 h. The composite scaffolds were then dried using a vacuum freeze-drying technique. Eventually, the ZOL loaded gelatin NPs carrying different concentrations of ZOL were synthesized on the scaffolds by means of pDA-mediated electrostatic self-assembly multilayer (figure 1).

2.1.1. Scaffold characterization

The surface morphology, thickness, and elemental composition of the ZOL loaded gelatin NPs composites were analyzed using a scanning electron microscope (SEM) (S-4800, HITACHI, Japan). Prior to observation by the SEM, a sputter coater was used to coat the samples with a thin layer of oxygen. Note that it is spontaneous for polydopamine to form self-adherent polydopamine coatings on the surface of various types of material, e.g. noble metals and polymers. Previous studies have shown that these polydopamine coatings played an optimum role in distributing and attaching biomolecules onto the surface of the scaffolds [19–21]. In addition, ZOL loaded gelatin NPs degrade over time after implantation, thus allowing new bone to grow into the scaffolds. We investigated the effect of the composite scaffolds on promoting osteogenesis as well as inhibiting excessive resorption in vitro. The promoting effects of the composite scaffolds on osteoporotic defects were further verified through in vivo implant assays in the femoral condyle of osteoporotic rabbits. We postulate that the new type of scaffold offers an effective and practical procedure option for patients suffering from osteoporotic bone defects.
gold for better electric conductivity (SHINKKU VD MSP 1S, Japan).

2.1.2. In vitro drug release
Scaffolds loaded with different concentrations of ZOL were immersed into 4 ml of phosphate buffered saline (pH = 7.4) and placed in a shaker at a constant temperature of 37 °C. After immersing them for predetermined time intervals (1 d, 4 d, 7 d, 14 d, 21 d, and 28 d), 3 ml of the supernatant was used for analysis. The concentration of released ZOL in the medium was measured using a UV/VIS spectrophotometer (AH1260, Agilent, USA) at a wavelength of 210 nm. The release curve of the ZOL was generated according to the release of ZOL concentration at different time instances.

2.1.3. Preparation of sample extracts
The ZOL loading scaffolds were incubated in α-MEM, which contains 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The incubation condition was set at 5% CO2, 95% humidity, and 37 °C for 24 h to obtain the extracts. According to ISO 10993-5, specimen mass to solution volume ratio was 0.2 g ml⁻¹ [24]. The extracts of samples were subsequently used for the following in vitro cell assays.

2.2. In vitro cellular assessments
2.2.1. Cell attachment and proliferation
For the osteoblast attachment assays, the osteoblasts were isolated and expanded as previously described [25]. The osteoblasts were enzymatically isolated from the calvariae of a 2 d old Sprague Dawley (SD) rat and cultured in α-MEM supplemented with 10% FBS, and 1% penicillin/streptomycin. Upon reaching approximately 90% confluence, the cells were dissociated with trypsin. Briefly, cell suspension (2 × 10⁵ cells ml⁻¹, 500 µl) was dropped into the scaffold placed in a 24-well plate and cultured for 6 h. Then, the medium was replaced with α-MEM supplemented with 10% FBS, 1% antibiotic/antimycotic, 50 mM ascorbic acid (Sigma-Aldrich, USA), 10 mM b-glycerophosphate (Sigma-Aldrich, USA), and 100 nM dexamethasone (Sigma-Aldrich, USA) (for osteoblast differentiation, as described previously [26]). The medium was replaced every 2 d.

For the osteoclast attachment assays, osteoclast precursors (bone marrow monocytes (BMMs)) were isolated and expanded as previously described [27]. The BMMs were isolated from the femora and tibiae of a 2 d old SD rat and cultured in α-MEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 25 ng ml⁻¹ macrophage colony-stimulating factor (M-CSF, Pepro Tech, Rocky Hill, NJ) at 37 °C.
in a 5% CO₂ humidified incubator for approximately 3 d. During cell culture, the medium was replaced once to remove the non-adherent cells. Upon reaching approximately 90% confluence, the cells were dissociated with trypsin. 50 000 cells ml⁻¹ were seeded onto scaffolds in a 50 μl drop and incubated for 2 h to allow attachment. All cultures were maintained in the same medium, α-MEM supplemented with 10% FBS, 1% antibiotic/antimycotic, 25 ng ml⁻¹ M-CSF, (Pepro Tech, Rocky Hill, NJ) and 25 ng ml⁻¹ receptor activator of nuclear factor kappa-B ligand (RANKL, R&D, Sweden) (for osteoclast differentiation, as described previously [28]). The medium was replaced every 2 d.

After 7 d of culture, samples were rinsed with phosphate buffered solution (Invitrogen, USA) to remove non-adherent cells and then fixed with 2.5 wt.% glutaraldehyde for 8 h. The samples were dehydrated with a series of gradient ethanol solutions (50%, 70%, 90%, 95%, 100%) and then replaced by pure isopropyl acetate. The morphology of cells was observed by SEM (S-3400, HITACHI, Japan). Prior to SEM observation, a sputter coater was used to coat the samples with a thin layer of gold for superior conductivity.

2.2.2. In vitro osteogenic differentiation and osteoclastogenesis assays

Osteoblasts isolated from a 2 d old SD rat were seeded at 2 × 10⁵ cells ml⁻¹ in 24-well plates at 37 °C in 5% CO₂ for 7 d. After 6 h culture, the culture medium was replaced with the sample extracts supplemented with 50 mM ascorbic acid, 10 mM β-glycerophosphate, and 100 mM dexamethasone (all Sigma-Aldrich, USA). The supplemented extracts were refreshed every 2 d. After 7 d of incubation, alkaline phosphatase (ALP) staining was performed as described in the previous work [29]. The activity of ALP was determined using an ALP microplate test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturer’s instructions.

For the osteoclastogenesis assays, BMMs (2 × 10⁵ cells well⁻¹) were plated and incubated in 24-well plates in triplicate. After 6 h culture, the medium was removed from each well and replaced with the extracts of the different implants supplemented with 25 ng ml⁻¹ M-CSF (Pepro Tech, Rocky Hill, NJ, USA) and 25 ng ml⁻¹ receptor activator of nuclear factor kappa-B ligand (RANKL, R&D, Sweden) at a constant temperature of 4 °C for 15 min. Protein concentrations were measured using a BCA kit (Pierce, Rockford, IL, USA) complying with the manufacturer’s instructions. For Western blot analysis, 20 μg proteins from each sample were loaded onto 8%–15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). Gel separated proteins were transferred into nitrocellulose membrane (Millipore, Bedford, MA, USA) at 230 mA 1 h and incubated with a mouse anti-rat TRAP monoclonal antibody (1:1000 in blocking buffer, Abcam) and a rabbit anti-rat β-actin monoclonal antibody (1:5000 in blocking buffer, Invitrogen) separately at 4 °C for 12 h, followed by reacting

2.2.3. Cell differentiation and quantitative real time polymerase chain reaction (RT-PCR)

Cells attached to the scaffolds were dissociated with trypsin and collected for differentiation analysis. The total RNA was then extracted and reversed into cDNA using a cDNA synthesis kit (TaKaRa, Japan), following the manufacturer’s instructions. The expression levels of osteoblastic markers ALP and Runx2 and osteoclast markers cathepsin K (Ctsk) and TRAP were quantified using an SYBR Green Master mix (TaKaRa, Japan) and a Step One Plus RT-PCR instrument (ABI7500, USA). Forward and reverse primers were designed by Primer Express Software (Applied Biosystems). The sequences of the forward and reverse primers were as follows: osteoblast: Runx2, 5’-ATCCAGCCACCTTCACTTAC3’-3’ and 5’-GGGACCATGGAACTGATAGG3’-3’; osteocalin (OCN), 5’-GCCCTGACTCTTCTGCTT-3’ and 5’-TCACCCATTGCTCCCTCTG-3’; osteoclast: CathepsinK, 5’-CGGCTATATGACCACCTGCTTTC-3’ and 5’-TTTGCGCGTTTATACATACT-3’; TRAP, 5’-GCACATTGCGTTTACATGGA-3’ and 5’-TGTCATTCTTTGGGCTTATC-3’; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5’-GCCACGATCAAGGCTGAGATG-3’ and 5’-ATGGTGGTGAAGACGCCAGTA-3’. The expression of GAPDH was examined as the endogenous control. Relative transcripts levels were calculated from the relative standard curve constructed from stock cDNA dilutions and divided by the target quantity of the calibrator following the manufacturer’s instructions.

2.2.4. Osteoclast (OC) differentiation and Western blot analysis

The effects of ZOL on OC differentiation were further verified from the protein expression level. BMMs (1 × 10⁵ cells well⁻¹) were plated and incubated in six-well plates in triplicate. After 6 h culture, the medium was removed from each well and replaced with the extracts of the different implants supplemented with 25 ng ml⁻¹ M-CSF (Pepro Tech, Rocky Hill, NJ, USA) and 25 ng ml⁻¹ receptor activator of nuclear factor kappa-B ligand (RANKL, R&D, Sweden). The extracts medium was replaced every 2 d. After 7 d of incubation, the total proteins of the OC cultured with sample extracts were isolated using a protein extraction reagent (1% PMSF, RIPA lysis) and then centrifuged at 12 000 rpm under a constant temperature of 4 °C for 15 min. Protein concentrations were measured using a BCA kit (Pierce, Rockford, IL, USA) complying with the manufacturer’s instructions. For Western blot analysis, 20 μg proteins from each sample were loaded onto 8%–15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). Gel separated proteins were transferred into nitrocellulose membrane (Millipore, Bedford, MA, USA) at 230 mA 1 h and incubated with a mouse anti-rat TRAP monoclonal antibody (1:1000 in blocking buffer, Abcam) and a rabbit anti-rat β-actin monoclonal antibody (1:5000 in blocking buffer, Invitrogen) separately at 4 °C for 12 h, followed by reacting
with hors eradish peroxidase-conjugated anti-mouse (1:2000 in blocking buffer, Pierce), respectively. Protein expression was visualized by reacting with diaminobenzidine tetrahydrochloride (DAB, Santa Cruz, CA, USA). The semi-quantification of the protein was based on three experiments performed independently. The densitometric intensities of protein bands were quantified using the software ImageJ (Ver. 1.6, NIH, Bethesda, MD, USA) and values were normalized to the values of β-actin of each sample.

2.3. Pit formation assay
Osteo assay surface plates (Corning, MA) were used for the pit formation assay; refer to previous research for details [30]. Briefly, BMMs (2 × 10⁵ cells) were seeded onto the plate for 6 h. Then, the medium was replaced with the sample extracts supplemented with M-CSF (25 ng ml⁻¹) and RANKL (25 ng ml⁻¹). The medium was replaced every 2 d. After 10 d incubation, the osteoclasts were lysed with 5% sodium hypochlorite solution. The images of the resorption pits were obtained using optical microscopy. The areas of the resorption pits were measured using ImageJ software (Ver. 1.6, NIH, Bethesda, MD). The incisions were then closed in layers and dressed with bandages.

2.4. In vivo assessment of new bone formation
2.4.1. Implantation procedure
Twenty-four mature female New Zealand White rabbits (aged 12 weeks; weight range: 3.0–3.6 kg), which were skeletally healthy, were acquired following the National Institute of Health Guidelines for the Use of Laboratory Animals, and all procedures were approved by the Fourth Military Medical University Committee on Animal Care. All the animals were housed in an environment with temperature of 22 C ± 1 C, relative humidity of 50 ± 1% and a light/dark cycle of 12/12 h. All animal studies complied with AAALAC and the IACUC guidelines.

Animals in the research first underwent a bilateral ovariectomy. The bone mineral density was measured by dual-energy x-ray (Hologic Discovery, Waltham, MA, USA) absorptiometry six months after the surgery to confirm the establishment of OP in the rabbits. The rabbits were then randomly divided into six groups, with four members in each group. Each group were treated with one of the following doses with porous titanium implants: nothing (control); scaffolds with ZOL loaded gelatin NPs = 1 μmol l⁻¹; scaffolds with ZOL loaded gelatin NPs = 10 μmol l⁻¹; scaffolds with ZOL loaded gelatin NPs = 50 μmol l⁻¹; scaffolds with ZOL loaded gelatin NPs = 100 μmol l⁻¹; scaffolds with ZOL loaded gelatin NPs = 500 μmol l⁻¹. The rabbits were anesthetized with 10% chloral hydrate (1.2 ml kg⁻¹ intravenously). A 5 mm in diameter defect was made at the bilateral femoral condyle using a bone drill copious saline irrigation. The composite scaffolds were implanted through press-fitting into the defect of the bilateral femoral condyle.

2.4.2. Micro-CT evaluation
Bone regeneration was measured by ex vivo micro-CT scans (micro-CT, YXLON, Y. Cheetah, Germany) on isolated grafted radius at eight weeks postoperatively. Individual bony blocks containing the implants and the surrounding tissues were obtained after euthanizing the rabbits, and fixed in 4% formaldehyde solution for two weeks. Ex vivo micro-CT images were acquired at 13 μm resolution (voltage: 90 kV, current: 55.6 μA, rotation step: 0.5) and then reconstructed using volumetric reconstruction software. Bone regeneration was expressed as bone volume within the pore of the titanium scaffold (BV), total volume of titanium scaffold (TV) and percent bone volume (BV/TV) using Vgstudio max3.0.

2.4.3. Histological processing
After micro-CT evaluation, histological analysis was performed on all specimens to investigate bone morphology and the interface of bone titanium. Samples were dehydrated in a graded ethanol solution, and finally embedded in methyl methacrylate. Sections with thickness of 100 μm were obtained using a hard tissue microtome (Leica Microtome, Wetzlar, Germany) and stained with 1.2% trinitrophenol solution as well as 1% acid fuchsin solution (Van Gieson staining). The image was obtained using a Leica LA automatic microscopy study microscope (OLYMPUS, BX53, Japan). The images were histopathologically analyzed and the volume of bone growing into the scaffolds was calculated.

2.5. Statistical analysis
SPSS Statistics 21.0 (SPSS, Inc., Al Monk, NY, USA) was used for statistical analysis. Data were presented as means ± standard deviations. Statistical analysis was performed using either one-way analysis of variance (ANOVA) or Student’s t-test. Values of P < 0.05 were considered statistically significant. Statistical significance was declared as (*) at p < 0.05, (**) at p < 0.01, (*** ) at p < 0.001.

3. Results
3.1. ZOL loaded gelatin NPs composite scaffolds characterization
As shown in figure 1, the porous titanium scaffolds were fabricated with a porosity of 57 ± 4.2% and a strut size of 6 mm in height and 6 mm in diameter. The elasticity modulus of the scaffolds was 1.85 GPa, which was close to human cancellous bone. Macroscopic inspection and SEM analysis verified that the pores of the porous titanium scaffolds were completely coated with polydopamine. The ZOL loaded
gelatin NPs were loaded and distributed evenly on the surface of the scaffolds.

3.2. In vitro drug release
In figure 2, we plotted the kinetics of ZOL release in a ZOL loaded gelatin NPs composite titanium scaffold over a period of 28 d, illustrating a controlled and sustained drug-release profile. The total release of ZOL, delivered from the scaffold loaded with ZOL loaded gelatin NPs, was approximately 9.6 ± 1.1% in the initial days, presumably occurring in a diffusion-controlled manner. The drug release gradually increased to 34.7 ± 1.1% in the subsequent 27 d, most likely attributable to a degradation-controlled mechanism. After an initial burst-release of ZOL, the composite scaffold maintained a stable release rate for at least several weeks in vitro, suggesting that the ZOL loaded gelatin NPs composited titanium scaffold can serve as an effective carrier for local drug delivery.

3.3. Cell attachment and apoptosis
At day 7, cells attached to the scaffold were observed with different distributions by SEM (figure 3). For the osteoblast assays, the cells presented an elongated and plump morphology with abundant filopodial and noticeable filopodia in the control group. From the 1 µmol l⁻¹ group to the 50 µmol l⁻¹ group, OB cells exhibited a significant increase in morphological elongation and filamentous filopodia. In the 100 µmol l⁻¹ and 500 µmol l⁻¹ group, the number of cells attached to the scaffolds decreased and presented as atrophied and with less pseudopodium. For the osteoclast assays, there was no significant difference in cell number and morphology between the control group and the ZOL loaded gelatin NPs groups 1 µmol l⁻¹ and 10 µmol l⁻¹. Significant atrophy in the morphology of the cell occurred in the 50 µmol l⁻¹ group. For the 100 µmol l⁻¹ and 500 µmol l⁻¹ group, nearly all the residual cells on the scaffolds were apoptotic.

Cells were inoculated and cultured for 7 d. Annexin-v-fc/PI double fluorescence labeling was used to detect the cells by flow cytometry (FACSAria, USA) (figure 4). The effect of porous ZOL loaded gelatin NPs titanium alloy scaffolds at different concentrations on the apoptosis of osteoblasts is shown in figure 4. For the osteoblast assay, the apoptosis rates of the 1, 10, and 50 µmol l⁻¹ groups on day 7 (3.27% ± 0.79%, 3.71% ± 0.82%, 3.39% ± 0.52%) were significantly lower than that of the control group (5.71 ± 0.37%, P < 0.05). The apoptosis rates (14.00% ± 1.64%, 21.7% ± 1.41%) in the 100 µmol l⁻¹ and 500 µmol l⁻¹ groups were significantly higher than that in the control group (5.71% ± 0.37%, P < 0.05). However, for OC, the cell apoptosis rate increased linearly with the ZOL loaded gelatin NPs loading concentration. From 1 µmol l⁻¹ to 500 µmol l⁻¹, the cell apoptosis rates were (15.77% ± 0.99%, 18.57% ± 4.08%, 54.77% ± 0.62%, 82.57% ± 3.01% and 91.23% ± 5.80%), respectively, significantly higher than that of the control group (5.67% ± 0.53%, P < 0.01).

3.4. Cell differentiation
3.4.1. Osteogenic differentiation and osteoclastogenesis
To determine the potential osteogenic effect, calvarial cells were cultured in sample extracts supplemented with ascorbic acid, glycerophosphate, and dexamethasone. At day 7, analysis of ALP staining and activity indicated that ZOL at concentration of 1 µmol l⁻¹, 10 µmol l⁻¹ and 50 µmol l⁻¹ could enhance the activity of ALP, and the optimized ZOL concentration was 50 µmol l⁻¹, compared with that of the control. From the 100 µmol l⁻¹ group, ZOL started to exhibit a suppression effect on ALP activity. A statistically significant difference was noticed among the 50 µmol l⁻¹ group, the control and 500 µmol l⁻¹ group (figure 5).

Differentiation of murine BMMs into mature osteoclasts was evaluated by quantifying the number and size of TRAP-positive cells that had more than three nuclei. Figure shows the morphology of TRAP-positive multinucleated osteoclasts cultured in extracts of different ZOL concentration groups. At day 7, analysis of TRAP-positive cells indicated that the ZOL at concentration of 1 µmol l⁻¹ and 10 µmol l⁻¹ could enhance the differentiation of OC (osteoclast index, 31.34 ± 3.09 and 33.67 ± 1.25, respectively), though the difference was not significantly compared with the control group. In groups of 50 µmol l⁻¹, the amount of TRAP-positive multinucleated osteoclasts decreased significantly compared to that of the control group (osteoclast index, 31.34 ± 3.09 vs 35.34 ± 1.70, P < 0.01). In the 100 µmol l⁻¹ and 500 µmol l⁻¹ groups, both TRAP-positive and non-positive cells were nearly apoptotic (figure 5).

3.4.2. Cell differentiation evaluated by quantitative RT-PCR and Western blot (WB)
In the RT-PCR test, correspondingly, gene expressions of Runx2 and ALP were increased in the 1 µmol l⁻¹ and 10 µmol l⁻¹ group, and optimized in the 50 µmol l⁻¹ group, compared with those in control group. Meanwhile, in the 100 µmol l⁻¹ group and beyond, gene expression began to decrease sharply (figure 6).

For osteoclast differentiation, the RT-PCR test demonstrated that gene expression of TRAP from 1 µmol l⁻¹ to 500 µmol l⁻¹ (0.69 ± 0.03, 1.01 ± 0.06, 0.11 ± 0.01, 0.07 ± 0.01) reached the highest level for the 50 µmol l⁻¹ group. Additionally, the expression levels decreased in the 100 µmol l⁻¹ group and beyond. Gene expression of Ctsk reached the highest level in the 10 µmol l⁻¹ group and
Figure 2. ZOL release profiles of the ZOL loaded gelatin NPs samples over a period of 28 d. ZOL release profiles for samples at concentration of (A) 10 µmol l⁻¹, (B) 50 µmol l⁻¹, and (C) 100 µmol l⁻¹.

Figure 3. Cell attachment and proliferation on the composite scaffolds. OB: morphology of osteoblasts attached on different ZOL-loading scaffolds group (ZOL loading concentrations from control to 500 µmol l⁻¹); OC: morphology of osteoclasts attached on different ZOL-loading scaffolds group (ZOL loading concentration from control to 500 µmol l⁻¹).

Figure 4. Effects of different ZOL loaded gelatin NPs implant extracts on cell apoptosis in vitro. OB: apoptosis rate of osteoblast under the culture of extracts from ZOL loaded gelatin NPs (from 0 µmol l⁻¹ to 500 µmol l⁻¹); OC: apoptosis rate of osteoclast under the culture of extracts from ZOL loaded gelatin NPs (from 0 µmol l⁻¹ to 500 µmol l⁻¹).

decreased sharply for the 50 µmol l⁻¹ groups and above. The WB assays testing the protein expression levels further verified the concentration-dependent effects of ZOL on OC differentiation (figure 7). The expression levels of osteoblastic markers TRAP exhibited similar trends from 0 µmol l⁻¹ to 100 µmol l⁻¹.

3.5. Osteoclast-mediated bone resorption
Bone resorption activity was evaluated by quantifying the size of the resorption pit on the osteo assay surface plate. Extracts from ZOL of 1 µmol l⁻¹ and 10 µmol l⁻¹ both enhanced the formation of resorption pits by 18.3% and 25.2%, respectively. In the 50 µmol l⁻¹ group, the formation of resorption pits by RANKL-induced osteoclasts were significantly inhibited and the difference of the resorption pit was statistically significant compared with that of the control group (figure 8).

3.6. Micro-CT evaluation
Representative 3D-reconstructed micro-CT images of scaffolds at eight weeks after implantation are shown in figure. Statistical analysis indicated that bone ingrowth started from the host bone bed towards the scaffold loaded with ZOL loaded gelatin NPs at 1 µmol l⁻¹, 10 µmol l⁻¹ and 50 µmol l⁻¹, resulting in higher percent bone volume (BV/TV) than in the control group (figure 9). From 1 µmol l⁻¹ to 500 µmol l⁻¹, the BV/TV was 10.2%, 12.2%, 21.2%, 7.7% and 7.2%, respectively. Note that the BV/TV was the highest in the 50 µmol l⁻¹ group (21.2%), significantly higher than that of the
Figure 5. Effects of different ZOL loaded gelatin NPs implants extracted on osteogenic differentiation versus osteoclastogenesis in vitro. (A) OB: ALP staining for osteoblast formation; OC: TRAP staining for osteoclast formation (ZOL loading concentration from control to 500 µmol l⁻¹) (magnification, ×100). (B) Osteoclast index of different ZOL loaded gelatin NPs groups quantified based on cell amount of OC. TRAP-positive multinucleated cells were counted using light microscopy (magnification, ×100). The data value is expressed as the mean ± SE of triplicate experiments. * * P < 0.01.

Figure 6. Osteogenesis differentiations of attached cells for 7 d were dissociated with trypsin and collected for quantitative RT-PCR. OB: the expression levels of osteoblastic markers ALP and Runx2; OC: the expression levels of osteoclastic markers cathepsin K (Ctsk) and tartrate resistant acid phosphatase (TRAP). * P < 0.05, ** P < 0.01.

control group (9.5%, P < 0.01). In the 100 µmol l⁻¹ and 500 µmol l⁻¹ group, the bone ingrowth ratio decreased significantly.

3.7. Histological evaluation
New bone formation and bone remodeling were evaluated by Van Gieson staining. Histology of the titanium scaffolds of different ZOL concentration groups revealed formation of a major plug of new bone at the bone–titanium bonding. As shown in figure 10, no significant difference was observed in terms of bone morphology and bone–titanium bonding among groups of different concentrations. The newly formed bone extended from the bone–titanium interface into the porous titanium and the inner space of the scaffold in composite scaffolds loaded with ZOL loaded gelatin NPs. The size of the new grown bone was largest in the test group.
50 µmol l⁻¹. Rare bone regeneration could be seen in the 500 µmol l⁻¹ group. Apart from bone tissue, the inside of the porous composite scaffold was filled with fibrous tissue.

4. Discussion

Osteoporotic bone defects have been linked with both excessive osteoclastic bone absorption and suppressed osteoblastic bone formation, inducing a major challenge in clinical treatment. Although autologous bone transplantation is recognized as the gold standard for repairing bone defects, approximately 20%–30% of patients who received autologous bone transplantation have been found to experience delayed union or malunion [31, 32].

In the process of developing tissue-engineered bone substitutes, Ti-6Al-4V is widely used in clinics and is fabricated as porous scaffolds to construct extraneous tissue-engineered bones [33]. However, pure porous Ti-6Al-4V scaffolds have limited osteoconduction, which may lead to unexpected
osseointegration and repair effects in local bone defects [34]. Recent research suggested that porous Ti6Al4V scaffolds coated with polydopamine can promote fast osteogenesis [35]. In the present study, the research group designed a polydopamine-coated Ti-6A1-4V scaffold with 57% porosity, rendering vascular ingrowth and new bone formation as well as a stable mechanical support. The porous titanium scaffold possesses a compressive strength of 1.83 GPa, which is comparable to the mechanical properties of natural bones [36, 37]. Notwithstanding this property, under the osteoporotic condition that is characterized as impaired osteogenesis and excessive remodeling, the polydopamine-coated Ti-6A1-4V scaffold is insufficient in promoting integration between bone and implant. Therefore, ZOL, currently used as one of the most potent inhibitors of osteoclastic bone resorption [38], was introduced in the scaffold. Traditionally, ZOL has been systemically injected in the treatment of postmenopausal OP, which can lead to side effects such as myalgia, bone pain, severe influenza-like symptoms, etc [39]. Various methodologies for local delivery of the drug are still under development and the optimal loading concentration of ZOL for promoting bone regeneration has not yet been determined. In the present study, we used NPs as a ZOL-carrying mediator to achieve a continuous release of ZOL locally for more than four weeks (figure 2). In addition, the polydopamine coating also improves the loading efficiency of ZOL loaded gelatin NPs onto the scaffold. To our knowledge, this is the first time that the osteoconduction and biocompatibility of pDA-coated Ti-6A1-4V scaffold and long-term anti-OP abilities of ZOL loaded gelatin NPs have been incorporated.

In this study, we prepared a series of ZOL solutions with concentrations ranging from 0 µmol l⁻¹ to 500 µmol l⁻¹. NPs were added to the solutions and incubated rocking, rendering a full integration of the ZOL and NPs. The ZOL loaded gelatin NPs allow a sustainable release of ZOL locally for several weeks despite the degradation of the NPs. The in vitro assays showed that ZOL could enhance OB differentiation and such a promoting effect was optimized at a ZOL loaded gelatin NPs concentration of 50 µmol l⁻¹. However, in the osteoclastogenesis assays, we discovered a concentration-dependent effect of ZOL loaded gelatin NPs on osteoclast formation. Analyses of osteoclastogenesis indicated that ZOL enhanced OC formation in the 1 µmol l⁻¹ and 10 µmol l⁻¹
groups, whereas in the groups with concentrations of 50 \( \mu \text{mol l}^{-1} \) and above, OC formation was significantly suppressed. In the test groups of 100 \( \mu \text{mol l}^{-1} \) and above, cells cultured with extracts were almost at apoptosis at day 7. To further verify the validity of this phenomenon, we detected the differentiation markers of OC, TRAP and Ctsk, from gene to protein expression levels. Results showed that extracts from lower concentrations (1 \( \mu \text{mol l}^{-1} \) and 10 \( \mu \text{mol l}^{-1} \)) of ZOL loaded gelatin NPs significantly enhanced both TRAP and Ctsk expressions compared with the control group, while both TRAP and Ctsk expressions decreased sharply at 50 \( \mu \text{mol l}^{-1} \) and above. It is worth noting that in most previous studies, ZOL had been mistakenly presumed to suppress the OC formation and its resorption ability \([40, 41]\). Only in the research of Vegger et al was it reported that systemic injection of ZOL increased the amounts of mature osteoclasts as well as expression of Ctsk in mice \([42]\). On the other hand, OC in our assays was induced from BMMs, which contained a large proportion of bone marrow stromal cells (BMSCs) and pre-OB in the culture system. Besides this, another explanation for the promoting effects of low concentration ZOL on OC were resulted from the pre-OB. Felix Peter Koch’s research suggested that ZOL could enhance osteoclast activity through a high RANKL/OPG ratio. The high ratio accelerated the osteoclasts’ metabolism, which promoted mineral-bound bisphosphonate liberation; therefore, it accelerated apoptotic cell death \([42, 43]\). This hypothesis was also consistent with the results that apoptotic ratio increased with the concentration of extract concentration of ZOL loaded gelatin NPs, from 15.77\% \pm 0.99\% at 1 \( \mu \text{mol l}^{-1} \) to 91.23\% \pm 5.80\% at 500 \( \mu \text{mol l}^{-1} \).

Ovariectomy in a rabbit is a standard OP model with high bone-turnover activity \([44]\). The in vivo effects of ZOL loaded gelatin NPs composite porous titanium scaffolds on osteoporotic defect healing were evaluated in the OP rabbit models. The results from the micro-CT and histological assays indicated that the bone trabeculae of the femoral condyle in all groups was very sparse. In the control group of no ZOL loaded gelatin NPs, bone regeneration was mainly located at the interface between the scaffold and bone, suggesting that the osteoconduction capacity of porous titanium was limited under osteoporotic condition. For scaffolds of the 10 \( \mu \text{mol l}^{-1} \) and 50 \( \mu \text{mol l}^{-1} \) groups, however, new grown bone was obvious in the pores of the porous scaffold, forming a staggered arrangement of bone and porous titanium scaffolds after implantation for 12 weeks. The results suggested that ZOL loaded gelatin NPs loaded on the scaffold would promote new bone growth into the porous scaffolds (enhance osteoconduction), thus facilitating the integration between the implant and bone tissue.

In summary, a concentration of 50 \( \mu \text{mol l}^{-1} \) ZOL loaded gelatin NPs was recognized to be the optimal amount for promoting osteogenic differentiation and suppressing osteoclastogenesis through pro-apoptotic function. This conclusion was also confirmed by the in vivo assays from micro-CT and histological evaluations.

There are several limitations in our study: (1) in this study, the concentration of ZOL was differed significantly between the test groups. It is worth performing further research to investigate the optimal drug-loading concentration; (2) more in vivo studies are needed to discover the comprehensive effects of a ZOL loaded gelatin NPs integrated porous titanium scaffold; (3) the mechanisms of sustained release of ZOL on repairing osteoporotic bone defects on the molecular level are barely understood; in particular, how ZOL affect osteoclastogenesis in a concentration-dependent manner remains unknown.

5. Conclusions

In this study, a new type of ZOL loaded gelatin NPs integrated porous titanium scaffold was designed by combining the excellent mechanical properties and biocompatibility of porous titanium and anti-OP of ZOL loaded gelatin NPs loaded on a scaffold for promoting healing of OP bone defects. The combination was designed to regulate bone formation and resorption simultaneously in the bone defect area. The efficacy of dual promotion of osteogenesis and anti-osteoclastogenesis was systematically demonstrated in vitro. In addition, a ZOL loading concentration of 50 \( \mu \text{mol l}^{-1} \) was recognized as the optimum for the composite scaffold, at which bone regeneration and bone callus size were both considerably promoted and bone resorption was suppressed simultaneously.

This novel porous titanium scaffold integrated with ZOL loaded gelatin NPs is a promising alternative for reconstruction treatment of OP-related defects compared to the traditional porous titanium implant; the new type of scaffold would be more effective and practical for treating patients suffering from osteoporotic bone defects.

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Author contributions

Yang Zhang and Hong-xun Sang conceived and designed the experiment. Xiao-jiang Yang and Chang-bo Lu performed the experiments. Xiao-jiang Yang, Chang-bo Lu and Jiwei Zou contributed the acquisition of data. Fa-qi Wang, Zhao Yang and Jin-bo...
Hu analyzed and interpreted the data. Xiao-jiang Yang and Chang-bo Lu wrote the manuscript. Fa-qi Wang performed the supplementary experiments according to the reviewers’ comments and revised the manuscript. All authors contributed to the study in significant ways and have approved the final manuscript.

Conflict of interest

There are no ethical/commercial or any other relationships that might lead to conflict of interest in the article.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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