Antibacterial and pro-osteogenic effects of β-Defensin-2-loaded mesoporous bioglass

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The human antimicrobial peptide beta-defensin-2 (hBD2) shows broad antibacterial activity and infrequent bacterial resistance. Here mesoporous bioactive glass (MBG) was loaded with hBD2, forming hBD2-loaded MBG (BD-MBG). The antibacterial and osteogenic effects of BD-MBG were investigated in comparison with MBG and the blank control (BC). The result showed that BD-MBG yielded a sustained hBD2 release for more than 7 weeks in vitro, and resulted in significantly lower amounts of viable bacteria and colony forming units, and significantly higher levels of bacterial protein release compared with those in the BC and MBG groups (all \( p < 0.05 \)). Compared with that in the BC group, significantly higher bone marrow stromal cell (BMSC) proliferation rates, alkaline phosphatase (ALP) activity, calcium nodule formation, and expression levels of early and late osteogenic makers were observed after MBG and BD-MBG treatments (\( p < 0.05 \)). Thus, BD-MBG inhibited bacterial growth, damaged their membrane, and promoted early and late osteogenic BMSC differentiation.

Keywords: β-Defensin-2, Mesoporous bioglass, Antibacterial, Bone regeneration

INTRODUCTION

Despite the ongoing development of bone tissue engineering, the occurrence of infections remains very common and has become one of the main causes of bone graft failure. For example, initial bacterial contamination occurs in 78.7% of cases of open fractures, which results in a mean bone infection rate of 10.2%, and the failure of 10–20% of cases in surgery for mandible bone grafts is caused by infection. Orthopedic infection caused by bacterial colonization could cause prolonged hospital stays, unsatisfactory functional outcomes, and increased patient dissatisfaction; however, their treatment is still a clinical challenge. Therefore, the development of osteogenesis-inducing bioactive bone grafts that can inhibit the adhesion of bacteria and the subsequent formation of biofilms are important scientifically and clinically.

Antimicrobial peptides (AMPs) play fundamental roles in innate immunity. The secretion of AMPs is markedly upregulated after microorganism stimulation, indicating that they may play important protective roles during microorganism invasion. The rapid development of bacterial resistance to conventional antibiotics has stimulated interest in the clinical use of AMPs, mainly because of their antimicrobial activity against multidrug-resistant bacteria. Human β-Defensin-2 (hBD2) is one of the most important AMPs, showing killing activity against both methicillin sensitive and resistant Staphylococcus aureus (S.a.) in a concentration-dependent manner. Moreover, hBD2 also shows potent antimicrobial activity against multi-drug resistant S.a. Interestingly, hBD2 is secreted by cultured osteoblasts, and its expression increased after stimulation using S.a. supernatants. The expression of hBD2 is detectable in non-infected bones and is upregulated in chronically infected bone. Conditioned medium derived from the hBD2-overexpressing bone marrow stromal cells (BMSCs) could reduce the viable numbers of S.a. in a concentration-dependent manner. More importantly, after implantation into S.a.-contaminated calvarial defects of rats, hBD2-overexpressing BMSCs could dramatically reduce the number of viable S.a., and ameliorated S.a. contamination’s negative effects on bone healing. However, whether incorporation of hBD2 into bioactive bone grafts could deliver synergistic antibacterial and osteogenic effects remains unknown.

Bioactive glass is widely used in bone regeneration grafts, where it forms a bonding interface with the host bone at its surface. In addition, the dissolution products of bioactive glass, such as phosphate ions silicon (Si), calcium (Ca), and sodium (Na), promote bone regeneration and ingrowth. Previous studies from our group and others described the fabrication of three-dimensional mesoporous bioactive glass (MBG) together with calcium sulfate or strontium. These MBG compounds showed very good osseointegration capability and increased mechanical strength. In addition, the mesoporous silica within the MBG could provide an excellent drug-loading capability. However, whether MBG could be successfully loaded with hBD2 as a novel bioactive bone graft, and whether the resulting bone graft has synergistical antibacterial and osteogenic effects has not been determined.

In the present study, we aimed to construct MBG loaded with hBD2 (BD-MBG), and to investigate its antibacterial and osteogenic effects. We hypothesized that when the hBD2 was integrated into the MBG, it could
be gradually released together with the components of the MBG, and would thus show synergistic antibiotic and pro-osteogenic effects. The results of the present study are expected to lead to the development infection-resistant bone regeneration biomaterials.

**MATERIALS AND METHODS**

**Synthesis of MBG and hBD2-loaded MBG (BD-MBG)**

Highly-ordered MBGs were synthesized as previously described\(^14,15\), in which the template comprised nonionic block copolymer P123, and the silica source was tetraethyl orthosilicate (TEOS, 99%). Briefly, P123 (8 g) was dissolved in ethanol (120 g) by stirring at room temperature for 2 h. Then 13.4 g of TEOS, 1.46 g of triethyl phosphate, 2.8 g of calcium nitrate tetrahydrate, and 2 g of 0.5 M HCl were added to the above mixture. The solution was stirred overnight at room temperature and filtered to obtain the precipitate, which was then washed and centrifuged. The precipitate was subjected to evaporation-induced self-assembly (EISA) for 24 h at 40°C under a vacuum to obtain dry powders. To form the final MBG powder (Si/Ca/P molar ratio: 80/15/5), the samples were calcined for 6 h at 600°C. All the chemicals mentioned above were obtained from Sinopharm Chemical Reagent (Shanghai, China). Initially, a 0.5 mg/mL stock solution of human \(\beta\)-Defensin-2 (hBD2; Reproline, Valley Cottage, NY, USA) in deionized water was prepared. Then, 0.5 g of MBG was added to 5 mL of hBD2 solutions (1 mg/mL) and mixed using gentle shaking for 24 h at 4°C. Thereafter, the samples were subjected to centrifugation for 10 min at 10,000 rpm to separate supernatant and the MBG. The produced BD-MBG was used for subsequent experiments.

**The loading efficiency of hBD2 and its release profile from BD-MBG**

To estimate loading efficiency, 0.5 g of MBG scaffolds were immersed in 5 mL of hBD2 solution (0.5 mg/mL) at 4°C for 24 h, and then removed from the solution. The scaffolds were then subjected to centrifugation for 10 min at 10,000 rpm, and the pellet was retained. An hBD2 ELISA kit (MBS2023556, MyBioSource, San Diego, CA, USA) was used to determine the amount of the free hBD2 in the solution, following the manufacturer’s instructions. The following equation was used to calculate the drug loading efficiency: Loading efficiency (%)=(Total hBD2 amount-free hBD2 amount)/total hBD2 amount\(^17\). The release of hBD2 in vitro was assessed as follows: 0.5 g of BD-MBG was added to 10 mL of phosphate-buffered saline (PBS, pH 7.4) and shaken at 120 rpm in a water bath set at 37°C. One milliliter of supernatant was sampled at each time point, which was replaced with the same volume of fresh PBS. An enzyme-linked immunosorbent assay (ELISA) was then used to detect the cumulative release of the loaded hBD2. The cumulative release of hBD2 (%) was normalized to the total amount of the peptide. The in vitro drug-release test assayed six samples under each condition (\(n=6\))\(^17\).

**Determining the antibacterial effect on bacterial planktonic growth**

*Staphylococcus aureus* (ATCC25923) and *Staphylococcus epidermidis* (ATCC35984) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured as described previously\(^19\). The effects of MBG and BD-MBG on bacterial planktonic growth were also assessed as described previously\(^19\). Briefly, gamma-radiation at 25 kGy was used to sterilize the samples. The bacterial cultures (2×10\(^4\) colony forming units (CFU)/mL) were treated for 1 or 3 days with 20 mg of MBG or BD-MBG. A blank control group (BC) was also set. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used to detect cell proliferation\(^19\). The absorbance at 570 nm of the samples was obtained using a panel counter. The results were presented as absorbance per well minus the optical density of the blank wells (\(n=6\)).

**Determination of bacterial CFUs**

To assess the antibacterial effects of MBG or BD-MBG against the bacterial strains, the spread plate method was used, as described previously\(^19\). Briefly, the bacterial density has set at 1×10\(^6\) CFU/mL and the incubation temperature was held at 37°C for 24 h. Sterilized MBG or BD-MBG powders (0.1 g) were mixed into the bacterial solution separately. The mixtures were diluted 100 times, and 100 μL were subsequently inoculated onto LB agar plates and incubated at 37°C for 24 h; a blank control group (BC) was also set. Thereafter, the number of bacterial colonies was counted, and the number of colonies was multiplied by the dilution ratio to obtain the CFU (\(n=6\)).

**Leakage of protein from bacteria**

Bacterial suspensions (1×10\(^8\) CFU/mL; 10 mL) were cocultured with sterilized MBG and BD-MBG samples (20 mg) for 3 and 5 h at 37°C, as described previously\(^19\). The cultures were centrifuged and 1 mL of the supernatant was frozen at −20°C instantly. A Bicinchoninic Acid Protein (BCA) Assay Kit (Thermo Scientific, Waltham, MA, USA) was used to assess the total protein content, in which the optical density (OD) at 570 nm was read using a plate reader. The different OD values were divided by the reaction time and the cumulative protein content to obtain the relative protein value.

**Cell proliferation**

Mouse bone marrow mesenchymal stem cells (BMSCs; Texas A&M Health Science Center, Bryan, TX, USA) were cultured in α-MEM medium comprising 100 U/mL penicillin/streptomycin and 10% fetal bovine serum. The sterilized MBG and BD-MBG samples (20 mg) were placed in separate Transwell inserts (3 μm pore size, Corning, Corning, NY, USA) and co-cultured with BMSCs (1×10\(^5\) cells/cm\(^2\)) in osteogenic inductive medium (American Type Culture Collection); a blank control group (BC) was also set. The osteogenic medium was refreshed every 3 days. Samples were co-cultured with BMSC for 1, 3, 5, and 7 days (\(n=6\)). Cell Counting
Kit 8 (CCK-8; Beyotime, Jiangsu, China) assays were used to determine the cell proliferation ratios, in which an enzyme-linked immunosorbent assay plate reader (Titertek, Helsinki, Finland) was used to determine the optical density (OD) at 450 nm<sup>18</sup>.

**Alkaline phosphatase (ALP) assay**
Sterilized MBG and BD-MBG samples (20 mg) were co-cultured separately in osteogenic differentiation medium with BMSCs (1×10<sup>4</sup> cells/cm<sup>2</sup>) for 7 and 14 days (n=6). A QuantiChrom ALP test kit (BioAssay Systems, Hayward, CA, USA) was then used to measure the ALP activity. A Synergy HT microplate reader (Bio-Tek, Winooski, VT, USA) was used to evaluate the quantity of ALP in the cell lysates at 520 nm. The ALP level as then normalized to the total protein content as determined using a BCA protein assay kit (Thermo) according to the manufacturer’s protocol<sup>18</sup>.

**Alizarin red assay**
Sterilized MBG and BD-MBG samples (20 mg) were co-cultured separately in osteogenic differentiation medium with BMSCs (1×10<sup>4</sup> cells/cm<sup>2</sup>) for 21 and 28 days (n=6). The cells were fixed and then stained for 1 h using 1% alizarin-red solution (Sigma-Aldrich, St. Louis, MO, USA), and then the non-specific staining was washed away using PBS. Acetic acid (10%) was added to the stained wells and then their absorbance was determined spectrophotometrically at λ=405 nm for quantitative analysis. A calibration curve that correlated the absorbance of alizarin red with known concentrations of calcium was used to determine the amount of extracted calcium (in millimoles)<sup>18</sup>.

**Oil Red O staining**
Sterilized MBG and BD-MBG samples (20 mg) were co-cultured separately with BMSC (1×10<sup>4</sup> cells/cm<sup>2</sup>) in adipogenic differentiation medium (American Type Culture Collection) for 21 days (n=6). The cells were fixed and stained with 1% Oil Red O solution (Sigma) for 15 min according to a previously published method<sup>18</sup>. To remove background staining, 70% alcohol was used to wash the cells for 5 s. The cells were then mounted for observation. The amount of lipid droplets by was quantified using a microscope image analyzer (Olympus, Tokyo, Japan). The area of the lipid droplets was measured relative to the size of the cell nucleus. This ratio was then used to quantitate lipids (n=6).

**Real-time polymerase chain reaction (RT-PCR)**
Sterilized MBG and BD-MBG samples (20 mg) were co-cultured in osteogenic differentiation medium separately with BMSCs (1×10<sup>4</sup> cells/cm<sup>2</sup>) for 7, 14 and 21 days (n=6), and the expression levels osteogenic differentiation-related genes, including Col1 (collagen I), Opn (osteopontin) and Ocn (ostocalcin), were determined using RT-PCR. Gapdh (glyceraldehyde 3-phosphate dehydrogenase) was used as the housekeeping gene. Table 1 lists the primers used. The TRizol reagent (Ambion, Grand Island, NY, USA) was used to extract total RNA, which was reverse transcribed using a PrimeScript<sub>®</sub> RT reagent kit (Takara Biotechnology, Dalian, China). An ABI 7500 machine (Applied Biosystems, Foster City, CA, USA) was used to perform qPCR using the SYBR premix EX Taq PCR kit (Takara). The formula 2<sup>-ΔΔCT</sup> was used to calculate the amount of target cDNA relative to that of GAPDH. Comparison with the normalized Ct value of the BC control, which was set at 1, was used to calculate the fold differences. Data were collected from three independent pooled samples<sup>18</sup>.

**Western blotting**
Sterilized MBG and BD-MBG samples (20 mg) were co-cultured separately with BMSCs (1×10<sup>4</sup> cells/cm<sup>2</sup>) for 14 days in osteogenic differentiation medium (n=6). Total protein extracted from cells was fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked using 5% non-fat milk at room temperature for 1 h. The blots were then incubated overnight with primary antibodies recognizing COL1 (1:400, sc-59772, Santa Cruz Biotechnology, Santa Cruz, CA, USA), OPN (1:400, sc-73631, Santa Cruz Biotechnology), OCN (1:400, sc-390877, Santa Cruz Biotechnology), and β-actin.

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**Table 1** Primer sequences used for RT-PCR

| Primer | Sequences          |
|--------|--------------------|
| OCN-F  | CTGCTCAGCTGCTGACCT |
| OCN-R  | GGAAGGGCAATCAAGGT  |
| OPN-F  | TAATAGCAGCAGCTATAGG |
| OPN-R  | TACAGGAGCGATCGAGG  |
| COL1-F | GCGAGTGCTGCTTTCT   |
| COL1-R | AGCAAGTGTTCCTCAGAGG |
| GAPDH-F| TCAACGGGCAAGTCAGAGG |
| GAPDH-R| GATGTTAGGGGTCTCAGC  |
(1:1000, 3700, Cell Signaling Technology, Danvers, MA, USA). The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (1:5000, Zhongshan Goldenbridge Biotechnology, Beijing, China). Immunoreactive proteins were visualized using enhanced chemiluminescence. A densitometer (Syngene Bioimaging System; Frederick, MD, USA) and Scion Image software (Frederick) were used to quantify the density of the bands on the blots. The level for β-actin was used to normalize the protein levels for each sample.

**Statistical analysis**

The data analyses were accomplished using SPSS 17.0 (IBM, Armonk, NY, USA). Quantitative data are reported as the mean of at least three independent experiments (±standard deviation (SD)). The Shapiro-Wilk test assessed the normality of the data distribution, with 95% confidence and the homogeneity of variance was assessed using Levene’s test. The data fulfilled the assumptions of parametric tests and analysis of variance was used to evaluate the statistical significance among groups. Tukey’s test was used to perform post-hoc comparisons between the groups. Statistical significance was accepted at \( p < 0.05 \).

**RESULTS**

**In vitro loading and release behavior of hBD2**

The characterization of MBG and BD-MBG were shown in the Table 2. The loading efficiency of hBD2 in the MBGs was calculated as 31.4±3.4%. The hBD2 release profile from the BD-MBGs demonstrated a burst release followed by cumulative release of approximately 20% after 48 h (Fig. 1a). In Fig. 1b, it was shown that 27% of hBD2 was released from BD-MBGs at the first 3 days. The samples released hBD2 rapidly within the first 12 days, after which the release was slower, with a gentle slope (Fig. 1b). The cumulative release gets to 42.1% at 12 day and 50.8% at 50 day (Fig. 1b).

| Materials   | Surface area (m²/g) | Pore volume (cm³/g) | Average pore size (nm) |
|-------------|---------------------|---------------------|------------------------|
| MBG         | 213                 | 0.251               | 4.870                  |
| BD-MBG      | 196                 | 0.245               | 4.898                  |

MBG: mesoporous bioactive glass, BD-MBG: hBD2-loaded mesoporous bioactive glass

**Table 2 Parameters of scaffolds**

![Fig. 1](image1.png)  
**Fig. 1** *In vitro* release behavior of hBD2 from BD-MBG.  
BD-MBG: hBD2-loaded mesoporous bioactive glass.

![Fig. 2](image2.png)  
**Fig. 2** Antibacterial properties of MBG and BD-MBG samples against ATCC25923 (a) and ATCC35984 (b).  
BC: blank control, MBG: mesoporous bioactive glass, BD-MBG: hBD2-loaded mesoporous bioactive glass.  
For all charts, groups designated by different uppercase letters are significantly different (\( p < 0.05 \)).
**Fig. 3** Representative images of viable bacterial growth and the quantification of colonies of viable ATCC25923 and ATCC35984 bacteria on different samples.

BC: blank control, MBG: mesoporous bioactive glass, BD-MBG: hBD2-loaded mesoporous bioactive glass. For all charts, groups designated by different uppercase letters are significantly different ($p<0.05$).

**Fig. 4** Effects of MBG and BD-MBG on the membrane leakage of proteins from ATCC25923 (a) and ATCC35984 (b) after 3 and 5 h of incubation.

BC: blank control, MBG: mesoporous bioactive glass, BD-MBG: hBD2-loaded mesoporous bioactive glass. For all charts, groups designated by different uppercase letters are significantly different ($p<0.05$).

**Antibacterial efficacy of BD-MBG**

Compared with that in the BC and NBG groups, BD-MBG treatment caused a significant decline in the bacterial survival rate (Figs. 2a, b). According to the results of the spread plate method, BD-MBG treatment significantly reduced the number of viable bacteria (CFU) of the two strains compared with those in BC and MBG groups ($p<0.01$), and no differences were observed between the BC and MBG groups ($p>0.05$) (Fig. 3). In addition, the concentration of released proteins was much lower in the pure MBG and BC groups compared with that in the BD-MBG group (Fig. 4).

**Osteogenic effects of BD-MBG on BMSCs**

In all groups, BMSC proliferation was observed for 7 days in culture. On days 1 and 3, the cell proliferation rate was similar among the three groups ($p>0.05$). At days 5 and 7, the proliferation rate of the BMSCs treated with BD-MBG was significantly higher than that in the MBG and BC groups ($p<0.05$). The cell proliferation ability in the MBG groups was significantly higher than that in the BC group at day 7 ($p=0.02$); however, it did not show a significant difference at other time-points (Fig. 5). In addition, the ALP activity was significantly higher...
in the MBG and BD-MBG groups compared with that in the BC group on days 7 and 14 \((p<0.01)\), while it did not show any difference between the MBG and BD-MBG groups on either time point \((p>0.05)\) (Fig. 6a). Alizarin-red staining followed by quantitative analysis indicated that the calcium nodule formation was significantly higher in the MBG and BD-MBG groups than that in the BC group on days 21 and 28 \((p<0.01)\) (Fig. 6b). However, there was no significant difference in BMSC adipogenic differentiation among the BC, MBG, and BD-MBG groups on day 21 \((p>0.05)\). Moreover, at day 7 in the MBG and BD-MBG groups, only the \(\text{Col1}\) expression level was significantly elevated compared with that in BC group \((p<0.01)\), while the expression levels of \(\text{Opn}\) and \(\text{Ocn}\) did not exhibit any difference among the BC, MBG, and BD-MBG groups \((p>0.05)\). At days 14 and 21, the gene expression levels of \(\text{Col1}, \text{Opn},\) and \(\text{Ocn}\) all increased significantly in the MBG and BD-MBG groups compared with those in the BC group \((p<0.01)\). At all time points, the expression levels of \(\text{Col1}, \text{Opn},\) and \(\text{Ocn}\) were similar between the MBG and BD-MBG groups \((p>0.05)\). Consistently, the levels of the \(\text{COL1}, \text{OPN},\) and \(\text{OCN}\) proteins were significantly elevated in the MBG and BD-MBG groups at 14 days compared with those in the BC group \((p<0.01)\), while they showed no difference between the MBG and BD-MBG groups \((p>0.05)\) (Fig. 7).

**DISCUSSION**

To prevent or treat infected or contaminated bone defects, bone grafts should be developed that exhibit osteoconductivity and antimicrobial activity\(^{20,21}\). The presence of multi-antibiotic-resistant organisms means that the local use of antibiotic-loaded bone substitutes is frequently unsuccessful. Moreover, antibiotic use risks increasing the appearance of multi-drug resistant bacteria, which in turn, complicates further treatment options\(^{22}\). Defensins are important AMPs that do not induce significant bacterial resistance. In the present...
study, we incorporated human β-defensin-2 (hBD2) into MBG to produce a novel hBD2-loaded MBG (BD-MBG). The results showed that BD-MBG effectively inhibited bacterial growth and increased their protein leakage, as well as promoting the early and late osteogenic differentiation of BMSCs, which support the application of BD-MBG in the promotion of bone healing in bacteria-contaminated bone defects.

hBD2 was first identified as being produced by epithelial cells, and has mainly been studied in the treatment of infected wounds8. Previous reports showed that the minimal bactericidal concentration of hBD2 was ≤12 μg/mL against gram-negative bacteria and ≥10 μg/mL against gram-positive bacteria20. In particular, hBD2 is expressed in bone and may promote bone remodeling21. Previously, recombinant hBD2 was successfully coated onto dental and orthopedic titanium implants at 10 and 20 μg/mL, which not only exhibited excellent antimicrobial activity, but also promoted the proliferation of keratinocytes, osteoblasts, and BMSCs24,25. In addition, our previous studies have shown that MBGs provides excellent drug-loading and pro-osteogenic capabilities14,15. Based on this evidence, in the present study, we incorporated hBD2 into MBGs, and the resultant BD-MBG released hBD2 in an initial fast burst manner, followed by slower release as the MBG slowly degraded. BD-MBG effectively inhibited bacterial growth and accelerated their loss of internal constituents, thus increasing cell death. The initial fast release of hBD2 from BD-MBG would benefit cases of acute infection because it would lead to a rapid and high local concentration of the antibacterial peptide. BD-MBG showed prolonged and the effective release of the drug for up to seven weeks. The cumulative total release was more than 50%. The two-phase release characteristics of hBD2 indicates the enhanced antibacterial effect of the BD-MBG scaffolds, which could kill planktonic bacteria in the short-term and then inhibit bacterial colonization in the long-term, making them ideal to treat infected osseous defects. The results of bacteria protein leakage showed that the concentration of the released protein from BD-MBG was significantly higher than pure MBG and BC. These results suggest that cell membranes of ATCC25923 and ATCC35984 may be damaged by BD-MBG, which could lead to the loss of cell internal constituents and thereby accelerating cell death19. However, during bone healing, the pathogenesis of infection is a complex process that comprises interactions among the host, pathogen, and biomaterial; however, the in vitro assays used here could not account for in vivo factors, such as host defense. Therefore, in vivo studies are required to confirm the antibacterial actions of BD-MBG using bacterial infection defect models22.

The osteogenic activity and biocompatibility of BD-MBG should also be assessed. Our results indicated that osteogenic differentiation and cell proliferation were promoted significantly by BD-MBG treatment compared with those in the BC control group. Furthermore, the results of the quantification of ALP and alizarin-red staining, and the gene and protein expressions of osteogenic factors, suggested that MBG and BD-MBG has similar pro-osteogenic effects on the early and late stages of osteogenesis. These results demonstrated hBD2 incorporation had a positive antibacterial effect without compromising MBG’s osteogenic potential. However, it should be pointed that the brittleness and low fracture resistance of MBG make it less than ideally suited for use as a bone scaffold, i.e., it falls short of the mechanical strength requirements for clinical bone repair26. These problems could be solved in a future study by incorporating MBG particles into biodegradable organic scaffolds, such as poly-(L-lactic-co-glycolic acid) (PLGA), which would combine the polymer’s flexibility with the osteoconductivity of the inorganic material27.

Comparing to the blank control group, BD-MBG could not only significantly inhibited bacterial growth and damaged their membrane, but also increase ALP activity, calcium nodule formation, and expression levels of early and late osteogenic makers by MSCs. We thus conclude that BD-MBG synergistically possess antibacterial and osteogenic effects. The bacterial resistance to conventional antibiotics developed rapidly, and β-Defensin-2 has been reported as a very promising agent to resolve this issue owing to its reliable antimicrobial activity against multidrug-resistant bacteria. Therefore, the superiority of the present BD-MBG is the sustained hBD2 release for more than 7 weeks and the subsequent strong anti-bacteria effects. Further studies are needed to verified the antibacterial effects of BD-MBG against more multidrug-resistant bacteria lines. It should be note that all osteogenic parameters of MSCs did not show any significant difference between BD-MBG and MBG treatment groups. This result suggested that, although β-Defensin-2 released from the MBG may not have promoting effects on the osteoblastic differentiation, it did not inhibit osteogenic differentiation of MSCs. This could be another advantages of BD-MBG because several effective antibacterial agents, such as silver ions has been shown to cause inhibition of osteoblast proliferation and differentiation28.

It is known that the MBG have mesoporous structure, larger specific surface area and aperture capacity, facilitating the non-specific adsorption of β-Defensin-2 within its mesoporous channels14,18. In addition, β-Defensin-2 belongs to the small cationic peptides, and it might have specific binding with the anions within MBGs, but one of the limitation of the present study is that we did not analyze the specific interaction mechanism of β-Defensin-2 and MBG. Further study using ultraviolet-visible spectroscopy, scanning and transmission electron microscopy associated with energy-dispersive X-ray spectroscopy and electron energy loss spectroscopy are needed to characterize the specific interactions between the β-Defensin-2 and MBG29.

CONCLUSION
The results of the present study showed hBD2 could be successfully incorporated into MBG to produce a novel bone graft, termed BD-MBG, which synergistically
possess antibacterial and osteogenic effects. Thus, BD-MBG could represent a candidate material to treat peri-implant infection and to repair bones via its combined properties of bone ingrowth stimulation and biofilm prevention. Further animal experiments are required to evaluate the ability of BD-MBG to prevent or treat infection, and to fully determine their release kinetics and biocompatibility.

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CONFLICT OF INTEREST
The authors have no conflict of interest to disclose.

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