

# Bio-Oss<sup>®</sup> modified by calcitonin gene-related peptide promotes osteogenesis *in vitro*

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**Abstract.** Bio-Oss<sup>®</sup> and  $\alpha$ -calcitonin gene-related peptide (CGRP) are involved in osteogenesis. However, it has remained to be assessed how  $\alpha$ -CGRP affects the effect of Bio-Oss. In the present study, primary osteoblasts were incubated with  $\alpha$ -CGRP, Bio-Oss,  $\alpha$ -GGRP-Bio-Oss or mimic- $\alpha$ -CGRP. The proliferation rate, mineralization nodules, alkaline phosphatase (ALP) activity and the expression of osteogenic genes were measured by a Cell Counting Kit-8 assay, Alizarin Red-S staining, ALP activity detection and reverse-transcription quantitative PCR as well as western blot analysis, respectively. The proliferation rate, ALP activity and the number of mineralization nodules were significantly increased in the  $\alpha$ -CGRP-modified Bio-Oss group compared to that in the Bio-Oss group. The mRNA and protein levels of osteocalcin, Runt-related transcription factor-2 and ALP were significantly upregulated in the  $\alpha$ -CGRP-Bio-Oss group compared with those in the Bio-Oss group. Furthermore, the effect of mimic- $\alpha$ -CGRP on osteogenesis was reduced as it carried a mutation. In conclusion, the present study was the first to demonstrate that Bio-Oss modified with CGRP contributed to osteogenesis and may provide a novel formulation applied in the clinic for restoration of large bone defects.

## Introduction

It has been demonstrated that autogenous bone grafting has limited availability and accompanying risks include infection and pain as well as injury to nerves and vessels (1-3). Thus, bone graft substitutes, such as Bio-Oss<sup>®</sup> blocks were

considered for bone transplantation in the clinic. Bio-Oss, a natural and porous bone mineral matrix with no antigenic properties, is produced by removing all organic components from bovine bone. Bio-Oss is physically and chemically comparable to the mineralized matrix of human bone due to its natural structure (4). Moreover, Bio-Oss provides a scaffold for osteogenic cell migration due to its macroscopic and microscopic structure with an interconnected pore system (4). It also has been reported that Bio-Oss is a non-resorbable bone substitute (5,6). Several studies have clearly suggested that Bio-Oss contributes to osteogenesis by providing a scaffold for osteogenic cells (7,8). However, Bio-Oss does not have osteoinduction capability and does not significantly promote the proliferation and differentiation of osteoblasts. These disadvantages have limited its clinical application in restoration of large bone defects.

Calcitonin gene-related peptide (CGRP), a neuropeptide abundant in sensory neurons, regulates processes associated with migraine and colitogenic responses (9,10). CGRP also has an important role in bone innervation and has two isoforms,  $\alpha$ -CGRP and  $\beta$ -CGRP (11).  $\beta$ -CGRP is not considered osteogenic (12). Han *et al* (13) showed that  $\alpha$ -CGRP promotes rat osteoblast proliferation and indicated that it may be essential in bone remodeling.  $\alpha$ -CGRP has also been shown to be involved in the physiological activation of bone formation (13). Moreover,  $\alpha$ -CGRP may inhibit apoptosis of human osteoblasts, thus favoring local bone regeneration (14). Overexpression of  $\alpha$ -CGRP in mice increased the rate of bone formation due to osteoblast activity, which further resulted in the increase of trabecular bone volume (15).  $\alpha$ -CGRP knock-out in mice leads to decreased bone formation and osteopenia (16).  $\alpha$ -CGRP has a crucial role in inhibiting bone resorption of osteoclasts and stimulating the division of osteoblasts (17,18). In addition,  $\alpha$ -CGRP promotes osteogenesis by increasing the number and the size of bone colonies in cultured rat bone marrow leukocytes (19). Thus,  $\alpha$ -CGRP has a potential application in promoting osteogenesis.

Vacuum freeze-drying is a technique of freezing water-bearing material to form a solid, followed by dehydration under low temperature and pressure. Moreover, vacuum freeze-drying does not affect the physical, chemical and biological properties of certain materials. It has been reported that it is feasible to load biological activity factors onto the

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surface of bone substitute materials by using a vacuum freeze-drying technique (20).

In general, modified bone tissue engineering has great potential for repairing bone defects that result from trauma, surgical resection and congenital deformity corrections (21-23). In the present study,  $\alpha$ -CGRP was loaded onto the surface of Bio-Oss using a vacuum freeze drying technique to modify the biological activity of Bio-Oss in order to promote the activity of osteoblasts. By evaluating osteogenesis for bone formation by  $\alpha$ -CGRP-Bio-Oss, the present study provided a potential clinical application of Bio-Oss in restoring large bone defects.

## Materials and methods

*Bio-Oss modified by  $\alpha$ -CGRP.* The CGRP used in the present study was  $\alpha$ -CGRP (Anaspec, Fremont, CA, USA), which differs from  $\beta$ -CGRP by one amino acid. CGRP was dissolved in distilled water to a final concentration of  $10^{-5}$  M, and was stored at  $-20^{\circ}\text{C}$ . CGRP was diluted to the appropriate concentration in culture medium prior to use.

A total of 100 mg Bio-Oss (Geistlich Biomaterials, Sweden, Switzerland) was added to 2 ml phosphate-buffered saline (PBS; BestBio, Inc., Shanghai, China) containing the optimal concentration of  $\alpha$ -CGRP ( $10^{-7}$  M), followed by reaction at room temperature for 24 h. Then Bio-Oss was then washed with distilled water and freeze-dried.  $\alpha$ -CGRP consists of 37 amino acids and amino acids 2 and 7 are involved in the formation of a disulfide bridge (24), which is essential for biological activity (25,26). Numerous peptide and non-peptide CGRP antagonists have been described. These were initially based on peptide fragments lacking the N-terminal disulfide-bonded loop, of which the best characterized is CGRP8-37 (27-29). In the present study, a mimic- $\alpha$ -CGRP, in which the disulfide bond was disturbed, provided by GL Biochem Ltd. (Shanghai, China), was used. Amino acids 1-7 of the mimic- $\alpha$ -CGRP were H-Thr-Thr-Thr-Thr-Ala-Thr.

*Scanning electron microscopy (SEM).* The samples were placed on a conductive object stage, sprayed with gold sputter in a vacuum spray apparatus (MSP-1S; Hitachi, Ltd., Tokyo, Japan) and then scanned by SEM (Hitachi S-3400N; Hitachi, Ltd.) at different magnifications. The surface morphology of Bio-Oss bone substitute in different groups was observed with the electron acceleration voltage set to 10 kV.

*Primary osteoblast isolation and cell culture.* Primary osteoblast isolation from the calvaria of neonatal Sprague Dawley rats (Medical Animal Experimental Center of Guangdong, Guangzhou, China) was performed as previously described (27). According to the rules of Committee on Animal Research and Ethics of Guangzhou Medical University (Guangzhou, China), the present research project was reviewed and approved to be appropriate and humane by the Animal Care and Use Committee of Guangzhou Medical University. In brief, 4 neonatal rats (age, 3 days old) were sacrificed and sterilized with 75% ethanol. The skin and brain tissue were carefully removed from the skull, the jaw was cut and excess connective tissue and vascular tissue was scrapped from around the edge of the calvaria. The calvaria was washed three times with PBS and cut into  $1 \times 1$  cm<sup>2</sup> blocks. The calvaria was

then incubated in 1% trypsin (4 ml; containing EDTA; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 10 min at  $37^{\circ}\text{C}$ , followed by three washes with PBS and subsequent incubation with 0.1% collagenase II (4 ml; Sigma-Aldrich; Merck KGaA; Darmstadt, Germany) at  $37^{\circ}\text{C}$  for 30 min. The collagenase solution was then replaced with fresh solution, followed by incubation for a further 20 min at  $37^{\circ}\text{C}$ . Cells digested by collagenase were collected and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml; Gibco; Thermo Fisher Scientific, Inc.), streptomycin (100 mg/ml; Gibco; Thermo Fisher Scientific, Inc.), ascorbic acid (10 mg/ml; Sigma-Aldrich; Merck KGaA) and  $\beta$ -glycerophosphate (10 mM; Sigma-Aldrich; Merck KGaA) in a humidified atmosphere at  $37^{\circ}\text{C}$  with 5% CO<sub>2</sub>.

*Immunofluorescence staining.* At the second passage (P2), the osteoblasts cultured in the 6-well plates were washed three times with PBS and then fixed with 4% paraformaldehyde (BestBio, Inc.) for 15 min, followed by washing with PBS containing 0.1% Triton X-100 (BestBio, Inc.) for 5 min. Rhodamine phalloidin (300  $\mu\text{l}$ /well; Sigma-Aldrich; Merck KGaA) was used for F-actin staining and Hoechst 33342 (30  $\mu\text{g}$ /ml; Sigma-Aldrich; Merck KGaA) for cell nuclear staining. The fluorescence images were captured using a fluorescence microscope (Olympus Corp., Tokyo, Japan).

*Alkaline phosphatase (ALP) staining.* The osteoblasts (P2) were cultured on slides (that were not treated with lysin) for 1 week. For ALP staining, the Gomori method was applied, following the instructions of the ALP stain kit (D0001-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

*Detection of ALP activity.* Measurement of ALP activity was performed with an ALP assay kit (A059-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In brief, osteoblasts were loaded onto a 24-well culture plate containing Bio-Oss,  $\alpha$ -CGRP-Bio-Oss, CGRP or mimic-CGRP, respectively, at  $2 \times 10^4$  cells per well. The blank group consisted of osteoblasts that were loaded onto a 24-well culture plate at  $2 \times 10^4$  cells per well. After culturing for 7, 14 or 21 days, the media was removed. A total of 0.2 ml 0.1% Triton-100 was added and 30  $\mu\text{l}$  cell lysate was used for ALP activity detection. The optical density value of the culture media was measured with a microplate reader (Thermo Fisher Scientific, Inc.) at 490 nm. The protein concentration was determined by bicinchoninic acid and bovine serum albumin (BestBio, Inc.) was used as a standard. ALP activity was expressed as a percentage over the negative control.

*Alizarin Red-S staining.* Osteoblasts at P3 were cultured in 6-well plates with a seeding density of  $1 \times 10^5$  cells/well if Bio-Oss (1 mg) was added or  $2 \times 10^4$  cells/well if  $\alpha$ -GGRP-Bio-Oss (1 mg) was used. When the cells reached 80% confluency, the cell culture media was changed to mineralized induction solution, which contained ascorbic acid (10 mg/ml),  $\beta$ -glycerophosphate (10 mM) and dexamethasone (0.1  $\mu\text{mol/l}$ ; all from Sigma-Aldrich; Merck KGaA) for culturing osteoblasts for up to 21 days. Subsequent to three washes in PBS, cells were fixed with 4% paraformaldehyde for 10 min.

Table I. Gene primers used for quantitative polymerase chain reaction.

Gene	Direction	Sequence, 5'-3'	Product size (bp)	GenBank accession no.
ALP	Forward	CATCGCCTATCAGCTAATGCACA	150	NM_013059.1
	Reverse	ATGAGGTCCAGGCCATCCAG		
OCN	Forward	CCCTCTCTCTGCTCACTCTGCT	119	NM_013414.1
	Reverse	CTTACTGCCCTCCTGCTTGG		
Runx2	Forward	CATGGCCGGGAATGATGAG	148	NM_001278483.1
	Reverse	TGTGAAGACCGTTATGGTCAAAGTG		
GAPDH	Forward	GGCACAGTCAAGGCTGAGAATG	143	NM_017008.4
	Reverse	ATGGTGGTGAAGACGCCAGTA		

ALP, alkaline phosphatase; OCN, osteocalcin; Runx2, Runt-related transcription factor 2.

Alizarin Red-S solution (BestBio, Inc.) was added and cells were cultured for 30 min at 37°C.

**Analysis of cell proliferation.** The effect of different concentrations of CGRP on the proliferation of primary osteoblasts was determined by using a Cell Counting Kit-8 (CCK-8; BestBio, Inc.). CCK-8 proliferation analysis was performed according to the manufacturer's procedure. In brief, osteoblasts were seeded into 24-well plates at a seeding density of  $1 \times 10^4$  cells/well and cultured with CGRP at concentrations of  $10^{-7}$ ,  $10^{-8}$ ,  $10^{-9}$ ,  $10^{-10}$ ,  $10^{-11}$  or 0 M, and the medium was changed every 2 days. In another experiment, the wells contained Bio-Oss,  $\alpha$ -CGRP-Bio-Oss, CGRP or mimic-CGRP, respectively, at  $10^{-9}$  M, and the incubation time was 1, 3 or 7 days. The cells were then treated with CCK-8 reagent for 2 h in the dark to assess the osteoblast proliferation rate. The absorbance of the culture media was measured with a microplate reader (Thermo Fisher Scientific, Inc.) at 450 nm.

**RNA extraction and reverse-transcription quantitative polymerase chain reaction (RT-qPCR).** The seeding density of the osteoblasts was  $10^5$ /well in 6-well plates. RNA was extracted from the control, Bio-Oss, CGRP-Bio-Oss, CGRP and mimic-CGRP group using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). DNA was further digested with gDNA Eraser (Takara, Otsu, Japan). The mRNA expression levels of the osteogenic genes ALP, osteocalcin (OCN) and Runt-related transcription factor-2 (RUNX2) were assessed by RT-qPCR (Bio-Rad CFX96; Bio-Rad Laboratories, Inc., Hercules, CA, USA). For the reverse-transcription step, the reaction mixture contained Prime Script RT Enzyme mix I (1.0  $\mu$ l), RT Primer mix (1.0  $\mu$ l), 5X Prime Script Buffer 2 (4.0  $\mu$ l), RNase Free dH<sub>2</sub>O (4.0  $\mu$ l) and the reaction solution from step 1 (10.0  $\mu$ l). Reverse-transcription was performed according to the to the manufacturer's instructions of the reverse transcription kit (RR047; Takara Bio, Inc., Otsu, Japan). The reaction condition was as follows: 15 min at 37°C, followed by 5 sec at 85°C and a holding temperature of 4°C, using a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primers of these genes are presented in Table I. SYBR® Premix Ex TaqII (12.5  $\mu$ l), forward primer (1.0  $\mu$ l), reverse primer (1.0  $\mu$ l), DNA (2.0  $\mu$ l) and ddH<sub>2</sub>O (8.5  $\mu$ l) were used to provide a total reaction volume of 25  $\mu$ l and a SYBR-Green I fluorescence

quantification PCR kit (RR820; Takara Bio, Inc.) was used for qPCR. The reverse-transcribed nucleotides were amplified using a CFX96™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.) and the following thermocycling conditions: 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 30 sec at 60°C. The relative expression level for each gene was normalized to GAPDH and relative quantification of gene expression was performed using the  $2^{-\Delta\Delta C_q}$  method (30).

**Western blot analysis.** To study the effects of CGRP on osteoblasts, western blot analysis was used to determine the expression of ALP, OCN and Runx2 on day. Briefly, osteoblasts were washed with ice-cold PBS three times. A total of 100  $\mu$ l radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology), containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40 and 0.1% SDS, was added into each well. After incubation on ice for 15 min, the osteoblasts were scraped and briefly centrifuged (12,000 x g at 4°C for 5 min). The concentration of protein in the samples containing ALP, OCN and Runx2 was measured by using G250 (Beyotime Institute of Biotechnology). Radioimmunoprecipitation assay buffer was used to adjust the concentration, for consistency. The nuclear extract was collected, boiled with 5X SDS sample buffer (Beyotime Institute of Biotechnology), and then subjected to SDS. A total of 10  $\mu$ l protein from each sample and 7  $\mu$ l Marker (26616; Thermo Fisher Scientific, Inc.) were loaded and separated using 10% SDS-PAGE. Samples were subsequently blotted onto a polyvinylidene difluoride microporous membrane (Beyotime Institute of Biotechnology) and the blot was blocked for 1 h in 5% non-fat dry milk in Tris-buffered saline with 0.5% Tween-20 (PBST). Furthermore, the blot was incubated with rabbit polyclonal anti-ALP (1:3,000; ab95462; Abcam, Cambridge, UK), mouse monoclonal OCN (1:2,000; ab13418; Abcam) and rabbit polyclonal Runx2 (1:2,000; ab23981; Abcam) antibodies at 4°C overnight. GAPDH (1:8,000; ab181602; Abcam) was used as a control. After washing in PBST, the blots were incubated in goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (diluted in 1:5,000; ab6721; Abcam) for 1 h at room temperature. All protein concentrations were determined according to the recommendation of Abcam. The membrane was then washed three times (10 min each time) with Tris-buffered saline with 0.5% Tween-20 and exposed

to film following chemiluminescence reagent treatment with the enhanced chemiluminescence and western blotting reagents (Beyotime Institute of Biotechnology). Bands were quantified using densitometry of digitalized images and the results were analyzed by Quantity One software 4.52 (Bio-Rad Laboratories, Inc.). The results of the present assay were confirmed by repeating the experiment three times.

**Statistical analysis.** Values are expressed as the mean  $\pm$  standard error of the mean from three independent experiments. Statistical analysis was performed using SPSS statistical software v20.0 (International Business Machines, Corp., Armonk, NY, USA). Data were evaluated using analysis of variance followed by a least-significant differences test or Bonferroni's post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Bio-Oss modification.** The surface morphology of the Bio-Oss, PBS-Bio-Oss and CGRP-Bio-Oss was examined by SEM. No significant difference in surface morphology was observed at different magnifications (Fig. 1). These results demonstrated that the vacuum freeze-drying technique did not affect the physical, chemical and biological properties of the material.

**Osteoblastic characteristics of primary osteoblasts.** The osteoblastic characteristics of primary osteoblasts were demonstrated by light microscopy, F-actin staining, ALP and alizarin red staining. The morphology of cells was mostly irregular, polygonal and had protrusions (Fig. 2A and B). Fluorescence staining further confirmed the above results. The cell skeleton marker F-actin was stained red and the nuclei were stained blue color (Fig. 2C and D). ALP is the specific marker enzyme of osteoblasts (31). ALP-positive osteoblasts were displayed as black or gray black particles in the cytoplasm after Gomori staining. As displayed in Fig. 2E and F, a vast majority of cells were positive. The mineralized nodules were dyed red after Alizarin Red-S staining. As presented in Fig. 2G and H, red positive nodules were variable in size with deep staining in the center, which gradually became shallow towards the edges. Primary osteoblasts were passed to the third passage for the subsequent experiments.

**Effect of CGRP on osteoblast proliferation.** The effects of CGRP on osteoblast proliferation were assessed using the CCK-8 assay. CGRP at different concentrations ( $10^{-7}$ ,  $10^{-8}$ ,  $10^{-9}$ ,  $10^{-10}$ ,  $10^{-11}$  or 0 M) was tested on osteoblasts after incubation for 1, 3 or 7 days. As shown in Fig. 3,  $10^{-9}$  M  $\alpha$ -CGRP resulted in the highest increase in the proliferation rate compared to that in the control group ( $P < 0.01$ ).

**Effects of CGRP on ALP activity in osteoblasts.** Effects of CGRP on ALP activity in osteoblasts were assessed using the ALP Kit assay. Various concentrations of CGRP ( $10^{-7}$ ,  $10^{-8}$ ,  $10^{-9}$ ,  $10^{-10}$ ,  $10^{-11}$  or 0 M) were tested on osteoblasts at days 7, 11 and 14. As shown in Fig. 4,  $10^{-9}$  M  $\alpha$ -CGRP resulted in the greatest increase in the ALP activity rate at day 7 ( $P < 0.01$ ) and 11 ( $P < 0.05$ ), compared to the control groups. Thus,  $10^{-9}$  M  $\alpha$ -CGRP was selected to modify Bio-Oss. Furthermore, the concentration at which mimic- $\alpha$ -CGRP was used was  $10^{-9}$  M.

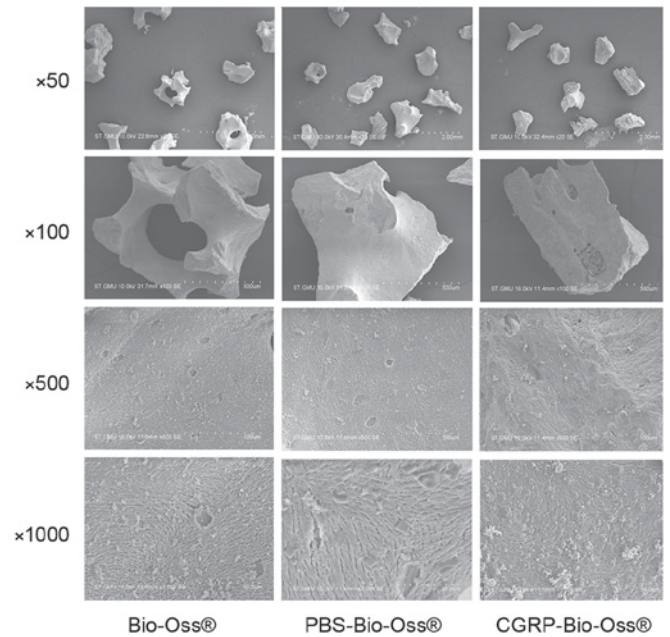


Figure 1. Surface morphology of the Bio-Oss<sup>®</sup> and CGRP-Bio-Oss<sup>®</sup> by scanning electron microscopy (magnification, x50, x100, x500 or x1,000 as indicated). No marked difference of surface morphology was observed. CGRP, calcitonin gene-related peptide.

**Assessment of osteoblast proliferation rate.** Compared with the control group, the cell proliferation rate in the  $\alpha$ -CGRP group was significantly increased at days 3 ( $P < 0.01$ ), 5 ( $P < 0.001$ ) and 7 ( $P < 0.001$ ). However, in Bio-Oss groups, the cell proliferation rate significantly increased at day 5 and 7 ( $P < 0.05$ ; Fig. 5) compared with the control group. Of note, the proliferation rate in the  $\alpha$ -CGRP-Bio-Oss group was significantly higher than that in the Bio-Oss group at days 5 and 7. By contrast, mimic- $\alpha$ -CGRP did not influence the osteoblast proliferation (Fig. 5).

**Effect of CGRP on osteoblast mineralization capability.** The effects of CGRP on osteoblast mineralization capability were assessed using the Alizarin Red-S staining. As shown in Fig. 6, the amount of mineralization nodules in the CGRP-Bio-Oss group was obviously higher than that in the Bio-Oss group.

**Assessment of osteogenesis-associated genes and proteins.** The expression of genes associated with osteogenesis, including the osteogenic transcription factor Runx2, the early osteogenic marker ALP and the late osteogenic marker OCN, were evaluated by RT-qPCR on days 7, 14 and 21. The addition of CGRP ( $10^{-9}$  M) to the culture media significantly increased osteoblastic expression of ALP, as well as that of OCN at day 14. Runx2 was also increased at days 7 and 14, but not at day 21 (Fig. 7). A significant upregulation of ALP and OCN was also found in osteoblasts cultured with  $\alpha$ -CGRP-Bio-Oss compared with that in the Bio-Oss group. Gene expression of Runx2 increased at day 7 but not on days 14 and 21 in the  $\alpha$ -CGRP-Bio-Oss compared to the Bio-Oss group. The detected protein levels of Runx2, ALP and OCN corresponded to the mRNA expression, which are presented in Fig. 8. Significant upregulation of Runx2, ALP and OCN was found in osteoblasts cultured with  $\alpha$ -CGRP-Bio-Oss compared to those in the Bio-Oss group ( $P < 0.05$ ; Fig. 8).

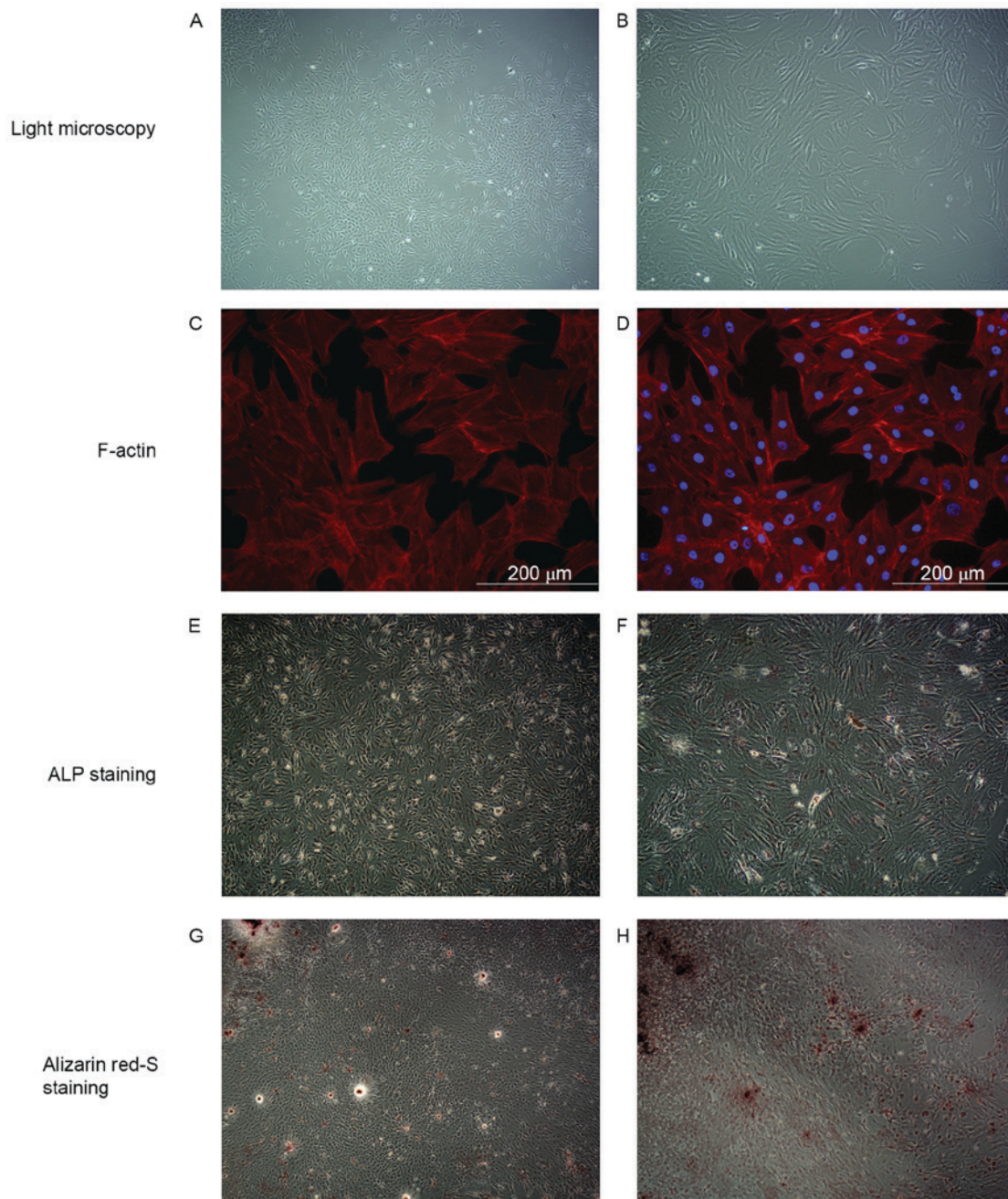


Figure 2. Osteoblastic characteristics of primary osteoblast. (A and B) The profile of cells was mostly irregular, polygonal and had protrusions [magnification, x40 in (A) and x100 in (B)]. (C and D) Fluorescence staining further confirmed the above results. Cell skeleton marker F-actin is shown in red color and the nuclei were counterstained in blue (magnification, x400; scale bar, 200  $\mu\text{m}$ ). (E and F) ALP is the specific marker enzyme of osteoblasts. ALP-positive osteoblasts displayed as black or gray black particles, in the cytoplasm after Gomori staining [magnification, x40 in (E) and x100 in (F)]. The vast majority of cells were positive. (G and H) Mineralized nodules were dyed red after Alizarin Red-S staining [magnification, x40 in (G) and x100 in (H)]. Positively stained nodules were variable in size with deep staining in the center, which gradually became shallow towards the edges. ALP, alkaline phosphatase.

## Discussion

The present study was the first, to the best of our knowledge, to modify Bio-Oss with  $\alpha$ -CGRP to analyze the specific effect of  $\alpha$ -CGRP-Bio-Oss on osteogenesis *in vitro*.

$\alpha$ -CGRP was loaded onto the surface of Bio-Oss using a vacuum freeze-drying technique in order to provide Bio-Oss with biological activity in order to promote the activity of osteoblasts. The samples were scanned by SEM, revealing no significant difference in surface morphology at different magnifications. These results demonstrated that the vacuum

freeze-drying technique did not affect the physical, chemical and biological properties of the material.

Due to its greatest effect on cell proliferation and ALP activity,  $10^{-9}$  M CGRP was chosen as the optimal concentration to modify Bio-Oss. It was found that the proliferation rate was significantly increased in the osteoblasts cultured with  $\alpha$ -CGRP-Bio-Oss compared to those in the Bio-Oss group on days 5 and 7. In previous studies, three different concentrations of CGRP ( $10^{-12}$ ,  $10^{-10}$  and  $10^{-8}$  M) were tested in bone marrow mesenchymal stem cells at day 4 post-seeding, but only the  $10^{-10}$  M concentration significantly increased

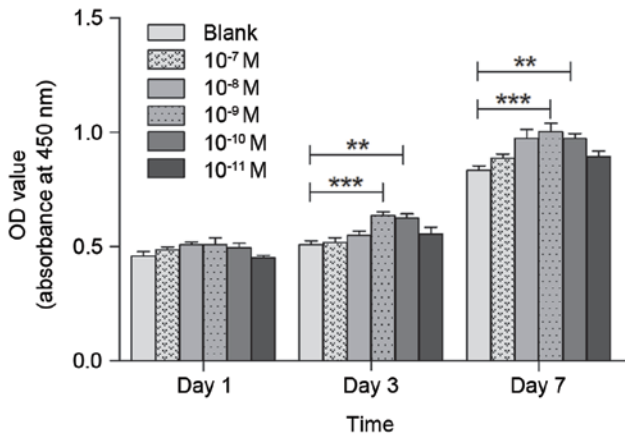


Figure 3. Effects of CGRP on osteoblast proliferation were assessed using the Cell Counting Kit-8. Treatment with 10<sup>-9</sup> M α-CGRP resulted in the most significant increase in the proliferation rate compared with that in the control groups. \*\*P<0.01, \*\*\*P<0.001. CGRP, calcitonin gene-related peptide; OD, optical density.

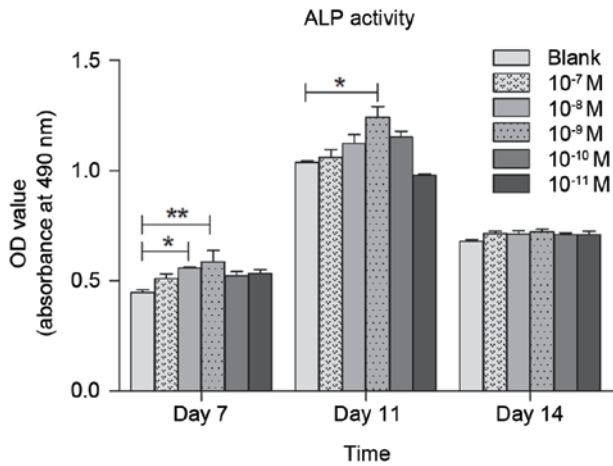


Figure 4. Effects of CGRP on ALP activity in osteoblasts were assessed using an ALP kit assay. Treatment with 10<sup>-9</sup> M α-CGRP resulted in the highest increase in the ALP activity rate at day 7 (P<0.01) and 11 (P<0.05), compared to the control groups. \*P<0.05, \*\*P<0.01 vs. blank. CGRP, calcitonin gene-related peptide; ALP, alkaline phosphatase; OD, optical density.

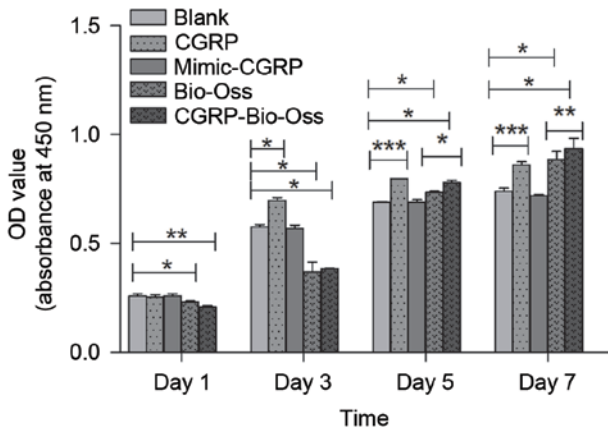


Figure 5. Proliferation of osteoblasts in different groups. The proliferation rate in the α-CGRP-Bio-Oss<sup>®</sup> group was significantly higher than that in the Bio-Oss group at day 5 and 7. However, mimic-α-CGRP did not influence osteoblast proliferation. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. CGRP, calcitonin gene-related peptide; OD, optical density.

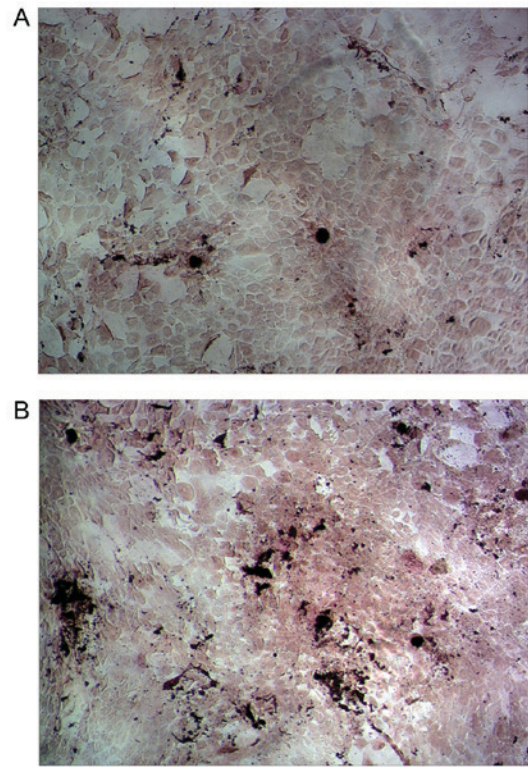


Figure 6. Effects of CGRP on the mineralization capability of osteoblasts were assessed using Alizarin Red-S staining. (A) Bio-Oss<sup>®</sup> group; (B) CGRP-Bio-Oss group. The CGRP-Bio-Oss group exhibited obviously more calcified nodules (magnification, x100). CGRP, calcitonin gene-related peptide.

bromodeoxyuridine incorporation by 36% (P<0.05), compared with vehicle-treated control cultures (32). Cornish *et al* (33) used different concentrations of CGRP (10<sup>-8</sup>, 10<sup>-9</sup> and 10<sup>-10</sup> M) on primary rat osteoblasts, and the proliferation activity was tested after a 24-h period. Their findings indicated that 10<sup>-9</sup> M CGRP was able to promote cell proliferation. A similar observation was indicated in the present study.

ALP activity has been widely used to demonstrate the early and late differentiation of osteoblast-like cells (34,35). ALP activity was enhanced in osteoblasts cultured with α-CGRP-Bio-Oss. The expression levels of ALP, OCN and Runx2 were increased in the α-CGRP-Bio-Oss group. However, in the mimic-α-CGRP group, disulfide breakage in α-CGRP led to the reduction of proliferation, ALP activity and expression alteration of osteogenic markers based on the same culture conditions between α-CGRP and mimic-α-CGRP, which suggested that this motif was essential for the function of α-CGRP in osteogenesis. It was thus concluded that α-CGRP significantly improved the osteogenesis function of Bio-Oss. However, α-CGRP-Bio-Oss should be further assessed *in vivo* in future studies.

In conclusion, the present study was the first, to the best of our knowledge, to demonstrate that Bio-Oss modified by α-CGRP significantly improved osteoblastic function. The strategy of applying α-CGRP-Bio-Oss may be a promising method of regenerating bone tissue in the clinic.

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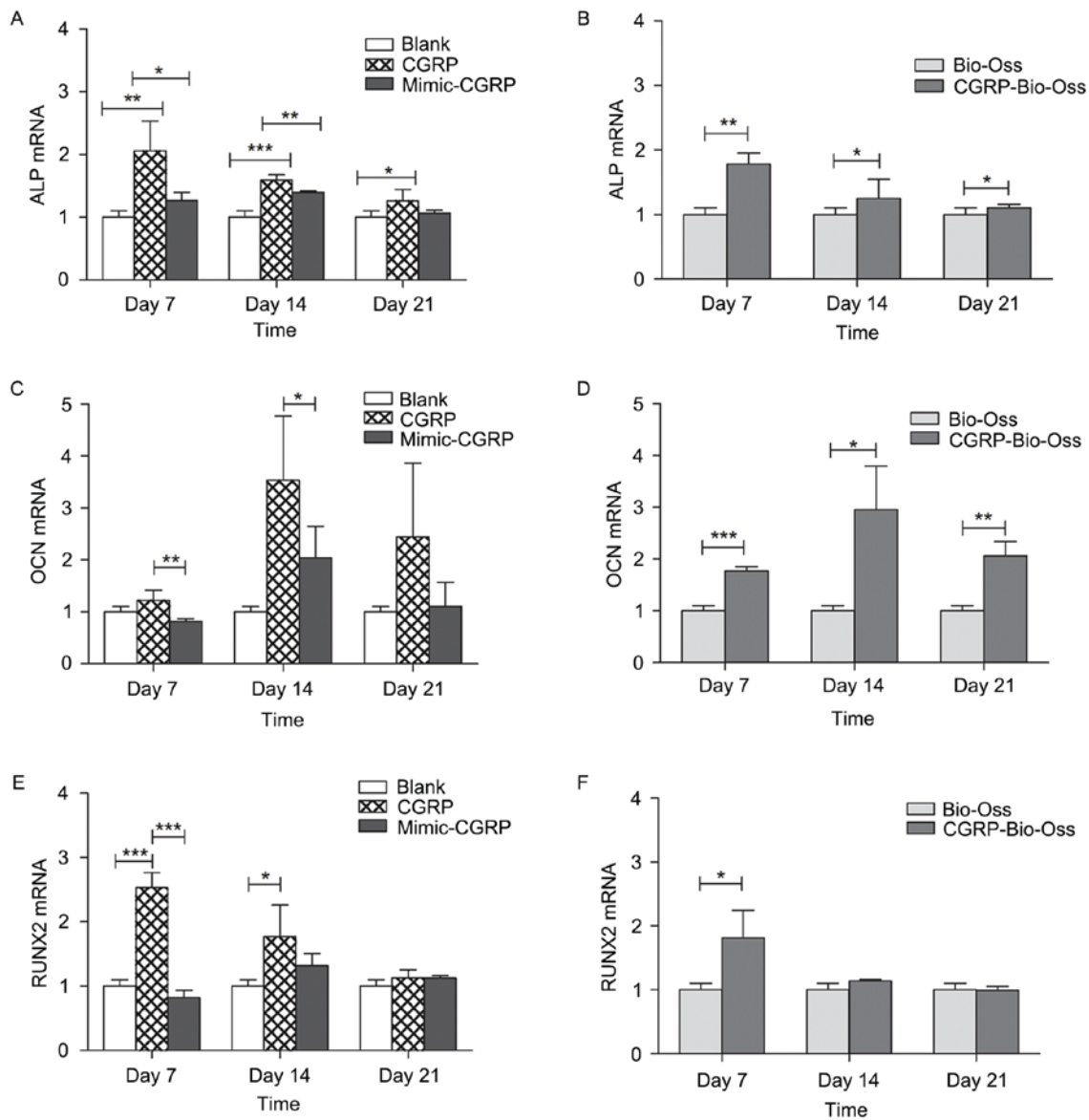


Figure 7. Expression of the osteogenesis-associated genes (A and B) ALP, (C and D) OCN and (E and F) RUNX2 was evaluated by reverse-transcription quantitative polymerase chain reaction analysis on days 7, 14 and 21. Addition of CGRP ( $10^{-9}$  M) to the culture media significantly increased osteoblastic expression of ALP, OCN at day 14 and Runx2 at days 7 and 14, but not on day 21. Significant upregulation of ALP and OCN was found in the osteoblasts cultured with  $\alpha$ -CGRP-Bio-Oss<sup>®</sup> compared to that in the Bio-Oss group. Gene expression of Runx2 increased at day 7 but not on day 14 and 21 in the  $\alpha$ -CGRP-Bio-Oss compared to that in the Bio-Oss group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . ALP, alkaline phosphatase; OCN, osteocalcin; Runx2, Runt-related transcription factor 2; CGRP, calcitonin gene-related peptide.

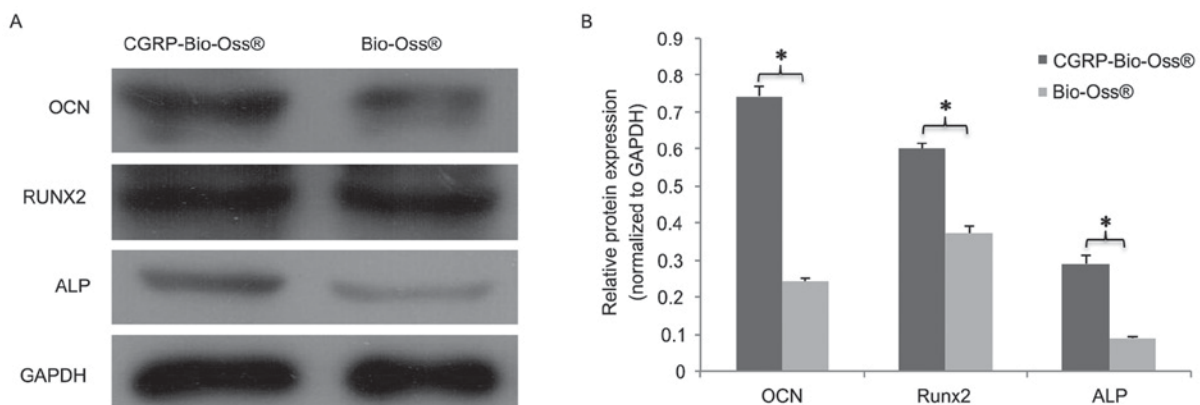


Figure 8. Protein levels of ALP, OC and Runx2 on day 14. (A) Representative western blot images and (B) quantified expression levels are presented. Significant upregulation of Runx2, ALP and OCN were found in the osteoblasts cultured with  $\alpha$ -CGRP-Bio-Oss<sup>®</sup> compared with those in the Bio-Oss group. \* $P < 0.05$ . ALP, alkaline phosphatase; OCN, osteocalcin; Runx2, Runt-related transcription factor 2; CGRP, calcitonin gene-related peptide.

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