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# Graded Porous $\beta$ -Tricalcium Phosphate Scaffolds Enhance Bone Regeneration in Mandible Augmentation

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**Abstract:** Bone augmentation requires scaffold to promote forming of natural bone structure. Currently, most of the reported bone scaffolds are porous solids with uniform pores. The aim of the current study is to evaluate the effect of a graded porous  $\beta$ -tricalcium phosphate scaffolds on alveolar bone augmentation. Three groups of scaffolds were fabricated by a template-casting method: (1) graded porous scaffolds with large pores in the center and small pores at the periphery, (2) scaffolds with large uniform pores, and (3) scaffolds with small uniform pores. Bone augmentation on rabbit mandible was investigated by microcomputed tomography, sequential fluorescent labeling, and histologic examination 3 months after implantation. The result presents that all the scaffold groups maintain their augmented bone height after 3-month observation, whereas the autografting group presents an obvious bone resorption. Microcomputed tomography reveals that the graded porous group has significantly greater volume of new bone ( $P < 0.05$ ) and similar bone density compared with the uniform pores groups. Bone substance distributes unevenly in all the 3 experimental groups. Greater bone volume can be observed in the area closer to the bone bed. The sequential fluorescent labeling observation reveals robust bone regeneration in the first month and faster bone growth in the graded porous scaffold group than that in the large porous scaffold group. Histologic examinations confirm bone structure in the aspect of distribution, activity, and maturity. We conclude that graded porous designed biodegradable  $\beta$ -tricalcium phosphate scaffolds are beneficial to promote bone augmentation in the aspect of bone volume.

**Key Words:** Bone regeneration, graded pore size,  $\beta$ -TCP, bone augmentation

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Titanium dental implants are rapidly becoming a routine procedure in today's dentistry and are widely used for a variety of cases, which includes surgical replacement of lost teeth or restoration of oral function.<sup>1,2</sup> A significant factor that limits the clinical application of a titanium dental implant is the restrictive necessity for alveolar bone mechanical support.<sup>3,4</sup> New advances brought by bone tissue engineering, in particular, by bone grafts, may surmount the current deficits of titanium dental implants.

One approach to designing bone grafts for alveolar bone augmentation or repair of critical size defects is biomimicking the chemical composition and structural features of natural bone, thus achieving similar regenerative properties.<sup>5-8</sup> Scaffolds fabricated with calcium phosphate ceramics such as hydroxyapatites and beta-tricalcium phosphate ( $\beta$ -TCP) have been shown to repair bone defects in clinical applications because of their biocompatibility and resemblance in chemistry composition to natural bone.<sup>9,10</sup> In addition, an interconnected pore structure and high porosity are essential for scaffolds, as they permit the exchange of nutrient transport within the scaffold and facilitate the ingrowth of cells and tissues. Currently, most of the reported bone scaffolds are porous solids with uniform pores.<sup>11-13</sup> However, in nature, the bone has a graded architecture. Both long bones and flat bones change spatially and gradually from a dense, stiff external structure (cortical bone) to a porous internal structure (cancellous bone).<sup>14</sup> Therefore, adhering to the cues from nature, graded-based strategies can be used in the design and fabrication of scaffolds for mimicking bone structure to promote bone repair and regeneration.<sup>15,16</sup>

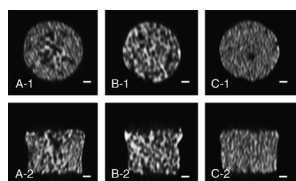
New research investigating the effect of assorted pore diameters in  $\beta$ -TCP scaffolds (150, 260, 510, and 1220  $\mu$ m) has demonstrated an inherent variance in bone formation and resorption rate among each group, suggesting that the spatial distribution of different pore sizes directly affects bone structure of the engineered bone.<sup>17</sup>

Creating a suitable graded porous ceramic scaffold that has adequate pore spatial distribution and interconnected pore structure has been a challenge.<sup>16</sup> We addressed this problem by developing a template-casting method to easily fabricate porous ceramic scaffolds with controlled spatial variation of chemistry and interconnected pore structure.<sup>18</sup> In addition, through this method, the desirable prerequisites of shape customization and pore size distribution could be fulfilled to achieve a graded pore distribution in a spatial manner. In this study, we fabricated 3 groups of scaffolds—(1) graded porous scaffolds, (2) large porous scaffolds, and (3) small porous scaffolds—and evaluated the effect of the scaffolds with recombinant human bone morphogenetic protein-2 (rhBMP-2) on osteogenesis and vertical augmentation in a rabbit mandibular defect model. By analyzing the difference of generated bone in the aspects of bone volume (BV) and bone density among the groups after 3 months of observation, we tend to find a suitable design of bone substitute.

## MATERIALS AND METHODS

### Scaffold Preparation

The following 3 groups of scaffolds with completely interconnected pores were prepared by our template-casting method<sup>18</sup>: (1) graded porous scaffolds (600- to 800- $\mu$ m pores in the center and 350- to 500- $\mu$ m pores at the periphery), (2) large porous scaffolds (600- to 800- $\mu$ m uniform pores), and (3) small porous scaffolds (350- to 500- $\mu$ m uniform pores). The fabrication process of the scaffolds included  $\beta$ -TCP slurry preparation, molding, casting, solidifying, drying, and sintering.<sup>19</sup> Gross and microcomputed tomography ( $\mu$ CT) examinations of their transverse section were performed as described previously.<sup>20</sup> The structural patterns of the  $\beta$ -TCP scaffolds were imaged as described in Figure 1. All the scaffolds were molded into cylinders with a height of 6 mm and a



**FIGURE 1.**  $\mu$ CT images. A to C show images of the graded porous scaffolds (GP), the large pore scaffolds (LP), and the small pore scaffolds (SP). Scale bars = 1000  $\mu$ m.

diameter of 8 mm. As for the graded scaffolds, the central region containing the larger pores was 4 mm in diameter, whereas the outer layer containing the smaller pores had a thickness of 4 mm.

### Scanning Electron Microscopy

Morphology of the scaffolds was observed using a scanning electron microscope (SEM; FEG-Philip XL30 ESEM, 2525 V). Before the observation, the samples were coated with a thin layer of Pt-Au.

### In Vivo Study of Osteogenesis Ability

#### Surgical Process

A total of 10 male New Zealand rabbits (8 w, 2.5–3.0 kg) with 20 mandibular sites were used to evaluate the *in vivo* osteogenesis ability of the 3 groups of  $\beta$ -TCP scaffolds (graded pores, large pores, and small pores) loaded with rhBMP-2, 1 positive control group (autogenous graft) and 1 negative control group (identical surgery without implantation) ( $n = 4$  per group). All animal studies were performed in accordance with procedures approved by the Animal Care and Ethics Committee of Peking University Health Science Center. The scaffolds were sterilized by gamma irradiation before rhBMP-2 was applied. The rhBMP-2 solution (Peprotech, 100  $\mu$ L) (>95% of purity, 0.1 mg/mL, 10  $\mu$ g per scaffold) was applied to the scaffolds. Animals were quarantined for a minimum of 1 week before experimentation and had *ad libitum* access to standard rabbit chow and water at all times.

To start the surgical process, the rabbits were anesthetized with sodium pentobarbital (30 mg/kg) by injection into the lateral ear vein. After hair shaving, a horizontal incision (25 mm) was made over the submandibular region to expose the buccal ramus of the mandible. A bone defect (8-mm diameter, 2-mm depth) was then created on the buccal ramus. Autogenous bone grafts from the iliac bone (8 mm in diameter, 4 mm in height) were fixed with titanium screws (1.5 mm in diameter, 8 mm in length) onto the mandible surface. The scaffolds (8 mm in diameter, 6 mm in height) embedded in the defect protruded the same height with the autografts did (4 mm) from the bone surface. All the blocks were covered by biodegradable membranes (Shenzhen Lando Biomaterials Co, Ltd). For the negative control group, a bone defect was created, without material implanted. The entire procedure was performed under sterile conditions. After the surgery, all rabbits received antibiotics (penicillin G, 200,000 units) intramuscularly daily for 3 days. Animals were housed individually, kept in a 12-hour light/dark cycle at controlled temperature (21°C) and fed *ad libitum* with standard laboratory diet.

The animals were killed 3 months after the surgery with an overdose of sodium pentobarbital, and the samples were prepared for examinations.

### Fluorochrome Labeling and Observation

Sequential fluorochrome labeling was used to determine the onset time of mineralization and the speed of new bone (spicule) formation. Polyfluorochrome tracers (tetracycline, alizarin red,

calcein) were subcutaneously injected as indicated in Table 1. After sample processing, polyfluorochrome tracers were identified by fluorescence microscopy. Imagepro-Plus 6.0 was used to analyze the proportion of each fluorochrome labeled area. Six regions of interest were selected from each sample according to different locations within the implanted scaffolds: outside-upper (O-U), outside-middle, outside-lower (O-L), inside-upper, inside-middle, and inside-lower. The level of mineralization of each region was blindly analyzed by an examiner who was not aware of the study design.

### Histomorphometric Examination

After fixation with 10% of phosphate-buffered formalin, the samples of mandibles were cut into 2 halves. One half of each sample was dehydrated in ethanol and then embedded in acrylic resin. The embedded blocks were trimmed by cutter and further ground to a final thickness of approximately 40  $\mu$ m. Then, the hard tissue slices were stained with toluidine blue and microscopically examined (Zeiss, LSM 5 EXCITER). The other half of each sample was decalcified with 5% of formic acid for 2 weeks<sup>21</sup> and then embedded in paraffin parallel to the sectioned surface. Serial cross-sections of decalcified samples were sectioned for hematoxylin and eosin (H&E) stain, toluidine blue staining, Masson staining, and osteocalcin (anti-osteocalcin antibody, ab13420; Abcam) with immunohistochemistry staining.

### $\mu$ CT Measurement

To quantify the bone regeneration in the implantation site, BV and bone mineral density (BMD) of each specimen were measured by  $\mu$ CT scans and analysis. All specimens were cut into rectangular prisms (1 cm<sup>3</sup>) containing the tissue-engineered construct. Samples were examined with a Skyscan 1076 high-resolution desktop  $\mu$ CT system (Skyscan, Kontich, Belgium). Taking into account the camera definition and the source-object-camera distance, two-dimensional images (pixel size, 8.665  $\mu$ m) were obtained. The corresponding three-dimensional images were produced by stacking all the two-dimensional cross-sections.

To study the scaffold pore size effect on bone formation, 6 regions of interest from different locations within the implanted blocks were selected, and each region was shaped into a cylinder (2 mm in diameter and 2 mm in length). After selection, upper and lower threshold values were decided to separate the bone from other tissues, and then the BV, tissue volume (TV), BV/TV, and bone density were calculated and analyzed using CTAn version 1.11.8.0+ (64 bits). The BV was defined as the volume of newly formed bone in the selected region with a threshold between 50 and 130, and the TV was defined as the overall volume in the selected region with a threshold below 130, which excluded the scaffold. We used the BV/TV as the index for new bone formation for comparison. The BMD was defined as the mineral density of the newly formed bone in pores of the scaffolds.

### Statistical Analysis

To further compare the regeneration ability among different sites in the scaffolds, 6 locations in each sample were selected and analyzed. Both the BV and the BMD were calculated. Statistics of

**TABLE 1.** Injection Schedule and Dosage of Polyfluorochrome Markers

Time, d	Substance	Doses, mg/kg
14	Tetracycline	60
44	Alizarin red (C <sub>14</sub> H <sub>8</sub> O <sub>4</sub> )	30
74	Calcein (C <sub>3</sub> O <sub>7</sub> H <sub>26</sub> N <sub>4</sub> O <sub>13</sub> )	10

each scaffold groups were analyzed in SPSS 17.0 software (IL). Two-way analysis of variance with Tukey post hoc test for multiple comparisons was used. The level for significance was set at  $P < 0.05$ .

## RESULTS

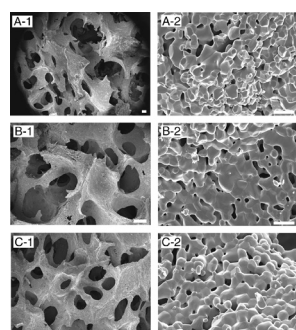
### SEM Examination

An SEM micrograph (Fig. 2) of the  $\beta$ -TCP porous scaffolds prepared from the template-casting method shows the interconnected structure of pores. Under low magnification, graded porous scaffolds showed the change of pore sizes, from central large pores (600–800  $\mu\text{m}$ ) to peripheral small pores (350–500  $\mu\text{m}$ ). No cracks or delamination was observed at the interface between zones in the samples. In uniform-size scaffolds, interconnected windows in the pores were seen. Under high magnification, micropores were observed in the struts of the 3 types of scaffolds.

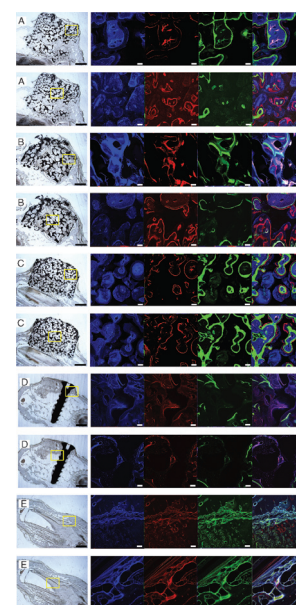
### In Vivo Osteogenic Ability

#### Polyfluorochrome Labeling on Nondecalfied Samples

The nondecalfied hard-tissue slices showed that the height of experimental groups was stable, and the scaffolds maintained their original shape (Figs. 3A–C). On the other hand, the shape of the transplanted iliac bone changed. Although the autogenous implants were stable and integrated to the bone, protruding bone appeared as a hemisphere rather than a cylinderlike shape. Moreover, their height decreased (Fig. 3D). As for the negative control, no vertical augmentation was observed, but dense tissue was formed bridging the bone defect (Fig. 3E). The fluorescence of the slices showed continuous mineralization through the entire 3 months after surgery. The blue, red, and green fluorescent colors indicate the newly formed bone within the first, second, and third months, respectively. The intensity and region of colors suggested the extent and location of the newly formed bone. In the scaffold groups, the areas of blue, red, and green color regions were different, whereas in the control groups, all colors covered the same area when superimposed. By comparing the 3 colored areas in the scaffold groups, we found that the blue regions (tetracycline-labeled for the newly formed bone within the first month) were the largest and that the red and green regions adjacent to the blue regions were much smaller. Our analysis showed that the tissue area labeled for the newly formed bone decreased over time (Fig. 4A), and no significant difference was found between the core area and the peripheral area. The newly formed bone areas labeled in the first month were significantly greater than those in the second and third



**FIGURE 2.** The SEM examination of the 3 kinds of scaffolds. A to C show images of GP, LP, and SP, respectively. A-1 shows the junction between large and small pore regions. A-2, B-2, and C-2 show high-magnification scans of their respective surfaces. Scale bars = 200  $\mu\text{m}$  for A-1, B-1, and C-1; scale bars = 10  $\mu\text{m}$  for A-2, B-2, and C-2.

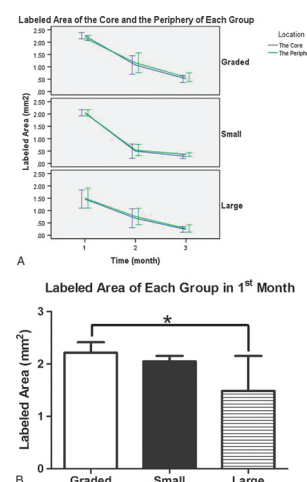


**FIGURE 3.** Fluorescent labeling of new bone formation over time. A to E show images of GP, LP, SP, the positive control group, and the negative control group. In each sample, 2 fluorescent labeling areas are presented: one in the center of the slices and the other in the outer ring of the slices. Each result includes a general view of the abrasive slice and a series of fluorescent-labeled images captured after excitation at different wavelengths. There are 4 fluorescent-labeled images in each series. Images in blue, red, and green present the calcified tissue in the first, second, and third months, respectively. The last column in each series shows the computer generated merged images. Scale bars = 1000  $\mu\text{m}$  in abrasive slices; scale bars = 100  $\mu\text{m}$  in fluorescent-labeled images.

months. Moreover, the bone areas colored in blue in the graded porous scaffolds were significantly larger than those in the large porous scaffolds (Fig. 4B) ( $P < 0.05$ ).

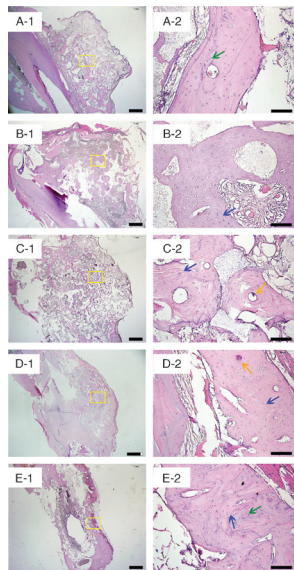
### Histologic Evaluation

The H&E staining of the samples revealed that, in all the groups, implanted blocks were tightly bound and integrated to the



**FIGURE 4.** Quantitative analysis of fluorescent-labeled areas in each group (GP, SP, and LP). A shows the areas in the first, second, and third months. The violet lines present areas in the core of scaffolds (inside-upper, inside-middle, and inside-lower), whereas the green lines present the area in the periphery of the scaffolds (O-U, outside-middle, and O-L). B shows the statistic analysis result in the first month.





**FIGURE 5.** The H&E staining of the decalcified samples. A to E show images of GP, LP, SP, the positive control group, and the negative control group. Blue arrows indicate bone matrix spotted with osteocytes, green arrows indicate osteoid, and yellow arrows indicate Haversian canals. Scale bars = 1000  $\mu$ m in A-1, B-1, C-1, D-1, and E-1; scale bars = 100  $\mu$ m in A-2, B-2, C-2, D-2, and E-2.

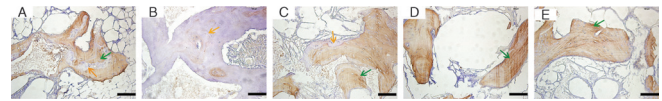
mandible bed, and no inflammation was found in any of the samples after surgery (Fig. 5). From the general view, it was obvious that the distribution of regenerated bone was uneven in the scaffolds. For all groups, BV near the bone bed seemed to be greater than those far from the base area. However, all the scaffold groups demonstrated a well-formed bone structure. At the magnification of 200 $\times$ , both bone tissue and soft tissue could be detected. Empty spaces interspersed in the tissues indicate areas where the scaffold was before demineralization. Immunohistochemistry staining of osteocalcin revealed active bone mineralization in all the groups, although the ranges of brown-colored tissue were different in each group (Fig. 6). Toluidine blue staining of the hard tissue slices clearly demonstrated the distribution of bone tissue in blue and the scaffold structure in black (Fig. 7). The gross view of the toluidine blue staining was in accordance with that of H&E staining, characterized with uneven bone forming from the base to the top of the scaffolds. Images showed close integration between bone tissue and scaffolds. At a high magnification of 100 $\times$ , intimate integration was found both in the bone-scaffold interface and implant-bone interface. Masson trichrome staining of all the samples presented a red color for cytoplasm, scattered with bits of blue for the bone (Fig. 8), which indicated wide range of bone tissue in the form of collagen. Collagen was mostly scattered around the bone lacunae.

### $\mu$ CT Observation and Measurements

Figure 9 shows two-dimensional profile  $\mu$ CT images of the mandible explants. Comparing the coronal image series of each sample of scaffold groups, identifiable differences in pore patterns could be observed. For the graded porous scaffold group (Fig. 9D), the large pore scaffold group (Fig. 9E), and the small pore scaffold group (Fig. 9F), the difference in pore diameters was obvious as designed. High-density tissue deposition was observed in pores. The distribution pattern varies among different tomography slice levels.

### BV and TV Measurements

The BV/TV ratio was used as the most important index for new bone formation in this study. The BV/TV ratios were different

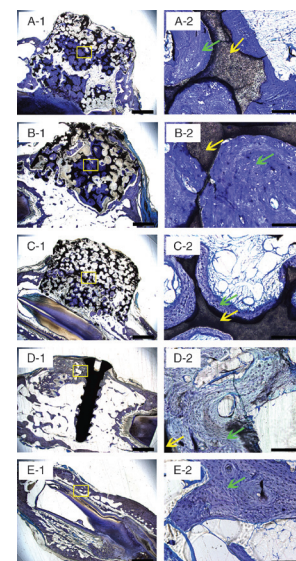


**FIGURE 6.** Immunohistochemistry staining of osteocalcin. A to E show images of GP, LP, SP, the positive control group, and the negative control group. Green arrows indicate the mineralized tissue, and yellow arrows indicate the tissue not mineralized. Scale bars = 100  $\mu$ m.

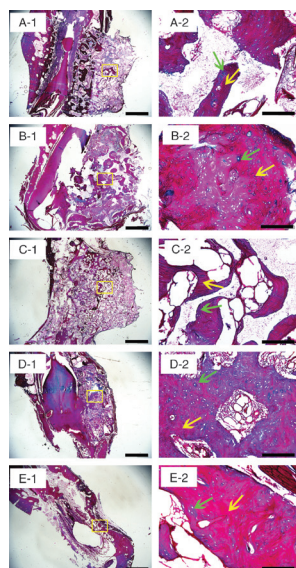
among the 3 groups, with statistical significance as shown in Figure 10. The ratio of BV/TV in graded porous scaffolds was significantly higher than those with large pores ( $P < 0.01$ ) and small pores ( $P < 0.05$ ), suggesting much greater new bone formation. No statistical difference was found between the 2 groups with uniform pores (Fig. 10A). In consistence with H&E staining, the ratio of BV/TV of different regions presented a slight decreasing trend from bottom to top of the blocks. Statistical differences were found between not only the O-U and O-L areas but also the central-upper and O-L areas (Fig. 10C). The bone density showed no significant difference among the groups (Fig. 10B) or between different regions (Fig. 10D).

## DISCUSSION

With the purpose of improving the osteogenic potential of  $\beta$ -TCP porous ceramics, we evaluated bone enhancement of porous scaffold with the addition of growth factor in bone defect models.<sup>22</sup> Porous scaffolds have been studied for decades, the uniform pore architecture of scaffolds faces a significant challenge in inducing the growth of new tissue in the way of natural bone acts.<sup>23–26</sup> The architectural motif of natural bone can be described as having a stiff, dense external layer of cortical bone, transitioning to a thin and porous cancellous bone internally.<sup>15</sup> There have been attempts to establish a fabrication method to manufacture a kind of scaffold that is both graded and highly interconnected.<sup>27–30</sup> Graded porous scaffolds have been proven to be advantageous compared with uniform pore design in both ectopic and orthotopic<sup>31,32</sup> osteogeneses, overcoming the current limitations associated with uniform pore design. The purpose of this study was to demonstrate the advantage of the designed graded porous scaffolds fabricated by a template-casting technique on bone augmentation in orthotopic bone defect.

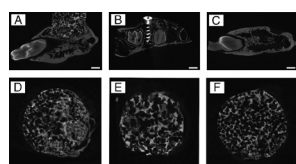


**FIGURE 7.** Toluidine blue staining of the hard tissue slices of all the samples. A to E show images of GP, LP, SP, the positive control group, and the negative control group. Green arrows indicate bone tissue, and yellow arrows indicate the scaffolds. Scale bars = 1000  $\mu$ m in A-1, B-1, C-1, D-1, and E-1; scale bars = 100  $\mu$ m in A-2, B-2, C-2, D-2, and E-2.

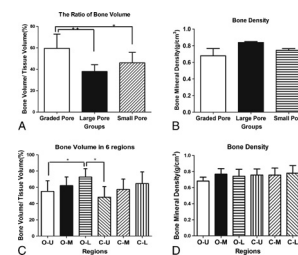


**FIGURE 8.** Masson trichrome staining of the samples. A to E show images of GP, LP, SP, the positive control group, and the negative control group. Green arrows indicate the immature bone tissue, and yellow arrows indicate the mature bone tissue. Scale bars = 1000  $\mu$ m in A-1, B-1, C-1, D-1, and E-1; scale bars = 100  $\mu$ m in A-2, B-2, C-2, D-2, and E-2.

Our *in vivo* study with New Zealand rabbits confirmed our hypothesis that the graded porous scaffold loaded with rhBMP-2 promoted bone augmentation at a higher rate compared with scaffolds with uniform pore sizes with the same loading of rhBMP-2 in orthotopic osteogenesis. The  $\mu$ CT analysis revealed superior BV augmentation in the graded porous scaffold group, with no decrease in BMD compared with uniform-size scaffolds, which excluded the possibility of impaired bone quality. The decreasing trend of BV from the base to the top of the scaffolds implied blood supply effect on bone regeneration. The obvious difference of augmented bone height between the experimental groups and the positive control group further demonstrated the advantages of engineered bone over autogenous iliac grafts in the aspect of bone configuration, which proved that the mechanical support provided by the scaffold can withstand pressure from the surrounding soft tissue, maintaining a stable bone height during the 3-month implantation period, whereas iliac grafts underwent a dramatic reconstruction, which lead to a bone height reduction. Although bone tissue in scaffolds presented an uneven distribution and different degrees of maturity, the structure of the mature part was familiar with that of the natural bone in the positive control group, such as typical lamellar bone mingled with woven bone, spotted osteocytes, and Haversian canals. As for the immature part, experiments with longer observing period need to be conducted. Masson staining revealed the maturity of the engineered tissue in most of the observed areas, whereas active osteogenesis seemed to be around the fringe, indicating the presence of a stable bone structure with active growth.



**FIGURE 9.** The  $\mu$ CT sagittal images of samples of augmented mandibles. A is the experimental group, B is the positive control group, and C is the negative control group. D to F are the  $\mu$ CT coronal images of the samples of the 3 experimental groups. D shows the GP group, E shows the LP group, and F shows the SP group. Scale bars = 1000  $\mu$ m.



**FIGURE 10.** The  $\mu$ CT quantitative analysis of the newly formed bone. A and B show the statistical analysis of  $\mu$ CT measurements of the augmented mandibles in the 3 experimental groups for BV/TV and BMD. C and D show the statistical analysis of  $\mu$ CT measurement of the augmented mandibles in 6 different areas in the scaffolds.

The polyfluorescent labeling is to visualize the process of new bone formation by identifying mineralized tissue during the drug injection period.<sup>33</sup> Because the injecting time interval of the 3 labeling drugs was the same, the larger the labeled areas are, the larger area the mineralized bone occupied. In our experiments, the bone growth in the first month was the fastest. Moreover, the difference of regenerated bone between the graded porous scaffolds and the uniform scaffolds had already emerged during this time, which provides further proof of the improved bone regeneration in the graded porous scaffolds, although the difference is not as significant as the  $\mu$ CT examination due to the limitation of information sample slices could ever offer. During the following 2 months, the bone formation/augmentation declined. Similar observation was found in other ectopic and orthotopic animal models as well.<sup>34,35</sup> The surgically created bone defect control groups showed little regeneration as evidenced by the lack of calcium ion deposition in the implantation area.

In our study, the intrinsic mechanism of the advantage of graded porous scaffolds seems to be various rather than single-effect. Because no significant difference was found between the core area and the peripheral area in this study, we believe that the graded scaffold act as a unique, single functional unit that the different configuration of the scaffold cause interstitial fluid diffusion changes and scaffold degradation profile changes, which lead to the different cell adhesion and different cell behavior as a response to the scaffold degradation. Considering that the living organism is such a complex system that a graded porous design is far from biomimicking, further investigations on scaffold development about scaffold degradation, growth factor release, and blood supply improvement should be considered. However, based on our study on the osteogenesis of porous scaffolds, the graded porous design could be proved to be beneficial in bone augmentation in orthotopic osteogenesis.

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