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ORIGINAL ARTICLE

TERM

Prefabrication of Axially Vascularized Bone by Combining β-Tricalciumphosphate, Arteriovenous Loop, and Cell Sheet Technique

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The repair of bone defects poses a great challenge for reconstructive surgeons. Although the development of tissue engineering has exhibited promise in replacing damaged bone, the fabrication of large constructs with functional blood vessels remains an obstacle. From the orthopedic surgeon's point of view, the generation of axially vascularized bone, which can anastomose with the recipient vessel, might be a solution to this medical problem. In this study, we aimed to prefabricate an axially vascularized bone by combining a β-TCP scaffold, arteriovenous loop (AVL), and cell sheet in a bioreactor *in vivo*. Twelve rabbits were randomly allocated into two groups: the experimental group (presence of AVL) and the control group (absence of AVL). The constructs were explanted at 8 weeks postoperatively. The histomorphometric results showed 42.8±5.9% of the bone area in the AVL group and 26.6±3.5% in the control group. Similarly, vessel analysis revealed the average vessel density in the AVL group (12.5±3.3) was significantly more than that in the control group (6.1±1.5, $p<0.05$). Our research indicated that the combination of a β-TCP scaffold, AVL and cell sheet might engineer vascularized bone. This prefabrication strategy might facilitate clinical translation of bone tissue engineering in reconstructing large bone defects.

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Key Words: Prefabrication; Vascularization; Tissue engineered bone; Arteriovenous loop; Cell sheet

INTRODUCTION

The repair of serious osseous defects poses a difficult medical problem that requires the development of novel treatment methods. Engineered bone tissue is a promising alternative to autologous bone graft. However, due to poor vascularization inside the large construct, its use is usually associated with poor graft survival and integration [1-3].

The generation of vascularized tissue engineered bone (TEB) might be a promising solution to this problem. Various *in vitro* strategies, including growth factor delivery, coculturing systems, mechanical stimulation, biomaterial design, and microfabrication techniques, have been attempted to enhance vascularization within engineered bone constructs [3]. However, after transplantation into bone defects *in vivo*, TEB usually needs a certain period of time (several days or weeks) to realize complete vascularization and thus its survival is still low [1,2,4]. Therefore, strategies that can achieve immediate perfusion between the TEB and recipient sites are ideal. According to some research, the *in vivo* arteriovenous loop (AVL) model may be able to fabricate vascularized tissue with an axial vascular pedicle and then connect with host vessels through microsurgical technique [2].

Our previous study showed that a cell sheet could act as a cell delivery system to revitalize the β-TCP scaffold [5]. However, both bone-like and fibrovascular tissue were mainly found at the edge of the scaffold. Osteogeneration and vascularisation are required to be facilitated, especially in center of the composite. We thus hypothesize that AVL may be a potential approach, and that the combination of AVL, β-TCP, and an osteogenic cell sheet may develop an axially vascularized bone (Fig. 1). To test this hypothesis, we first performed anastomosis between the saphenous artery and vein to creat an AVL in a rabbit model. Then we used AVL to circle the β-TCP graft and wrapped a cell sheet around the composite. Finally we evaluated its *in vivo* osteogenic potential ectopically.

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MATERIALS AND METHODS

Experimental animals and material

Twelve 6-month-old New Zealand rabbits (average weight of 2.6 kg) were included. The animal welfare and ethics committee of Lanzhou General Hospital approved all laboratory animal protocols (LZZYKY2014005). Porous β-TCP scaffold (provided by Bio-lu Biomaterials Company Limited, Shanghai, China) was custom made in the shape of a cylinder (radius of 3 mm and height of 8 mm) with two grooves on both sides (Fig. 2A and B). The scaffold was sterilized by ^{60}Co irradiation before use.

Tissue culture dishes were bought from Falcon (San Jose, CA, USA). DMEM and FBS were obtained from Gibco (Invitrogen Corp, Grand Island, NY, USA). Other chemicals were purchased from Sigma (Aldrich, St. Louis, MO, USA).

Preparation of osteogenic cell sheet

Rabbit bone marrow mesenchymal stem cells were isolated and cultured as described previously [5]. Briefly, iliac bone was split and fresh bone marrow was flushed out with a basic medium consisting of low glucose DMEM, 10% FBS, and 2 mM L-glutamine. To get a homogeneous cell suspension, clumps of bone marrow were pipetted repeatedly. The suspension was then placed in 100-mm culture dish. Adherent cells were harvested by a complete medium change after 48 hours. Until 85% confluence, cells were trypsinized and subcultured. The passage-one cells were replated in 60-mm dish at 5×10^4 cells/cm^2 and cultured with an osteogenic induction medium continuously for 14 days. The conditioned medium is mixture of DMEM (high glucose), FBS (10%), dexamethasone (100 nM), β-glycerophosphate (10 mM), and L-ascorbic acid (50 mg/L). Then a thick cell sheet was detached from the dish bottom with a cell scraper. The average thickness of the cell sheet is 98+14 um and the average cell number of each cell sheet (60 mm) was roughly 1.2×10^7 (with a cell density of 2.2×10^5/cm^2). Finally, an oblong cell sheet (25×10 mm) was prepared for use (Fig. 2C).

In vivo implantation

After skin preparation and disinfection, a incision (5 cm in length) was performed from the groin to the knee. The rabbit saphenous artery and vein were then dissected and exposed. The distal ends of the vessels were cut and an AVL was micro-

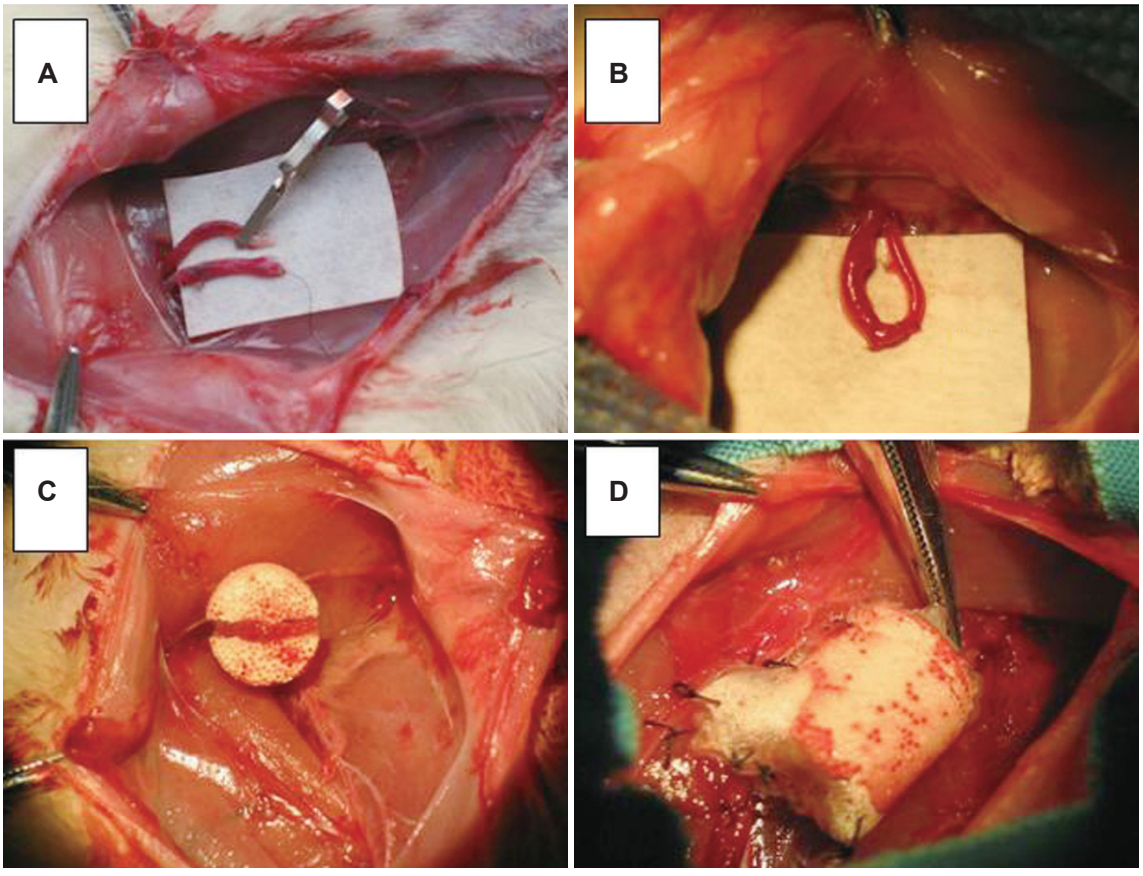

Figure 1. *In vivo* implantation protocol and intraoperative view. (A) The femoral vessels were dissected and exposed. (B) AVL was microsurgically generated between the proximal ends of the rabbit saphenous artery and vein. (C) The AVL was put in the grooves to circle the β-TCP graft. (D) The graft was wrapped around with an oblong cell sheet. AVL: arteriovenous loop.

surgically generated by a connection between the proximal part of the vein and artery. The AVL was inserted into the lateral grooves to circle the β-TCP scaffold. The scaffold was then wrapped around an oblong cell sheet to create a graft (Fig. 1). The graft was transplanted in the thigh muscles of the cell-donor rabbit. The vessel anastomosis site was carefully checked for hemostasis before wound closure.

As control, β-TCP cylinders wrapped with cell sheet without AVL were also implanted into the muscular pockets. Totally 12 rabbits were included and 6 specimens were collected in each group. After 8 weeks of *in vivo* implantation, the rabbits were euthanased and all specimens were collected for gross evaluation and histological examination.

Histological and histomorphometric examinations

After fixation in 4% buffered paraformaldehyde (ph 7.4 at 4°C) for 48 hours, decalcification of the samples was performed using 5% formic acid solution for 2 weeks. Each sample was divided into two parts in transverse plane, dehydrated in graded alcohol, embedded in paraffin. Four 5-μm sections were cut from each part through the long axis, mounted on slides, stained with hematoxylin and eosin, and observed under light microscopy. For histomorphometric examination, four photomicrographs were randomly selected from each section and analyzed twice by a single-blind examiner. The cross-sectional area of bone-like tissue was measured and the relative percentages of bone to the total section area was calculated using Image J Analysis software. At the same time, The average density of blood vessels (lumen structures containing red cells) of each photomicrograph was also analyzed and recorded as the number of blood vessels in per photomicrograph.

Statistical methods

The histomorphometric data were expressed as means±standard deviations and analysis was done by a Mann-Whitney-U test using SPSS Version 20.0 software (SPSS Inc., Chicago, IL, USA). $p<0.05$ was considered significant.

RESULTS

Experimental animals and gross examination

The surgical procedures were tolerated well by all rabbits and

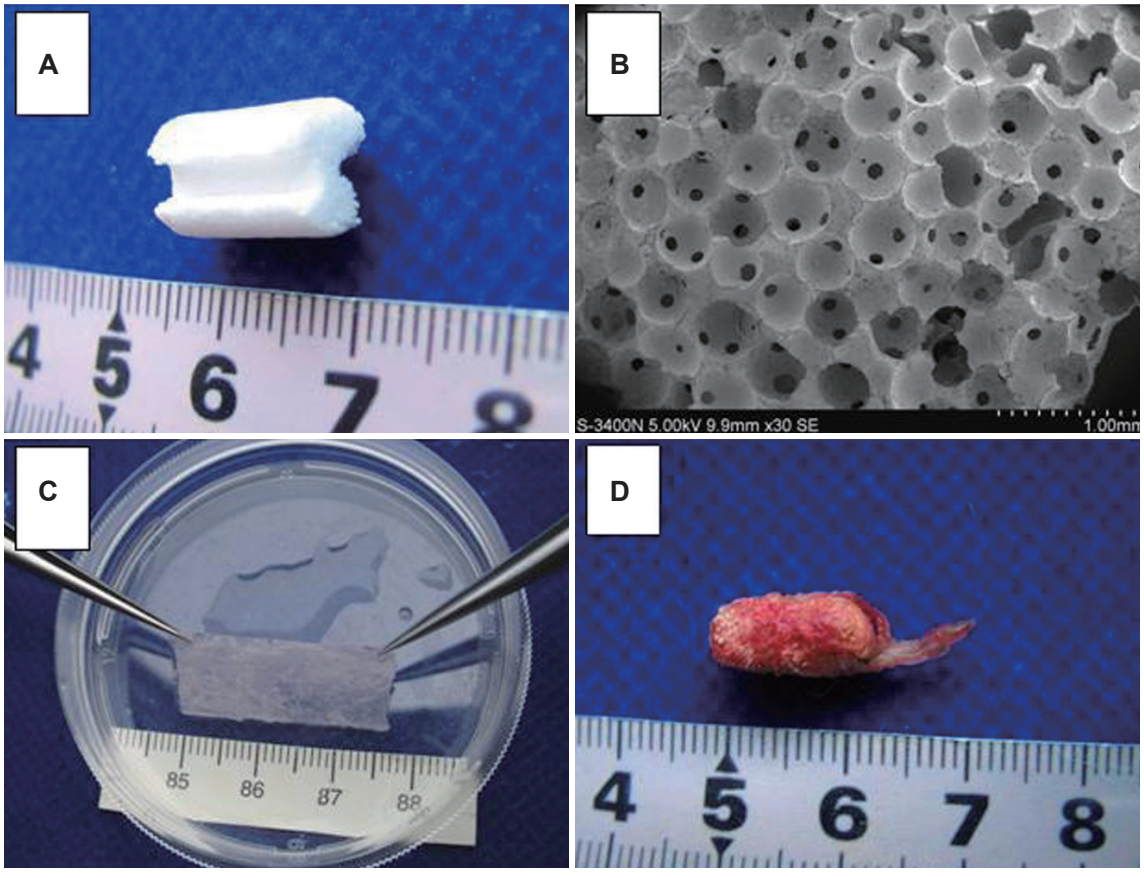

Figure 2. Appearance of the β-TCP cylinder and harvested sample. (A) Gross appearance of the custom-made β-TCP scaffold. (B) SEM photographs of surface architecture of β-TCP. (C) Gross appearance of an oblong cell sheet. (D) Gross view of a representative explant with a vascular pedicle in the arteriovenous loop.

no major complications occured. A hematoma was confirmed by puncture in one animal, and the vascular anastomotic leak was repaired. After eight weeks of implantation, gross evaluation of the explanted graft showed evidence of a hard tissue flap with a vascular pedicle in the AVL group (Fig. 2D). However, a layer of fibrous tissue covered the scaffold, and a few slender blood vessels were found in the control group.

Histological and histomorphometric examinations

As shown in Figure 3A and B, typical bone tissue was present at the edge of composites in the AVL group, with evidence of osteoblasts lining the matrix surface, osteocytes buried in the lacunae, and irregular marrow cavity-like structures surrounded by a dense matrix. Furthermore, new trabecular bone, a shell of matrixs scattered in connective tissue, formed in the neighbor

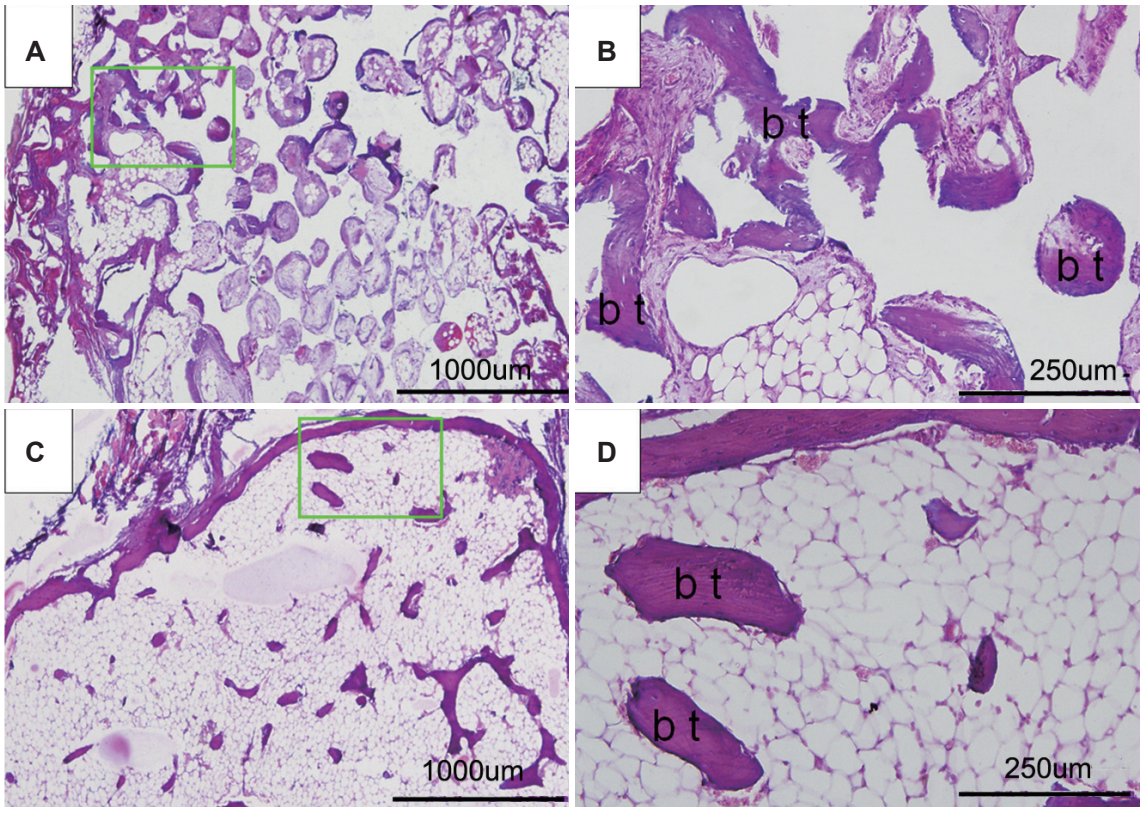

Figure 3. Representative histological examinations (H&E staining) of harvested 8-week constructs. (A) Typical bone tissue was present at the edge of composite in the AVL group, with the evidence of osteoblasts lining matrix surface, osteocytes burried in the lacunae, and irregular marrow cavity-like structure. (B) Trabecular bone, a shell of matrix scattered within connective tissue was detected in the middle of the construct in the AVL group. (C) A layer of lamellar bone tissue formed at the edge of composite in the control group. (D) Sporadic bone was found in the control construct. AVL: arteriovenous loop.

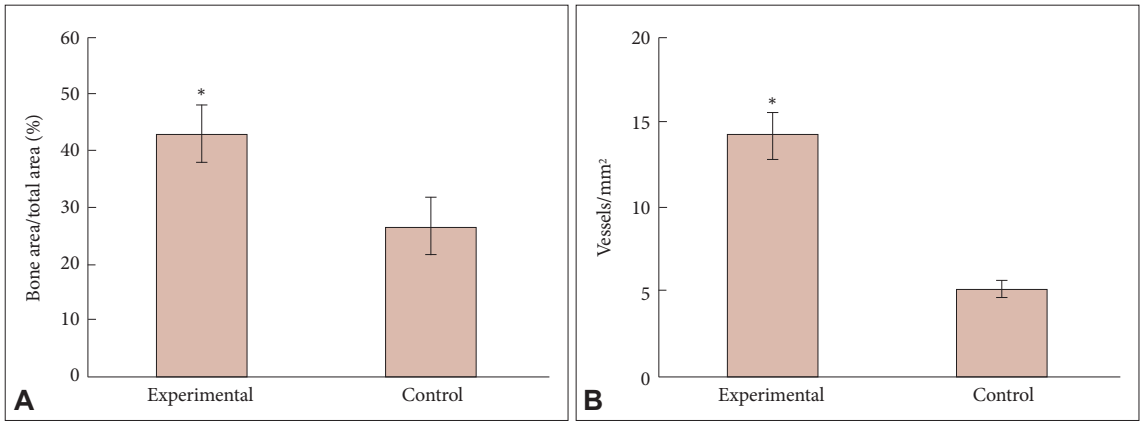

Figure 4. The area ratio of bone-like tissue to the total image (A) and the average vessel density (B) of explants in both the experimental and the control group (n=6, *p<0.05).

space of β-TCP implants. In contrast, in the control group, a layer of lamellar bone tissue formed mainly at the edge of graft (Fig. 3C and D), with sporadic bone only forming inside the graft.

The quantitative results of histomorphometry (Fig. 4) showed 42.8±5.9% of the bone area in the AVL group and 26.6±3.5% in the control group. The experimental group had higher area ratio than the control group ($p<0.05$). Similarly, analysis of the vessel revealed the average vessel density in the experimental group (12.5±3.3) was significantly more than that in the control group (6.1±1.5, $p<0.05$).

DISCUSSION

Our study indicates the feasibility of generating axially vascularized bone tissue by combining a β-TCP scaffold, an AVL, and a cell sheet. Like vascularized autologous bone flaps, the transplantation of the engineered bone substitute could be performed by vascular anastomosis between the TEB and recipient sites. Immediate blood perfusion would be realized. Thus, our strategy exhibits promising potential for translation into clinical practice [1,4].

In this study, we used the cell sheet technique, rather than cell suspension, to combine the cells with the scaffold for several reasons. Firstly, the cell sheet wrapped around the scaffold might be more likely to survive after *in vivo* transplantation. As shown in our previous study [5], cells in the sheet might get initial nutrient supplies since the thickness of the cell sheet is less than 150 μm and thus is within the optimal oxygen diffusion distance of 150–200 μm [6,7]. As a commonly used cell harvesting and application technique, cell suspension has the advantage of being seeded into both the edge and the center of the porous scaffold. However, cells located in the center usually had low *in vivo* survival [5,7]. Therefore, it is inappropriate for our approach. Arkudas et al. [7] suggested that the secondary injection of osteoblasts into the prevascularized scaffold can promote cell survival and facilitate bone formation. An apparent limitation of their method is the increase in the prefabrication time and surgical trauma. Secondly, because the cells were harvested together with their self-secreted extracellular matrix, and subsequently, the cell sheet was lager than an equal number of isolated cells, the utility of the cell sheet technique could enhance cell retention at the transplantation site [5,8]. Although many materials, such as fibrin gel and collagen, have been used as a carrier for cell immobilization, they are far from ideal because of their inherent drawbacks [9]. Moreover, the preservation of the extracellular matrix and intercellular connection has been proven to facilitate differentiation and osteogenesis [5,8]. Accordingly, these factors suggest that the cell sheet could not only act as a useful cell delivery system, but also improve the efficacy of cell therapy.

It is known that osteogenesis and vascularisation are two integrated parts of bone regeneration. Osteogenesis is essentially featured by capillary ingrowth to the mesenchymal niche and the differentiation of target cells into osteoblasts. Osteoblasts synthesize the organic matrix, leading to trabecular bone formation. Activated by trauma or pathophysiological response, angiogenesis also initiates and promotes ossification in bone healing [10]. Therefore, vascularization plays a critical role in the development of functional TEB. Currently, two main approaches have been explored to engineer vascularized bone: the *in vitro* pre-vascularization strategy and the *in vivo* prefabrication model [2,4,7]. Although various *in vitro* methods have been attempted and great advancements have been achieved, *in vitro* vascularized constructs usually need a certain period of time to integrate with the surrounding native tissue after *in vivo* transplantation [1]. Graft survival is still a challenge during this period [6,7]. As a result, some researchers focus on the *in vivo* prefabrication method to resolve this problem. Several highly vascularized *in vivo* bioreactors, including intraperitoneal and intramuscular bioreactors, have been used as potential environments to prefabricate vascularized TEB [1,7,11]. For example, Warnke et al. [11] transplanted a mesh cage filled with bovine bone particles, BMP, and fresh bone marrow in the human latissimus dorsi muscle to creat vascularized TEB. They successfully repaired a patient's mandibular defect using the prefabricated heterotopic graft and muscle flap. In 1980, Erol and Sira [12] revealed that using AVL on a skin flap could result in abundant neovascular formation. Since then, AVL has been widely used in tissue prefabrication. Arkudas et al. [7] established a combined AVL model in the field of bone tissue engineering using a rat model. After achieving success in a preclinical large-animal model, Horch et al. [13] and Beier et al. [14] even used AVL-based bone to repair bone defects in a clinical scenario. The mechanisms of AVL in enhancing vascularization might involve three factors: the local inflammatory response in vessel trauma, shear stress during vein arterialization, and hypoxia response at the local site [2,7].

The histological findings of our study show that ingrowth of fibrotic tissue into the β-TCP scaffold is taking place at the same time as bone formation. Osteogenesis speed in this model still did not match with the scaffold degradation rate, particularly in the center area. Therefore, further studies are needed to optimize the prefabrication of bone flap. For example, secondary combination of osteoblasts after pre-vascularization has been shown to facilitate bone formation greatly [1,7]. In addition, adding osteoinductive factors such as BMP could be an effective method fore increasing bone formation [11]. Moreover, the biomechanical properties and repairing abilities in orthotopic

defect models need to be investigated.

In conclusion, the cell sheet was a useful cell delivery system to revitalize the β-TCP scaffold. Moreover, the use of AVL could further facilitate new bone formation during the prefabrication period. In conclusion, the current research demonstrated the feasibility of prefabrication approach to develop axially vascularized constructs based on a β-TCP scaffold, an AVL, and a cell sheet. Transplantation of the vascularized bone substitute could be performed by vascular anastomosis, and immediate blood perfusion would be realized. Thus, our strategy can facilitate the clinical translation of bone tissue engineering in reconstructing large bone defects.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

The animal welfare and ethics committee of Lanzhou General Hospital approved all laboratory animal protocols (LZZYKY2014005).

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