Improved Repair of Bone Defects With Prevascularized Tissue-Engineered Bones Constructed in a Perfusion Bioreactor

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Abstract: Vascularization of tissue-engineered bones is critical to achieving satisfactory repair of bone defects. The authors investigated the use of prevascularized tissue-engineered bone for repairing bone defects. The new bone was greater in the prevascularized group than in the nonvascularized group, indicating that prevascularized tissue-engineered bone improves the repair of bone defects. [Orthopedics. 2014; 37(10):685-690.]

Repairing bone defects resulting from trauma, tumors, or inflammation remains challenging for orthopedic surgeons. Current treatments for bone defects mainly include autografts and allografts. However, these methods have several drawbacks, including limited availability and donor-site morbidity for autografts and issues related to immune responses, infection, and disease transmission for allografts.

The promotion of angiogenesis and vascularization of tissue-engineered bones must fulfill several important prerequisites (eg, ease of acquisition, good expansion capacity, and stable endothelial cell [EC] phenotypes and osteoblast cell [OC] phenotypes) before these bones can be considered for clinical applications. Bone marrow mesenchymal stem cell (BMSC)-derived ECs and OCs satisfy these prerequisites.

The repair of bone defects with vascularized tissue-engineered bones has been reported in previous studies mainly focused on small animal experiments involving rats and rabbits. For better application in clinical practice, the repair of bone defects needs to be studied in larger animals.

In this study, the authors constructed prevascularized tissue-engineered bone with BMSC-derived OCs and BMSC-derived ECs co-cultured in porous β-tricalcium phosphate ceramic (β-TCP) in a perfusion bioreactor to repair bone defects in sheep. The hypothesis was that prevascularized, compared with nonvascularized, tissue-engineered bone could improve the repair of bone defects in a large animal.

Materials and Methods

Cell Isolation and Culture

Sheep BMSCs were isolated and expanded using a modification of a previously reported method. Briefly, 20 mL of bone marrow aspirates was harvested through a bone marrow biopsy needle inserted through the iliac crest. Then, 2 mL of the bone marrow aspirates was immediately inoculated onto a 100-mm culture dish. The aspirates were cultured in a growth medium containing α-minimal essential medium (α-MEM; Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, California), 100 IU/mL of penicillin, and 100 mg/mL of streptomycin (Hyclone, Logan, Utah) at 37°C in a humidified 5% CO₂ incubator. After 3 days, nonadherent cells were removed by washing with phosphate-buffered saline, and adherent cells were further cultured in α-MEM until...
80% to 90% confluence. The remaining attached cells were mainly BMSCs. The obtained BMSCs were enzymatically lifted and subcultured at a density of $5 \times 10^3$ cells/cm$^2$. The second passage of BMSCs was used for this experiment.

**Differentiation of Bone Marrow Mesenchymal Stem Cells**

Bone marrow mesenchymal stem cells were induced to differentiate into osteoblasts in α-MEM supplemented with 10% FBS, 100 IU/mL of penicillin, 100 mg/mL of streptomycin, and osteogenic supplements consisting of 50 mg/mL of ascorbic acid, 10 mM of β-glycerophosphate, and 10 nM of dexamethasone (all from Sigma, St. Louis, Missouri). Cells from the second passage were used for the experiments.

To promote the EC phenotype differentiation, BMSCs from the second passage were cultured on dishes coated with 1 µg/cm$^2$ of bovine fibronectin (Sigma) in EC growth medium (EGM2-MV; Clonetics Corp, Walkersville, Maryland) containing 0.4% human fibroblast growth factor with heparin, 0.04% hydrocortisone, 0.1% vascular endothelial growth factor (VEGF), 0.1% human recombinant insulin-like growth factor (R3-IGF-1), 0.1% ascorbic acid, and 5% FBS at 37°C in a humidified 5% CO$_2$ incubator. Cells from the second passage were used for the experiments.

**Scaffold Materials for Tissue Engineering**

The critical-size porous cylindrical β-TCP scaffold (Shanghai Bio-Lu Biomaterials Company Limited, Shanghai, China) used in this study had a homogeneous porosity of 75%, spherical pores 530±100 µm in diameter, and interconnections of 150±50 µm in diameter (Figure 1). The cylinder has a diameter of 10 mm and a height of 30 mm, creating a blind end tunnel situated in the scaffold’s center. The tunnel was 2.5 mm wide and 25 mm long.

**Repair of Bone Defects in Sheep**

Eight 2-year-old sheep weighing 50.0 to 62.0 kg were used in this study. Once the sheep were anesthetized, an approximately 4-cm lateral incision was made inferior to the greater trochanter along the right femur, exposing the proximal shaft of the femur (Figure 3A). A K-wire was drilled into the femoral neck inferior to the greater trochanter along the right femur, exposing the proximal shaft of the femur (Figure 3A). A K-wire was drilled into the femoral neck inferior to the greater trochanter. A cannulated reamer with an outer diameter of 10 mm was drilled along the K-wire to create a bone defect approximately 3 cm deep (Figure 3B). The constructed pre-
vascularized tissue-engineered bone was grafted into the bone defect, and then the incision was closed (Figures 3C-D). The nonvascularized tissue-engineered bone was grafted into the left femur. Postoperatively, 40,000 IU/kg of penicillin was administered for 5 days as antibacterial prophylaxis. Four sheep were sacrificed at 8 and 16 weeks postoperatively, and specimens were prepared for examination.

**Gross View and Radiographic Examination**

The animals’ postoperative activities, food intake, and wound healing were observed. After sacrifice, the animals’ bilateral femurs were removed and anteroposterior radiographs were obtained to observe bone healing. The status of bone repair and new bone formation were studied in samples collected through the original surgical site after radiographic examination.

**Histological Observation and Histomorphometry**

For histological analysis, the samples were fixed in 70% ethanol for 10 days and then dehydrated in a series of increasing graded ethanol, cleared with toluene, and embedded in methylmethacrylate. After polymerization, approximately 150-µm-thick sections were achieved via a Leica SP 1600 saw microtome (Leitz, Wetzlar, Germany). This software distinguishes between different tissue fractions based on their individual color spectra, marks them in a specific color, and assigns them to a metric variable that allows for the calculation of different bone indices. As an evaluation parameter, the area of the newly formed bone was calculated in proportion to the area of the pore in the scaffold. Data were gathered from 3 high-powered fields—×40 per specimen—on 4 specimens per group.

**Statistical Analysis**

The results were reported as mean±standard deviation. A paired t test was performed using SAS version 6.12 software (SAS Institute, Inc, Cary, North Carolina). P<.05 was considered statistically significant.

**RESULTS**

**Gross View and Radiographic Examination**

All of the sheep had normal diets after implantation of the tissue-engineered bone. Their activity and ability to stand returned to normal approximately 3 weeks postoperatively. All of the sheep survived until the scheduled time of sacrifice without apparent complications such as infection, skin necrosis, or fracture. To evaluate bone defect repair and scaffold degradation, radiographs were obtained at 8 and 16 weeks after tissue-engineered bone grafting (Figure 4). At 8 weeks, the scaffold of β-TCP was clearly observed on radiographs for all sheep. However, the density of the β-TCP constructed for the prevascularized tissue-engineered bone was lower than that for the nonvascularized tissue-engineered bone. For all of the sheep, the density of β-TCP was much lower after 16 weeks than after 8 weeks, and the density of the β-TCP was lower for the prevascularized tissue-engineered bone than for the nonvascularized tissue-engineered bone.

**Histological Observation and Histomorphometry**

At 8 and 16 weeks postoperatively, samples were extracted for staining with Stevenel blue and van Gieson picrofuchsin (Figure 5). At 8 weeks, the sections showed that soft tissues had grown into the porous β-TCP scaffold and a small amount of new bone had formed in both the prevascularized and the nonvascularized groups. In both groups, stained sections showed that more new bones were formed at 16 weeks than at 8 weeks after implantation of the tissue-engineered bone. In the prevascularized group, many vessel-like structures containing numerous red blood cells had formed. The bone tissue was immature around the vessel-like structures, and the mature bone tissue was far from the vessel-like structures.
and close to the immature bone tissue. In the nonvascularized group, no vessel-like structures and only immature bone tissues had formed. At 8 and 16 weeks, the new bone formed in the porous scaffold was calculated (Figure 6). After 8 and 16 weeks, the area of new bone in the prevascularized group was more than that in the nonvascularized group ($P<.05$).

**DISCUSSION**

Cells play the most important role in the formation of new bone tissues. In numerous studies, tissue-engineered bones have been constructed using single cells with osteogenesis function (eg, BMSCs, adipose-derived stem cells, and embryonic stem cells) to repair bone defects. In addition, sufficient blood supply is necessary for new bone formation. Considering the close relationship between angiogenesis and osteogenesis, neovascularization is an important element in repairing bone defects. Recent studies have used BMSC-derived ECs for vascularization of tissue-engineered bones, with several achieving satisfactory results. Most of these studies used static culture, which limits the survival of seeding cells in the deep parts of the porous scaffold, especially in critical-size scaffolds. In addition, previous studies have focused on the repair of bone defects in small animals such as rats and rabbits. In the current study, the authors constructed prevascularized tissue-engineered bone using BMSC-derived OCs and BMSC-derived ECs in a perfusion bioreactor system to improve the repair of bone defects in sheep.

Radiographs showed that the ß-TCP scaffold was more obvious in the nonvascularized group, no vessel-like structures and only immature bone tissues had formed. At 8 and 16 weeks, the new bone formed in the porous scaffold was calculated (Figure 6). After 8 and 16 weeks, the area of new bone in the prevascularized group was more than that in the nonvascularized group ($P<.05$).

**Figure 5:** Histological sections stained with Stevenel blue and van Gieson picrorhuchsin (original magnification ×40) at 8 weeks and 16 weeks postimplantation of tissue-engineered bone. There was little new bone formation at 8 weeks postoperatively in both the nonvascularized group (A) and the prevascularized group (B). At 16 weeks postoperatively, new bone tissue had formed in both the nonvascularized group (C) and the prevascularized group (D). The area of new bone tissue was much greater in the prevascularized group than in the nonvascularized group. In the prevascularized group, some microvessel-like structures had formed.
group than in the prevascularized group at each time point. Furthermore, the normal structure of bone trabeculae could be seen on radiographs in the prevascularized group at 16 weeks postoperatively, with no such structure seen in the nonvascularized group. These findings are in agreement with those previously reported.8,11 The results suggested that mature bone tissues had formed in the prevascularized group. Furthermore, the β-TCP scaffold was absorbed more in the prevascularized group than in the nonvascularized group. This phenomenon may be attributed to the fact that more blood vessels can supply more blood and clear more metabolic products, including the components of the β-TCP scaffold. In addition, an increased blood supply can transport more macrophages into the porous scaffold to participate in removing the β-TCP scaffold.

Histological staining showed that more new bone tissues had formed in the prevascularized group than in the nonvascularized group at 8 and 16 weeks postimplantation of the tissue-engineered bone. However, new bone formation was dramatically increased in the prevascularized group compared with the nonvascularized group. The area of new bone formation was far greater in the prevascularized group than in the nonvascularized group. These results were in agreement with those previously reported.24 In addition, in the prevascularized group, some microvessel-like structures containing many red blood cells were found, demonstrating that these structures were connected with preexisting blood vessels around the bone defect. These findings were similar to those of a previous study.8 These structures may be neovessels formed from BMSC-derived ECs or from preexisting blood vessels around the bone defect. The origin needs to be investigated further.

On the histological sections, new immature bone tissues had formed around the microvessel-like structures, and some mature bone tissues were located far from the microvessel-like structures and around the immature bone tissues. These results suggest that new bone began to form around the neovessels. Thus, the vascularization of the tissue-engineered bone was extremely important for bone defect repair. Moreover, some interactions may be present between the BMSC-derived OCs and the BMSC-derived ECs. Bone marrow mesenchymal stem cell–derived ECs possibly enhanced the osteogenesis of BMSC-derived OCs. Furthermore, signals from ECs may have positively affected the osteogenic potential of BMSC-derived OCs, which eventually influenced the maturation of the tissue-engineered bone. Bouletreau et al25 found that ECs secrete bone morphogenic proteins to promote osteogenesis and stimulate osteoblasts as well as their precursor cells to secrete VEGF. This condition can significantly promote the proliferation of ECs, vascularization, and osteogenesis.26 Villars et al27 showed that co-culturing MSCs and ECs promotes the proliferative activity of osteoblasts and accelerates the vascularization of tissue-engineered bones. Therefore, co-culturing BMSC-derived OCs and BMSC-derived ECs had similar effects.

The current study had 2 major limitations. First, this study only quantified organic matrix. The staining method did not detect bone mineral. Although radiographs detect bone mineral, these observations were not quantified. Second, there was no evaluation of the vascular connection to the host tissue circulation. These issues will be investigated in the future.

**Conclusion**

This study demonstrated that prevascularized tissue-engineered bone constructed by co-culturing BMSC-derived OCs and BMSC-derived ECs in porous β-TCP in a perfusion bioreactor improved vascularization and osteogenesis in vivo. Prevascularized, compared with nonvascularized, tissue-engineered bone mediated by BMSC-ECs could enhance the ability to repair bone defects.

**References**


