

Biomimetic Construction of Large Engineered Bone Using Hemoperfusion and Cyto-Capture in Traumatic Bone Defect

Fei Liu,¹ Shaofen Yu,¹ Zhengguo Wang,² and Xinjun Sun¹

Abstract

Due to lack of blood vessel systems, only a few tissues, such as skin, cartilage, and cornea, have been successfully constructed *in vivo*. Anticoagulative scaffolds have been used in drug-eluting stent systems both in animal studies and clinical therapies, as in the medicinal leech therapy used to salvage venous-congested microvascular free flaps improved perfusion inspired us to tackle this hurdle in bone tissue engineering. We hypothesize that a combination of bone marrow as the blood supply and a heparin/chitosan-coated acellular bone matrix that acts like hirudin, together with a vacuum-assisted closure therapy system, would provide blood perfusion to the scaffold. Using these methods, a biomimetically engineered bone construct would facilitate clinical translation in bone tissue engineering and offer new therapeutic strategies for reconstructing large bone defects if the hypothesis proves to be practical.

Introduction

PREVIOUS STUDIES in tissue engineering have provided new information about replacing or restoring function in bone defects due to infection, trauma, or resection of tumors.^{1,2} However, up to now, most of the successes in tissue engineering have been limited to avascular and thin tissues that can survive by receiving a supply of oxygen and nutrients via diffusion, without the need for an additional vascular supply.^{3,4} Today, only *in vitro*-engineered tissues, such as skin and cartilage, are successfully used in the clinical setting, and very few real tissue-like test systems have been developed.^{5,6} Therefore, the main hurdle in the field of tissue engineering is how to provide a sufficient blood supply to grafted tissue substitutes during the early post-transplantation period.⁷

Manufacture Prevascularized Tissue *In Vitro*

Angiogenic factors are commonly used to induce neovascularization in engineered tissues. These factors activate the endothelial cells (ECs) or endothelial progenitor cells (EPCs) and stimulate them to migrate toward the factor gradient. Furthermore, they promote vessel formation and maturation. The main factors involved in upregulating the angiogenic processes are vascular endothelial growth factor (VEGF),

basic fibroblast growth factor (bFGF), and hepatocyte growth factor. A complex network of cytokines, including platelet-derived growth factor and transforming growth factor beta, participate in the regeneration of endothelial tubes.⁷ Incorporating localized and long-term delivery of angiogenic factors at the implantation site into the biomaterial design can overcome the naturally high degradation rate of growth factors.⁸ Previous studies have used biomaterials with degradable porous reservoirs or pre-encapsulated microspheres.⁹ An alternative to biomaterial control of growth factor release is the use of transfected cells to provide sustained growth factor release. Geiger et al. compared bone substitute scaffolds coated with VEGF-encoding plasmid DNA versus those seeded with mesenchymal stem cells (MSCs) transfected with VEGF.¹⁰ They found that the latter led to greater vascularization and faster resorption of the bone substitute. Similar results have been reported for the application of MSCs transfected with VEGF after myocardial infarction, which resulted in improved blood perfusion.¹¹

Scaffolds seeded with ECs and other cell types can be cultured *in vitro* to form three-dimensional prevascularized structures. The construct containing the vascular network is then implanted into the ischemic area to anastomose with the host. Tremblay et al. found that the connection between the prevascularized construct and the host tissue required 4 days to form after transplantation; however, a

¹Department of Orthopedics, 89th Hospital of People's Liberation Army, Weifang, China.

²Department 4, Research Institute of Field Surgery, Daping Hospital, Third Military Medical University, Chongqing, China.

14-day period was necessary to achieve a similar result with a nonendothelialized control.¹² Another technique, cell sheet engineering, combines biomaterials with ingrowing cells that are harvested from temperature-responsive cell culture dishes. After being layered, a three-dimensional structure is created and transplanted into ischemic tissues. Increases in blood perfusion could be found with this approach.¹³

Prefabrication of Large Vascularized Bone Grafts *In Vivo*

Most tissues *in vivo*, with the exception of cartilage and the cornea, receive nutrients and oxygen via blood vessels separated by less than 200 μm .¹⁴ Previous studies have described large tissue-engineered constructs that can be sufficiently supplied with oxygen and nutrients in perfusion bioreactors.¹⁵ However, diffusion is limited by the distance from host capillaries once these constructs are implanted *in vivo*.

In vivo prevascularization is an alternative to preseeding scaffolds with cells. In contrary to the strategy with the preseeded scaffold, *in vivo* prevascularization is based on angiogenesis: a sequence of events between ECs and other cell types in the surrounding tissue to form new blood vessels from pre-existing vessels. During the preliminary implantation into a host body, *de novo* vascularization of the construct occurs. The process was first demonstrated by Fontaine et al. in 1972, and since then, various studies have demonstrated that host cells are able to build a perfusable vascular network in suitable artificial structures.^{16–18} After successful vascularization, the vascularized bone grafts is explanted again into the ischemic target site. The disadvantage of this approach is the need for polysurgeries: the implantation of the cell-free scaffold, followed by the removal and the proximate insertion of the prevascularized biomaterial. Warnke et al. used intramuscular tissue as an *in vivo* bioreactor to prefabricate a large mandible replacement for clinical use during a 7-week prefabrication period.¹⁹ Other studies have assessed ectopic and orthotopic bone formation in cell-based constructs. These studies report abundant bone formation throughout the intramuscular implantation. Orthotopic bone apposition was limited to the interface of surrounding bone, with negligible osteogenesis occurring at the center of the implant.²⁰ The arteriovenous (AV) loop chamber is another strategy for prevascularization using *in vivo* anastomoses. This intrinsic vascularization model forms a shunt loop between an artery and a vein. Polykandriotis and Kneser obtained vascularized calcified tissue constructs by inserting an AV bundle into a disk of processed bovine cancellous bone matrix.^{21–24} This approach requires several weeks to anastomose with the host vasculature *in vivo* and does not address the risk of blood coagulation upon implantation.

The strategies mentioned above provide promising advancements for the clinical use of vascularized constructs. However, one major limitation is the survival of cells in large constructs through nutrient and oxygen diffusion, which is related to the limited hemoperfusion in the early post-transplant phase.³ The need for multiple surgeries is also a major disadvantage.²⁵

Hemocompatibility and Endothelialization of Anticoagulant Biomaterials

Blood contact with the surface of a foreign material may result in protein adsorption, activation of the complement system and the clotting cascade, and eventually thrombus

formation.²⁶ The thrombus makes oxygen and nutrient diffusion more difficult. The surface properties of a biomaterial are crucial to determine its hemocompatibility *in vivo*. These surface properties are often supplemented with active anticoagulant molecules, such as heparin, a glycosaminoglycan that acts as a strong polyanion, or chitosan, a polycation structurally similar to glycosaminoglycans and compatible with a variety of mammalian cell types.

Layer-by-layer (LBL) assembly of polyelectrolytes is one method to modify the surface of a biomaterial.^{27,28} Drug delivery systems made using LBL assembly of heparin and chitosan can store heparin-binding growth factors, such as FGFs and BMPs.²⁹ Heparin-chitosan LBL-coated coronary stents have been tested *in vitro* and *in vivo*. These stents safely and efficiently promote re-endothelialization and intimal healing, in addition to their anticoagulant properties.³⁰ Our group has shown previously that the LBL assembly of heparin-chitosan coats on acellular bone matrix (HC/ACBM) significantly increases blood perfusion compared to blood diffused into the scaffold in ACBM.³¹

Endothelial-Like Differentiation and Homing of Bone Marrow Stem Cells Are Crucial Aspects of Tissue-Engineered Therapy Design

MSCs can differentiate into a variety of cell types, including osteoblasts, chondroblasts, adipocytes, and hematopoiesis-supporting stroma. This cell type has been widely studied for its therapeutic applications in regenerative medicine. MSCs subjected to fluid shear stress (SS) differentiate toward ECs, which are a key cell type in vasculogenesis and cardiac repair.³² MSCs can also be differentiated to ECs using VEGF stimulation and hypoxic environments.³³

Another cell source option to regenerate function in ischemic tissue is EPCs. These cells reside in the bone marrow primarily and in low concentrations in the circulating peripheral blood. EPCs stimulate the re-endothelialization of injured blood vessels and can support vasculogenesis and angiogenesis in hypoxic areas.³⁴ EPCs deposit and remodel the extracellular matrix to a greater extent than ECs, which could be an important advantage for tissue engineering applications.³⁵ Due to these numerous advantages, EPCs have an enormous potential in vascular regenerative medicine. However, the peripheral blood of healthy adults contains very low concentrations of circulating EPCs. Several transducers can boost the mobilization of these progenitor cells from the bone marrow into the peripheral blood to accelerate *in situ* endothelialization, such as the stromal cell-derived factor-1, VEGF, granulocyte-colony stimulating factor (G-CSF), and erythropoietin.³⁶ EPCs represent a promising tool for generating an endothelium on materials. Thus, in recent years, the interest to capture EPCs on surfaces has gained much attention. Synthetic vascular grafts coated with capture molecules can also be used to attract circulating EPCs, mimicking natural homing factors, including antibodies, peptides, and oligosaccharides.³⁷

Stem cell sources for bone tissue engineering are often expanded *in vitro* using bioreactor systems.³² Bioreactor systems can create an environment similar to the *in vivo* environment of bone and provide adequate nutrition and oxygen to cells throughout the scaffold. Bioreactors can also provide fluid SS cues *in vitro* similar to those acting on MSCs *in vivo*. Additionally, fluid SS plays a crucial role in

the differentiation of MSCs. Previous studies have shown that different SS, ranging from 0.3 to 15 dynes/cm², can induce osteogenic, endothelial, or cardiomyocyte-like differentiation of MSCs.^{14,27} Recently, microscale computational fluid dynamics models have been developed to predict local velocity and shear profiles throughout the microarchitecture of the scaffold.³⁸ Cioffi et al. applied a combined macroscale/microscale model to quantify the hydrodynamic SS throughout a perfused porous scaffold and predict the oxygen profiles within a cell-seeded construct during the initial stages of bioreactor culture. The average SSs calculated with their model within a scaffold perfused at 0.03–0.3 mL/min, were consistent with the magnitudes predicted by previous microcomputed tomography-based models.³⁹

Presentation of the Hypothesis

The significant improvement in tissue perfusion observed when using medicinal leeches to salvage the venous-congested microvascular free flaps or reattached limbs inspired us to try a similar method to improve perfusion when constructing tissue *in vivo*. The beneficial mechanism of this therapy relies on secretion of the anticoagulant hirudin from the salivary glands of the leech. This mechanism also relies on the proboscis through which blood is absorbed. Thus, eliminate microcirculation disorders, restore damaged vascular permeability, eliminate hypoxia, and increase the bioenergetic status of the organism. Vacuum-assisted closure (VAC) can promote blood perfusion similar to the leech proboscis.⁴⁰

We integrated the current knowledge of bone tissue engineering, anticoagulation scaffold prefabrication techniques, blood perfusion, and vascularization in soft tissue enabled by the VAC therapy system. We hypothesize that a combina-

tion of MSCs form the bone marrow, controllable release of heparin and cell capture molecules and VAC therapy system can improve the blood perfusion and vascularization of the engineered tissue.

The protocol to test this hypothesis used bone marrow as a blood supply, delayed heparin release from the HC-coated ACBM as an anticoagulant, and a VAC therapy system to promote blood perfusion throughout the scaffold (Fig. 1). Biomimetic-engineered bone generated using this leech-inspired therapy enabled blood perfusion and captured stem cells for neoangiogenesis and bone formation. To verify that the construct works *in vivo*, we established a 30-mm tibia traumatic bone defect model in pigs. The surgical procedures are as follows: The pigs were anesthetized by intravenous injection of ketamine (50 mg/kg body weight), and bilateral surgical procedures were conducted under aseptic conditions. The external fixation was put on the anterolateral of the tibia. Then, a 50-mm incision was made at the anteriomedial within 2 pins, and the tibia was isolated through the interspace of the tendons and muscles. Then, a complete bone defect of 30 mm, which included the periosteum, was made in the middle segment of the tibia. The fibula was left intact for mechanical stability. The implanted materials were inserted into the bone defect and bound to the fibula by thread without other fixation devices. The drainage tube was inset into the hole of the scaffold, placed through the muscle and cutaneous and inosculation with the VAC system. After implantation, the wounds were carefully washed with saline, and the incisions were sutured in full thickness (Fig. 2). The blood flow in this model, which ranges from 0.05 to 0.1 mL/(min·g), can transport oxygen and other nutrients as predicted by macroscale and microstructured models *in vitro*.³⁹ This strategy opens new possibilities for reconstructing large segment bone defects and facilitates clinical

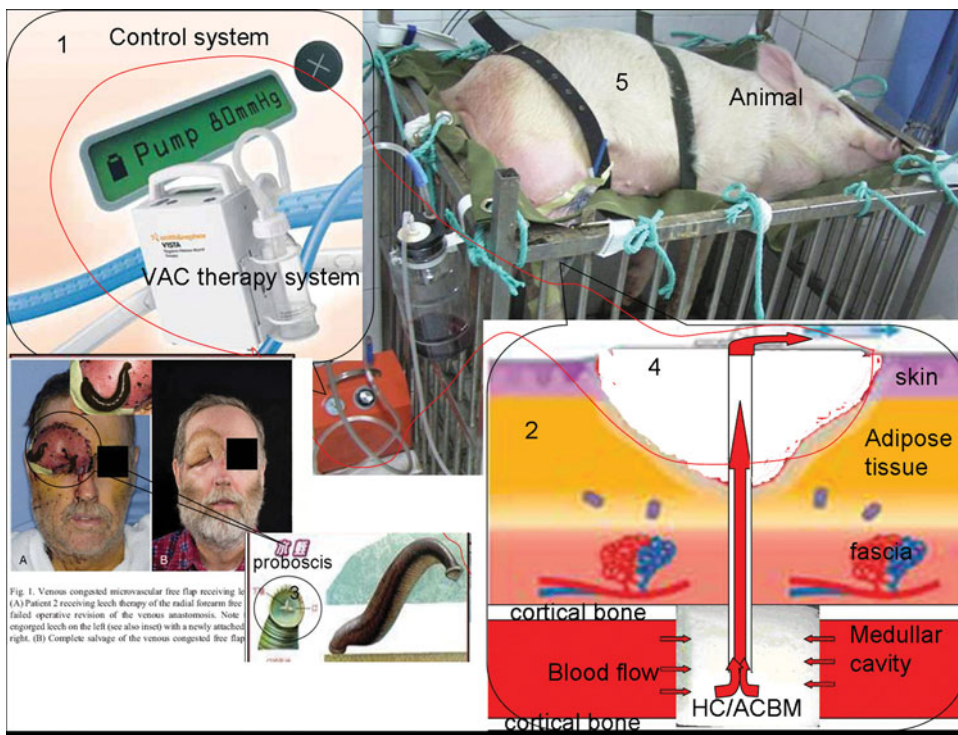
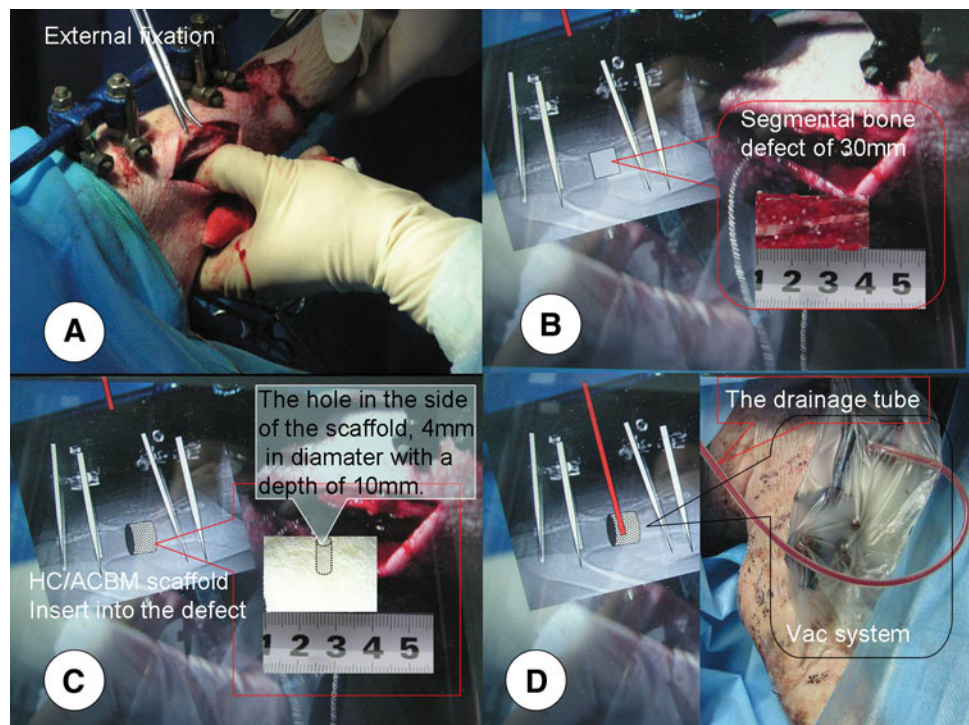


FIG. 1. Biomimetic construction of tissue-engineered bone. Biomimetic bone engineered using the medicinal leeches therapy enabled blood perfusion. The vacuum-assisted closure (VAC) therapy system draws the blood from medullar to perfuse into heparin–chitosan coats on acellular bone matrix (HC/ACBM) act as the proboscis promoting blood flow within the venous-congested microvascular free flaps. The heparin released from the HC/ACBM keep the blood from coagulation as that of hirudin. 1, control system of VAC system; 2, biomimetic-engineered bone (bottom right inset; red arrow indicates direction of blood flow); 3, proboscis of leech; 4, VAC therapy system (top left and bottom right insets, encircled in red); 5, experimental animal.

Fig. 1. Venous congested microvascular free flap receiving leech therapy. (A) Patient 2 receiving leech therapy of the radial forearm free flap failed operative revision of the venous anastomosis. Note: engorged leech on the left (see also inset) with a newly attached right. (B) Complete salvage of the venous congested free flap.

FIG. 2. Surgical procedures to construct biomimetic-engineered bone. **(A)** The external fixations were put on the anterolateral of tibia. Then, a 50-mm incision was made at the anteriomedialis, and the tibia was isolated through the interspace of the tendons and the muscles. **(B)** A complete bone defect of 30 mm, including the periosteum, was made in the middle segment of the tibia. **(C)** HC/ACBM materials were inserted into the bony defect. **(D)** The drainage tube was inset into the hole of the scaffold, placed through the muscle, cutaneous, and inosculation with the VAC system. Blood could be drawn immediately after incision sutured.



translation in bone tissue engineering. This strategy has three advantages compared to conventional reconstruction methods. First, the VAC system allows blood to be perfused, rather than only diffuse, into the anticoagulation construct during the early postimplantation period. Second, the volume and shape of tissue-engineered constructs can be customized, and MSCs can be integrated within the scaffold to form capillaries. Third, this strategy promotes bone healing at the site of the bone defect, without requiring additional surgeries.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Xinjun Sun, MD
Department of Orthopedics
89th Hospital of PLA
Beigongxi Street, 3770 Weifang
Shandong 261021
China

E-mail: 13637895821@163.com