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Research Article

A Porcine Model for Repair of Long Bone Non-Union Defects Using Three-Dimensionally Printed Scaffolds

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Abstract

Background: Approximately 5-10% of bone injuries in the United States result in non-union, costing an estimated \$10.4-26.1 billion annually for treatment. While several orthopedic strategies to address non-unions in long bones are available, all have drawbacks and risks of complications. A possible alternative is the recent advances in tissue engineering treatments that are improvements over current treatment options.

Methods and Findings: We have developed a porcine long bone segmental defect model for testing osseous non-union repair using 3D-printed biodegradable scaffolds. We generated a 3.5 cm mid-diaphyseal defect in the right radius of a six-month-old pig. A Polycaprolactone (PCL) scaffold implant designed from radius Computed Tomography (CT) scans and produced using solid form fabrication was implanted into the defect and stabilized with a stainless-steel plate. The animal could stand and place weight on the limb at six hours post-surgery and ambulating without a noticeable limp by four weeks. CT imaging post-surgery show bone fill in the defect space by two weeks, significant defect closure by eight weeks and complete union by 24 weeks.

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This healing has taken place on a PCL scaffold with no other treatments in conjunction with the implant.

Conclusion: The porcine radius segmental defect model provides a viable platform for testing printed scaffolds and bioengineered composite scaffold constructs for the efficacy of healing segmental defects and other forms of non-union in long bones.

Keywords

Bone Injuries; Bone; Three-Dimensional (3D) Printing; Polycaprolactone

Introduction

There are roughly 6.3 million bone fractures annually in the United States. Of these, 5-10% require bone grafting or other surgical intervention due to non-union healing at the cost of approximately \$2.5 billion [1-3]. Unfortunately, complications and revision surgeries to address failure of initial fracture healing treatments are common. Factoring in patient morbidity, quality of life issues and lost work opportunity costs, total costs for these injuries swells to four to six times this amount [4]. Currently, there are no consent guidelines to determine treatment of non-unions after failed surgery attempts [5].

Long bone injuries present unique challenges for the field of regenerative medicine. Historically, treatment of long bone injury resulting in non-union was limited to amputation [6]. In recent decades, the development of treatments relating to augment and accelerate bone regeneration include:

1. Bone autografts (from the same human) [7,8]
2. Bone allografts (from cadavers) [9,10]
3. Bone transport by distraction osteogenesis (also known as the Ilizarov technique) [11,12]
4. Direct bone deposition utilizing tissue engineering techniques [13-15]

Despite successful results with these methods, significant drawbacks are associated with many of these approaches and partial or complete failure rates are substantial. These treatment methods can be lengthy, leading to issues of patient fatigue and maintenance of compliance. Harvesting of bone autografts is painful and leads to donor-site co-morbidities. Grafts can fail to vascularize and integrate with recipient bone requiring new grafting attempts [16-18]. Repeated failures eventually may necessitate amputation [19].

Bone tissue engineering tends to restore bone tissue function through cell biology, material science and engineering [16,19]. With the advent of three-dimensional (3D) printing technology to fabricate stiff polymeric biodegradable materials into complex shapes, tissue engineering strategies have been devised to replace or augment current therapies for non-unions. The fabrication process for bone engineering is desirable due to the ability to control

the geometry and internal structure of tissue scaffolds [19]. Typical strategies utilize a printed bone insert scaffold coupled with growth factor treatments to impart osteoconductive or osteoinductive competence to the scaffold [20]. Dozens of biomaterial 3D printing strategies are available today, consisting of biodegradable polymers such as Polycaprolactone (PCL), softer biomatrix protein-based material, biodegradable synthetic polymers, or ceramic material [21]. PCL is common due to the low toxicity of degradation products [21]. Multiple studies have proven the advances in new tissue engineering treatments, however, the translation of this research into clinical therapies for bone tissue and non-osseous tissue has mostly been unsuccessful [22,23]. Implementation of tissue engineering approaches in humans using osteobiologics has been limited and restricted to only a few growth factors [10,13].

The investigation of tissue engineering work in small animal models has been extensive over two decades. Focus on the use of scaffold materials loaded with single or multiple growth factors and stem cells in different combination has had extensive research including chitosan nanoparticles, hydrogel scaffolds, 1,6-hexanediol diacrylate scaffolds, bisphosphateloading scaffolds and mesenchymal stem cells infused in scaffolds [5,22,24,26,21,13,20,27,28]. The transition of this research into clinical therapies has struggled since models for long bone segmental defects require mechanical stabilization and large animal test subjects must be able to utilize the limb during recovery and healing for care and housing to be feasible. Large animal models provide appropriate mass transport and volume challenges for scaffold-based tissue regeneration that small animal models or in vitro models cannot.

The mammalian model for bone tissue engineering studies has typically been porcine since they are immunologically and physiologically more like humans than other non-primate species. The porcine bone biology, microstructure and healing properties resemble humans, providing a reasonable model for this study. We have chosen the radius bone of six-month-old Yorkshire pigs as a model site for long bone repair studies, as it is easily accessible for surgical manipulation and the tight association with the ulna provides a natural anchoring for orthopedic stabilization of surgically generated defects without the need of pins or external fixation. Here, we describe the initial development of a large animal long bone critical size defect model for the study and testing of 3D-printed scaffold implants for healing long bone defects.

Materials and Methods

Initial Scaffold Design

Using CT scans of a six-month-old Yorkshire pig, a PCL implant was designed (Fig. 1). PCL was chosen due to low toxicity, durability and FDA approval. Mimics[®] software utilized CT scans to generate the external scaffold geometry [21]. The internal pore architecture was designed using a custom MATLAB[®] program. The pore architecture was merged with the external defect shape to create the final scaffold design. The PCL cage portion of the construct was designed to fit a defect in the radius bone comprising approximately 30% of the length of the bone, centered around the mid-diaphyseal shaft. Scaffolds were fabricated from

Polycaprolactone (PCL) using solid form fabrication. The bridgeable PCL pin portion of the scaffold was designed to provide sufficient support (Fig. 1) to keep the two ends of the fracture immobilized but flexible enough to allow compression and pending to promote fracture healing through endochondral ossification. Details of the specific design and fabrications methods are described in these reviews [29,30].

Figure 1: Initial radius scaffold design. (A) CAD file showing initial radius scaffold implant design. (B) 3D-printed PCL scaffold (C) Schematic of scaffold design shown in position inserted into the radius.

Secondary Scaffold Design

Similar to the initial scaffold design, a PCL implant was designed from CT scans of the radius of six-month-old Yorkshire pigs. (Fig. 2). CT scans were utilized by Mimics[®] software with the MATLAB[®] designed pore architecture to generate the external scaffold geometry and the internal marrow space. The design incorporated an attached sleeve to fit over the intact portion of the radius and provide a surface to stabilize the construct and anchor it into the bone using a stainless-steel orthopedic plate. Details of the specific design and fabrications methods are described in these reviews [29,30].

Figure 2: (A) Image of bisected radius shows dimensions of the bone and location of the growth plates (blue text). The radius is tightly connected to the ulna. A 3.5 cm defect is approximately 30% of the length of the radius. (B) CAD file showing the secondary scaffold design. (C) Final 3D-printed secondary scaffold device. A PCL sleeve without radius insert and with two slots is seen above the scaffold.

Surgical Implantation

Experiments involving the use of animals were conducted under protocols approved by the University of Illinois Animal Care and Use Committee (IACUC #13389). After administering general anesthetic and intubation, a six-month-old pig was placed in dorsal recumbency on the operating table. The left front leg was prepped and draped and a curvilinear incision made along the cranial aspect of the leg, between the elbow and the carpus, using a #10 scalpel blade. The extensor muscles and tendons were retracted to expose the cranial surface of the radius. The radius was transected at the approximate center of the diaphysis, using a Stryker surgical saw under irrigation to generate a diaphyseal defect of 3.5 cm, approximately 30% of the radius length. The PCL scaffold was placed between the cut ends of the bone and anchored into the proximal and distal marrow cavities by using a narrow 4.5 mm stainless-steel dynamic compression plate along the scaffold by cortical screws through the sleeve portion of the scaffold and the radius into the ulna. The soft tissues were then closed with three suture layers.

Monitoring of Healing: Computed Tomography Imaging and Histology

The bone repair was monitored via Computed Tomography (CT) imaging at 2, 4, 8 and 24 weeks post-implantation. At 24 weeks, the animal was euthanized and the radius-ulna complex of the left leg was excised and examined. Portions of the repaired radius tissue were cut from the bone complex with a band saw, fixed in 10% neutral buffered formalin, then decalcified

using CalciClear (National Diagnostics, Atlanta, GA). After decalcification, the bone tissue was embedded in Neg50 embedding medium and sections were cut on a Leica CM2000 cryostat. Sections were stained using hematoxylin and eosin and were immunostained using a monoclonal antibody for vimentin (monoclonal clone V9, ICN, Biochemicals). Histological images were captured using a Nanozoomer digital pathology system slide scanner (Hamamatsu, Shizuoka, Japan), while immunostained images were captured using a Leica DMI2000 inverted fluorescent microscope.

Results

Scaffold Design and Surgical Implantation

The initial scaffold design failed. The scaffold was unable to remain immobilized in the marrow cavity of the bone and popped out. This led to the secondary scaffold design and using the shape of the radius as a guide, we designed an implant (Fig. 2) for porcine radius defects, consisting of a macroporous body shaped like the central diaphysis segment of the radius and incorporating an attached sleeve to fit over the intact portion of the radius and provide a surface to stabilize the construct and anchor it into the bone using a stainless steel orthopedic plate.

We tested this design by implanting the scaffold in six-month old male Yorkshire pigs (Fig. 3). A 3.5 cm defect was generated in the radius and the device was implanted into the defect and stabilized with a stainless-steel narrow dynamic compression plate. By six hours post-surgery, the animal was able to stand and place weight on the limb. By four weeks, the animal was walking without a limp and mobility was not lost for the duration of the study.

Monitoring of Bone Healing

The pig was allowed to heal for 24 weeks. During this time period, CT imaging of the forelimb was performed at 2, 8, 12 and 24 weeks post-surgery. Imaging showed gradual filling in of the radius defect (Fig. 4). By 8 weeks, the dynamic compression plate became engulfed by bone and was pushed away from the surface of the radius. By 24 weeks, the radius defect was bridged and the plate was almost completely engulfed by ectopic bone. The animal was euthanized at 24 weeks and the radius-ulna complex was excised.

Examination of the complex (Fig. 5) showed that the scaffold had not been integrated with the forming radius and filled with new bone, but rather had been pushed up away from the radius and had been almost surrounded by ectopic bone. Histological examination of samples of tissue from the excised radius-ulna complex showed that the interior of the scaffold itself was filled with a fibrous and fatty tissue. The surfaces of the scaffold were not directly attached to bone tissue, but rather surrounded by a bed of fibrous connective tissue that connected to bone tissue (Fig. 6). This fibrous tissue connected to the underlying bone through a thickened layer of periosteal tissue that stained positive for vimentin (Fig. 6) [31].

Figure 3: (A) Generation of a 3.5 cm radius diaphyseal defect and surgical implantation of the scaffold. The scaffold was stabilized via stainless-steel plate and screws. (B) The pig six hours post-surgery, standing and walking. (C) The pig 13 months post-surgery, standing and walking with full weight on limb.

Figure 4: CT images taken 2, 4, 8 and 24-weeks post-surgery show bone fill in the gap defect space (indicated by red arrow). By 2 weeks the 3.5 cm gap of the radius has bone fill, 8 weeks is nearly closed and by 6 months the gap is filled. Note the steel plate (indicated by yellow arrows) is pushed away from the radius surface and embedded in bone.

Figure 5: (A) Comparison of implanted bone (left) to the radius-ulna of control limb (right). Note the increased thickness of implanted radius-ulna. (B) Scaffold and plate not fully encased in new bone (indicated by purple arrow). (C) Removal of excess bone and the plate shows that the scaffold had been pushed up and away from the regenerated radius bone and is surrounded by fibrous soft tissue.

Figure 6: Cross sections of tissue surrounding implant. (A) From the center of the regenerated bone showing H&E stained trabecular bone of the reformed radius (indicated by “R”). The scaffold (indicated by “S”) lies above the reformed radius (out of view of the section but direction is indicated by yellow arrow) on a bed of fibrous tissue and is surrounded by fatty and fibrous material. (B) Concentrations of blood cells (black arrows). (C) Magnified region of yellow box in (A) Showing a presumptive periosteum (indicated by “P”) making connections (yellow arrows) with new bone tissue (indicated by “b”). (D) Presumptive periosteum demonstrates a dense population of vimentin-positive cells (green stain) by immunostaining.

Discussion

It has been a longstanding goal in orthopedic research to develop novel, tissue engineering-based therapies to address non-union bone healing that take advantage of the increasing availability of 3D-printed materials that can potentially be utilized for weight-bearing, osteocompatible implants [15]. This case study demonstrated that porcine radius is an acceptable model to test 3D-printed scaffolds for use in repairing long bone defects. We produced a significant CSD of in the radius of a pig and implanted with a 3D-printed PCL scaffold design. While the initial scaffold design was unable to bear the weight of the pig, the secondary design proved successful. The PCL scaffold itself was not integrated into the newly forming bone but rather pushed aside and surrounded by fibrous tissue and encased in ectopic bone. Even with the imperfect healing, the pig was able to maintain use of the limb though the duration of the study and the defect was healed. The formation of periosteum (Fig. 6) occurs when injured bone reforms, therefore, the defect is responding as that of naturally injured bone. While the results were not of perfect healing, it showed that progression of healing occurred. The results are aligned with that of Feng, et al., (2011), thus proving that such research is translatable to larger scale models [5,32].

Conclusion

Despite their cost and logistical challenges, large animal models are critically important for bone tissue engineering studies. Large animal models provide more appropriate mass transport and volume challenges for scaffold-based bone tissue regeneration that cannot be investigated appropriately in small animal models or *in-vitro*. The future goals are to develop and test patient-customizable 3D-printed, bioresorbable, osteoinductive artificial callus scaffolds that can be used to treat and heal segmented long bone defects from a variety of orthopedic injuries. An animal model such as our porcine radius defect system may provide enough mechanical stability to the limb to test softer, non-weight bearing materials for their ability to promote long bone defect healing materials that mimic the properties of softer bone tissue healing intermediates that are generated during long bone fracture healing. While further research and modification of the scaffold is needed, this case study demonstrates that the porcine radius segmental defect model provides a viable platform for testing printed scaffolds and bioengineered composite scaffold constructs for the efficacy of healing segmental defects and other forms of non-union in long bones.

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