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| Original Article |

Comparison of Bone Regeneration Rate in Flat and Long Bone Defects: Calvarial and Tibial Bone

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Abstract : An ideal scaffold for bone tissue regeneration should be dissolved at the same rate of host bone growth into the defect. Therefore, to produce such a scaffold, it is necessary to obtain a standard healing rate of bone defects. In this study, we compared healing rate of bone defects in calvarial and long bones, which have differential developmental and regenerative mechanisms. In the calvaria and tibia, 3 mm defects were made, and healing was analyzed using microcomputed tomography (microCT) and histology up to six weeks. MicroCT analysis showed that in calvarial defects, an unhealed gap remained until six weeks, whereas tibial defects had healed after three weeks. H&E and Trichrome staining consistently showed that calvarial defects were not completely healed by six weeks, however, a tibial defect started to heal from three weeks. Results of histomorphometric analysis showed that 60% of calvarial defects had healed at six weeks after surgery, whereas 80% of tibial defects showed regeneration at three weeks. Cartilage formation was detected only in tibial defects, suggesting endochondral regeneration in long bone, but not in flat bone. Collectively, these results demonstrate that healing of a long bone defect is faster than that of flat bone by approximately two folds. Therefore, our data suggest that dissolution of scaffold should be optimized based on the type of bone defect.

Key words: *bone regeneration, calvarium, tibia, healing rate*

1. Introduction

Bone defects can occur by a variety of causes, such as trauma and cancer metastasis. In treatment of bone defects, particularly large bone lesions, the defective bone requires a scaffold that can support bony architecture and mechanical load. Recently, use of a tissue engineering approach encompassing scaffold and mesenchymal stem cells (MSCs) has become an attractive technology for regeneration of large bone defects. Manipulation of stem cell function and scaffold performance is a central issue in enhancement of bone regeneration. In particular, scaffold performance has been improved by modulation of biocompatibility and osteoconduction property. In addition to pro-osteogenic property, scaffolds need to be dissolved as the host bone grows. Since inert properties of biomaterials may hinder host bone growth and replacement, and render healed bone fragile, control of dissolution of scaffold in the body is of importance in regeneration of bone tissue defects.

Typical approaches to regeneration of bone defects include simple implantation of biocompatible scaffolds to the defect region. Recently, implantation of scaffolds combined with MSCs has also been considered for clinical treatment of bone defects. However, since healing with host bone is an ultimate goal of bone regeneration, healing rate of bone and dissolution rate of scaffold should be tightly controlled. In fact, healing rate of bone varies according to morphology and region of bone in the body. During development, cranial vault and facial bones with flat bone morphology undergo membranous ossification, while long bones in the limbs are formed by endochondral ossification. Consistent with differential developmental processes, these bones show differential fracture healing properties. Fracture healing of long bone undergoes intramembranous and endochondral ossification.[1] Damaged periosteum provides cells for cartilaginous and osteoid bone during the fracture healing process.[2] Periosteum plays a role in differential healing of fractures of flat and long bones.[3] In addition to fracture, bone defects can be

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healed with a similar mechanism. Alberius reported that calvarial bone defects healed by intramembranous ossification, finding no chondrogenic cells.[4] Therefore, bone defects in different types of bone may heal with a differential regeneration rate and thus require scaffolds that have a differential dissolution rate. In this study, we made bone defects in calvaria and tibia, and examined bone regeneration rate.

2. Materials and Methods

2.1 Animal Experiment

Male Sprague-Dawley (SD) rats, aged six weeks, were obtained from Daehan Bio Link (Korea). Animals were acclimatized for one week before surgery. Surgical procedures were performed under anesthesia. Rats were divided into two groups, flat or long bone defects, and each group was split into four time points: one, three, five, and six weeks. Five mice were used for each time point. For calvarial defects, a midline incision was made on the skin and periosteum was carefully excised (Fig 1). A 3 mm hole was drilled on one side of the calvarium using a trephine burr (Fig 1). Periosteum flaps were wrapped and sutured, and the skin was then sutured. A similar procedure was used for tibial bone defects. A tibial bone defect (3 mm) was made using a trephine, then covered with periosteum again and sutured. Antibiotics and anti-inflammatory reagents were applied on the skin and injected for three days to prevent inflammation. The animals were housed in a controlled environment; temperature 22-24°C, relative humidity 45~65%, and 12/12h light/dark cycle. The animal experiment was approved by the Institutional Animal Care and Use committee of Kyungpook National University.

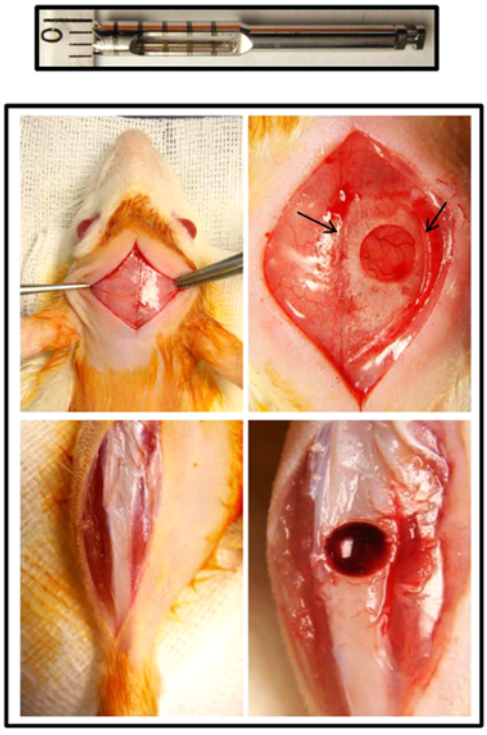

Figure 1. Surgical procedures showing calvarial and tibial defects. A trephine bur with a diameter of 3 mm was used to make bone defects.

2.2 MicroCT

At the indicated time point, the rats were sacrificed by cervical dislocation, and skull and legs were cut and fixed with 10% formalin for 3-4 days. Specimens were examined using the microCT system (X-eye MCT system, SEC, Korea). X-ray tube voltage was 70 kV, current was 70 μA. Samples were scanned through 180° at a spatial resolution of 30 μm. Projection number was 400. X-ray image data were reconstructed for creation of 3D images using the iCAT-3D imaging program.

2.3 Histology and Histomorphometry

Calvaria and tibia were fixed in 10% buffered formalin for 24 hr and demineralized in 10% ethylenediaminetetraacetic acid solution. Paraffin blocks were sectioned with 6 μm thickness, and the sections were stained with H&E. For measurement of regenerated bone area, iSolution DT analysis system (iMTechnolog, Daejeon, Korea) was used. Briefly, in H&E images with 40X magnification, percentage of newly formed bone area over total bone defect area was calculated. For analysis of collagen and cartillage synthesis, the sections were also stained with Masson's Trichrome staining[5] and Alcian blue staining[6], respectively, as described previously.

3. Results

3.1 Microcomputed Tomography

Flat and long bones have differential healing processes for wounds like fracture and defects. However, bone regeneration rate has not been thoroughly investigated. For comparison of bone regeneration rate in flat and long bone, defects with a diameter of 3 mm were made at calvaria and tibia, as described in the Materials and Methods section. At one, three, five and six weeks later, mice were sacrificed, and bone regeneration was evaluated using microCT. In calvarial defects, an unhealed area was still observed at five or six weeks after surgery (Fig 2 A). Of particular interest, regeneration pattern of calvarial defect showed that the front line of ingrowing bone was not even (Fig 1A). Some part of the front line was protruded and fused together. On the other hand, a 3D reconstructed image of the tibial defect showed that healing was prominent from three

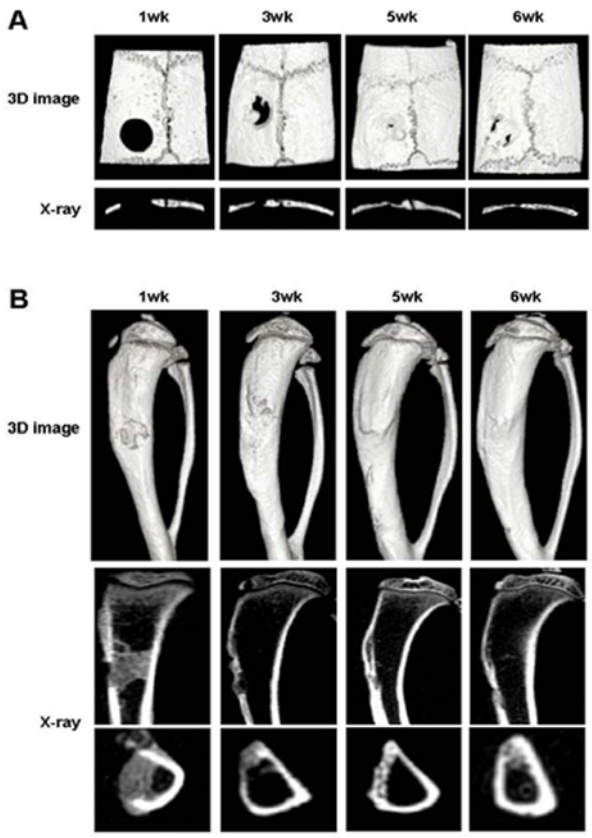

Figure 2. MicroCT images of calvarial and tibial bone during regeneration. Calvaria and tibia were drilled with a trephine and sutured. After the indicated time, calvaria and tibia were scanned using microCT. 3D images were reconstructed using the iCAT-3D imaging program.

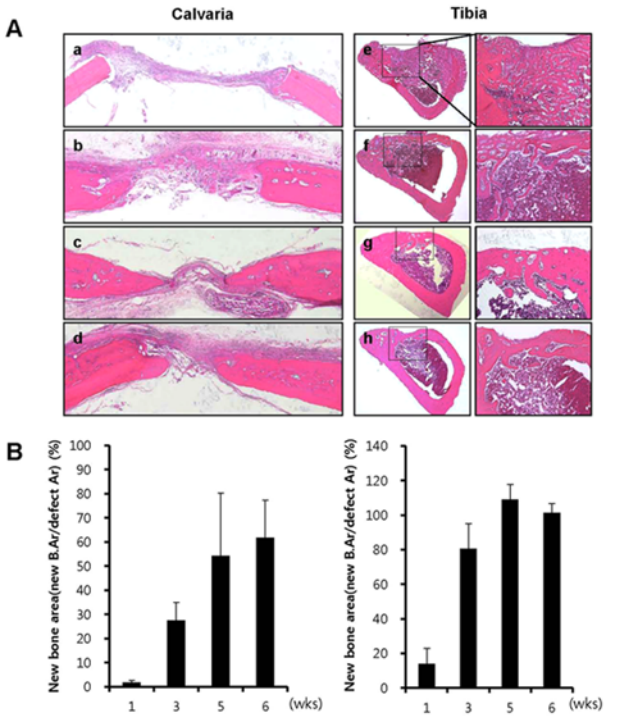

Figure 3. H&E staining of calvarial and tibial bone defects during regeneration. At one week (a and e), three weeks (b and f), five weeks (c and g), and six weeks (d and h), calvaria (a~d) and tibia (e~h) bone were collected and serially sectioned and stained with H&E. New bone area was calculated as percentage of new bone over the defect area (B).

weeks (Fig 2B). Cross-sectional 2D images showed that tibial regeneration was almost complete at three weeks (Fig 2B). These results suggest that regeneration of a tibial defect is faster than that of a calvarial defect.

3.2 Hematoxylin and eosin staining and histomorphometry

For analysis of regeneration pattern and to confirm bone regeneration, calvarial and tibial bone was cross-sectioned and stained with H&E. As shown in Fig 3, calvarial bone had an unhealed gap until six weeks (Fig 3A), although the gap was becoming narrowed. However, in the tibial defect, trabecular bone was formed by three weeks in the region of the defect (Fig 3f). Lacunae were formed and osteocytes were included, suggesting that bone was under maturation. By five weeks, trabecular bone became thickened and marrow was connected, and lamination was also obvious (Fig 3g). At six weeks after surgery, trabecular was further shortened (Fig 3h). To quantify bone regeneration, newly formed bone in calvaria and tibia was analyzed using histomorphometry. Calvarial defect was healed in 1.68% at one week, 27.4% at three weeks, 54.21% at five weeks, and 61.83% at six weeks. In tibial defects, regeneration was achieved in 13.88% at one week, 80.59% at three weeks, 109.04% at five weeks, and 101.17% at six weeks. These results demonstrate that bone regeneration of tibial defects was faster by more than two folds than calvarial defects after three weeks.

3.3 Trichrome Staining

To further confirm bone maturation, Trichrome staining was performed. In calvarial defects, collagen deposition, shown as blue color, was not obvious until three weeks (Fig 4A), suggesting that bone matrix synthesis is not active until three weeks. On the contrary, in tibial bone defects, collagen deposition was prominent at one week (Fig 4B), indicating that bone matrix is actively synthesized from an early time. From three weeks, the newly formed bone turned to a red color, indicating that the new bone had

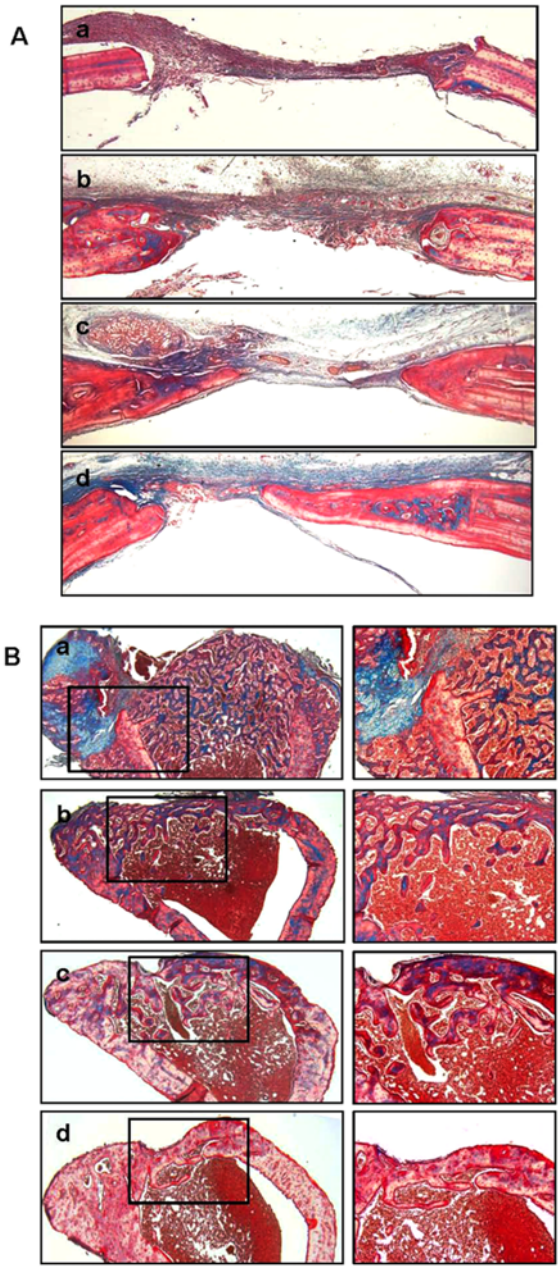

Figure 4. Masson's Trichrome staining of calvarial and tibial bone defects during regeneration. At one week (Aa and Ba), three weeks (Ab and Bb), five weeks (Ac and Bc), and six weeks (Ad and Bd), calvaria (Aa~Ad) and tibia (Ba~Bd) bone were sectioned and stained with Trichrome. Collagen fiber was stained with blue and bone was stained with red color.

undergone maturation (Fig 4Bb-Bd).

3.4 Alcian Blue Staining

Because fracture healing of long bone is associated with transient cartilage formation, we next attempted to determine whether cartilage is associated with bone defects. Alcian blue staining did not show any positive signal in calvarial defects (Fig 5A). However, in tibial defects, alcian blue staining was observed at one week (Fig 5Ba), suggesting an association of long bone regeneration with cartilage formation.

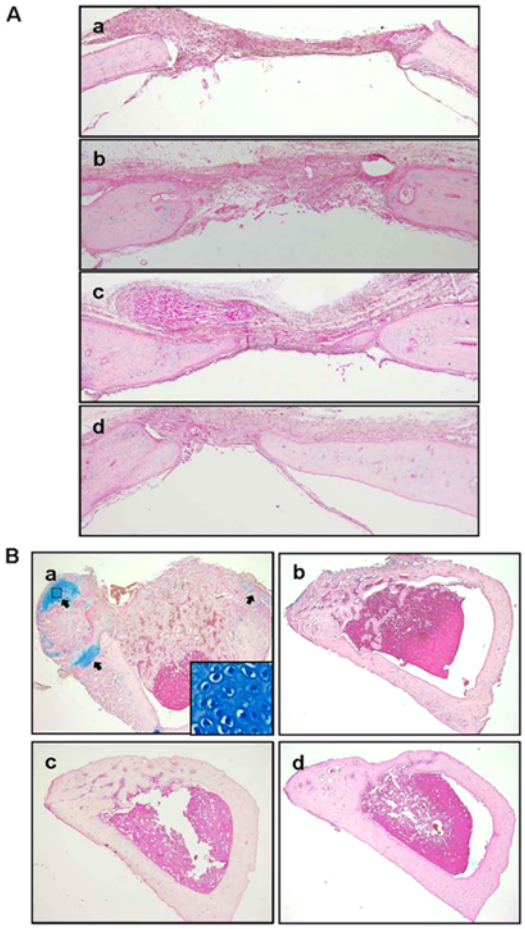

Figure 5. Alcian blue staining of calvarial and tibial bone defects during regeneration. At one week (Aa and Ba), three weeks (Ab and Bb), five weeks (Ac and Bc), and six weeks (Ad and Bd), calvaria (Aa~Ad) and tibia (Ba~Bd) bone were sectioned and stained with alcian blue. Cartilage was stained with blue color.

4. Discussion

The current study showed that healing time of long bone defects is much faster than that of flat bone. In addition, we found that tibial bone regeneration was accompanied with early trabecular bone formation in the cortical bone region followed by remodeling and maturation. Therefore, we believe that bone regeneration rate and pattern demonstrated in this study may be helpful to design and construction of a scaffold that has a similar dissolution rate with host bone ingrowth.

Several previous reports have suggested that the fracture healing process is differentially regulated by periosteum of different regions of bone. Although we now know that periosteum of different regions of bone has differential potential for bone formation, determination of bone regeneration rate in flat and long bone is still necessary for tissue engineering application. We measured host bone ingrowth rate, and found that, in a given defect size, calvarial bone defect took more than six weeks to heal, however, tibial bone took only a couple of weeks (Fig 2 and 3). These results suggest that healing of long bone is much faster than that of flat bone. In fact, tibial bone regeneration rate was faster by almost two fold, compared to calvarial bone regeneration. Rapid healing of a long bone defect may be attributed to potential of tibial periosteum. In fact, previous reports have shown that cell differentiation patterns in periosteum may differ between the tibia and calvaria. Grafted tibial periosteum formed a larger area of bone than grafted calvarial periosteum,[3] suggesting that tibial periosteum has a higher potential for formation of bone regeneration than calvarial periosteum.

Consistent with previous reports, showing that calvarial bone defects are healed by intramembranous ossification,[4] we did not observe callus formation in calvaria. However, in tibial defects, cartilage tissue was observed at one week after surgery (Fig 5), indicating that endochondral regeneration occurred only in tibial defects.

In addition to periosteum, an osteoconductive substrate functioning as a scaffold between bone ends is important, enabling active ingrowth of bone and fibrovascular tissue.[7] At one week after surgery, calvarial defect still had a clear end, while tibial defect showed fibrous tissue around the area of the defect (Fig 3A and 3B, Fig 4A and 4B). These results suggest that endogenous matrix substrate is well formed and that the matrix is functioning as a scaffold for bone ingrowth in tibia.

Mechanical loading may also contribute to rapid bone regeneration in tibia compared to that in calvaria. Mechanical strain is a fundamental physiologic factor regulating bone formation and renewal.[8] Lack of mechanical load causes reduced production of matrix-protein.[9,10] Mechanical stress induces expression of osteoblast differentiation markers, such as Runx2 ALP, COLI, and OPN in bone marrow mesenchymal stem cells.[8-11]

This study demonstrated a differential regeneration rate in flat and long bone, however, application of these results for tissue engineering has some limitations. Implantation of scaffold may affect regeneration rate, so that overall dissolution of scaffold and host bone ingrowth can be changed. In addition, bone healing may also depend largely on the size of the defect and integrity of soft tissue.[12] Nevertheless, differential bone regeneration rate and pattern determined in this study may provide information for use in decisions on scaffold. For example, β-tricalcium phosphate (β-TCP), which can be dissolved within a couple of weeks, may be the first choice for long bone defects less than 3 mm in diameter, whereas β-TCP/hydroxyapatite composite scaffold, which can be dissolved over several weeks, could be used for a calvarial defect. Investigation of this suggestion may be needed in the future.

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