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A Review of Mouse Critical Size Defect Models in Weight Bearing Bones

Jonathan S. Harris, **Thomas B. Bemenderfer**, **Alexander R. Wessel**, and **Melissa A. Kacena***
Department of Orthopaedic Surgery, Indiana University School of Medicine, Indianapolis

INTRODUCTION

The history of Orthopaedic surgery can be traced back to the first time Orthopedia was penned by Nicholas Andry in 1741; taken literally, it means “Straight Child”, and most of the efforts from then until now have been aimed at changing or altering the macrostructure of bone architecture [1]. However, a paradigm shift has occurred relatively recently whereby the focus of altering bone architecture and healing has shifted from hardware application, such as plates and screws, to the microscopic microcosm of the cellular environment. As Henry Mankin, Professor of Orthopaedics at Harvard, stated"future changes in orthopaedics will be based in biology and more specifically in our ability to understand and alter the basic unit, the cell.” This endevour has led to some exciting discoveries over the past several decades, including elucidating the osteoinductive potential of bone morphogenetic proteins (BMPs) in the 1970’s. The need to better understand this microscopic world of bone repair cannot be over-emphasized at this epoch in our history. The advent of the cannon shot in the 14th century would usher in a new era of “high-energy” skeletal injury, and as one could imagine, high-energy injuries have only become more commonplace in today’s world. From the battlefields to the highways, skeletal trauma has only increased in severity and incidence, and this has necessitated advances in fracture treatment.

For many fractures and bone defects, the use of hardware fixation alone is not enough to ensure fracture healing. The “gold standard” for nonunions and high-energy fracture treatment is autograft; however, this option is fraught with limitations and complications. Donor site pain is the most commonly stated side effect, plaguing 18% of patients at two years who underwent iliac crest bone grafting in one series [2]. Additionally, autograft is limited both in the size and shape available, making large defects impractical to treat. These large defects can arise from skeletal trauma as well oncologic procedures. Solutions to these problems have been sought in both synthetic scaffold designs as well as in allografts, and this has spawned proliferation in research designs and animal models. A need to study fracture nonunions and large defects has recently led to the development of murine models employing CSDs, or better put, defects that would not normally heal in the lifetime of the animal [3].

It is important to note that these murine models do not readily recapitulate into the clinical scenarios, except in the more esoteric realm of orthopaedic oncology. It is exceedingly rare,

*Corresponding Author: **Melissa A. Kacena, Ph.D.**, Department of Orthopaedic Surgery, Indiana University School of Medicine, 1120 South Drive, FH115, Indianapolis, IN 46202, Tel.: 317-278-3482, Fax: 317-278-9568, mkacena@iupui.edu.

if ever, that a patient would experience a high-energy trauma resulting in a large mid-diaphyseal segment of bone being completely and cleanly removed from the body. More often, a high-energy trauma can result in a comminuted fracture that will eventually fail to heal, resulting in a nonunion. This situation is difficult to recreate in an animal model in a reliable, reproducible fashion; therefore, investigators recreate the ingredients that cause nonunions to occur. Nonunions are generally classified broadly as viable and non-viable [4]. Viable nonunions, or hypertrophic and oligotrophic nonunions, occur due to inadequate fixation from poor surgical technique or improper hardware, either of which causes excessive motion at the fracture site. In this type of nonunion, the blood supply, and therefore the osteogenic and osteoinductive potential, is adequate, giving rise to the characteristically larger callus formation. Non-viable nonunions, or atrophic nonunions, lack sufficient blood supply to support adequate callus formation and the characteristic absence of callus is seen. The factors leading to an inadequate blood supply are likely multifactorial and not fully understood. Certain traumatic injuries, such as Gustilo Type IIIB open tibial fractures, experience frank stripping of soft tissue and periosteum, an obvious cause of devascularization at the fracture site, but many circumstances exist where the cause of nonviability is not readily known [5]. It is important to note that fracture healing via callus formation is inherent to the indirect bone healing pathway of endochondral ossification--this is in contrast to the direct bone healing pathway that involves absolute rigid fixation without callus formation. Discussion of the direct bone healing pathway is beyond the scope of this review. Additionally, intramembranous ossification will also be mentioned as it occurs in select circumstances, and these will be further outlined.

The CSD model creates an environment where fracture healing is impeded due to lack of blood flow to the fracture site (non-viable nonunion) or lack of proper fixation (viable nonunion), or both. This allows researchers to study various pathways involved in fracture healing and various therapies to augment healing. Several prerequisites must be met to have a successful murine CSD model: the surgical procedure must be straightforward and easily reproducible, the defect must be large enough to ensure reproducible nonunions in control groups, the intercalary graft must have sufficient strength to support an ambulatory animal (if load-sharing fixation is used), the graft must be immunocompatible to avoid host rejection, the graft must have an osteoconductive matrix if incorporation is a desired outcome, and the graft must be secured in place over the duration of the study [6–8]. Load-sharing fixation includes the use of an intramedullary device, such as a Kirchner wire, which results in the graft having to share the mechanical load [9]. Most studies, though not all, utilize this type of fixation and thus require intercalary grafts to have sufficient strength to support the weight of the animal. The specifics of each of the above criterion vary in different designs, and each variable will be further discussed throughout this review; but the overall goal remains the same in with CSD experiments: create a circumstance that will result in a nonunion. CSD models are an attractive way to study fracture healing because they stress the organism’s ability to repair a fracture, allowing researchers to determine if experimental interventions are capable of inducing successful bone healing in a difficult scenario.

DEFECT SIZE

In order to stress the bone healing ability of an organism to nonunion, the segmental resection must be large enough to create a non-viable space that inhibits indirect bone healing to bridge the defect. This model has been studied extensively in the mouse species, and with the exception of a few study designs, most researchers have arrived at the same dimension: 4mm. In 2004, Tiyapatanaputi et al. designed one of the first mouse models to utilize a CSD that resulted in consistent nonunions when devitalized allografts were placed between the intercalary defects [6]. In this study, 4mm mid-diaphyseal femoral defects were

created and bridged with either autograft (femur was cut but not removed), or bridged with devitalized allograft. This model showed consistent lack of bridging over the full length of the graft, though the graft-host junction did undergo endochondral ossification callus formation. With regard to the autograft, the graft-host junctions united via endochondral ossification with callus formation, and the middle portion of the graft underwent intramembranous ossification, validating the tremendous healing potential contained within periosteum as had been postulated in previous studies [10]. The conclusion from this study is that a smaller defect would have allowed endochondral bridging to occur in the devitalized allograft group, illustrating the importance of maintaining the appropriate defect size. Subsequent studies have further supported that a 4mm femoral defect size in a mouse model reliably leads to nonunion, both with scaffold designs and with bone graft designs, when no other experimental intervention is performed [11–16].

Notable exceptions to the 4mm femoral CSD exist in the literature and deserve attention as they highlight the salient features of nonunions. Garcia et al. demonstrated that 1.8mm femoral mid-diaphyseal defects in mice led to reliable nonunions if the periosteum was also stripped to a distance of 2mm from the cut proximal and distal femoral edges [17]. In this study, 0.8mm and 1.8mm defects were created and stabilized with a pin-clip fixation, with some groups undergoing 2mm of periosteal resection from the cut edges and the other groups retaining periosteum up to the cut edges. Though nonunions occurred in every group, the 1.8mm group with periosteal resection experienced a 100% atrophic nonunion rate. The obvious question is why did these models experience nonunions with a much shorter defect? The pin-clip fixation maintained the gap length and supported the weight of the ambulatory animal, leaving a complete absence of osteoconductive matrix. Additionally, the periosteal stripping effectively left an even larger “gap” that was void of any osteogenic potential. These two actions together prohibited any new cartilage formation and subsequent endochondral ossification. This study demonstrates that an appropriate sized gap lacking osteoconductive matrix juxtaposed with periosteal stripping, which effectively increases the osteogenic “gap”, can result in consistent nonunions. Most weight-bearing mouse designs involve femoral defects, though some have explored creating defects in other long bones. Fu et al. described a mouse model where 2.5mm tibial resections were created and bridged with composite fibrous scaffolds, some of which were treated with BMP-2, and others that served as the control group that resulted in nonunions or delayed unions [18]. In addition, larger animal designs have contributed to the understanding of bone healing and elucidating novel methods or materials to aid in bone healing [7, 19, 20].

From these study designs, it is apparent that various methods have been developed to achieve nonunions in animal models. A few standards can be extrapolated from the recent literature concerning mouse models, especially with respect to defect size and location. The mouse femur is relatively easy to access surgically, ensuring reproducible operation results, and it is large enough to precisely create segmental defects thereby minimizing variations in defect size. The segmental mid-diaphyseal defect size of 4mm with an intercalary load-sharing construct has repeatedly been shown to not heal in the lifetime of the animal when no additional treatments are used. Using a biomechanically sound intercalary construct is advantageous because it allows for ambulation as well as providing a matrix for endochondral ossification to occur, and, in select circumstances, for intramembranous ossification to occur. Intercalary constructs also can serve as vectors to deliver experimental substances, such as BMP-2, or can serve as a surface on which to fix experimental cells or viruses. The evolution of these intercalary constructs has resulted in two main types of grafts: scaffold grafts and bone grafts. The requirements of these constructs are stringent and, in the case of scaffold grafts, have called for novel manufacturing materials and methods.

SCAFFOLD GRAFTS

In the field of orthopaedic oncology, large long-bone defects are surgically created secondary to en bloc resection of sarcomas or metastatic cancers, and filling these defects has been an orthopaedic conundrum. Allografts are currently used but face several drawbacks including infection, microfracture propagation, and catastrophic failure in some cases [21]. This has led researchers to explore alternative synthetic graft designs. An *ideal* scaffold graft would be: i) bio-compatible, not eliciting an immune response by the host organism; ii) biomechanically supportive, allowing the animal to ambulate throughout the experiment duration; iii) made of porous matrix of the appropriate size, allowing for vascular ingrowth and resorption if graft integration is desired; and iv) able to sustain or deliver experimental biomaterials, allowing researchers to effectively study the biomaterials in question. Though it has been shown that cells can be seeded onto nonporous biomaterials and some neovascularization can occur, ample evidence suggest that porous matrices are advantageous and experience a greater degree of vascular ingrowth-- some even consider a porous size of at least 100μm to be a minimum requirement for proper vascularization and bone cell colonization [22–26]. A scaffold fulfilling these requirements would offer an attractive alternative to current allografts, as well as offer an alternative solution for limb salvage in cases of high-energy trauma.

As with most medical advances, the search for this ideal scaffold design began in animal research. In 2007, Chu et al. reported the development of cylindrical scaffolds that were manufactured from poly(propylene) fumarate/tricalcium phosphate (PPF/TCP) composites and employed dicalcium phosphate dehydrate (DCPD) as a carrier for BMP-2 [7]. These 5mm scaffolds were secured between surgically created, equal sized femoral mid-diaphyseal defects in rats using intramedullary Kirshner wire. After fifteen weeks of healing, the scaffolds augmented with BMP-2 demonstrated trabecular formation between the periosteal callus and scaffold surface, with some degradation of the scaffold. None of the scaffolds in the study collapsed, proving they can support an ambulatory rat for the duration of 15 weeks. Yu et al. designed a porous, degradable polyester cylindrical scaffold composed of polycaprolactone-hydroxyapatite (PLA-HA) to determine if seeding endothelial cells onto the scaffold would prevent necrosis of engineered bone via neovascularization. 4mm femoral mid-diaphyseal segmental defects were created in mice and interposed with scaffolds. The mice were divided into three groups: those without any treatment (control), those with scaffolds which were seeded with endothelial cells, and those with scaffolds which were seeded with both endothelial cells and osteoblasts. The study found that pre-seeding the scaffolds with endothelial cells resulted in neovascularization, as evidenced by a widely distributed capillary network, which prevented ischemic necrosis of the newly formed bone. The porous nature of the scaffolds seems to be advantageous in supporting cell biocompatibility and neovascularization [23]. As mentioned previously, a porous matrix is not absolutely necessary for cellular adhesion or vascular ingrowth, but it does seem to enhance these processes, making it an ideal characteristic in scaffold designs [24–26]. These scaffold designs also contained calcium orthophosphates in their matrices, which is an important component, as other studies have pointed out [7, 27].

The addition of calcium orthophosphates to the basic scaffold matrix does appear to enhance new bone formation by recapitulating the nanodimensional and nanocrystalline nature of *in vivo* hard tissue, allowing for greater viability and better proliferation of various cell types (notably osteoblasts), resulting in excellent biocompatibility [28]. Several studies have demonstrated this by comparing bone healing techniques using scaffolds that contained calcium orthophosphates and scaffolds that did not. Fu et al. constructed scaffolds made from poly(D,L-lactide-co-glycolide), or PLGA, and added hydroxyapatite (HAp) to several scaffold groups to study release kinetics in a tibial segmental defect mouse model [18]. The

purpose of the study was to determine if adding HAp to the scaffold matrix would result in a more controlled release of BMP-2; the study confirmed that the fibrous PLGA/HAp composite scaffolds maintained biomechanical integrity throughout the experiment while delivering BMP-2 in a more controlled manner. This resulted in improved formation of new bone compared with scaffolds containing only PLGA. Jacobson et al. constructed scaffolds made of 100% polylactic acid (PLA), or a mixture of 85% PLA and 15% beta-tricalcium phosphate (β-TCP), or PLA/βTCP, to test the effectiveness of intermittent teriparatide systemic injections in a femoral CSD mouse model [15]. In their study, the scaffolds that contained βTCP demonstrated significantly higher bone volume and mineral content at 9 weeks post-surgery compared with the 100% PLA scaffolds when both groups were treated with intermittent systemic teraparitide injections. This suggests that the scaffolds containing calcium orthophosphates enhanced bone healing. It should be noted, however, that calcium orthophosphates carry the disadvantage of being brittle and possess poor fatigue resistance, relegating their use to primarily serve as fillers in scaffold designs [28]. Furthermore, not all studies incorporated calcium orthophosphates into the scaffold matrices. Kanczler et al. studied the importance of vascular endothelial growth factor (VEGF) in a mouse femoral CSD model by comparing new bone formation using PLA scaffolds, none of which contained calcium orthophosphates. Some of the PLA scaffolds were encapsulated with VEGF using supercritical CO_2 fluid technology in combination with human bone marrow stromal cells (HBMSC), resulting in an average pore size of 250 μm and an average pore density of 70%. The mouse femora interposed with scaffolds encapsulated with VEGF and HBMSC showed significant bone regeneration, including increased bone volume, trabecular number, and reduced trabecular separation. Though the lack of calcium orthophosphates in the scaffolds was not a main focus of this study, it does highlight the fact this material is not essential for new bone formation. However, a plethora of literature does suggest that adding calcium orthophosphates to the matrix can augment new bone formation [15, 18]. A brief discourse of calcium orthophosphates has been provided; however, this topic that cannot be scrupulously reviewed here, and the reader is referred to the review by Dorozhkin for a more thorough discussion [28].

It is apparent from this brief overview of the current literature that many scaffold designs exists. What seems to be constant, however, is that successful scaffold designs maintain structural integrity throughout the experimental timeframe and possess a matrix that facilitates osteoconduction and neovascularization. The literature suggests that adding calcium orthophosphates can enhance bone formation. From the preceding discussion, it is clear that many factors must be considered when designing a scaffold that can fulfill the previously mentioned ideal characteristics. Current scaffold designs are moving toward this goal, though it may be some time before an ideal scaffold is available for use in patients. To borrow from a colloquial phrase, such a scaffold would be the “holy grail” in treating patients with segmental defects, whether arising from orthopaedic oncologic procedures or from high-energy trauma injuries. It is with this goal in mind that current research continues toward the ideal scaffold.

BONE GRAFTS

After blood transfusions, bone is the second most commonly implanted material in patients, comprising an estimated 600,000 grafting procedures annually [4]. The history of allograft procedures largely began with the work of the Frenchman Ollier in the mid-1800s when he demonstrated the osteogenic potential of transplanted bone [29]. The understanding of bone biology slowly progressed until Urist’s work in the 1960s where he discovered that devitalized bone matrix can induce heterotopic ossification, and he later discovered the most widely studied and used osteoinductive agents: BMPs [30]. The terms osteoconduction, osteoinduction, and osteogenicity emerged from this earlier research and have provided the

foundation on which current allograft procedures are built. A thorough review of all allograft materials and procedures is beyond the scope of this paper; therefore, for the purposes of this review, *cortical* allografts will be the main focus given that CSD models in animals incorporate these types of allografts.

Although cortical allografts can be effective in many situations, there are several major drawbacks inherent with their use. Modern techniques in obtaining these materials from cadavers attempt to remove or neutralize pathogens that may potentially harm the host patient; however, these methods are not universally standardized or completely effective. Proprietary processing techniques usually employ some combination of irradiation, antibiotics, detergents, ultrasonics, or hydrogen peroxide that can minimize the risk of transmitting pathogens to the host, but it should be noted that none of these techniques result in sterility, in the strict use of the term, given that sterilizing techniques would have a deleterious effect on the mechanical integrity of the graft [31]. Furthermore, because cortical allograft is avascular, it becomes a site for subsequent infection even if the graft is free of pathogens at the time of implantation. Another consequence of necrotic bone is the inability to remodel, an integral component of bone homeostatis in keeping with Wolf's law; this can lead to the development of microfractures which can propagate and ultimately cause catastrophic failure. Some series have found a failure rate ranging from 16–27% secondary to infection, fracture, or nonunion [11]. Though these potential complications raise concern for cortical allograft implantation, the overall success rate is promising when compared to the alternative use of metallic implants. In one series, patients implanted with a cortical allograft after bone resection for high-grade extremity osteosarcomas enjoyed a 70% satisfaction rate [21]. Additionally, the risk of disease transmission (HIV, hepatits B, hepatitis C, West Nile virus, bacteria, and prions) among all bone allografts is extremely low, with the overall incidence estimated at 0.014% [31]. Current research in animal models utilizing cortical allografts is aimed at discovering novel techniques and materials to augment bone healing, with the hope of improved graft-host union, graft incorporation, and ultimately, complete graft replacement with new bone. A brief overview of recent research employing cortical allografts in animal models will highlight salient techniques and discoveries. Before moving forward, it is important to review terminology specific to bone graft animal research-- at the risk of being pedantic, this will hopefully avoid any potential confusion. Autografts are segments of bone taken from an animal and implanted back into that same animal, whether or not they are devitalized. Allografts are segments of bone taken from an animal and implanted into a genetically different animal of the same species (segment of C57BL/6 mouse femur implanted into C3H mouse). Isografts, which are particular to animal research and specifically murine models, are devitalized segments of bone taken from an animal and implanted into another animal that is genetically identical (segment of C57BL/6 mouse femur implanted into another C57BL/6 mouse). This situation allows for a unique scenario to study bone healing, but it should be noted that this scenario does not readily exist in clinical practice. Admittedly, while this could happen, it would be exceedingly rare to find monozygotic twins where one was in need of allograft and the other was available for cadaveric harvesting.

Tiyapatanaputi et al. created 4mm mid-diaphyseal femoral segmental defects in a mouse model to compare the bone healing response of allografts and autografts [6]. The study found that the autograft implants, with intact periosteum, consistently formed new bone at the host-graft junction via endochondral ossification and new bone along the shaft via intramembranous ossification, while the allograft implants only formed new bone at the host-graft junction via endochondral ossification. This suggests the importance of periosteum and the progenitor cells contained in the cambium layer. Furthermore, none of the allograft group experienced complete callus bridging, much less any appreciable resorption and replacement of the necrotic bone. It should be noted, however, that

devitalized autograft was not used in this study (though devitalized isograft was used), making it difficult to accredit all new bone formation to intact periosteum. Zhang et al. more clearly suggests the importance of periosteal cells by engrafting BMP-2 transfected stromal cells onto the surface of devitalized allografts and implanting them in 4mm mid-diaphyseal femoral segmental defects in a mouse model [32]. Compared with acellular allograft, the cellularized allograft exhibited a 3-fold increase in callus formation and 7-fold increase in neovascularization allowing for extensive graft resorption, providing a promising strategy for engineering a functional periosteum in the clinical setting. Xie et al. sought to understand the differences between systemic versus local cyclooxygenase-2 (COX-2) with regard to the inflammatory stage of bone healing [33]. Utilizing wildtype (WT) and cyclooxygenase-2 (COX-2) knockout mice, they were able to show that prostaglandin E2 (PGE-2) is integral to initiating the bone healing process. Though the main focus of this study was aimed at determining the differences between systemic versus local COX-2, it does highlight the importance of signaling molecules, such as prostaglandins, in initiating bone formation. Xie et al. designed another mouse allograft model to recapitulate the cellular processes inherent to intact periosteum [13]. To engineer a “pseudo-periosteum”, the group either seeded mesenchymal stem cells transfected with human BMP-2 onto devitalized isografts and allografts or onto a membranous small intestinal submucosa which was wrapped around naked isografts/allografts. The grafts were then implanted into 4mm intercalary mid-diaphyseal femoral defects. Both the isografts and allografts that were directly seeded and those wrapped with the seeded membrane displayed significant new bone formation, as well as biomechanical strength equivalent to intact femora, though the graft cortex was never completely resorbed and replaced by new bone. This study provides another potential route for augmenting allograft procedures in humans. Collectively, these studies suggest that the periosteum, and ergo the cells that it contains, are vital to initiating bone formation, and furthermore, that the signaling molecules responsible for recruitment and activation of these cells are vital to this process. The microcosm of the cells and signaling molecules is complex, and many studies have sought to elucidate additional interactions that may be potentially exploited to enhance bone repair.

Ito et al. employed a rather sophisticated method of lyophilizing recombinant adeno-associated viruses (rAAV) transfected with VEGF, receptor activator of nuclear factor κB ligand (RANKL), or both, onto femoral allografts and implanting them into 4mm mid-diaphyseal segmental femoral defects in mice [34]. Marked remodeling and neovascularization occurred with the allografts coated with both VEGF and RANKL, leading the group to conclude that both signaling molecules are necessary for sufficient remodeling. The study was developed under basis that CSD models using allografts exhibit new bone formation, but remodeling ceased once the callus calcified on the allograft cortex, leaving a permanent necrotic bone core. By stimulating creeping substitution to continue, this study successfully demonstrated that adding VEGF to the local milieu caused increased neovascularization; furthermore, adding RANKL stimulated osteoclastic activity, which is vital for necrotic bone resorption as evidenced by up to 50% of the graft cortex being resorbed and replaced by new bone. Koefoed et al. also utilized rAAV by coupling activin receptor-like kinase-2 (ALK2), a molecule that generates signals similar to BMPs, and lyophilizing the viruses to allograft surfaces which were implanted into 4mm femoral defects in a mouse model [14]. By expressing ALK-2, the viruses were capable of transducing adjacent inflammatory cells and osteoblasts, resulting in osteoclastic resorption of the graft cortical surface accompanied by endochondral bone formation with evidence of remodeling, supporting a potential method to revitalize allografts *in vivo*. Kitaori et al. sought to better understand the role of stromal cell-derived factor 1 (SDF-1), a potent chemokine in bone marrow, and its receptor, CXCR4, in a mouse bone healing model [35]. Exchange grafting in various combinations with either anti-SDF-1 neutralizing antibody or an antagonist for CXCR4 led the group to conclude that SDF-1 increased mesenchymal stem

cell migration leading to endochondral bone formation. This displays another potential pathway that could be exploited to enhance fracture repair.

To this point, the studies reviewed have sought to manipulate or augment the local environment around the CSD, but other studies have experimented with systemic treatments to enhance bone graft healing. Reynolds et al. exploited the bone healing potential of teraparatide therapy to enhance devitalized isograft osseointegration in a 4mm mid-diaphyseal femoral defect mouse model [12]. Briefly, 4mm devitalized isografts were implanted into 4mm intercalary defects, then after one week the mice were given daily injections of teriparatide. This treatment resulted in enhanced host-graft integration, evidenced by increased trabeculated callus formation and biomechanical strength, illustrating the systemic use of teriparatide therapy as a potential adjunctive treatment for fracture healing. O'Keefe et al. attempted to further understand the effects of PGE2 on bone healing by creating 4mm mid-diaphyseal femoral segmental defects in mice, transplanting devitalized isografts into the intercalary defect, and administering either daily COX-2 inhibitors or PGE2 by direct minipump infusion for 4–5 weeks. Results indicated that there was a significant reduction in bone formation in the group receiving COX-2 inhibitors, suggesting that PGE2 production is a prerequisite for efficient skeletal repair. This study validates the notion that inhibition of COX-2 can have deleterious effects on bone healing, potentially guiding clinical analgesic treatments.

Several salient points deserve mentioning in lieu of the proposed methods that have been discussed thus far. The biological environment has profound effects on new bone formation, whether endochondral or intramembranous, and correct manipulation of this environment could potentially lead to complete resorption of the necrotic bone graft cortex, allowing replacement by new bone, resulting in a completely regenerated bone identical to native bone. Understandably, the road from animal models to clinical applications is a long one, even so, progress continues in the development of promising new therapies for potential applications in humans. Developing an allograft with an adjunctive treatment that would allow for eventual complete resorption and replacement by host bone, while allowing immediate weight-bearing without an external fixation device, would greatly improve the quality of care for patients and potentially decrease healthcare costs. Such a treatment would provide a solution to many orthopaedic conundrums, revolutionizing the way surgeons approach these conditions.

CSD OUTCOME MEASURES

Along with the advances in CSD models and their respective therapeutic interventions, the way in which researchers measure various bone healing parameters has also made advancements. The evolution of detecting bone healing in animal models holds its foundation in X-ray imaging and has progressed since that time. The introduction of micro-computed tomography (μCT) has greatly enhanced the ability to calculate specific aspects of bone healing. With many studies aimed at understanding the cellular environment involved with bone healing, the use of histology and immunohistology have become ubiquitous in CSD studies, allowing researchers to better understand complex cellular interactions. Another very important aspect of bone healing, given that one of the functions of bone is structural support, is the mechanical strength of the CSD model, and many methods for assessing biomechanics have been developed. These modalities are complex and involved—indeed entire books have been written on each method alone; however, a review of CSD would be remise without a brief overview.

X-rays are useful because of the relative ease in obtaining images and the ability to image *in vivo*. This allows serial images to be easily taken, which can be invaluable to understanding

how bone callus mineralization is occurring over time. Goldberg et al. developed a scoring system for fracture healing which has been adopted and modified by several other studies as well [36]. X-rays are limited in the detail of data that can be extrapolated, but remain a valuable tool due to the relative ease in obtaining films and the ability to obtain serial films on live animals.

The need to further evaluate bone healing and to objectively quantify callus volumes has led to the widespread use of μCT in CSD studies. High resolution imaging allows direct measurement of bone microarchitecure that is superior to histologic analysis [37]. The images can be broken down into single two dimensional slices, reconstructed into three dimensional cross-sections, or reconstructed into solid three dimensional objects that can be rotated around any axis to view incredible detail. Additionally, correlations can be made between bone architecture and mechanical properties. μCT-based finite elemental analysis has been validated as a useful biomechanical analysis tool to quantify the effects of stress and strain on bone tissue [38]. This is largely based off the fact that the attenuation coefficient values (Hounsfield values) can predict the Young's modulus of bone tissue [39]. Furthermore, μCT cross-sections can be used to predict the response to torsion by calculating the polar moment of inertia, or angular displacement of bone when subjected to torque (found by taking the integral of all the bone pixels on a given cross-section). Other aspects of bone healing, such as neovascularization, can also be studied in great detail. Zhang et al. employed a vascularization assay in their CSD study where a contrast of silicone rubber with lead chromate (Microfil) was introduced into the circulation at the time of euthanasia and allowed to perfuse the CSD area and polymerize; the samples were then decalcified to allow image segmentation between bone and the intra-vascular contrast [40]. Several studies have developed novel methods to quantify bone healing. Reynolds et al. developed a novel algorithm to compute the "union ratio" between host bone and graft bone by contouring the periosteal and endosteal surfaces [41]. This ratio was developed by comparing the surface area of the graft that was in contact to the new callus and the surface area that was void of callus. The group further validated the "union ratio" by carrying out biomechanical testing, where the ratio correlated significantly with ultimate torque and torsional rigidity, concluding that this method could provide a novel tool for noninvasive assessment of functional strength and failure risk of allografts in the clinical setting [11]. A more recent use of μCT involves i*n vivo* serial scanning, offering the distinct advantage of tracking bone formation over time. It does, however, have its drawbacks. As an example, serial scanning subjects the tissue to large amounts of ionizing radiation which can effect bone formation [42]. Also, from a practical standpoint, it can be difficult to properly anesthetize the animal considering that the scanning resolution is in the micrometer range [15]. That being said, in vivo μCT is likely to become more commonplace in the future as the machines become more available [37]. A committee was formed to put forth guidelines in response to the lack of consistency in reporting outcomes, and the reader is encouraged to review this report for better understanding [37]. Briefly, a study should report a minimal set of image acquisition parameters (voxel size, X-ray tube potential, and integration times); a minimal description of trabecular bone (bone volume fraction, trabecular number, trabecular thickness, and trabecular separation); and a minimal description of cortical bone (total cross-sectional area, cortical bone area, cortical area fraction, and average cortical thickness). Reporting these parameters is essential, as different μCT machines do not give comparable results [43]. It is easy to see why the use of μCT is becoming commonplace in evaluating CSD models, and this modality will continue to evolve.

Plain X-rays and μCT offer a wealth of information; however, many studies require additional analysis of cellular activities, molecular interactions, and microscopic structures. Basic histology, histomorphometry, and immunohistology offer very detailed, specific information that can be invaluable to the researcher. Indeed, the study of hard tissue

histology is an entire field unto itself, and for a more exhaustive discussion on calcified tissue histology, the reader is referred to Dickson's "Methods of Calcified Tissue Preparation" [44]. Here, a general overview will suffice to discuss a few salient points. Basic histologic staining allows for delineation between bone, cartilage, and fibrous tissue. Furthermore, osteoblasts and osteoclasts, as well as other pertinent cells, can be identified and counted to determine their prevalence. Cellular activity can be quantified by staining for specific enzymes or structural proteins, such as alkaline phosphatase and osteocalcin, respectively. In addition, some studies have called for more specific immunohistologic staining such as the COX-2 enzyme [33]. What can be stained for and quantified is limited only by the availability of reagents, and this availability continues to expand. Histomorphometry adds the ability to quantitatively analyze the microscopic structure and organization of bone tissue, giving insight to how well new bone is forming. Histologic analysis can determine if bone formed via endochondral ossification or intramembranous, and in many cases, both occur simultaneously. Most, if not all, current CSD research employ some form of histology, solidifying its ability to greatly enhance the understanding of how experimental interventions effect bone healing and to what extent.

The ultimate goal in developing bone healing therapies is to restore the bone to its original mechanical properties, and this can only be directly evaluated through biomechanical testing. Imaging, whether by x-ray, μCT, or histology, cannot provide direct measurement of physical properties such as torsional strength or elastic modulus, though limited correlations can be made [41]. It is well known that callus volume does not necessarily correlate to mechanical strength, nor does bone density correlate to mechanical strength. Ultimately, to understand how well a CSD models has healed, stressing the bone to failure is required. It should be noted that the field of biomechanical testing is involved and cannot be thoroughly reviewed here; please see the following articles for more information [45, 46]. Several basic types of biomechanical testing exist and will be briefly reviewed here. Tensile testing is relatively simply to perform on cortical bone, but has several limitations. It is not physiologically relevant, as *in vivo* bone is not loaded in pure tension, and some bending can occur during testing giving measurement errors; therefore, most studies do not utilize this type of testing. Compression testing is best used with cancellous bone as it mimics *in vivo* loading. It, too, is relatively easy to perform, but a major limitation is in achieving accurate results due to the end effects during testing. Bending tests cause both tensile and compressive forces to the bone, more closely mimicking physiologic conditions. Stress, strain, and Young's modulus can all be calculated using this method. Torsional testing measures the biomechanical properties of bone in shear, with maximum shear being experienced at the outer cortical surface. This test is often used to assess bone repair in CSD models because four biomechanical stages of fracture healing can be assessed: stage I (fractures through the original bone defect site with low stiffness); stage II (failure through the original bone defect site with high stiffness); stage III (failure through original defect site and through native bone with high stiffness); and stage IV (failure entirely through native bone with high stiffness) [47]. Variations of this classification can be made. Reynolds et al. classified the modes of failure as "pre-union", where the graft was pulled out from the host bone, "early-union", where a fracture involved the graft-host interface, and finally "mature union", where the failure occurred largely through the graft [11]. In either interpretation, torsional testing can provide a high correlation between the fracture pattern and the torsional properties of healed bone. Notched fracture mechanics testing is a newer way to test fracture resistance that mitigates the confounding factors inherent in bone, namely, the anisotropic nature of the bone matrix juxtaposed with internal pre-existing flaws. By creating a notch on the cortex and subjecting the bone to biomechanical testing (say three-point bending), the fracture propagates through the "worst-case flaw", which has been reproduced on each specimen to provide consistency [45]. It should be noted that all of the above mentioned testing methods are limited in that they are highly dependent on the specimen shape, and

irregularities make it difficult to interpret the intrinsic material properties of the healed bone [46]. These tests can give valuable information on the mechanical properties of the healed bone, but they do not give information on the material changes in the healing region; furthermore, it is difficult to compare results across studies given that the final mechanical properties are dependent on the models used, test conditions, animal species, techniques employed, and the amount of time the animals were allowed to heal after surgery [46]. From a more practical standpoint, biomechanical testing results are results based off of laboratory techniques that are very controlled and consistent, making it difficult to know the exact physiological relevance of these tests. That being said, biomechanical testing does yield useful information that can help researchers evaluate experimental interventions.

CONCLUSION

From the early days of fracture care in Switzerland, researchers have been challenging putative doctrine to advance the field of orthopaedics. Today, this research delves far into the microscopic environment, where the future of orthopaedic care lies. CSD models create an ideal environment to test the limits of bone healing potential and, in doing so, allow researchers to discover new therapies to powerfully augment bone healing. It can readily be seen that there are many musculoskeletal problems that do not currently have ideal solutions, such as segmental defects in long bones from oncologic resections and difficult nonunions that occur secondary to high-energy traumas. A scaffold graft that provides mechanical support, does not illicit an immune response, and ultimately allows for complete resorption and remodeling at a rate that does not compromise mechanical strength would certainly be a breakthrough in orthopaedics. Similarly, a bone graft model juxtaposed with an adjunctive treatment that allows complete resorption and remodeling of the graft, while maintaining mechanical integrity, would equally revolutionize orthopaedic care. The development of either an ideal scaffold graft or an ideal bone graft for clinical use will likely be founded in animal research, and to this end, CSD models serve a vital role and are a pivotal cog in the medical research machine.

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Highlights

- Defect sizes used in critical size defect (CSD) models are reviewed.
- Current scaffold designs are discussed, including manufacturing methods and results.
- Current cortical bone grafts are reviewed with regards to CSD models.
- Recent experimental interventions utilized in CSD models are discussed.
- Outcome measures, including μCT, histology, and biomechanical testing are reviewed.