Radioprotection and Cross-Linking of Allograft Bone in the Presence of Vitamin E

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by

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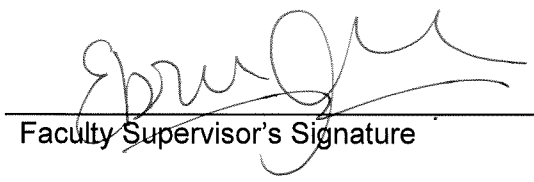
Author's Prior Degrees: SM, PhD

I have reviewed this thesis. It represents work done by the author under my supervision and guidance.



Faculty Supervisor's Signature

Dedication

To my parents Iwan Suhardi and Juliati Prajitno, to my wife, Anastasia Oktarina, and to my sister and brother for their relentless love and encouragement.

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Bone allografts are the preferred method for bone augmentation in about 500,000 orthopaedic surgical procedures in the US. Sterilization by ionizing radiation is the most effective method of minimizing the bioburden of bone allografts; however, radiation causes chain scission of collagen, resulting in the decrease of the mechanical strength of bone. In this study, we impregnated bone allografts with natural free radical scavenger vitamin E as radioprotectant using a novel two step process to protect the collagen architecture against radiation damage. In addition, we investigated combining the radioprotectant with a cross-linking agent, to further minimize collagen degradation. Both of these methods significantly improved the fracture toughness of bone allografts irradiated to 25 kGy and did not affect the osteoblast attachment on radiation sterilized bone. The two-step process also allowed complete impregnation of large bone within 30 hours, supporting the practical use of this technique.

Thesis Supervisor: Ebru Oral, PhD

Title: Assistant Professor in Orthopaedic Surgery

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Contents

List of Abbreviations

ALP : Alkaline Phosphatase

ASTM : American Society of Testing and Materials

BMP : Bone Morphogenic Protein

CCK-8 : Cell Counting Kit-8

CDC : Center for Disease Control

CT : Computed Tomography

DAPI : 4',6-diamidine-2'-phenylindole

DHLNL : dihydrolysinonorleucine

DI : Deionized water

DNA : Deoxyribonucleic acid

FBS : Fetal Bovine Serum

Gy : Gray

HCV : Hepatitis C Virus

HIV : Human Immunodeficiency Virus

IR : Infrared

kGy : kilo Gray

OD : Optical Density

PBS : Phosphate Buffered Saline

pNPP : p-nitrophenyl phosphate

Pyr : Pyridinoline

RANKL : Receptor activator of nuclear factor kappa-B ligand

SAL : Sterility Assurance Level

TGF- β : Transforming Growth Factor-β

TRAP : Tartrate-Resistant Acid Phosphatase

US : United States

Chapter I

Introduction

I.I Bone Graft

Bone augmentation using natural bone grafts is routinely used in orthopaedic fracture, tumor and joint replacement surgeries to support the repair of defects. In limb-salvage surgery, allograft reconstruction allows wide resection of bone tumor while maintaining skeletal continuity[1]. In spine surgery, bone grafts are commonly used as interbody grafts, strut grafts, or as corticocancellous chips to enhance spinal fusion[2]. In joint arthroplasty, bone grafts are used to fill bone deficiencies to provide better bone stock, to protect deficient or weak bone from fracture, to provide necessary support for implant components that are otherwise insufficiently supported by the native bone [1]. In the trauma setting, bone grafts are used for reconstruction of skeletal defects, treatment of nonunions, or for augmentation during fracture repair[3]. For example, cancellous or corticocancellous chips are often used as osteoconductive filler for metaphyseal defects that occur with tibial plateau fractures[3]. Forty eight percent of all bone grafts are used in spinal surgery[4]. Arthroplasty and trauma cases each account for about 20 % of bone grafts usage, whereas foot and hand surgeries account for the remaining 12 %[4].

Cortical bone grafts are often used as structural bone allografts for orthopaedic applications that require stability, mechanical strength and osteoconductivity [5-9]. The goal of bone graft is to provide immediate structural support, to initiate formation of new bone at the host-graft interface that will ultimately create integration between the host and the graft material. To achieve the aforementioned goal, several properties are necessary:

- Osteoconductivity: A material can be considered osteoconductive when it can serve as a matrix for cells that originate from the host and differentiate into osteogenic cells[10]. Clinically, osteoconductive material is one that permits blood-vessel incursion and bone growth on its surface and down its channels[11].
- Osteogenic: A material can be considered osteogenic if it contains live osteogenic cells. This usually occurs in one of two ways, either autogenous bone was harvested from a patient and implanted immediately to different site or when autogenous bone cells were cultured onto the material.
- Osteoinductivity: A material can be considered osteoinductive when it is able to induce migration of mesenchymal stem cells from the surrounding tissue, and subsequently differentiate into bone-forming osteoblasts[12]. This process is usually facilitated by presence of growth factors within the graft, such as bone morphogenetic proteins (BMP)[13].

I.II Sources of Bone Graft

Autogenous bone (autografts) is the gold standard of bone graft material because of its osteoconductivity, serving as a scaffold for new bone growth, osteogenicity, and osteoinductivity as it stimulates osteoprogregnitor cells to differentiate into osteoblasts. Autografts are also osteogenic by virtue of the presence of live bone cells in the graft material that contribute to bone remodeling [14]. Presence of viable osteoblast is crucial in healing with autograft ecause callus formation within the first 4-8 weeks after surgery is often dependent on bone formation by graft osteoblasts[15]. In addition, autograft does not cause immune response after implantation, thus further enhancing incorporation into host bone[16]. The major disadvantage of autografts is

donor site morbidity and infection. Complications of autograft harvesting such as increased postoperative pain, increased anesthesia time, and increased operative blood loss are observed in as high as 25 % of patients[17]. In addition, bone stock is limited and cannot be used for cases where large amounts of bone are required. After implantation, cortical autograft underwent resorptive phase (~18 mo in canine species), during which time nonviable bone is removed by osteoclasts[17]. As a result, the allograft loses approximately one-third of its strength before recovering its strengths as bone mass reaccumulates and the construct is remodeled along the lines of stress[17]. This process of removal of bone tissue and replacement with new bone is known as creeping substitution[17].

Allografts are defined as tissue harvested from one individual and then implanted in a different individual from the same species[18]. In the US, an estimated one million bone grafting procedures are performed annually, about half of which use cadaveric bone allografts [19]. Incorporation of allograft into host bone differs according to the type of graft that is used: Cortical strut grafts are incorporated to the host bone through intramembranous bone formation at the cortical junctions[20, 21]. Meanwhile, cortical grafts with exposed medullary canal are incorporated into the host bone through initial reabsorbtion of the cortical graft which weakens the initial structural strength, and subsequent enchondral bone formation[22]. In contrast, cancellous allografts are incorporated by both enchondral bone formation without initial reabsorption, thus the strengths increase over time[22]. Like autograft, allografts are biocompatibile, possess good mechanical strength, and osteconductivity [23]. However, in addition to its inferior osteoinductivity as compared to autograft, implanted allografts elicit immune response which may impede their integration with the host bone[24]. Furthermore, the

risk of disease transmission through implantation of allograft is currently small but not inconsequential[4].

Bone banks use comprehensive donor screening and selection [25] to minimize the risk of viral and bacterial disease transmission, which is a major concern with allograft use [26, 27]. Hepatitis C, HIV, and species of *Staphylococcus* are the most common contaminants of bone allografts [28]. Despite thorough donor screening for HIV, the risk of HIV transmission through bone allograft from screened donor is not zero (1:1.6 million)[29], with transmissions occur through cadaveric donor with viral titers below the limit of detection of the assay[30]. Similarly, in 2002, CDC reported 5 patients became infected with Hepatitis C secondary to subclinical viral titers in the donor[31]. Singh *et al*. [32] found that 60 % of 126 femoral heads obtained from living donors were contaminated with mostly gram-positive bacteria. Infection rates with massive structural allograft for oncologic use was reported to be as high as 12.8 %[33].

Fresh allografts are cleaned using solvents and/or solutions of various compounds such as emulsifiers and antibiotics [34, 35] to remove blood, bone marrow, cellular debris while also inactivating bacteria and viruses [36]. Either the cleaning and decontamination procedures are done in aseptic environment or terminal sterilization by ethylene oxide or gamma irradiation are used [37]. In fresh-frozen allograft, tissue is harvested under sterile condition, cultured, soaked in antibiotic solution, packaged and frozen for up to 5 years[38]. Unsurprisingly, fresh-frozen allografts have been documented as the most common sources of viral infection in recipients of bone grafts[39, 40].

During cleaning and decontamination process, chemicals such as ethanol, acetone, and ether are often used as they have been shown to inactivate viruses such as HIV and hepatitis[41]. Hydrogen peroxide, by virtue of its ability to form free radicals, has been shown to be both

viricidal and bactericidal. However, exposure to hydrogen peroxide for more than 60 minutes has been shown to negatively impact the graft's osteoinductivity[41]. While chemical sterilization has been shown to minimally impact the mechanical properties of bone allografts [37], the penetration of various chemicals into bone might not be enough to ensure complete sterility throughout the bone thickness, especially for structural allografts. In addition, infectious agents can be reintroduced during the subsequent handling and packaging of the grafts [42]. The use of ethylene oxide for sterilizing bone allografts is rare because of the incidence of inflammation of the grafted host tissue [43-45].

Radiation sterilization is the most effective method of decreasing the bioburden throughout any size bone allograft without leaving chemical residues [37]. Efficacy of irradiation in eradicating microorganisms is mainly dependent on the microorganisms' capability to repair DNA single strand breaks; strains which are less capable of repairing DNA single strand breaks are a lot more radiosensitive. Low dose radiation (1 kGy) is sufficient to kill 90% of S. *aureus* and E. *coli* [46], however, HIV can only be inactivated by using a terminal radiation dose of at least 25 kGy[47, 48]. As a result, 25 kGy is generally accepted for complete assurance of sterility (SAL = 10^{-6}) [49] despite its known detrimental effects on the allografts' mechanical strength [48].

I.III Mechanism of Host Bone-Allograft Integration

Intimate contact between host bone and allograft cortices are crucial in promoting and accelerating unionization. When the gap between host bone and allograft is > 5 mm, healing process between host and allografts are significantly compromised[21]. Two main processes occur during healing after implantation of bone allografts: surface repair and internal repair[23].

Surface repair was achieved by the deposition of a thin layer of appositional host bone, starting from the first 3-6 months, on the unresorbed surface of the graft that serves as the anchors for the attachment of host soft tissue. The surface repair process started with bone resorption of the cortical bone on the surface of the allograft. By 2 weeks, widespread resorption of the allografts occur, and progressively increases during the initial 6 months. Osteoclastic resorption provides the space for vascular invasion of the Haversian and Volkmann's canal by host capillaries and osteoprogenitor cells[50]. Compared to autograft, vascular penetration in allograft is more superficial and impded: Allograft revascularization is not as complete at 8 mo after implantation as compared to autograft at 1 month[51]. Within 6 months, a thin layer of apposition lamellar bone forms from the host, and cover about half of the outer cortical surface by the end of first year [20]. The other half of the surface is resorbed and covered by new lamellar bone by remodeling during the second year. The aforementioned resorption process only removes 1-2 mm of superficial cortical bone. [20]. Within 6 months, bridging external callous was formed from the periosteum of host bone and extend to the surface of allografts[50]. Ultimately, the allograft is not completely replaced by new bone, and its remaining tissue is surrounded by new lamellae[20]. By two years, almost the entire surface of the allograft was covered by a seam of viable lamellar bone [52].

Internal repair begins with invasion by fibrovascular tissue into the surface stoma of Volkmann and Haversian canal[20]. This invasion caused enlargement of the haversian canals by osteoclastic resorption. New appositional bone was subsequently deposited into the resorbed area[20]. The pace of internal repair is very slow, only few milineters per year[20]. The abundance of resorptive activity during the first two years suggests that 2 years post-implantation period is the critical period for cortical weakening of massive allografts[20]. In addition, the poor

revascularization of the cortical allograft resulted in increased quantity of necrotic bone that remains even after full incorporation of the allograft and poor ability of the allograft to heal from fatigue-generated microfractures [17]. These observations correlates with clinical studies that shows allograft fracture occurred more frequently during second year after implantation[53, 54].

I.IV Effect of Irradiation on Bone Graft

Radiation resulted in the degradation of collagen alpha chains, causing increased brittleness [23, 49, 55-57]. Bone tissue damage by radiation is caused by a combination of two mechanisms: the direct scission of polypeptide chains [58] and the radiolysis of water molecules. Radiolysis of water produces hydroxyl radicals, which then attack the collagen proteins, leading to scissioning [59]. It has also been shown that ionizing radiation causes a reduction in intermolecular cross-link density in human femoral cortical bone [60]. These changes in collagen architecture may be a primary reason resulting in the significant reduction in mechanical properties [19]. The collagens of bone matrix is most important on the post-yield properties of bone tissues[61]. Therefore, effect of irradiation on bone is commonly observed in the post-yield (plastic) region of cortical bone, suggesting primarily a loss of ductility, ultimate tensile strength, and toughness [57, 62]. During loading of bone into the plastic region, collagen fibers provide bridging and reinforcement to the bone matrix, therefore increasing resistance to crack propagation[19]. Akkus et al showed that after irradiation, collagen of bone failed to provide bridging and instead individual collagen molecules collapse under loading instead of transferring the load to the lamella[61]. In general, strength of cortical bone allograft decreases as irradiation dose increases [23]. For example, bending strength of cortical bone allograft decreased by 10%, 20%, and > 50 % as irradiation dose increased from 17 kGy, to 29.5 kGy, and 94.7 kGy

respectively[63]. From the same study, the work to failure decreased by 30-40 %, 50-60 %, and 90% for irradiation dose of 17 kGy, 29.5 kGy, and 94.7 kGy respectively. Irradiation also decreases the fatigue resistance of allograft (87% decrease in fatigue life at 36.4 kGy) and resistance to crack propagation[23]. On the other hand, cancellous bone is much more resistant to irradiation than cortical bon: Anderson et al found no significant difference in failure stress between irradiated cancellous bone at 10, 31, and 51 kGy when compared to non-irradiated cancellous bone[64]. Separate study by Zhang et al did not find any significant change in elastic modulus, compressive strength, and strain to failure of 20-25 kGy irradiated cancellous bone compared to non-irradiated cancellous bone[65].

In the dose range of 25-35 kGy used commonly for sterilization, mechanical properties impaired by irradiation include bending and torsion strength [66], tension fatigue strength [61], fracture resistance [57], and compressive strength [67, 68]. Lietman et al found that patients receiving irradiated massive bone allografts had significantly higher fracture rate than patients that received non-irradiated allografts (38 % vs 18%)[69]. It is believed that most fractures through allografts occur through areas where revascularization and ingrowth of host tissue are absent. As a result, micro- and macro-cracks will accumulate in the allografts and subsequently progress to failure.

In addition to the reduced mechanical strength and increased clinical fracture rate of radiation sterilized bone allograft, higher delayed union rate was observed with irradiated bone allograft as compared to unirradiated bone allograft[70]. Interestingly, the rate of delayed healing is positively correlated with the irradiation dose: Irradiation with 1 kGy, 5 kGy, and 25 kGy showed 16 %, 24 %, and 100 % delayed healing at 12 weeks compared to autogeneic fresh autograft respectively. Another study by Godette et al using implanted allograft in rabbits for 6

weeks showed that the non-union rate of irradiated allograft-host bone was positively correlated to the irradiation dose of the allografts (0%, 12.5 %, and 33 % rate of non-union with irradiation dose of 0 kGy, 25 kGy, and 75 kGy respectively)[71]. A separate in-vivo canine study comparing irradiated and unirradiated bone allograft also showed delayed bone remodeling in the irradiated group as compared to the unirradiated group[72]. The detrimental effect of radiation to bone healing are likely due to two main factors: First, irradiation degrades biologically active osteogenic protein[70]. Second, irradiation produces toxic compounds that are cytotoxic to the host's osteoblast and osteoclasts[73].

Delay in the host bone-allograft integration with irradiated bone can potentially be explained by decreased osteoconduction and onsteonduction properties of irradiated bone grafts and production of cytotoxic byproducts from irradiation. Irradiation degrades collagen, fibrillary network of bone matrix, and growth factors such as bone morphogenetic proteins (BMP) and transforming growth factor-β (TGF- β)[74, 75]. Interestingly, studies showed irradiation caused more destruction on the collagen but minimally impact the activity of BMP and TGF- β: In vivo implantation of non-irradiated BMP combined with irradiated collagen pellets showed no bone formation as compared to non-irradiated BMP and non-irradiated collagen pellets group[74]. However, implantation of irradiated BMP combined with non-irradiated collagen showed similar bone formation to control group (non-irradiated BMP combined with non-irradiated collagen pellets)[74]. Moreau et al found that osteoblast-like cells cultured with irradiated bone slices showed higher degree of cell death when compared to non-irradiated bone slices[73]. Further chemical analysis of the irradiated bone by Moreau et al showed higher degree of peroxidized lipids (2-3x higher) than in non-irradiated bone samples, which will subsequently form free

radicals that can induce necrosis and osteoblast death, induce giant cell reactions to release cytokines and prostaglandins, leading to inflammatory mediated bone resorption[73].

Impregnation of bone allografts using a free radical scavenger as a radioprotectant could protect bone allografts against radiation damage [19]. We proposed the incorporation of the natural free radical scavenger vitamin E as a radioprotectant through diffusion and supercritical CO_2 homogenization to allow maximum protection from ionizing radiation. We also impregnated the bone allografts with a biocompatible collagen crosslinking agent (genipin) prior to irradiation to increase mechanical properties of bone. We hypothesized that the mechanical strength of radiation-sterilized bone could be improved without sacrificing the potential for osteoblast attachment. Furthermore, several in vivo studies showed the beneficial effect of vitamin E in enhancing fracture healing [76-80]. Therefore, we also hypothesized that vitamin E impregnated bone allograft will enhance fracture healing in vivo.

Chapter II

Materials and Methods

Vitamin E, Tween 80, chloroform, methanol, calcium phosphate, and genipin were all purchased from Sigma-Aldrich, Inc., St Louis, USA. Bovine tibias were obtained from Animal Technologies, Texas, USA. Terminal sterilization using a 3MeV electron beam was conducted using a Van de Graaf generator at the MIT High Voltage Research Laboratory, Cambridge, MA, USA.

Bone graft machining and cleaning: The diaphysis of bovine tibia was machined into 3.7 mm x 3.7 mm x 55 mm blocks for bending testing (**Figure 1**) and to 4 mm x 10 mm x 50 mm for Izod impact testing. All samples were then washed with isopropanol for 2 hours and then delipidized in 1:1 chloroform:methanol for 48 hours. Samples were then divided into subgroups for subsequent treatment with the radioprotectant (vitamin E) and the radioprotectant + crosslinking agent (genipin) (**Figure 1**).

Preparation of emulsified vitamin E: In an Erlenmeyer flask, 1.875 gram vitamin E and 7.5 gram Tween 80 were mixed, heated at 70^{o}C and stirred for 30 minutes. The resulting mixture was added to 110 gram of deionized water (DI) at 70^{o}C. 7.5 gram ethanol was then added to the mixture and heated under reflux for 2 hours until a homogenous emulsion was obtained.

Treatment of allograft with the vitamin E emulsion: One group of delipidized bovine tibia was fully immersed in the vitamin E emulsion supplemented with 300 mg/L calcium phosphate for 2 weeks at 25^{o}C under constant stirring. The emulsion was replaced every 2 days. At the completion of the impregnation, samples were removed from the emulsion, washed with saline, and packaged in vacuum bags and irradiated to 25kGy.

Treatment of allograft with antioxidant doping followed by supercritical fluid homogenization: Another group of delipidized bovine tibia was fully immersed in the vitamin E at 55^{o}C for 6 hr. Samples were then transferred to a supercritical fluid chamber. The samples that were previously doped with vitamin E were then treated in supercritical CO_2 at 40^{o}C and 85 bar for 24 hr. Samples were packaged in vacuum bags and irradiated to 25kGy.

Treatment of allograft with cross-linking agent and with radioprotectant: Another group of delipidized bovine tibia was fully immersed in a 1.0 wt % genipin solution supplemented with 300 mg/L calcium phosphate at 0^{o}C for 2 weeks in a dark room. The solutions were replaced every 2 days. Then, the samples were further treated with vitamin E in supercritical CO_2 as mentioned above and then stored in vacuum bags and terminally irradiated to 25kGy.

Mechanical Testing: Samples were soaked in phosphate buffered saline (PBS) solution at room temperature for at least 30 minutes prior to mechanical testing. The bending test samples were notched to 1 mm depth and tested (Insight 2, MTS, Eden Prarie, MN) at a displacement rate of 10 mm/min. Fracture toughness and work-to-failure were calculated according to ASTM C1421-10.

The IZOD impact test samples were also notched in the middle of the sample to 1 mm depth and tested according to ASTM F658-07 (CEAST 9050, Instron, Norwood, MA).

Reflectance Fourier Transform Infrared Spectroscopy (Reflectance-FTIR) of bone samples: The bone samples were polished sequentially with 600 grit carbide papers for 3 min, 800 grit carbide papers for 3 minutes, and 1200 grit carbide paper for 3 minutes. Degree of collagen cross-linking was determined by specular reflection geometry at a resolution of 4 cm^{-1} as an average of 150 scans at every 200 um. Reflected IR light was gathered from bone surface at a near-normal angle [81]. To analyze the reflectance spectrum, we used Kramers-Kronig relationship. Previously, it was shown that irradiation resulted in decrease in ratio of peak at 1660 cm^{-1} to peak at 1690 cm^{-1}, which correspond to Pyr cross-linked peptides and DHLNL crosslinked collagen peptides respectively [82]. Therefore, we used a similar method to quantify change in collagen cross-linking by taking the ratio of the peaks at (1660 cm^{-1})/(1690 cm^{-1}).

Fluorescence Imaging and quantification of bacterial growth from spore infused bone allografts : Allografts prepared as detailed above were immersed in the 1:10 diluted *Bacillus subtilis* (*B.* subtilis) spore solution (110649, EMD Millipore) for 48 hr at 4ºC prior to terminal irradiation. After removing excess liquid, all samples were separately packaged in vacuum bags. Samples were then irradiated using an electron beam to 25 kGy. To assess the sterility of the samples, all samples were aseptically transferred to sterile 12-well plates. Two ml of Trypsin-soy broth was added to each good plate and subsequently incubated for 24 hr at 37ºC. The growth of bacteria on the bone was imaged using Live/Dead bacterial viability kit (L7012, ThermoFisher Scientific).

Quantification of bacterial growth from spores on the bone allografts were conducted on separate set of samples. For each sample, two ml of Trypsin-soy broth was added and was subsequently incubated for 24 hr at 37ºC. Samples were then sonicated in the original broth to detach any bone-adherent bacteria. Bacteria in the supernatant was then quantified by measuring absorbance at 600 nm (OD 600).

Osteoclast culture on bone : RAW 264.7 mouse macrophages (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified atmosphere containing 5% CO2. RAW 264.7 cells were seeded on the bones which were placed on 12-well plates at a density of $2x10^4$ cells/well in α-minimal essential medium (Gibco) containing 10% FBS for 12-24 h to allow cells to attach to the bone surface. For osteoclastogenesis, recombinant mouse RANKL (R & D Systems) was added to the wells for 4 days at a concentration of 30 ng/ml. Thereafter, cell viability assays and fluorescence imaging with fluorescence tartrate-resistant acid phosphatase (TRAP) stain, fluorescence actin stain, and fluorescence nuclear stain were performed.

Osteoblast culture on bone : 7F2 mouse osteoblasts (ATCC, Manassas, VA) were cultured in alpha minimum essential medium with 2 mM L-glutamine and 1 mM sodium pyruvate containing 10% FBS at 37°C in a humidified atmosphere containing 5% CO2. 7F2 cells were seeded on a bone allograft which was placed on a well in a 12-well plate at a density of $2x10^5$ cells/well for 3 days. Thereafter, cell viability assays, alkaline phosphatase (ALP) assay, and

fluorescence imaging with fluorescence ALP stain, fluorescence actin stain, and fluorescence nuclear stain were performed.

Fluorescent TRAP stain : To visualize the osteoclasts and TRAP activities on the bone allografts, we performed fluorescent TRAP staining on bones using ELF97 TRAP staining protocol[83] by combining TRAP assay kit (387A, Sigma-Aldrich, St. Louis, MO) and ELF97 phosphatase subtrate (Molecular Probes; Eugene, OR). A solution containing 110 mM acetate buffer, 1.1 mM sodium nitrite, 7.4 mM tartrate and 200-µM concentration of ELF97 were used for the fluorescence-based TRAP staining. Bones containing cells on the surface were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 for 60 s and incubated with the fluorescence-based TRAP staining solution for 1 h at 37°C. Thereafter, cells were counterstained with AlexaFluor546-labeled phalloidin (0.2 U/ml in PBS; Molecular Probes) and DAPI (4',6-diamidine-2'-phenylindole, 1µg/ml in PBS, Sigma-Aldrich).

Fluorescent Actin stain : Actin was stained with phallotoxins dye (Alexa Fluor®594 Phalloidin, Thermo Fisher Scientific) according to the manufacturer's recommended protocol. Briefly, cells adherent on the bone samples were washed twice with 37ºC phosphate-buffered saline, pH 7.4. The samples were then fixed with 3.7 % formaldehyde solution in PBS for 10 minutes. After washing with PBS two more times, samples were immersed in 0.1 % Triton X-100 in PBS for 5 minutes. Samples were then washed two times with PBS, and then diluted fluorescent phallotoxins solution (25 µL stock solution in 1000 µL PBS containing 1 % bovine serum albumin) was added to the bone samples. After 20 minutes of incubation in the dark at room

temperature, staining solution was removed from the sample. Samples were then washed with PBS for two more times.

Fluorescent ALP staining on osteoblasts: Osteoblasts on bones were fixed in 4% paraformaldehyde permeabilized in 0.1% Triton X-100 for 60 s. The ALP substrate working solution was prepared as indicated by the manufacturer's instruction (ImmPACT™ Vector® Red, vector laboratories, Burlingame, CA). Osteoblasts on bones were incubated with the substrate working solution for 20-30 minutes at 37°C, then counterstained with AlexaFluor488-labeled phalloidin (Molecular Probes) and DAPI.

Fluorescent Nuclei Stain : Cell nuclei were stained with 300 nM solution of 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO) solution in PBS. Staining was conducted for 5 minutes in the dark. Samples were then washed with PBS for two times.

ALP activity assay : ALP activities were measured by a colorimetric ALP kit (ab83369, abcam, Cambridge, MA) according to the manufacturer's instruction. Osteoblasts on the bones were harvested, washed in PBS and resuspended in 100 µL assay buffer. Samples were centrifuged at 4 °C for 15 min to remove the insoluble material. Samples were incubated with a phosphatase substrate p-nitrophenyl phosphate (*p*NPP) which turns yellow (λmax=405 nm) when dephosphorylated by ALP. Samples and *p*NPP were mixed well and incubated at 25 °C for 60 mins protected from light. After adding the stop solution to terminate the reaction, the optical

density (OD) was measured at 405 nm on a microplate reader. The ALP activity (U/L) was calculated based on a standard curve.

Cell viability assay: Viability of osteoclasts and osteoblasts on the allograft prepared as mentioned above were measured using a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Gaithersburg, MD) according to the manufacturer's instructions. The well supernatant was removed briefly, and 10 µl of the CCK-8 solution and 90 µl PBS was added to each well. The plates were incubated at 37°C for 1 h, after which the optical density (OD) was measured at 450 nm by using a microplate reader. The results were presented as the percentage of viable cells over the control.

Confocal fluorescence microscopy: For imaging of osteoclasts and osteoblasts on the surface of allograft, confocal fluorescence microscope was used (LSM 880, Zeiss, Germany). Osteoclasts were triple stained using fluorescent TRAP stain, fluorescent actin stain, and fluorescent nuclei stain (DAPI). Osteoblasts were triple stained using fluorescent ALP stain, fluorescent nuclei stain (DAPI).

Murine calvaria host bone-allograft unionization model: Study approval was granted from Pine Acres Rabbitry Farm Institutional Animal Care and Use Committee. The in vivo murine calvaria bone regeneration model consisted of two main steps: allograft extraction from rat calvaria and subsequent implantation of processed allografts into other rats. For allograft extraction, fifteen

Sprague-Dawley rats aged 8 weeks were euthanized using CO_2, and the rat calvaria was carefully isolated. Using 8 mm trephine saw, circular allograft was obtained from the mid-parietal bone along the sagittal suture. Allografts were then washed with isopropanol for 2 hours and then delipidated in 1:1 chloroform: methanol for 48 hours. Samples were then dried at 25^{o}C in the dark for 24 hr.The fifteen allografts were then randomized into three groups: a control group (Irr), VE/SC, and Gen/VE/SC groups. Allografts in the VE/SC and Gen/VE/SC groups were prepared according to the procedure mentioned above.

Implantation of the allograft into rats using critical size calvarial defect was adapted from Spicer PP et al, 2012[84]. Fifteen Sprague-Dawley rats aged 8 weeks were randomly assigned to either control (Irr), sterilized VE/SC allograft, or sterilized Gen/VE/SC allograft. Anesthesia was achieved using inhaled isoflurane (2 %) supplemented with oxygen (1.2 liters/min). Pre-emptive analgesia was administered before the procedure started (buprenorphine 0.05 mg/kg). No pre or postoperative antibiotics were administered. Around 1.5 cm incision down to the periosteum was made along the middle sagittal crest over the scalp. After pushing the periosteum laterally, 8 mm diameter calvarial defect was created using trephine drill. The circular bone inside the defect was removed and was replaced with either Irr, VE/SC, or Gen/VE/SC. The periosteum was then closed over the implant using 4-0 Monocryl sutures. The skin was then closed over the periosteum using 3-0 plain gut suture.

In vivo computed tomography was performed immediately after surgery and every four weeks until five months. The rats were anesthetized using inhaled isoflurane (2 %) supplemented with oxygen (1.2 liters/min) throughout the computed tomography scan (10 minutes). The computed tomography was performed using high-resolution CT (Inveon, Siemens), acquired with 80 kVp and 500 μA X-ray power, 256 projections, and 180 μm isotropic resolution.

Statistical analysis: Statistical analysis was performed using a Student's t-test for one-way analysis of variance to compare more than two groups. Significance was assigned to $p < 0.05$.

Chapter III

Results

III.I Antioxidant-treated and combined antioxidant-crosslinker treated allograft are mechanically stronger than irradiated-only allograft

Irradiation of native bone grafts decreased their fracture toughness and work-to-failure compared to native bone (34±2.7 % and 74±8.1 %, respectively; **Figures 2a and 2b**). The fracture toughness of samples doped using emulsion of the radioprotectant vitamin E and irradiated to 25 kGy (VE-e) showed a measurable but not statistically significant increase compared to the 25 kGy irradiated native bone (Irr) (**Figure 2a**). The fracture toughness of the sample doped in the pure antioxidant followed by supercritical homogenization and subsequently irradiated to 25 kGy (VE/SC) was comparable to that of unirradiated native control and higher than that of the irradiated control (**Figure 2a**). The work to failure of both VE-e and VE/SC samples were higher than Irr (**Figure 2b**) and were comparable to that of unirradiated native bone (**Figure 2b**).

Samples doped first with a cross-linking agent, then with the pure radioprotectant followed by supercritical homogenization and 25 kGy irradiation (Gen/VE/SC) had statistically significant higher fracture toughness (**Figure 2a**) and work to failure (**Figure 2b**) compared to that of control irradiated without treatment. Both the fracture toughness and the work to failure of all of these samples were comparable to that of the unirradiated native bone with complete recovery of fracture toughness and 84±9.8 % recovery for the work to failure over control irradiated without treatment.

III.II Antioxidant-treated and combined antioxidant-crosslinker treated allograft have higher crosslinking index than irradiated-only allograft

Irr samples showed low collagen crosslinking index ($1660cm^{-1}/1690cm^{-1}$) throughout the sample as compared to native bone (**Figure 3a**). VE-e samples had higher crosslinking index along the outer edge of the samples than the center of the samples (**Figure 3a).** Both VE/SC and Gen/VE/SC samples showed higher cross-linking index throughout the samples compared to Irr samples (**Figures 3b**). The mean crosslinking index of all treated samples was higher (14±1.4 %, 74±2.8 % and 78±6.7 %, respectively) than that of control irradiated without treatment (**Figure 3b**) and except for the VE-e, was comparable to that of unirradiated native bone.

III.III Antioxidant-treated and combined antioxidant-crosslinker treated allograft did not impede radiation-mediated killing of bacterial spore

Imaging of *Bacillus subtilis* spore-treated native bone after incubation in Mueller-Hinton media for 24 hr at 37ºC showed presence of live *Bacillus subtilis* (green elongated bacilli) (**Figure 4a-b**). On the other hand, no live *Bacillus subtilis* was observed on Irr, VE/SC, and Gen/VE/SC allografts after incubation for 24 hr; only the original spores (spheroids) were observed on those two samples (**Figure 4b**).

Sonication and reculturing of the *Bacillus subtilis* spore-treated native bone after incubation in Mueller-Hinton media for 24 hr at 37ºC showed significantly higher OD600 than Irr, VE/SC, and Gen/VE/SC (**Figure 4a**). There is no statistically significant difference in OD600 between Irr, VE/SC, and Gen/VE/SC (**Figure 4a**).

III.IV Antioxidant-treated and combined antioxidant-crosslinker treated allograft are less cytotoxic to osteoclast and osteoblast than irradiated only allograft

Osteoclast and osteoblast density on the surface of the unirradiated native bone were higher than on surface of Irr, VE/SC, and Gen/VE/SC samples (**Figure 5, Figure 6**). Higher density of osteoclast was observed on the VE/SC samples than Irr samples (**Figure 5b**). Multinucleated and elongated osteoclasts with ruffled border were observed on both VE/SC and Gen/VE/SC but not on the Irr sampes (**Figure 5a**). All of the attached osteoclasts produced TRAP (green) for all four samples (**Figure 5a**). Osteoblasts attached to the surface of all four samples were elongated and spread as shown by the actin stain (green) (**Figure 6a**). Deposits of products from alkaline phosphatase digestion of the VECTOR red alkaline phosphatase substrate (red-orange) were observed in the native unirradiated bone, VE/SC, and Gen/VE/SC, but not in the Irr samples (**Figure 6a**).

Relative amount of live osteoclast and osteoblast (viability) on the Irr sample was significantly lower than the native bone (**Figure 5b, 6b**). Viability of osteoclasts and osteoblasts on the VE/SC sample were similar to the native bone, and significantly higher than osteoclasts on the Irr sample (**Figure 5b, 6b**). Osteoclasts on the Gen/VE/SC sample was significantly higher than Irr samples but was lower than both the native bone and VE/SC sample (**Figure 5b**). Osteoblasts on the Gen/VE/SC sample showed higher trend than the Irr samples, but was not statistically significant (**Figure 6b**). ALP activity of osteoblasts on VE/SC samples was significantly higher than Irr and similar to the native samples (**Figure 6c**). ALP activity of

osteoblasts on the Gen/VE/SC sample showed higher trend than the Irr samples, but not statistically significant (**Figure 6c**).

III.V Antioxidant-treated and combined antioxidant-crosslinker treated allograft allowed faster host bone-allograft unionization than irradiated-only allograft

Faster unionization between allograft and the host bone was observed at post-operative day 120 (4 months) in rats receiving VE/SC or Gen/VE/SC allografts (**Figure 7**). Quantification of the volume between allograft and host bone (defect volume, **Figure 8**) at post-operative month 0, 1, 2, 3, and 4 showed significantly lower defect volume in rats receiving VE/SC or Gen/VE/SC as compared to rats receiving irradiated only allograft (Irr) (**Figure 8a**). The largest change in defect volume was observed at the first post-operative month: significant decrease in defect volume was observed in the VE/SC and Gen/VE/SC compared to their corresponding immediate post-operative defect volume, whereas the defect volume in Irr branches at the first post-operative month was relatively unchanged compared to its corresponding immediate post-operative defect volume (**Figure 8a**). Average relative density (ratio of density difference between voxel of interest and minimum density of surrounding soft tissue to the difference between maximum density of cortical bone and minimum density of surrounding soft tissue) of both VE/SC and Gen/VE/SC were both higher Irr samples (**Figure 8b**).

Goldner's trichrome and toluidine blue stain of the calvaria at post-operative month-4 showed formation of collagenous, non-mineralized tissue in between the host calvaria and Irr samples (**Figure 9**). New mineralized bone formation were observed between the host calvaria and the VE/SC and Gen/VE/SC samples (**Figure 9**). Complete unionization between the host

bone and the VE/SC samples was observed for the inferior half-thickness of the specimen (**Figure 9**). Mineralized bony spicule was observed between the host bone and the Gen/VE/SC samples (**Figure 9**). Histological grading of the area between the host bone and the allograft of all rats in the study (1: empty defect (no connective tissue) to 6: complete ossification) showed that VE/SC groups have significantly higher grade, followed by Gen/VE/SC, then Irr (**Figure 10**).

Chapter IV

Discussion

IV.I Vitamin E-infused allograft preserves irradiated bone allografts' mechanical strength

Minimal compromise in mechanical strength, osteoinduction, and osteoconduction of cortical allografts is crucial for repair of fractures, bone replacement during tumor removal, and reconstruction of skeletal defects. Sterilization through ionizing radiation is desirable to ensure deactivation of all unwanted bacteria and virus such as HIV that might accidentally be transmitted from the donor to the allograft recipient [48, 85]. However, the incidence of in vivo fracture of human cortical grafts is higher when allografts sterilized with ionizing radiation are used [69], suggesting that the mechanical properties of irradiated bone allograft need to be improved for efficient use of this method for sterilization. In addition, irradiation degraded osteogenic proteins within the allograft and produced toxic compounds that further impede unionization between host bone and the allograft [70] [73]. Therefore, formation of toxic byproduct need to be minimized to maximize unionization between host bone and the allograft.

The biocompatible free radical scavenger vitamin E can be used as a radioprotectant in bone allograft to minimize the adverse effects of gamma sterilization. Vitamin E is a hindered phenol chain breaking antioxidant, which can act very efficiently to scavenge free radicals caused by reactive oxygen species and protect cell membrane lipids against oxidation [86, 87]. Vitamin E has been shown to be the most important lipid-soluble anti-oxidant in vivo, and it protects cell membrane from oxidation by reacting with lipid free radicals that was produced by polymorphonuclear cells. Vitamin E has also been shown to withstand high dose of irradiation

and protect UHMWPE against oxidation when blended into this polymer during the manufacture of total joint implant bearing surfaces [88].

The diffusion of additives into cortical bone is severely limited due to the low diffusion coefficients of compounds through this dense structure. Previous attempts at incorporating addtives into allograft bone resulted in diffusion times on the order of several weeks, even for samples with an effective diffusion length (shortest dimension) of 1 mm [19], which is likely to be too thin for tumor reconstruction surgery or most fracture repair in humans. To decrease the time required to incorporate vitamin E into 3 mm-thick clinically relevant bone allografts [89], we proposed a two-step process comprising doping in pure vitamin E followed by homogenization in supercritical carbon dioxide to enhance diffusion.

Treatment of bone allografts with vitamin E doping followed by supercritical fluid homogenization before radiation sterilization (VE/SC) completely preserved the fracture toughness and work to failure of unirradiated, native bone (**Figures 2a and 2b**). This method was superior in preserving the fracture toughness over bone treated with vitamin E emulsions, presumably because the maximum concentration of vitamin E used in the emulsion technique (15 mg/mL) resulted in lower concentration of the radioprotectant in the bone. By contacting the bone with pure radioprotectant, a high surface concentration could be achieved, which could then be homogenized through the bone by the diffusion of the supercritical carbon dioxide. An additional benefit was the decreased diffusion time, which resulted in a total processing time of 30 hours compared to 2 weeks by the emulsion method with similarly sized samples. In fact, the higher collagen cross-linking around the edges of the samples treated by the vitamin E emulsion (**Figure 3a**) suggested that 2 weeks were not sufficient to drive the vitamin E throughout the components when using an emulsion. Although we have not specifically tested for the effects in

this study, supercritical carbon dioxide has also been used previously to obtain sterility [90], which may help decrease the risk of any microbial contamination during processing.

Bone allografts doped with vitamin E and homogenized by supercritical CO_2 (VE/SC) were superior in preserving the fracture toughness of the native bone when compared to samples treated using a vitamin E emulsion (VE-e) **(Figure 2a)**. This was most likely due to more homogeneous distribution of antioxidants through supercritical treatment as shown with higher overall crosslinking index throughout the samples (**Figure 3a-b)**. More importantly, the homogeneous distribution of the radioprotectant obtained using this technique resulted in the fracture toughness of native bone being completely preserved when sterilized at 25 kGy **(Figure 2a)**.

IV.II Combined crosslinker and Vitamin E-treated allograft did not give additional strength benefit as compared to Vitamin E only-infused bone allografts

Based on the knowledge that the disruption of the collagen network via chain scissioning during irradiation is the major mechanism behind the loss of the mechanical properties of bone allografts, we hypothesized additional cross-linking of collagen may be advantageous. We hypothesized that the addition of a cross-linking agent would increase collagen cross-linking after irradiation and further improve the mechanical strength of the treated bone allografts after radiation sterilization. Several chemicals, including formaldehyde, glutaraldehyde, and genipin have been previously used for collagen crosslinking[91]. Exposure of bone tissue scaffolds to glutaraldehyde has been shown to result in a significant increase in tensile strength [92]. Formaldehyde and gluteraldehyde reacts with the amine or hydroxyl functional group of collagen

through a Schiff-base reaction and connects the biopolymeric chains via intra and intermolecular reactions to form crosslinked network [93]. Unfortunately, the aldehyde moiety of formaldehyde and glutaraldehyde group are toxic for cells and cause severe inflammation in the body[94]. Previous study showed that live cells were not able to survive on the surfaces of glutaraldehyde-crosslinked tissues ([94, 95]. In vivo, this cytotoxicity has been associated with delayed healing, repair, incorporation, and remodeling[96]. Genipin, on the other hand is a natural crosslinker found as a hydrolytic product of geniposide extracted from the fruit of *Garnedia jasminoides*. Genipin acts by forming cross-bridges between free lysine and hydroxylysine residues through nitrogen-iridoid and aromatic monomer intermediates[96]. Uquillas et al found positive correlation between the genipin concentration and the Young's modulus and ultimate tensile strength (UTS) of the collagen threads[97]. Furthermore, genipin has very low cytotoxicity, ~10,000 time less cytotoxic than gluteraldehyde [93]. Cell proliferation after exposure to genipin is approximately 5,000 times greater than that observed following treatment with genipin [93].

Interestingly, we did not find significant improvement in mechanical properties for genipin treated vitamin E containing bone allograft (VE/SC/Gen) as compared to non-genipin treated, vitamin E containing bone allograft (VE/SC) (**Figures 2a-b, 3a-b**). There are several possible reasons to explain our findings: First, vitamin E was very effective in protecting the collagen structure and additional cross-linking, if any, did not result in further improvement of properties. Second, the additional crosslinking formed by genipin was not substantial enough to cause detectable improvement in the mechanical properties of the allograft. Ng et al found that the efficacy of genipin as crosslinker agent greatly depends on the density of collagen in tissue[98]: In their study, genipin-treated bovine patellar tendon showed significant improvement

in mechanical strength compared to untreated bovine patellar tendon [98]. However, genipin-treated human patellar tendon, which has less collagen density than bovine patellar tendon (27% collagen w/w in human vs 35 % w/w in bovine tendon), did not show statistically significant improvement in mechanical strength when compared to native human patellar tendon [98]. Bone tissues contains lower density of collagen (~25 % w/w[99]) than human patellar tendon, thus extrapolating from Ng et al's result, bone tissues' collagen density is too low for genipin to efficiently perform crosslinking.

IV.III Vitamin E and Genipin did not inhibit bacterial spore eradication by irradiation

In addition to preserving the mechanical strength of bone allograft upon irradiation, an ideal radioprotectant for bone allograft must not protect the pathogens from being inactivated by irradiation. Since vitamin E protects the bone allograft by scavenging free-radical produced by irradiation, it could also potentially prevent eradication of unwanted microorganism in the allograft by the same mechanism. Therefore, it was important to assess that doping of radioprotectant and the subsequent supercritical fluid treatment did not hinder radiation's ability to eradicate unwanted microorganism contaminant. Most common microbial contamination are low-virulence bacteria (91%), which includes coagulase-negative Staphylococcus (71.5%), Micrococcus (9.3 %), Corynebacterium (4.7%), and *Bacillus* cereus (4.7%)[100]. 7% of microbial contaminants are high-virulence bacteria such as Streptococcus (2.5 %), Acinetobacter (2.5%), and *Escherichia coli* (1.9%)[100]. Within the same bacterial species, bacterial spores are in general more resistant to radiation damage than its planktonic form[42]. Hence, for this study, we tested our material against the spore form of *B. subtilis*.

Both fluorescence live-dead imaging and quantitative analysis of the *B. subtilis* spore impregnated allograft showed that no live bacteria grew from the irradiated-only control, VE/SC samples, and Gen/VE/SC, thus indicating that radiation completely deactivated all the spores on those three types of allografts (**Figure 4**). More importantly, this result indicates that both of the radioprotectant and crosslinker did not impede eradication of the microorganism, at least to the one that we tested.

IV.IV Vitamin E and Genipin-treated irradiated allograft has superior biocompatibility as compared irradiated-only allograft

In addition to the mechanical strength of the allograft and its sterility, clinical outcome also depends on the ability of the allograft to unionize with the host bone. Irradiated allograft was shown to have inferior union rate than both unirradiated allograft and autograft, likely because of deactivation of osteogenic proteins in the allograft and formation of cytotoxic byproducts [70] [73]. Irradiation of residual fat within the medullary space of bone induced lipid peroxidation [73], which may cause further tissue damage by free radicals. Peroxidated lipids was found to induce osteoblast death on cultured cell around bone slices through necrosis pathway [73]. Therefore, it was important to assess the impact of radioprotectant and crosslinker on the viability of osteoblast, osteoclast, and the overall host bone-allograft union rate in animal model.

Both of the qualitative imaging analysis of live-dead assay stained surfaces and quantitative viability assay of the number of osteoblasts and osteoclasts attached to the surfaces revealed that irradiation without treatment significantly decreased the ability of osteoblasts to

attach to the bone surfaces (**Figures 5a, 6a**); therefore, it is likely that radiation sterilization alone could compromise not only the mechanical strength but also the integration of the graft with host bone. These results are in line with previous publications that showed irradiated allograft produced byproducts that were cytotoxic to osteoblasts and osteoclasts [73]: Kluger et al found that osteoclasts incubated with irradiated cortical bone slices had 57% reduced activity as compared to fresh frozen bone[101]. They also observed that the irradiation process damaged bone matrix proteins such as integrins, which lead to impairment of osteoclast attachment and resorption[101]. Hofman et al showed that osteoblasts incubated with irradiated cortical bone slices had reduced cell viability, lower alkaline phosphatase production, and lower osteocalcin production when compared to fresh frozen bone[102].

In contrast, superior osteoclasts and osteoblasts attachment were observed onto the VE/SC allografts and to a lesser extent, the Gen/VE/SC allografts (**Figure 5a, 6a)**. In fact, osteoclast and osteoblasts viability attached to the VE/SC was similar to the native untreated bone (**Figure 5b, 6b**). Furthermore, the osteoblast activity (as measured by the ALP activity) of VE/SC was superior to the Irr and similar to the native untreated bone (**Figure 6c**). Interestingly, Ulrich et al showed that culturing osteoblast with vitamin E did not show any increase in cell proliferation and percent viability[103]. Moreover, they did not observe statistically significant difference in production of collagen I, osteocalcin, and osteonectin whether vitamin E was present in the culture media or not[103]. Therefore, it is likely that the observed increased osteoblast attachment in Gen/VE/SC and VE/SC as compared to Irr samples are solely due to reduction in production of toxic byproducts that may impedes bone healing when compared to irradiated only allografts.

IV.V Vitamin E treated irradiated allograft has faster host bone-allograft integration than irradiated-only allograft

In our animal study using murine calvaria defect model, faster host bone-allograft unionization and mineralization was observed with rats receiving VE/SC allografts and Gen/VE/SC allografts (**Figure 7, 8**). The largest difference in defect healing was observed in the first month (**Figure 8**), where most toxic byproducts from irradiation would likely be eluted out from the allograft. As assessed radiologically and histologically (**Figure 7, 8, 9)**, the beneficial effect of vitamin E-impregnated allografts were manifested by earlier bridging fracture line and higher degree of early mineralization. In addition to decreasing the formation of toxic byproducts due to irradiation, previous in vivo studies have shown that vitamin E increased bone formation and fracture healing [76-80]. Early phase of bone healing (within the first 2 weeks) involves infiltration and activation of polymorphonuclear cells and production of superoxide free radicals[78]. The first 3 days of bone healing are considered to be ischemia period because of high production of free oxygen radicals by activated polymorphonuclear cells, while the remaining 2-3 weeks are considered as reperfusion period[104]. Turgut et al showed that the oxygen free radicals during the ischemia period can impede bone formation[105]. Subsequently, Kurklu et al found that that in lapine model, intravenous vitamin E supplementation increases bone formation by decreasing oxygen free radicals that are produced at the early stage of healing by polymorphonuclear neutrophils and impairment of blood supply to the bone ends. As a result, osteoblastic activity was increased and osteoclastic resorption of newly formed bone was decreased[76].

IV.VI Study limitation and future works

Several important limitations of the study should be considered when interpreting the result of this study. One limitation of our study is that we did not investigate the relationship between the amount of radioprotectant and radiation dose. Since the radioprotectant is so effective, it may be possible to increase the terminal sterilization dose to above 35 kGy, where the efficiency of eliminating viruses of interest such as the Hepatitis C and HIV is highest [48]. Future studies may be designed to test feasibility of performing terminal sterilization at dose >25 kGy to ensure more complete eradiation of Hepatitis C and HIV while maintaining sufficient mechanical strength. Another limitation is the measurement of any effects on the osteoinductivity of the resulting bone allografts. It is possible that vitamin E can also protect the destruction of proteins other than collagen in the allograft network and may contribute to an enhanced interaction with the host bone. Future studies may be designed to measure the activity of BMP and TGF-β within the bone allograft after terminal irradiation. Another limitation of our study is that we only assessed eradication of spores of an aerobic bacterial strain (*B. subtilis*) and have not tested our material against other types of spores, viruses, and prions. Because viruses such as HIV and HCV are harder to be inactivated compared to bacteria [47, 48], future studies should test radioprotectant-infused irradiated allografts against such viruses. Finally, while our study showed faster integration between host bone and allograft in rat model, the result might not be translatable to human. Therefore, future clinical trials are needed to see whether vitamin E impregnated allograft result in faster bone graft-host bone healing when compared to irradiated only bone allograft.

IV.VII Conclusion

In conclusion, incorporating the radioprotectant vitamin E into allograft bone by the novel method of doping and subsequent homogenization in supercritical carbon dioxide before radiation sterilization recovered any deleterious effect of irradiation on its mechanical properties, cytocompatibility, and host bone-allograft unionization rate. It appears that the mechanism by which vitamin E can protect bone is through the sparing of the collagen cross-links from free-radical mediated scission and through suppression of oxygen free radical formation during early stage of healing. The amount of time for diffusion of vitamin E into bone by traditional methods was largely decreased; presumably improving the feasibility of using such a method in the processing of structural grafts. The processing of other types of grafts such as cortical or cancellous chips is easily possible with this method, where processing times would be further reduced due to the small effective diffusion length required for these smaller samples.

Summary

Bone allograft is routinely used in orthopaedic surgery, ranging from bone chips for augmentation of spinal fusion to massive allografts for reconstruction in limb-salvage surgery. In the US, and estimated one million bone grafting procedures are performed annually, half of which uses bone allograft. To minimize disease transmission such as HIV, Hepatitis C, prion, and bacteria from donor to recipient, radiation sterilization up to 25 kGy is often utilized. Unfortunately, radiation of bone allograft resulted in reduction in its mechanical strength and produced cytotoxic byproducts that are toxic to osteoblast and osteoclasts. As a result, prior clinical studies showed that irradiated allograft has higher incidence of graft fracture and non-union. Bone tissue damage by radiation is caused by a combination of direct scission of polypeptide chains, radiolysis of water molecules which produces hydroxyl radicals, which then attack the collagen proteins, leading to scissioning, and reduction in intermolecular cross-link density. To address this problem, in this study we used radioprotectant such as Vitamin E and crosslinking agent such as genipin to minimize destruction of collagen and increases collagen crosslinking respectively.

We found that treatment of bone allografts with vitamin E doping followed by supercritical fluid homogenization before radiation sterilization (VE/SC) prevented collagen degradation and completely preserved the fracture toughness and work to failure of unirradiated, native bone. Combination of genipin crosslinking and vitamin E treated allograft (Gen/VE/SC) did not show significant improvement in mechanical properties as compared to VE/SC allograft. This is likely due to low density of collagen (25 % w/w) in the bone preventing effective collagen crosslinking by genipin. Both VE/SC and Gen/VE/SC samples did not impede spore

eradication by irradiation. We also found that osteoblasts and osteoclasts have better attachment, viability, and activity on VE/SC and Gen/VE/SC than irradiated only allografts.

Finally, using in vivo murine calvarial model, we showed that both VE/SC and Gen/VE/SC allografts integrated with the host bone faster than irradiated only allografts. Radiological and histological assessment showed that the beneficial effect of vitamin E-impregnated allografts were manifested by earlier bridging fracture line and higher degree of early mineralization. The largest difference in defect healing was observed in the first month, where most toxic byproducts from irradiation would likely be eluted out from the allograft. Vitamin E-treated allograft allows faster healing between host bone and allograft by decreasing production of cytotoxic products in the allograft by irradiation and by decreasing oxygen free radicals that are produced at the early stage of healing by polymorphonuclear neutrophils.

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Figures and Tables

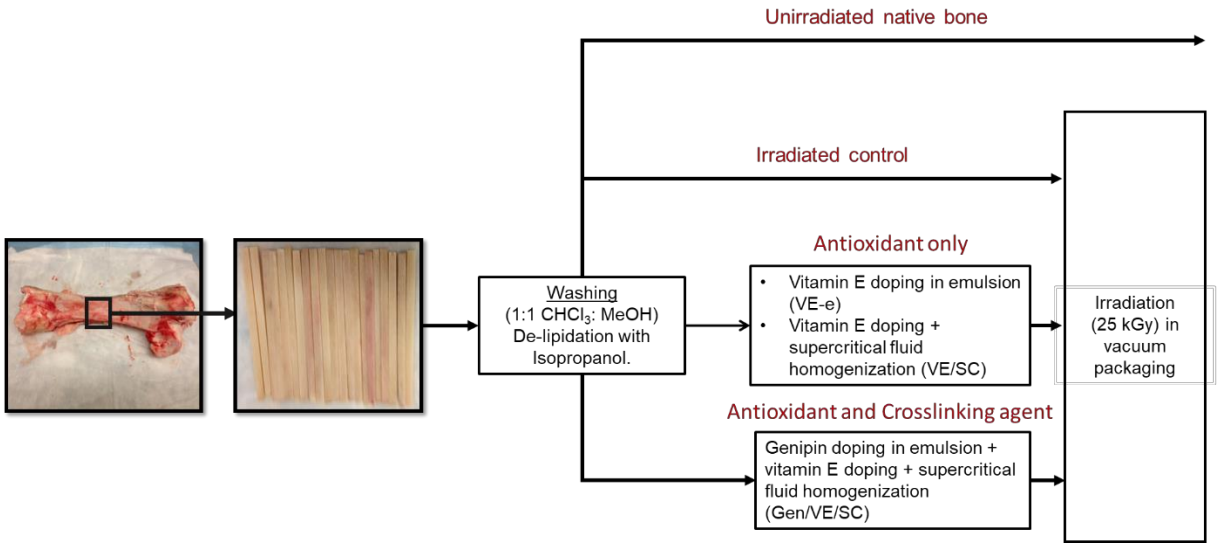
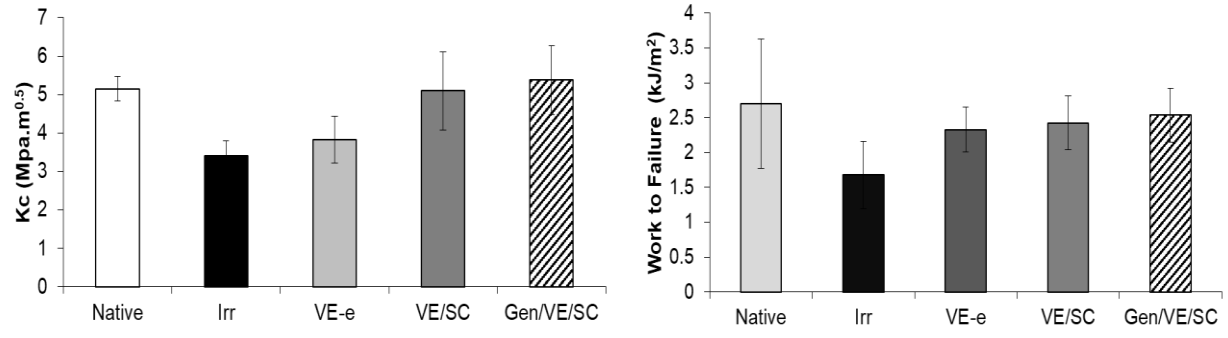
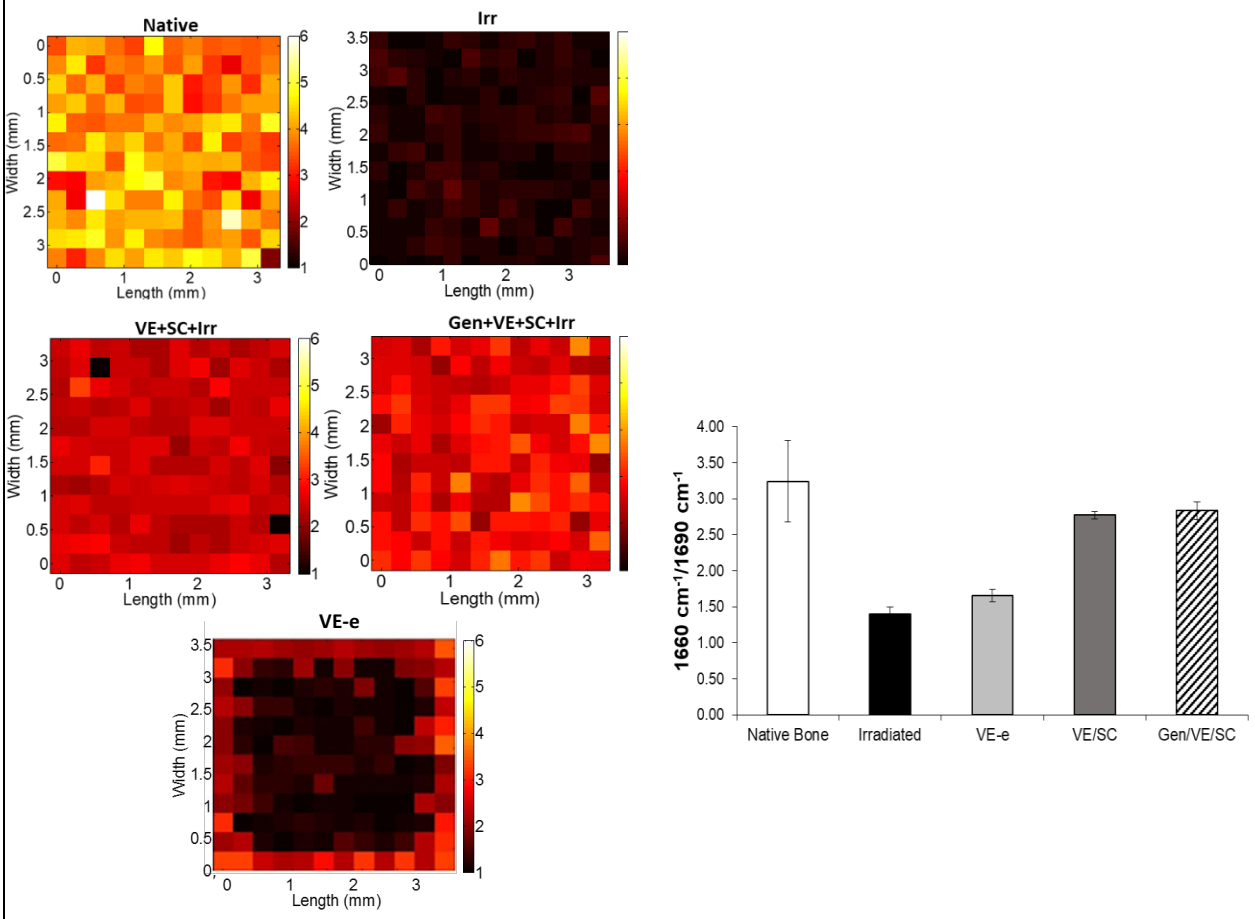


Figure 1. Schematic summarizing the processing of the bone allografts used in this study



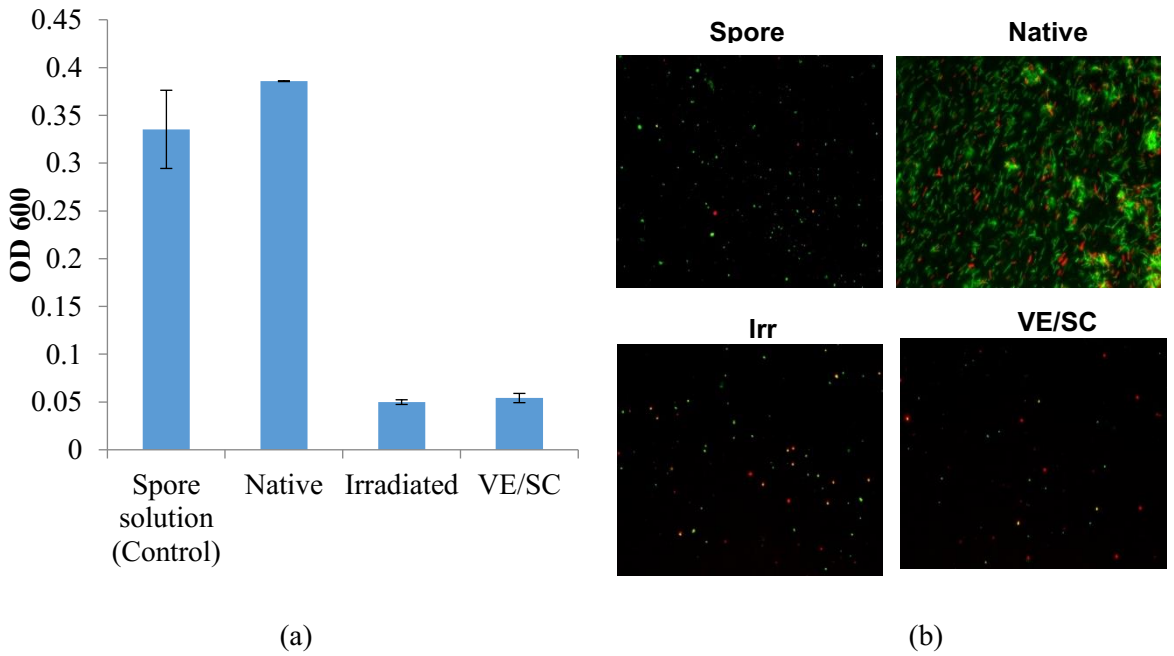
(a) (b)

Figure 2. Fracture toughness (a) and work to failure (b) of bone samples doped with radioprotectant (Vitamin E) and radioprotectant and cross-linking agent (genipin). **VE-e**: vitamin E emulsion; **VE/SC**: vitamin E doping and supercritical carbon dioxide homogenization; **Gen/VE/SC**: genipin diffusion followed by vitamin E doping and superciritical carbon dioxide homogenization



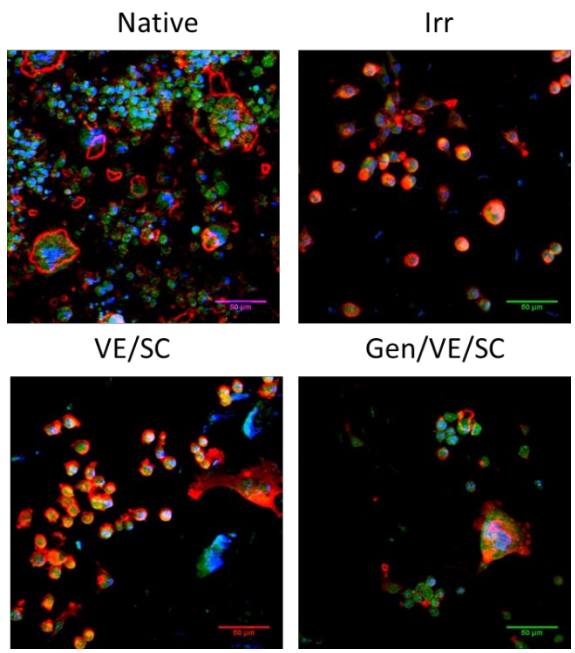
(a) (b)

Figure 3. **(a)** Representative maps of collagen cross-linking. Color map indicates cross-linking index as measured by the ratio of the absorbance of peaks at 1660 cm^{-1} and 1690 cm^{-1}. **(b)** Mean collagen crosslinking index. **VE-e**: vitamin E emulsion; **VE/SC**: vitamin E doping and supercritical carbon dioxide homogenization; **Gen/VE/SC**: genipin diffusion followed by vitamin E doping and superciritical carbon dioxide homogenization.

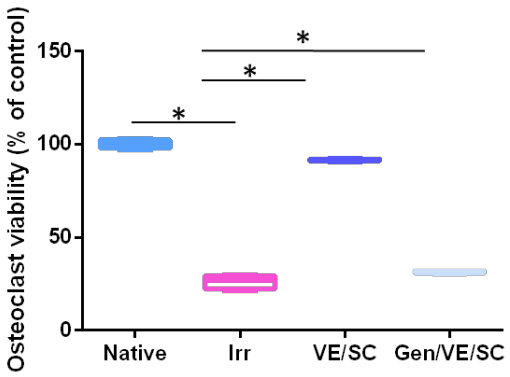


(a) (b)

Figure 4. (a) *B*. subtillis concentration in the media after 24hr incubation for native, irr, VE/SC, and Gen/VE/SC treated bone that has been immersed in *B*. subtilis spore for 48 hr prior to terminal irradiation. (b) Live-Dead bacterial stain of spore and/or bacteria containing media described in (a).



(a)



(b)

Figure 5. (a) Representative confocal fluorescence image of RANKL-stimulated RAW264.7 macrophages on the surface of bone allograft. Green=Tartrate-resistant acid phosphatase, red= actin, blue=nucleus. Scale bar = 50 um. (b) Relative viability of bone-adherent RAW 264.7 (relative to the mean of control-unirradiated samples). Data are presented as mean ± s.d. (n=6 per group). Statistical tests were performed using one-tailed Mann-Whitney non-parametric tests, *$p<0.05$

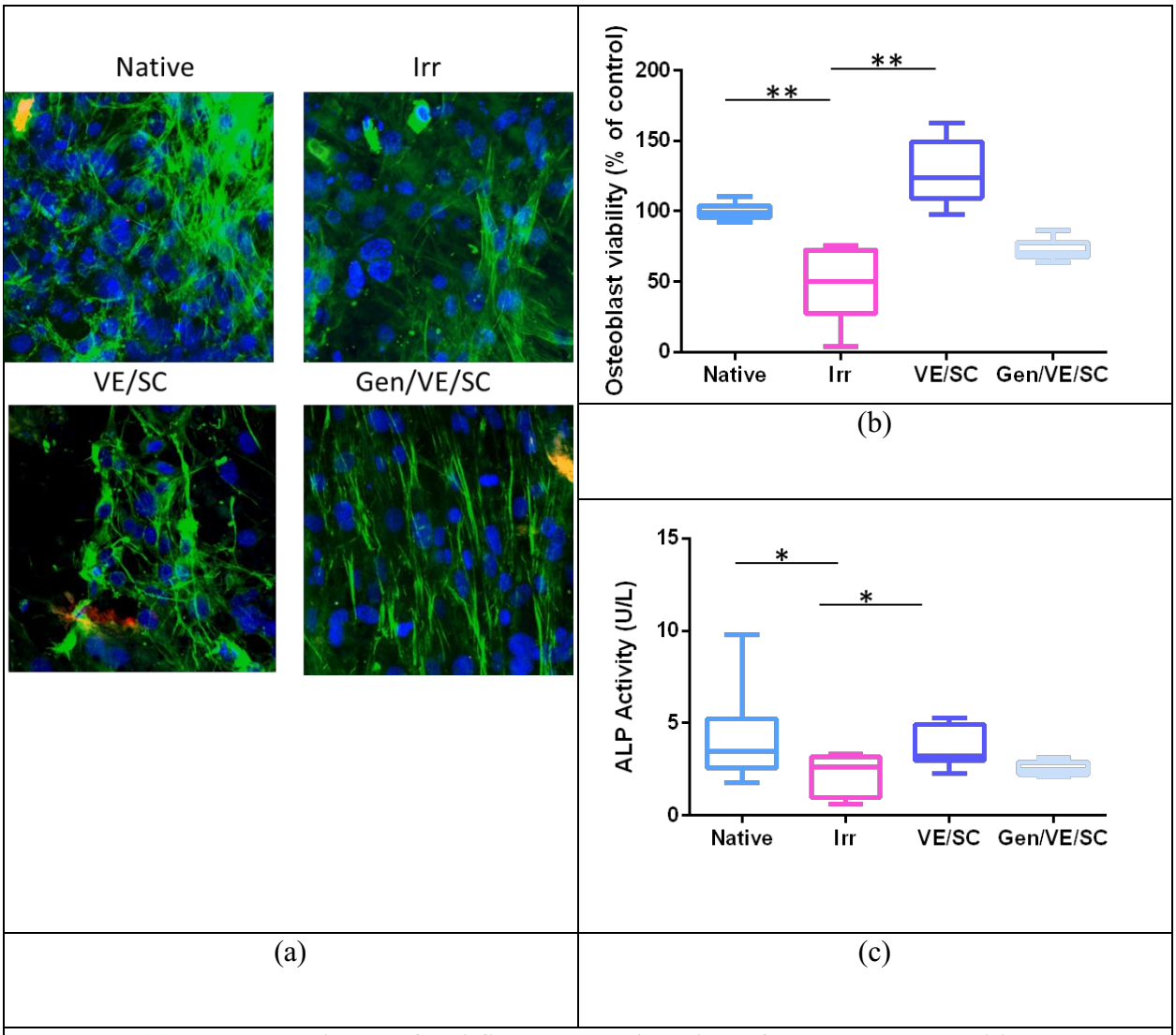


Figure 6. (a) Representative confocal fluorescence imaging of MC3T3-E1 osteoblast grown on the surface of bone allograft. Green=actin, red=ALP, blue=nucleus. Scale bar = 25 um. (b) Relative viability of bone-adherent MC3T3-E1 osteoblasts (relative to the mean of control-unirradiated samples). Data are presented as mean ± s.d. (n=6 per group). (c) ALP activity of bone adherent MC3T3-E1 osteoblasts. Data are presented as mean ± s.d. (n=6 per group). Statistical tests were performed using one-tailed Mann-Whitney non-parametric tests, *$p<0.05$, **$p<0.01$.

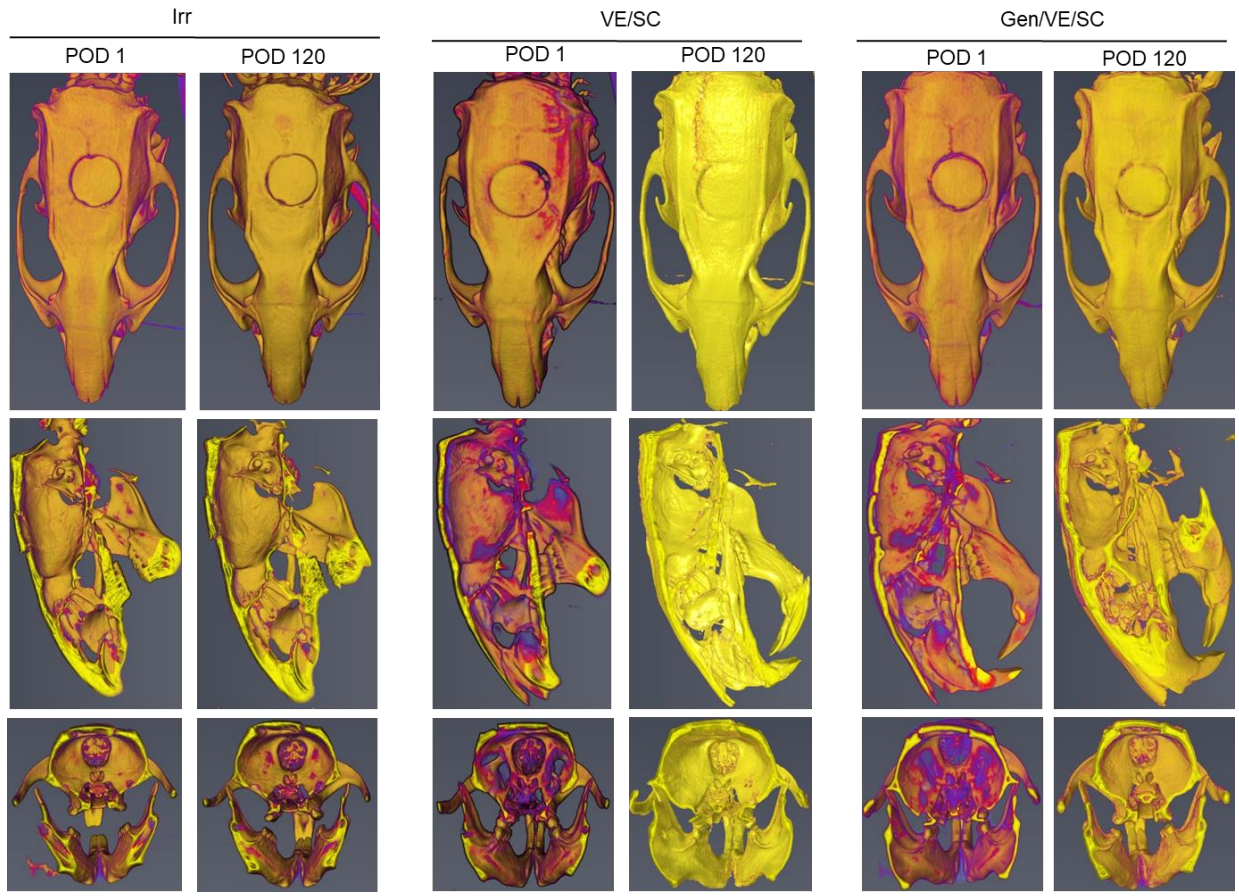
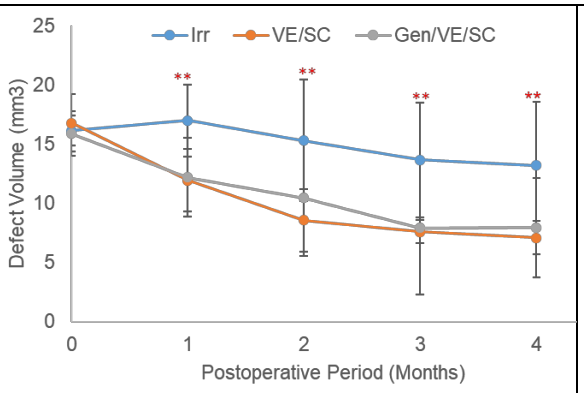
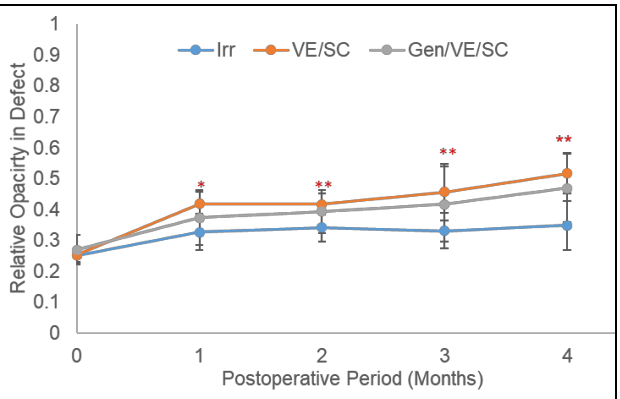


Figure 7. Radiographic representation of bone interface between the implant (Irr, VE/SC, or Gen/VE/SC) and the host's calvaria at post-operative day one (POD 1) and post-operative day 120 (POD 120).Top=aerial axial view, middle=mid-sagittal cut, right=mid-coronal cut.



(a)



(b)

Figure 8. (a) Volume of interface between the implant and the host's calvaria over the course of longitudinal study. Data are presented as means ± s.d. (n=5 per group). (b) Relative density of the defect volume between the implant and host's calvaria over the course of the longitudinal study. Relative density is calculated as the ratio of density difference between voxel of interest and minimum density of surrounding soft tissue to the difference between maximum density of cortical bone and minimum density of surrounding soft tissue. Data are presented as mean ± s.d. (n=5 per group).

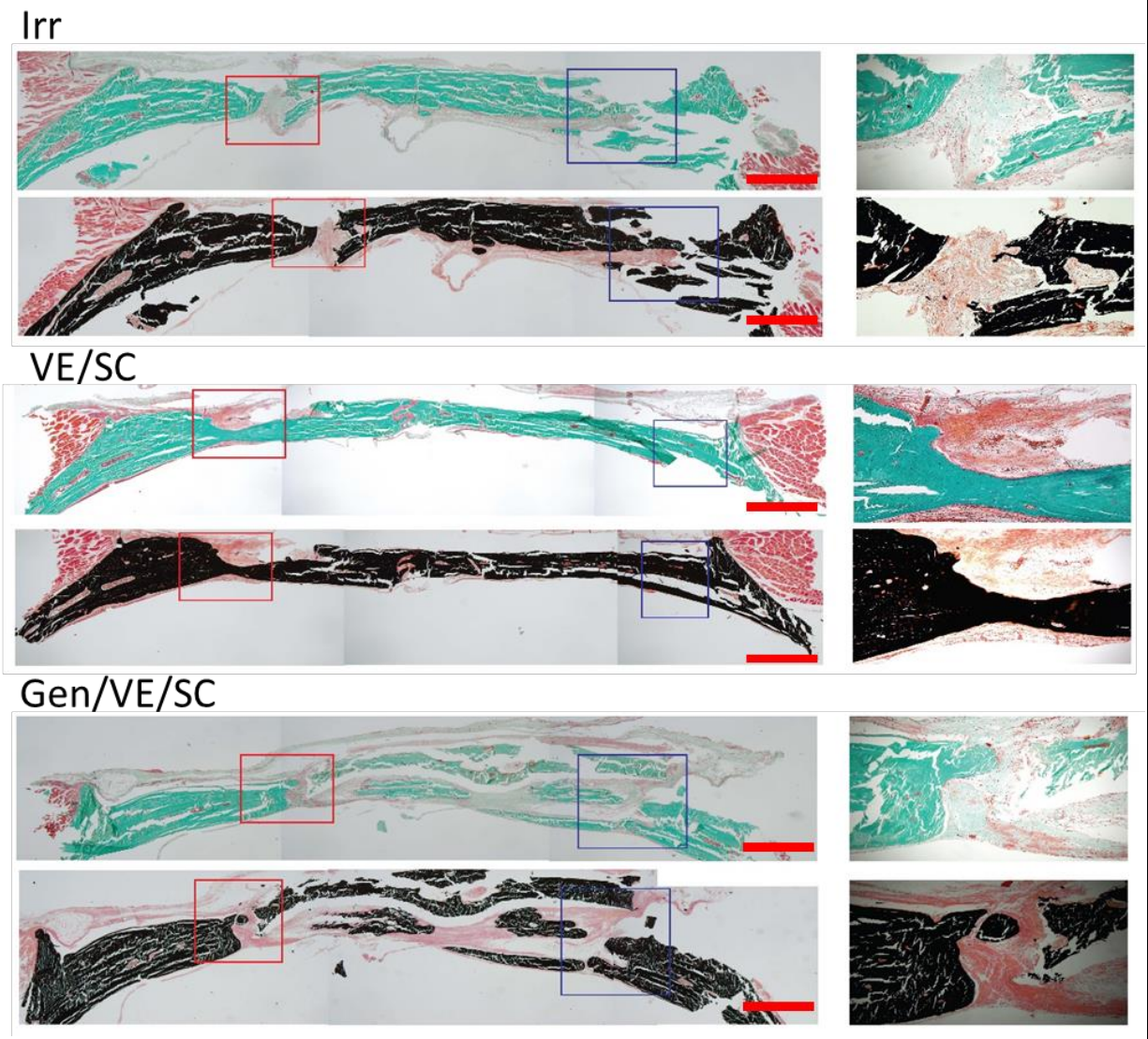


Figure 9. Representative cross-section of explanted calvaria at post-operative day 120 of Irr, VE/SC, and Gen/VE/SC**.** Samples are stained with Goldner's trichome (red-green stain) and toluidine blue (red-black). Images are taken at 2x magnification (left) and 10x magnification (right). The interface between the implant and host's bone are marked with orange and blue boxes. Magnified view from the orange boxes are shown on the right.

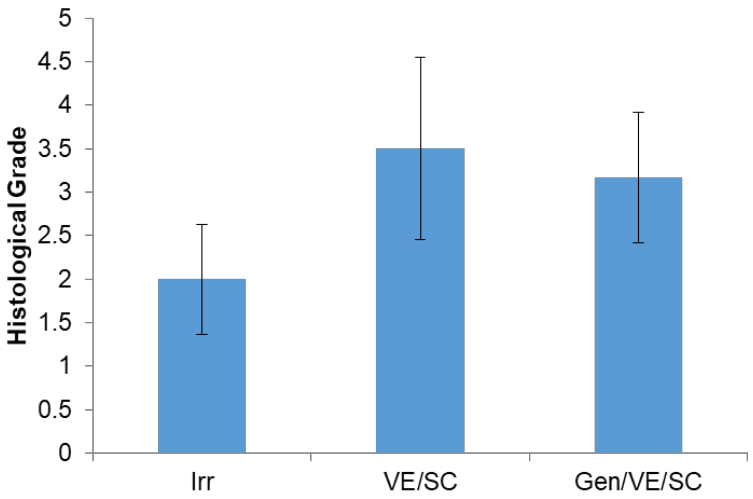


Figure 10. Histological grading (1-6) based on the degree of healing. 1=Empty defect (no connective tissue), 2= Fibrous tissue, 3= Collagenous connection (<50% of interface), 4= Collagenous connection (>50% of interface), 5= Ossicles, 6= Complete ossification. Data are mean ± s.d. (n=15 per group).