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Nonvascularized Bone Graft Reconstruction of the Irradiated Murine Mandible – An Analogue of Clinical Head and Neck Cancer Treatment

Kevin M. Urlaub, BS^a, Russell E. Ettinger, MD^a, Noah S. Nelson, MPH^a, Jessie M. Hoxie^a, Alicia E. Snider, MD^a, Joseph E. Perosky, MS^a, Yekaterina Polyatskaya, MD^a, Alexis Donneys, MD, MS^a, and Steven R. Buchman, MD, FACS^a

^aSection of Plastic and Reconstructive Surgery, Michigan Medicine, 4-730 C.S. Mott Children's Hospital, 1540 E. Hospital Dr., Ann Arbor, MI 48109, USA

Abstract

Nonvascularized bone grafts (NBGs) represent a practical method of mandibular reconstruction that is precluded in head and neck cancer patients by the destructive effects of radiotherapy. Advances in tissue-engineering may restore NBGs as a viable surgical technique, but expeditious translation demands a small-animal model that approximates clinical practice. This study establishes a murine model of irradiated mandibular reconstruction using a segmental iliac crest NBG for the investigation of imperative bone healing strategies. Twenty-seven male isogenic Lewis rats were divided into two groups; control bone graft (CBG) and irradiated bone graft (XBG). Additional Lewis rats served as graft donors. The XBG group was administered a fractionated dose of 35Gy. All rats underwent reconstruction of a segmental, critical-sized defect of the left hemi-mandible with a 5mm NBG from the iliac crest, secured by a custom radiolucent plate. Following a 60-day recovery period, hemi-mandibles were evaluated for bony union, bone mineralization, and biomechanical strength ($p < 0.05$). Bony union rates were significantly reduced in the XBG group (42%) compared to controls (80%). Mandibles in the XBG group further demonstrated substantial radiation injury through significant reductions in all metrics of bone mineralization and biomechanical strength. These observations are consistent with the clinical sequelae of radiotherapy that limit NBGs to non-irradiated patients. This investigation provides a clinically relevant, quantitative model in which innovations in tissue engineering may be evaluated in the setting of radiotherapy to ultimately provide the advantages of NBGs to head and neck cancer patients and reconstructive surgeons.

Keywords

radiation; nonvascularized bone graft; segmental mandibular defects; mandible; head and neck cancer

Corresponding author: Steven R. Buchman, MD, FACS, Michigan Medicine, Pediatric Plastic Surgery Section, 4-730 C.S. Mott Children's Hospital, 1540 E. Hospital Drive, Ann Arbor, MI 48109-4215, USA, Fax: 734-936-7815, Phone: 734-936-5881, sbuchman@med.umich.edu.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Introduction

Segmental mandibular defects secondary to trauma, infection, and tumor extirpation constitute a major clinical challenge in maxillofacial surgery. Nonvascularized bone grafts (NBGs) represent a practical and highly successful method of reconstruction that has been widely utilized since the early 1960's.¹ However, the use of NBGs for mandibular defects has since become limited by the implementation of adjuvant radiotherapy in the treatment of head and neck cancer. Radiation results in severely damaging effects on bone, including vascular and cellular depletion as well as impairment of osteogenesis and mineralization.²⁻⁴ These consequences manifest clinically in irradiated patients through significantly higher failure rates of NBGs, forming the basis for their contraindication.⁵ As a consequence, vascularized free tissue transfer of osseous or osteocutaneous flaps became the gold standard in the setting of radiotherapy, yet this technique presents significant drawbacks for both the patient and reconstructive surgeon. Among these disadvantages are prolonged operative procedures, notable donor site morbidity, and increased technical demand in comparison to traditional NBGs.^{6,7} Vascularized fibular free flaps are currently the most common method of mandibular reconstruction after oncologic resection, but have been shown to have inferior contouring ability, implant success, and quality of life outcomes in comparison to nonvascularized grafts from the iliac crest.⁷⁻¹⁰

Fortunately, recent advances in tissue engineering and pharmacologic enhancement of bone healing have highlighted the potential for surgical alternatives to vascularized free flap reconstruction and may restore the optimal characteristics and ease of use of NBGs in the setting of irradiated mandibular defects. Before clinical progress can move forward, robust animal models must first be created to allow for rigorous evaluation of promising new surgical techniques and therapies.^{11,12} To address this issue, this study establishes a murine model of irradiated nonvascularized bone graft reconstruction of segmental mandibular defects using the iliac crest donor site, creating an ideal platform for innovation in irradiated reconstruction. Mandibular defects have been extensively studied in large-animal models in order to simulate the biomechanical forces and approximate size of the human mandible.¹³⁻²² However, these investigations necessitate resource-intensive animal husbandry, increased cost, smaller sample sizes, and lengthy recovery periods in comparison to small-animal models. Murine models therefore permit rapid throughput of tissue engineering strategies that may reintroduce NBGs as a reconstructive option for head and neck cancer patients following radiotherapy. In addition to cost and efficiency, murine models offer greater genomic and phenotypic control, and have an extensive array of established antibodies. As the demand to elucidate the underlying mechanisms and genetic correlations of disease and treatment modalities continues to grow, the use of murine models has become increasingly prevalent in recent decades.²³

Despite the growing use of small-animal models, the literature available for bone healing evaluation within the craniofacial skeleton has several shortcomings. Most importantly, small-animal correlates of surgical procedures utilized in clinical settings are lacking. Clinically, autologous bone grafting is the current standard of care for segmental mandibular reconstruction, making the development of animal models that recapitulate this surgical technique essential for in-vivo investigation of novel therapeutics. Furthermore, mitigation

of the damaging effects of radiotherapy remains a secondary focus of animal research despite the fact that radiotherapy is widely implemented in cancer treatment and remains one of the most prevalent and significant obstacles to reconstructive surgeons and recovering patients.² Our laboratory has previously established murine models of mandibular fracture and distraction osteogenesis, and published extensively surrounding the ability of novel tissue-engineering strategies to remediate radiation-induced injury within these models.^{24–29} Notably, translation of the surgical and pharmacologic strategies investigated by our laboratory has been successfully demonstrated in human mandibular reconstruction.³⁰

This study aims to expand upon surgical approaches currently researched by our laboratory as well as previous literature surrounding small-animal models of craniofacial reconstruction. Specifically, this investigation utilizes the 5mm critical-sized defect defined by Deconde et al.³¹ to establish a murine model of non-vascularized bone graft reconstruction of segmental mandibular defects using an iliac crest donor site. Interpositional bone grafting with rigid fixation in the setting of radiotherapy was used in our study to replicate the clinical complications encountered in human head and neck cancer reconstruction. Herein, we seek to describe the surgical procedure as well as technical challenges associated with manipulation of the murine mandible to develop a reproducible and clinically-relevant model that can be utilized in future studies of bone healing. By establishing quantitative metrics of radiation-induced bone injury, this model serves as a platform for future investigations in which tissue engineering, stem cell, and pharmaceutical therapies may be applied and tested in-vivo in the setting of radiotherapy. Such innovations may ultimately expand the utility of non-vascularized bone grafting in the setting of radiation and fully restore this practical technique to the armamentarium of reconstructive surgeons.

Materials and Methods

Study Design

Twenty-seven male isogenic Lewis rats were divided into two groups; control bone graft (CBG) and irradiated bone graft (XBG). Six additional isogenic Lewis rats served as donors for the harvest of iliac crest bone grafts. Rats receiving radiation treatment were administered a fractionated, human-equivalent dose of 35Gy, comparable to 70Gy administered to head and neck cancer patients clinically and were allowed a 2-week recovery period prior to surgical treatment. All rats underwent creation of a segmental, critical-sized defect of the left hemi-mandible, which was subsequently interposed with a 5mm iliac crest bone graft and secured with a custom radiolucent plate (KLS Martin, Jacksonville, FL) using 4mm Sonic Weld pins (KLS Martin, Jacksonville, FL). Following a 60-day recovery period, hemi-mandibles were grossly evaluated for bony union upon dissection. All samples were assessed for bone mineralization through microcomputed tomography (micro-CT) and underwent subsequent biomechanical testing.

Animal

All animal experimentation was conducted in accordance with the guidelines published in the Guide for the Care and Use of Laboratory Animals: Eighth Edition. Protocols were

approved by the University of Michigan's Committee for the Utilization and Care of Animals (UCUCA) prior to implementation. Twelve-week-old isogenic Lewis rats weighing approximately 400g were paired in cages and maintained in a pathogen free vivarium on a 12-hour light/dark schedule and fed standard hard chow and water ad libitum. All animals were allowed a 7-day acclimation period prior to initiation of the radiation protocol. Following radiation, the rats were maintained on regular chow and water and observed for 2 weeks before surgery. The diet was changed to moist chow 48 hours preoperatively along with Hill's high-calorie diet (Columbus Serum, Columbus, Ohio).

Radiation Procedure

Rat hemi-mandibles in the XBG group were irradiated using a Philips RT250 orthovoltage unit (250 kV, 15 mA) (Kimtron, Inc., Oxford, CT). A fractionated dose of 35 Gy was given over 5 days. Rats were anesthetized using isoflurane/oxygen (2% and 1 L/min) and placed right side down to ensure that only the left hemi-mandible was irradiated. A lead shield with a rectangular window protected the pharynx, brain, and remainder of the animal. This radiation regimen has been performed for many years in the department of Radiation Oncology at the University of Michigan under ULAM/IACUC-approved protocols.

Iliac Crest Bone Graft Harvest

Rats utilized as donors were euthanized immediately prior to the harvest of segmental iliac crest bone grafts. Donor rats were shaved and depilated in a 5×5cm area over the thoracolumbar region. After sterile prepping and draping, manual palpation of the tips of the iliac crests was used to confirm the appropriate level for access. A midline incision was carried out and bilateral subcutaneous flaps were elevated laterally to each side of the animal. Skin flaps were then retracted to one side and intramuscular dissection was carried out through the paraspinous muscles to the level of the underlying iliac bone. A periosteal elevator was used to circumferentially free all muscle attachments to the iliac bone (Figure 1). A 5mm double bladed oscillating saw (Bien Air Dental, Biel, Switzerland) was then used to harvest three to four segmental grafts in series from the broadest portion of the iliac bone. The distal tip of the iliac crest and the proximal most extent of the iliac bone were excluded from all donor grafts due to anatomically suboptimal curvature relative to the mandibular osteotomy site. After harvest, iliac crest grafts were placed in saline moistened gauze for later interposition into the mandibular osteotomy sites.

Mandibular Osteotomy and Segmental Bone Grafting

Recipient rats were administered subcutaneous injections of gentamicin (5 mg/kg) in normal saline and buprenorphine (0.03 mg/kg) in lactated Ringer's solution prophylactically prior to surgery. Animals were then anesthetized and maintained on an inhalational mixture of oxygen and isoflurane for the duration of the surgical procedure. Protective ocular lubricant was applied following adequate induction of anesthesia. Anesthetized animals were placed supine on a warming blanket and their submandibular regions and necks were shaved and depilated prior to sterile prepping and draping. Midline submental incision was performed, and subcutaneous skin flaps elevated over the superficial masseter muscle bellies bilaterally. The inferior boarder of the mandible was visualized by grasping the lingual and buccal aspects of the superficial masseter muscle with Adson-Brown forceps. Intramuscular

dissection was then carried out with 15-blade scalpel between the tines of the Adson-Brown Forceps directly onto the inferior boarder of the mandible. A number 9 periosteal elevator was utilized to sweep the masseter muscle attachments along the buccal margin of the mandible and the submasseteric space was packed with a gauze. In a similar fashion, the lingual margin of the mandible was also cleared of muscular attachments and packed with gauze to allow for hemostasis.

The segmental iliac crest bone graft was then prepared for interposition. The iliac crest graft was stabilized with forceps and a central drill hole was created (Figure 2A). A custom cut three-hole Resorb-X (KLS Martin, Jacksonville, FL) plate was positioned over the central drill hole and secured in place with a 4mm Sonic Weld pin (KLS Martin, Jacksonville, FL) (Figure 2B).

With the iliac crest bone graft now prefabricated for interposition, the hemostatic gauze packs were removed from the buccal and lingual aspects of the mandible. Distal osteotomy and subsequent mesial osteotomy were then performed with a single blade reciprocating saw (Bien Air Dental, Biel, Switzerland) (Figure 3A). The prefabricated iliac crest bone graft and plate were then interposed into the osteotomy gap, spanning the 5mm critical-sized defect. Rigid fixation was achieved by predrilling the mesial hole first and securing the plate with a 4mm Sonic Weld pin. The distal hole was predrilled and fixated with a 4mm Sonic weld pin with care taken to not use undue force in the thinner bone at the angle of the mandible. The final construct consisted of the 5mm iliac crest bone graft interposed and rigidly fixed into the segmental osteotomy gap (Figure 3B). The submental incision was then closed with surgical staples and Silvadene (Monarch Pharmaceuticals, Inc., Bristol Tennessee) ointment was applied to the incision lines. Animals were recovered on a warming blanket to maintain normothermia and given blow by oxygen as needed until fully awake.

Postoperative Care

Animals were housed one per cage and fed moist chow with Hills high-calorie diet and water ad libitum. Two post-operative doses of gentamycin (5 mg/kg subcutaneously every 12 hours) were given as well as the continuation of buprenorphine (0.03 mg/kg subcutaneously every 12 hours) through postoperative day 4 or longer as needed as indicated by appropriate weight gain, porphyrin staining, and adequate food and fluid intake. All animals were allowed a 60-day consolidation period before sacrifice.

Micro-computed Tomography

Qualitative in-vivo micro-CT scans were obtained using a 65 kV, 385 μ A, and 87ms exposure (GE Healthcare Biosciences) (Figure 4). 392 projections were taken at a resolution of 35 micron voxel size. Following euthanasia and harvest, quantitative micro-CT scans were obtained using an 80 kV, 80 mA, and 1,100 ms exposure. 392 projections were taken at a resolution of 45 microns voxel size. After initial calibration of known density standards, each complete mandible was scanned in a chilled dH₂O solution. Utilizing MicroView 2.2 software (GE Healthcare, Milwaukee, WI), two regions of interest (ROIs) were defined for each hemi-mandible at the anterior and posterior bone graft interfaces as follows; the

coronal plane was centered at the interface between native bone and the iliac crest bone graft, which remained identifiable following graft incorporation (Figure 5). From this initial position, 20 frames were splined anteriorly and posteriorly for a total of 40 consecutive frames per ROI. Several rotations and cropping of non-bone space were undertaken to ensure uniform data measurement. The metrics of bone mineral density (BMD), bone volume fraction (BVF), and tissue mineral density (TMD) were obtained from each ROI. Following bone analysis, anterior and posterior ROI data from each animal were averaged for subsequent statistical analysis.

Biomechanical Testing

Bony union was determined clinically and defined as solid bony bridging and an absence of motion across the graft interfaces on palpation after plate removal. Mandibles were then potted and loaded to failure in uniaxial monotonic tension at 0.5 mm/s using a servohydraulic 858 Minibiox II testing machine (MTS Systems Corporation, Eden Prairie, MN). Crosshead displacement was recorded using an external linear variable differential transducer (LVDT; Lucas Schavitts, Hampton, VA), and load data was collected with a 100-lb load cell (Sensotec, Columbus, OH). Data was sampled at 200 Hz on a TestStar system (TestStar IIs System version 2.4; MTS Systems Corporation, Eden Prairie, MN). Load-displacement curves were analyzed for ultimate load, failure load, and stiffness using custom computational code (MATLAB 7.11; Mathworks Inc., Natick, MA).

Statistical Analysis

Group sizes were determined prior to experimentation with the use of nQuery Advisor 7.0 software. Under the assumption that data would be evaluated using a general liner model with associated analysis of variance with a desired power of 0.80 with a difference between groups of one standard deviation we required at least five animals per testing group. Due to the use of preoperative radiotherapy and need for post-consolidation biomechanical testing we judiciously increased group sizes to account for these variables. Statistical analysis of outcome variables was performed using SPSS 24.0 for Windows (SPSS, Inc., Chicago, IL) in consultation with the Center for Statistical Consultation and Research (CSCAR) at the University of Michigan. Chi Square analysis was used to compare union rates between groups. Independent samples T-test was used to compare means for all other reported metrics. Significance was defined as $p < 0.05$.

Results

Bony Union

Hemi-mandibles exposed to radiation exhibited grossly evident structural damage and anatomical changes. Upon dissection, 12 out of 15 hemi-mandibles (80%) in the CBG group exhibited bony union (Figure 6). Bony union rates were significantly reduced in the XBG group, in which 5 of 12 hemi-mandibles (42%, $p = 0.042$) achieved bony union, demonstrating the extent of radiation impairment to bone healing in this study.

Mineralization

In our investigation, radiation treatment diminished bone mineralization across all metrics (Figure 7). In comparison to non-irradiated controls, the XBG group exhibited significant decreases in BMD (383 mg/mL vs. 608 mg/mL; $p = 0.003$), TMD (648 mg/mL vs. 728 mg/mL; $p = 0.014$) and BVF (0.45 vs. 0.74; $p = 0.003$).

Biomechanical Strength

The diminished healing capacity of irradiated bone was further corroborated by biomechanical metrics. Compared to the control group, the XBG group exhibited a significant decrease in mean stiffness (41 N/mm vs. 323 N/mm; $p = 0.013$) (Figure 8). Additionally, the XBG group demonstrated significantly lower ultimate load (15 N vs. 65 N; $p = 0.011$) and failure load (15 N vs. 63 N; $p = 0.015$) in comparison to non-irradiated controls.

Discussion

NBGs represent a well-established and simpler alternative technique to vascularized free tissue transfer for mandibular reconstruction. In addition to offering expedited operative times, NBGs provide the distinct advantages of increased precision in bone symmetry, superior facial contour, and greater subsequent implant success in comparison to free flaps.^{1,6–8} Although the surgical practicality of nonvascularized grafts is preferred due to their simplicity, their application became limited by the increasing use of adjuvant or neoadjuvant radiation therapy in head and neck cancer treatment protocols. This has led to the relegation of NBGs to non-irradiated patients due to high failure rates secondary to vascular depletion and loss of cellular function in irradiated tissue beds.^{32–35} In the clinical setting, radiotherapy has effectively pushed vascularized free tissue transfer to become the gold standard, but this technique now has shortcomings due to a lack of secondary reconstructive options beyond repeat free flap procedures. Furthermore, vascularized free tissue transfer has inherent disadvantages of prolonged operations, increased donor site morbidity, and the requirement of specialized training and operative equipment.^{5,34,36–38} Despite this, little investigation has focused on the potential reengineering of bone healing that would allow the reintroduction of NBGs to afford irradiated patients their inherent advantages.² Such efforts would require an animal model that reflects the clinical reconstruction of head and neck cancer patients. However, no murine model of nonvascularized bone graft transplantation for segmental mandibular defects currently exists, creating the impetus for this study, which creates reproducible and quantitative metrics that can be used to determine the degree by which pharmaceutical and tissue engineering interventions are successful in remediating the effects of radiation.

In this investigation, radiation-induced injury was evidenced by decreased bony union, bone mineralization, and biomechanical strength. These results are in line with an abundance of scientific literature delineating bone healing impairment by radiotherapy both clinically and in animal models.^{2–5, 25–29, 32,39–41} The bony union rate of 80% (12/15) achieved by the control group in this study accurately reflects those observed in nonvascularized bone grafting of human mandibular defects.^{32,34,42} Radiotherapy decreased union rates to 42%

(5/12) in the XBG group, mirroring the pernicious effect of radiation on NBGs in humans that resulted in their discontinuation.^{5,7,39} Future investigations surrounding advancements in tissue-engineering and cellular therapies should utilize this model to test their ability to facilitate successful graft incorporation in the challenging environment of radiotherapy. Successful bone healing despite impairment by radiation would not only establish treatment efficacy more definitely, but also offer highly translatable interventions for patient populations with severely limited surgical options.

Recent murine investigations surrounding the reconstruction of segmental and partial thickness defects have focused on the development of various scaffolding materials coupled with stem cells or distinct growth factors such as bone-morphogenic-protein and VEGF.^{43–51} While useful for basic science evaluation of treatment efficacy, such models do not simulate human disease conditions nor offer clear avenues to direct clinical translation of these therapeutics. Moreover, investigations of the detrimental consequences of radiotherapy on bone healing are noticeably lacking despite their clinical significance.² In this study, a 5mm critical-sized defect of the mandible was successfully reconstructed using an NBG from the iliac crest. This provides a clinically relevant model that reflects current surgical techniques in craniofacial reconstruction for the evaluation of novel bone healing strategies that may reintroduce this practical reconstructive option in the setting of radiation.

Additionally, existing murine models utilize titanium plate fixation, which results in significant backscattering during micro-computed tomography.^{31,52,53} The radiolucent plate used in our model offers a unique and imperative testing ground for novel innovations in tissue engineering. Lack of scattering allows for unprecedented longitudinal *in-vivo* micro-CT evaluation of bone healing and thus provides an unparalleled tool for the establishment of optimal therapeutic and recovery time frames as well as the opportunity to study the effect of postoperative radiotherapy (Figure 4).³⁷ By obviating the need for numerous experimental groups to assess treatment efficacy at multiple time points, longitudinal evaluation further enhances the advantages in cost and efficiency inherent to murine models. Finally, the radiation procedure outlined in the current study has been utilized and published extensively by our laboratory, ensuring a reliable protocol for the investigation of quantifiable radiation-induced damage and remediation.^{25–29}

Mandibular reconstruction within small-animal models presents several challenges and limitations exemplified by this study. Mesial and distal drilling is necessary for adequate fixation and stabilization of segmental bone grafts, which poses a significant risk of fracturing the rat mandible, particularly at the posterior angle. Future investigators should therefore take particular caution when manipulating the posterior mandible. The use of Sonic Weld pins for plate fixation in our model reduces the risk of mandibular fracture in comparison to more commonly utilized titanium screws, but also makes stabilization of the bone graft more challenging due to their decreased rigidity. Rescue screws were necessary in select cases to secure graft interposition and present a learning curve for the surgeon. Additionally, tolerable blood loss in reconstruction of the rat mandible is limited to approximately 2mL. Control of hemorrhage was a key challenge noted by Deconde et al.³¹ in the segmental resection of the rat mandible and was observed in our study in two cases. Notably, electrocautery was not utilized in our surgical procedure and could be applied in

future investigations to help limit intraoperative blood loss. The duration of the operative procedure for creation and plating of a segmental mandibular defect has been reported at 45 minutes.³¹ Our operative times were 35 minutes, demonstrating that interpositional bone grafting with fixation can be completed efficiently, which may be attributed to the use of custom, three-hole resorbable plates as opposed to titanium plating with a greater number of screws. Finally, although murine models offer greater efficiency of time and resources, innovations in bone healing augmentation should be subsequently confirmed as proof of concept in humans or in large animal models that approximate the size and load bearing conditions of the human mandible. Indeed, advancements in mandibular reconstruction are highly translatable and have been successfully demonstrated by our laboratory through proof of concept in humans.³⁰

The advantages of non-vascularized bone graft transplantation are currently restricted to non-irradiated patient populations. In order to develop strategies to facilitate successful nonvascularized reconstruction in the setting of radiation, small-animal models that reflect clinical techniques in mandibular reconstruction are essential. The surgical procedure delineated in this study provides a necessary and optimal model in which to investigate translational advances in tissue engineering, biomaterials, and pharmaceutical therapies for the enhancement of bone healing. Translational application of such innovations may ultimately expand the application of NBGs and provide practical benefits for both reconstructive surgeons and head and neck cancer patients.

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Figure 1:
Exposure of the iliac crest of isogenic donor rat.

Figure 2:
Preparation of bone grafts through creation of central drill hole (A) and fixation of radiolucent plate (B).

Figure 3:
Creation of 5mm critical-sized defect of the left hemi-mandible (A) and final reconstruction with iliac crest bone graft (B)

Figure 4:
Progressive healing within the same non-irradiated rat at 10-day intervals. Radiolucent plating system circumvents backscattering encountered in titanium plate fixation.

Figure 5:
Regions of interest for quantitative evaluation of bone mineralization at graft interfaces.

Figure 6: Bony union rates were significantly reduced in the irradiated bone graft (XBG) group compared to the control bone graft (CBG) group. (* $p < 0.05$).

Figure 7:
Radiotherapy caused a significant reduction in all metrics of bone mineralization in the irradiated bone graft (XBG) group compared to the control bone graft (CBG) group. (* p < 0.05).

Figure 8:
Irradiated mandibles demonstrated significantly lower biomechanical strength compared the control group in each reported metric (* $p < 0.05$).