An Athymic Rat Model for Mandibular Osteoradionecrosis Allowing for Direct Translation of Regenerative Treatments

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. We aim to create a model of mandibular osteoradionecrosis in athymic rats. Athymic rats provide an immunsuppressed environment whereby human stem cells and biomaterials can be used to investigate regenerative solutions for osteoradionecrosis, bridging the gap between in vivo testing and clinical application.

Study Design. Prospective animal study.

Setting. Academic otolaryngology department laboratory.

Subjects and Methods. After Institutional Animal Care and Use Committee approval, 10 athymic nude rats were divided into 2 groups. The experimental group (n = 6) underwent irradiation (20 Gy), while the control group (n = 4) underwent sham irradiation catheter placement only. All 10 rats underwent extraction of the second mandibular molar 7 days later. The rats were sacrificed 28 days after dental extraction, and their mandibles were harvested. The mandibles were examined with histologic analysis and bone volume analysis based on 3-dimensional micro–computed tomography.

Results. All 10 rats survived the experiment period. Radiographic and histologic analysis revealed decreased bone formation in the experimental group compared with the control group. Jaw region volume ratio was 0.83 for the experimental group versus 0.97 in the control group (P = .003). The region-of-interest volume ratio was 0.75 in the experimental group and 0.97 in the control group (P = .005). Histologically, there were increased osteoclasts (P = .02) and decreased osteoblasts (P = .001) as well as increased fibrosis in the experimental group versus the control group.

Conclusion. Mandibular osteoradionecrosis can be effectively and reproducibly produced in an athymic rat model. This will allow further research to study regenerative medicine in an athymic rat model.

Keywords
osteoradionecrosis, animal model, mandible, translational research

Received April 30, 2015; revised May 20, 2015; accepted June 5, 2015.

Osteoradionecrosis (ORN) of the mandible is a devastating phenomenon confronted in patients after head and neck irradiation. ORN is encountered in approximately 5% to 15% of patients who receive head and neck radiation. With the advent of newer irradiation techniques, this rate is now trending more toward the 5% mark.¹⁻³ Patients may be asymptomatic, although pain, halitosis, odynophagia, trismus, fistulae, and infection are frequently encountered. Physical examination findings range from small areas of exposed bone to profound mandibular bone necrosis with pathologic fracture.

The pathophysiology is not well understood, and multiple theories have been proposed.⁴⁻⁶ As the understanding of the pathophysiologic basis of ORN improves, treatment options continue to evolve. Currently, there is no universally agreed-on treatment approach to patients with mandibular ORN. Hyperbaric oxygen therapy has been implemented with mixed results. While medical therapies continue to evolve and show promise, surgical therapy has been the definitive treatment.

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This article was accepted for presentation at the 2015 AAO-HNSF Annual Meeting & OTO EXPO; September 27-30, 2015; Dallas, Texas.

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We propose studying the effects of human mesenchymal stem cell therapy on mandibular ORN using a rat model. The University of California, Los Angeles, group has performed 2 experimental models of inducing mandibular ORN in Sprague-Dawley rats. In its updated model, the group compared rats irradiated with 20-Gy high-dose-rate (HDR) brachytherapy to sham irradiation. The researchers performed dental extractions 7 days later and sacrificed the animals 21 days after extractions for micro–computed tomography (micro-CT) and histologic analysis. They concluded that ORN was introduced histologically and radiologically and that their model provides a method of quantifying bone loss. Although this rodent model for the development of ORN has been established, it has yet to be validated at an outside facility and has not been performed in an animal that can support human cell growth or the use of human biomaterials. The decreased immune function in athymic rats can allow for such in vivo trials to be conducted without rejection of human products, thereby bridging the gap between the laboratory and the patient.

Before studying stem cell applications, we first need to ensure that mandibular ORN can be effectively and reproducibly achieved in an athymic animal model. Therefore, we aim to validate an experimental animal model of ORN as a potential platform for stem cell application.

Methods

Experimental Design

The research protocol was approved by the Mayo Clinic Institutional Animal Care and Use Committee (protocol A68713). Ten athymic nude male rats 7 weeks of age (150-200 g) were obtained from Charles River Animal Laboratories International (Wilmington, Massachusetts).

The rats were divided into 2 groups. The experimental group (n = 6) underwent left mandibular irradiation, followed by left second molar extraction. The control group (n = 4) underwent left mandibular sham irradiation, followed by left second molar extraction. Both groups were then euthanized for mandibular harvest 28 days after dental extraction.

Animal Care

Rats were kept individually and provided with a standard pelleted rodent diet and autoclaved water. The rats were weighed every Monday, Wednesday, and Friday. The standard diet was modified after the irradiation procedure and dental extractions. A 30-mL dose of Children’s Acetaminophen (160 mg/5 mL; Rugby Laboratories, Livonia, Michigan) per bottle of autoclaved water was supplied for analgesia to give a dose of 100 to 300 mg/kg. The acetaminophen-and-water solution was changed every other day. The diet was changed to Harlan irradiated rodent chow (Harlan Animal Laboratories, Madison, Wisconsin) and soaked in sterile water for 3 to 5 minutes, drained, and placed in the animal’s cage every other day along with the water change. After 2 weeks of the modified diet, the standard diet resumed.

Irradiation

The rats were anesthetized by intraperitoneal injection with a solution of ketamine (100 mg/kg; Ketaset, Fort Dodge Animal Health, Fort Dodge, Iowa), xylazine (20 mg/kg; Lloyd Laboratories, Shenandoah, Iowa), and acepromazine (3.3 mg/kg; Vedco, St Joseph, Missouri) for induction and half-strength solution for maintenance anesthesia.

After initiation of anesthesia, the rats were placed in a right lateral position on the radiation table. A gas-sterilized HDR catheter (Best Medical, Springfield, Virginia) was placed along the lateral aspect of the left mandibular body. The catheter was placed through the left lower gingivalabial sulcus and advanced through the skin below the external auditory meatus. The experimental group went on to receive mandibular irradiation. The control group underwent HDR catheter placement only.

Irradiation was performed with HDR 192-Ir brachytherapy via a single dwell point. Treatment planning was performed on a BrachyVision planning system and delivered with a VariSource iX afterloader (Varian Medical Systems, Palo Alto, California). Plans were constructed to deliver 20 Gy to the 5-mm isodose line, and the dwell times were verified with RadCalc (LifeLine Software, Austin, Texas). The brachytherapy source was delivered through the inserted HDR catheter. Dwell point localization was performed with an Acuity fluoroscopic simulator (Varian Medical Systems) during extension of the afterloader dummy wire. All systems were commissioned for clinical use and subject to regular quality assurance checks. The calculations were performed by, and the treatment delivered by, a board-certified medical physicist and radiation oncologist (American Board of Radiology). Figure 1 shows an example of the 20- and 10-Gy isodose lines superimposed on the fluoroscopic radiograph with the dummy wire in position.

Dental Extraction

All 10 rats underwent extraction of the left second mandibular molar 7 days after irradiation or sham irradiation. They were anesthetized by intraperitoneal injection with a solution of ketamine (100 mg/kg; Ketaset, Fort Dodge Animal Health), xylazine (20 mg/kg; Lloyd Laboratories), and acepromazine (3.3 mg/kg; Vedco) for induction and half-strength solution for maintenance anesthesia. At the end of the procedure, Buprenorphine SR LAB (0.6 mg/kg; ZooPharm, Fort Collins, Colorado) was administered subcutaneously for postoperative analgesia.

Extractions were accomplished with 2.5× surgical loupes to verify that the second molar was removed and that no fractures or remaining tooth roots occurred at the conclusion of the procedure.

Euthanasia

The rats were sacrificed 28 days after dental extraction, and the mandibles were completely resected and placed into 10% neutral buffered formalin (Sigma-Aldrich, St Louis,
Missouri). Standard protocol was followed by placing a single rat into an automated carbon dioxide chamber consisting of 4 stages:

**Stage 1**: Carbon dioxide flows into the chamber at a rate of 2.5 L per minute for 30 seconds.

**Stage 2**: Gas stops flowing, and a 40-second period is allowed for anesthetic effect of the gas to occur and labored breathing to begin.

**Stage 3**: Gas resumes flow for 1 minute at a rate of 10 L per minute. Breathing should stop by the end of this stage.

**Stage 4**: Gas stops flowing, and another 3-minute period is allowed to provide exposure of the gas for irreversible euthanasia. At this point, the lid is removed, and death is confirmed by physical examination.

**Radiographic Analysis**

The mandibles were mounted on a 360° rotating stage and scanned with a micro-CT scanner via an x-ray source with a tungsten anode and hafnium filter to produce 60-keV x-ray photons. The resulting 3-dimensional images consist of 20-µm cubic voxels and were analyzed with the Analyze 12.0 software package (Mayo Clinic College of Medicine, Rochester, Minnesota).

The teeth were segmented from the rest of the mandible and not used as part of the analysis. Dental volume was excluded from all measurements to ensure that measurement differences were not secondary to the presence or absence of teeth. Each mandible was split into hemimandibles, and a subregion of bone from each hemimandible around the molar sockets was measured for bone volume (Figure 2). This subregion extended from the anterior to posterior molar socket, with the mylohyoid groove as a marker to determine the depth. The volume measurements of the left hemimandible, which had the extraction, were normalized via the volume of the right hemimandible; then, averages of the normalized hemimandible volume and subregion volume were calculated.

**Histologic Analysis**

The 10 rat mandibles were embedded in methyl methacrylate and then sectioned in a sagittal plane with the Exakt cutting and grinding saw (Exakt Technologies Inc, Oklahoma City, Oklahoma). The specimens were cut to a thickness of 20 µm. The specimens were then stained with hematoxylin and eosin. Histologic analysis was performed by a dedicated head and neck pathologist (M.G.K.) using light microscopy (Olympus BX51 Research Microscope). The pathologist was blinded and unaware of the study groups being analyzed. Osteoclast and osteoblast cell counts were performed at 40×. The region of interest was standardized with the space between the first and second molars. The mean of 10 high-power fields was calculated for each specimen. Fibrosis was graded as mild (<33%), moderate (33%-66%), or severe (>66%) according to the percentage of fibrosis filling the extracted tooth socket between the first and third molars.

**Statistical Analysis**

Differences between the experimental and control groups were determined with a 2-tailed t test according to SPSS.
Results

Qualitative

All 10 rats survived the experiment period. Both groups of rats continued to gain weight throughout the study period. The experimental group had an increased weight gain of 65.8 g (SD, 45.0 g) compared to 44.3 g (SD, 28.9 g) for the control group. All experimental rats showed gross evidence of cutaneous changes consisting of dermatitis or shallow ulceration adjacent to the left mandible shortly after dental extractions, while none of the control rats developed ulceration. None of the wounds showed evidence of infection requiring treatment throughout the study period. There were no retained tooth roots, fractured teeth, or mandibular fractures after dental extractions.

Micro-CT Analysis

Three-dimensional computed tomography (CT) reconstructions were performed on each mandible. Normalized jaw region volume, jaw region CT value, region-of-interest volume, and region-of-interest CT value were then calculated on each rat mandible (Table 1). All 4 measurements were significantly lower in the irradiated rats as compared with the control group.

Histologic Analysis

The irradiated mandibles demonstrated a significant amount of fibrosis versus the control group (Table 1). The experimental group was found to have moderate (n = 4) to severe (n = 2) fibrosis, while the control group had mild (n = 3) to moderate (n = 1) fibrosis. There was also a significantly increased number of osteoclasts (P = .020) and decreased number of osteoblasts (P = .001) in the irradiated rats as compared with the control rats.

Discussion

ORN of the mandible secondary to irradiation of head and neck tumors can be a devastating condition. Patients often suffer from pain, chronic draining wound, fistula formation, decreased oral nutrition, and pathologic fractures. A typical presentation consists of head and neck irradiation, followed by dental extraction leading to ORN. There is little known of its pathophysiology; thus, treatment options remain limited. Treatment options include medical therapy, hyperbaric oxygen (HBO), and surgical resection.

Pharmacologic treatments of mandibular ORN have recently become accepted and consist of pentoxifylline and tocopherol. The addition of clodronate has led to the acronym PENTOCLO to describe the combination of medications. Preliminary results of PENTOCLO demonstrate the treatment to be safe, with improved mucosal healing and radiologic improvement and a reduction in the number of procedures. Delanian et al concluded in 2 phase II clinical trials that these medical therapies are safe and effective means of improving symptoms and decreasing bone exposure.10,11 However, in a study that did not include clodronate, 1 of 12 patients stopped treatment secondary to adverse effects. Three patients in this study ultimately failed treatment, requiring segmental mandibulectomy and bony free flap reconstruction.

The use of HBO in the treatment of mandibular ORN is widely used, but the effectiveness has recently been called into question.12,13 Annane et al performed a randomized control trial that showed no improvement with HBO. Their study was stopped early, secondary to HBO patients doing worse than the placebo group.14 In addition, a review of the literature showed low evidence to support the use of HBO.15

Surgical therapy remains the mainstay of treatment for advanced disease, especially with pathologic fracture of the mandible. Unfortunately, surgery typically entails a complex and multidisciplinary approach. Patients often require segmental mandibulectomy, especially if their ORN has

Table 1. Outcomes of 3-dimensional CT Reconstruction and Histology.

<table>
<thead>
<tr>
<th></th>
<th>Experimental Group: Rat No.</th>
<th>Control Group: Rat No.</th>
<th>P Valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 Mean ± SD</td>
<td>7 8 9 10 Mean ± SD</td>
<td>0.03</td>
</tr>
<tr>
<td>Jaw region</td>
<td></td>
<td></td>
<td>0.023</td>
</tr>
<tr>
<td>Volume</td>
<td>0.86 0.80 0.78 0.80 0.92 0.84 0.83 ± 0.05</td>
<td>0.95 0.99 1.02 0.92 0.97 ± 0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>CT value</td>
<td>0.85 0.78 0.75 0.78 0.96 0.82 0.82 ± 0.07</td>
<td>0.91 1.02 1.05 0.88 0.97 ± 0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>Region of interest</td>
<td></td>
<td></td>
<td>0.023</td>
</tr>
<tr>
<td>Volume</td>
<td>0.80 0.70 0.62 0.78 0.89 0.68 0.75 ± 0.09</td>
<td>0.86 1.03 1.03 0.95 0.96 ± 0.07</td>
<td>0.05</td>
</tr>
<tr>
<td>CT value</td>
<td>0.78 0.68 0.57 0.73 0.93 0.64 0.72 ± 0.12</td>
<td>0.81 1.07 1.06 0.90 0.96 ± 0.11</td>
<td>0.09</td>
</tr>
<tr>
<td>Fibrosisa</td>
<td>2 2 2 2 2 2 2.33 ± 0.52</td>
<td>2 1 1 1 1.25 ± 0.50</td>
<td>0.011</td>
</tr>
<tr>
<td>High-power field</td>
<td></td>
<td></td>
<td>0.005</td>
</tr>
<tr>
<td>Osteoblasts</td>
<td>5 3 7 9 11 8 7.2 ± 2.9</td>
<td>16 22 16 14 17 ± 3.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Osteoclasts</td>
<td>18 14 11 24 32 28 21.2 ± 8.2</td>
<td>8 14 6 5 8.3 ± 4.0</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Abbreviation: CT, computed tomography.

aScoring: 1 = mild, 2 = moderate, 3 = severe.
bBetween means.
resulted in pathologic fracture. This poses additional complexities regarding reconstruction, which is typically accomplished with composite free tissue transfer to restore the mandibular arch.

All these therapies have mixed results regarding effectiveness and morbidities. Therefore, we propose studying the application of mesenchymal stem cell therapy for the treatment of ORN. To successfully accomplish this, an athymic animal model is needed to reliably and reproducibly induce ORN of the mandible. Cohen et al laid the groundwork for an animal model for inducing ORN of the mandible. Their group at the University of California, Los Angeles, then standardized their protocols to improve upon the model to ensure its reproducibility to study the pathophysiology of ORN. Although their rodent model for the development of ORN has been established, it had yet been performed at our institution and had not been performed in athymic rats. The decreased immune function in these rats will allow for the study of human mesenchymal stem cell application in the ORN disease process. Therefore, we validated their method by performing a standardized protocol in our laboratory using 10 athymic nude rats.

The protocol was safe to the animals, as none died during the study period. All irradiated rats did suffer minor cutaneous changes, but none became infected or required topical antibiotic ointment. In addition, all rats did not fail to thrive, as they all gained weight during the study period.

Our protocol was standardized to eliminate sources of error and variation among the study subjects. Irradiation with a single 20-Gy insult was used to balance inducing ORN with animal safety. This dose has been shown to be effective in decreasing bone regeneration while minimizing animal morbidity. Other models have shown that higher doses are likely unnecessary for inducing ORN, while they may lead to increased animal mortality. Dental extractions 1 week after irradiation have been shown to be effective in producing ORN. Therefore, we extracted left second molars on all rats 7 days after irradiation and, too, found this to be reliable. Removing the middle molar has been shown to provide a consistent and reproducible landmark for further radiographic and histologic analysis. In our experiment, there were no fractured teeth or retained tooth roots, thus decreasing error in bone volume measurements.

At the conclusion of this experiment, ORN in an athymic rat model was reliably reproduced and was shown radiographically and histologically. Three-dimensional reconstructed CT showed a significant reduction in bone volume of the hemimandible of the radiated mandibles compared with the control group. There was also a significant reduction in volume of the standardized area of interest. Histologically, there was increased fibrosis as well as a quantifiable increase in osteoclasts and decrease in osteoblasts in the irradiated rats versus the control rats. These findings demonstrate reduction of bone in a region of interest that can be reproducibly measured in future studies.

Histologically, there was a significant difference in osteoclasts, osteoblasts, and fibrosis between the experimental and control groups. These histologic findings within a standardized anatomic landmark between the first and third left molars provide a reproducible area to study the cellular effects of ORN in the future with the application of stem cell therapy.

This model has several limitations. First, the small sample size of the experimental and control groups limits statistical analysis of the micro-CT and histlogic data. In addition, histologic analysis can be somewhat subjective. Therefore, the pathologist analyzing the slides was blinded and did not have knowledge of the treatment groups in effort to limit bias.

Conclusion

An athymic nude rat model can successfully and reliably produce ORN of the mandible in a systematic and measurable fashion. The current study validates prior rat models of ORN in an athymic rat model. Histologic and radiographic analyses both demonstrated decreased bone formation in those rats that were irradiated as opposed to the control group. This model will serve future research when an athymic rat is needed to study the effects of stem cell application in mandibular ORN.

Author Contributions

Ryan S. Jackson, conception and design, acquisition, analysis and interpretation of data, drafting, revising and approving the manuscript, and accountable for all aspects of the work; Stephen G. Voss, conception and design, acquisition of data, critically revising and approving the manuscript, and accountable for all aspects of the work; Zachary C. Wilson, conception and design, acquisition of data, critically revising and approving the manuscript, and accountable for all aspects of the work; Nicholas B. Remmes, conception and design, acquisition of data, critically revising and approving the manuscript, and accountable for all aspects of the work; Paul G. Stalboerger, conception and design, interpretation of data, critically revising and approving the manuscript, and accountable for all aspects of the work; Michael G. Keeney, conception and design, acquisition of data, critically revising and approving the manuscript, and accountable for all aspects of the work; Eric J. Moore, conception and design, interpretation of data, critically revising and approving the manuscript, and accountable for all aspects of the work; Jeffrey R. Janus, conception and design, interpretation of data, critically revising and approving the manuscript, and accountable for all aspects of the work.

Disclosures

Competing interests: None.
Sponsorships: None.
Funding source: Internal funding was provided by the Center for Regenerative Medicine, Mayo Clinic, Rochester, Minnesota.

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