Video Article

Retroductal Submandibular Gland Instillation and Localized Fractionated Irradiation in a Rat Model of Salivary Hypofunction

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Abstract

Normal tissues that lie within the portals of radiation are inadvertently damaged. Salivary glands are often injured during head and neck radiotherapy. Irreparable cell damage results in a chronic loss of salivary function that impairs basic oral activities, and increases the risk of oral infections and dental caries. Salivary hypofunction and its complications gravely impact a patient's comfort. Current symptomatic management of the condition is ineffective, and newer therapies to assuage the condition are needed.

Salivary glands are exocrine glands, which expel their secretions into the mouth via excretory ducts. Cannulation of these ducts provides direct access to the glands. Retroductal delivery of a contrast agent to major salivary glands is a routine out-patient procedure for diagnostic imaging. Using a similar procedure, localized treatment of the glands is feasible. However, performing this technique in preclinical studies with small animals poses unique challenges. In this study we describe the technique of retroductal administration in rat submandibular glands, a procedure that was refined in Dr. Bruce Baum's laboratory (NIH)[1], and lay out a procedure for local gland irradiation.

Video Link

The video component of this article can be found at https://www.jove.com/video/53785/

Introduction

Collateral destruction of healthy tissues accounts for a number of deleterious side-effects of cancer treatments. A part or whole of the major salivary glands that lie with the radiation fields are inevitably destroyed. Therefore, most patients undergoing radiotherapy for head and neck cancer, cervical lymphoma, or full-body radiation before bone marrow transplantation suffer one of the most common and persistent adverse effects of radiation, salivary gland hypofunction[2-6].

The fluid-producing acinar cells of the salivary glands are acutely sensitive to radiation. Damage to the salivary glands causes a drastic diminishing of salivary flow, a condition referred to as salivary hypofunction. The chronic reduction in salivary flow impairs key oral activities such as mastication, swallowing, speech, and taste, but the morbid sequelae of intense pain, mucosal tears, dysphagia, opportunistic infections, and dental caries worsens a patient's well-being and function[2,3].

Since radiotherapy-associated salivary cell loss is irreversible, there is no corrective treatment of xerostomia. Current treatment that focuses on assuaging symptoms with artificial salivary substitutes and prosecretory drugs is ineffective for long-term relief[6]. Although improved radiation delivery techniques have helped diminish the severity of the condition, normal tissue toxicity and its complications remain a limiting factor in cancer treatment[6,7]. Pre-emptive measures to prevent radiotherapy-associated complications are, therefore, becoming the norm. Radio-protective agents that scavenge free radical oxygen species, foster cell repopulation, or enhance DNA repair are being explored to avert salivary hypofunction [8-11].

Secretions of exocrine salivary glands drain into the mouth through the main excretory ducts. Intra-oral cannulation of the excretory ducts for injection of contrast agents is done routinely as an outpatient procedure. Utilizing a similar approach, salivary glands can be directly targeted for localized treatment[12]. Apart from reducing the risk of systemic side-effects, retroductal gland instillation has added benefits. The monolayer arrangement of salivary cells around the ductal tree allows targeting of all salivary epithelial cells, and the fibrous encapsulation of the gland acts as a barrier to reduce unwanted therapeutic spread. In essence, salivary glands are optimally suited for targeted treatment of gland afflictions such as radiation-induced salivary hypofunction.

Conventional radiation for cancer treatment is delivered in small doses (1.8 - 2.5 Gy/fraction/day, five days a week) for a period of weeks. Therefore, a radio-protective therapeutic that shows efficacy against a protracted radiation scheme in experimental models has greater clinical

bearing. Compromised salivary function after fractionated radiation has been recorded in small animals, but radiation source, dose fraction, and protocols used are varied[9,10,13].

This report establishes methods for retroductal delivery to and localized radiation of rat submandibular glands using patient-relevant radiation source and dose fraction.

Protocol

All procedures were approved by the LSU Health, Shreveport, Animal Care and Use Committee and were in accordance with the NIH guidelines for the care and use of laboratory animals.

1. Cannulation of Rat Submandibular Salivary Glands

1. **Preparation of Syringe-Tubing Assembly**
    1. Cut a 10 cm length of PE10 polyethylene tubing with a scalpel. Hold both ends of the tubing between the index finger and thumb. Heat the middle section of the tubing above a gentle flame, and gently stretch the softened tubing to double its length by pulling both sides.
    2. Cut the tubing in the middle with a scalpel at an angle of 45^{o} to get 2 cannulae - each with a tapered end. Expand the non-tapered end of the cannula by fitting it over a 29 G needle of 0.5 cc Insulin syringe.
    3. Remove the cannula, and draw 220 µl of saline solution into the syringe. Tap to dislodge air bubbles. Fit the cannula over the needle, and push the syringe plunger to expel air and ensure a free flow of solution through the cannula. Adjust the volume to 200 µl.

2. **Intra-Oral Cannulation of Submandibular Gland Duct**
   NOTE: Autoclave instruments prior to the procedure, and sterilize in-between procedures in a hot bead sterilizer.
    1. Weigh the Sprague Dawley rat, and dispense the calculated volume of ketamine (42 mg/kg)/ xylazine (8 mg/kg)/ acepromazine (1.4 mg/kg) mix in a hypodermic syringe.
    2. Restrain the animal by grasping the base of the tail between the index finger and the thumb of one hand and sliding the other hand over the body to grasp it. Rest the index and middle fingers along the sides of the head while hold the torso with the thumb and remaining fingers.
    3. Inject the anesthetic in the hind limb musculature. Confirm depth of anesthesia by toe pinch and palpebral reflex. Apply eye lubricant to prevent dryness while animal is under anesthesia.
    4. Place the animal on a specially-designed platform (**Figure 1**), and engage the upper incisor teeth on the transverse bar. Pull the lower jaw down by looping a rubber band around the lower incisors and anchoring it to the platform.
    5. Pass a sterile suture through the tongue and lift it up to raise the floor of the mouth. Clamp the sutures with a hemostat, and pass it over the transverse bar.
    6. Expand the cheeks with a custom-built cheek spreader (**Figure 1**), and under a dissecting microscope locate the sublingual papillae on the floor of the mouth.
    7. Grasp the tapered end of the preformed PE10 tubing using a delicate forceps. Gently manipulate the tip of the cannula into the ductal orifice on the sublingual papillae. Confirm placement of the cannula by threading it 3 - 5 mm into the duct; ensure that it passes without any obstruction.

3. **Submandibular Gland Instillation**
    1. Inject atropine (0.5 mg/kg) subcutaneously in the scruff of the neck, and wait 10 min for a reduction in salivary secretions.
    2. Secure the cannula to the duct orifice with a drop of cyanoacrylate (glue), and allow to dry. Instill the solution in the gland (200 µl/gland) by slowly pressing the syringe plunger at a rate of ~ 50 µl/min.
    3. Crush the tubing with a hemostat, and carefully remove the syringe. Retain the tubing in the duct for 30 - 60 min until the animal regains consciousness. Remove the suture that holds the tongue.
    4. Transfer the animal to a separate cage, and use a heat lamp to keep it warm during recovery. Do not leave the animal unattended until it has regained consciousness to maintain sternal recumbence.
    5. After the animal is fully ambulatory, house it at the vivarium with unrestricted access to food and water.

2. Localized Fractionated Irradiation of Submandibular Glands

1. Restrain the animal as described before, and anesthetize with intramuscular administration of ketamine (33 mg/kg) /xylazine (6 mg/kg)/ acepromazine (1 mg/kg) mix in the hind limb. Confirm depth of anesthesia, and apply lubricant to the eyes.
2. Place the animal supine on the linear accelerator tabletop, and extend its neck by tilting the head. Collimate the radiation field (3 cm slit width) to encompass the area from the lower border of the mandible to the top of the sternum.
3. Place a 1 cm tissue-equivalent bolus over the region, and adjust the distance between radiation source and top of the bolus to 100 cm.
4. Irradiate the animal (2.5 Gy) using a 6 MV photon beam of a linear accelerator. Dose rate, field size, and distance from radiation source to bolus surface will dictate the exposure time. In the current set up, animals were irradiated at a dose rate of ~ 1 Gy/min.
5. Repeat exposure; 2.5 Gy/day for a total of 8 days; 4 days/ week with a 2-day interval in between. Keep the animal warm during recovery. Transfer the animal to the vivarium after it is fully ambulatory.
6. Collect stimulated submandibular gland saliva 8 weeks after radiation to measure gland function[14]. Euthanize animals under anesthesia by cardiac perfusion of cold 4% paraformaldehyde/ phosphate buffered saline pH 7.2. Extirpate submandibular glands for histologic and immunohistochemical analyses[14].

Representative Results

Adapting a minimally invasive sialography technique, local treatment of major salivary glands is feasible. Retroductal administration in rat submandibular salivary glands was attempted by intra-oral cannulation of Wharton's ducts (**Figure 2**). The salivary ducts of Wharton open on the sub-lingual papillae located on the floor of the mouth, but the orifices are not readily visible. Insertion of the cannula was, therefore, done by gentle probing. To avoid untoward bleeding or duct perforation, no force was used while placing the cannula. Smooth resistance-free passage of the cannula in the duct was confirmed by gentle back and forth movement of the tubing.

Cannulation during initial experiments was confirmed by infusing the gland with hematoxylin or trypan blue solution, and evaluating gland staining in euthanized animals **(Figure 3**). Gland fill volume was ascertained by visualizing staining of the ductal tree in extirpated glands. For animals weighing 150 - 250 g, complete filling of the gland was achieved with 200 - 250 µl of solution. Rapid infusion can increase gland pressure and heighten the risk of injury and biospread. To avoid tissue destruction, the solution was administered gradually at a rate of ~ 50 µl/min

The rat submandibular glands are comparable to parotid glands in their sensitivity to radiation[15]. Submandibular glands were studied because they can be selectively irradiated without incurring severe off-target effects (**Figure 4**). Sparing of the oral cavity and a large volume of the parotid glands minimized the influence of oral mucositis and acute xerostomia on animal health and experimental outcome. Our earlier studies in rats were performed with single, large doses of radiation[11,16], but fractionated dosing is more pertinent to clinical practice. The neck region of the animal was, therefore, exposed daily to small doses of radiation. To achieve a uniform buildup of radiation dose within the submandibular glands located near the skin surface, a soft tissue-equivalent bolus made from a homogenous gel with a density of 1.03 g/cc was placed over the neck during radiation.

The effect of fractionated radiation scheme on salivary flow as determined at 8 weeks post-radiation show that daily exposure to increments of 2.5 Gy reduced salivary flow precipitously. A near 10-fold reduction in saliva output was recorded in irradiated animals in contrast to non-irradiated animals (2-sided Student's *t*-test, $p < 0.01$; **Figure 5**)[14]. The results confirmed the establishment of radiation-induced salivary hypofunction in animals.

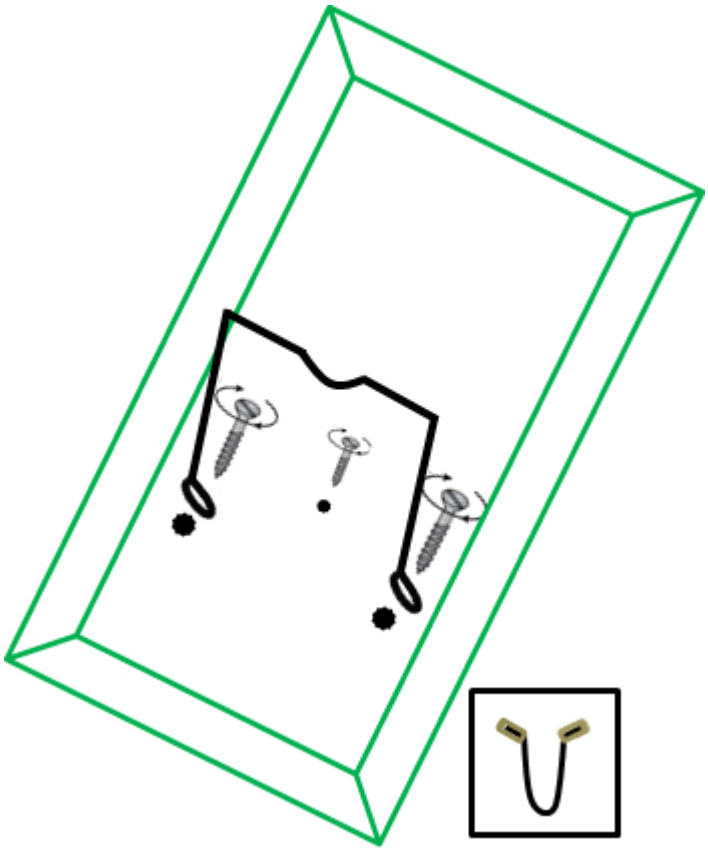

Figure 1. Construction of a Customized Platform and Cheek-Spreader. A stiff 4 mm wire was bent in a 3-sided rectangle (11 cm height X 13 cm width) as shown, and the transverse bar was bowed at the center. The ends of the wire were bent into loops, and screws were passed through them to secure the assembly to a plastic board (25 cm length x 15 cm width x 0.8 cm depth). A central screw was inserted 5 cm posterior to the wire assembly. It served to anchor the rubber band that pulled open the lower jaw. *Inset:* A custom-built cheek-spreader made of a disposable 1 mm thick wire bent in a "U shape" with flared ends. Plastic tubings were passed over the wire ends to prevent damage to the buccal mucosa. Please click here to view a larger version of this figure.

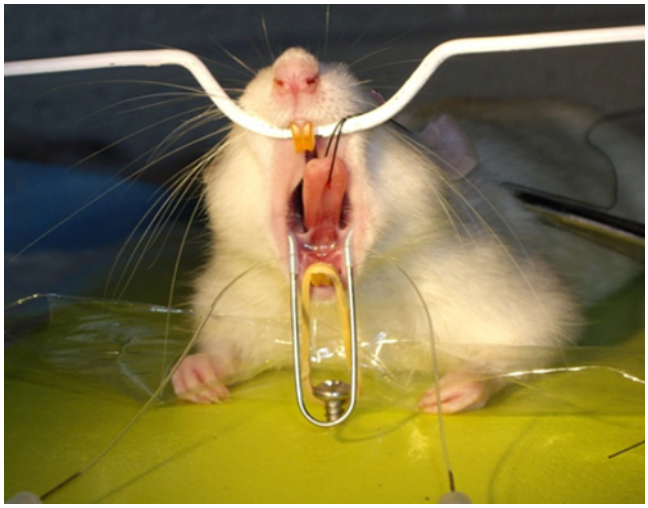

Figure 2. Cannulation of Rat Submandibular Gland Excretory Ducts. The tongue is raised to elevate the floor of the mouth, and polyethylene cannulae were inserted into the submandibular duct orifices located on the floor of the mouth. Please click here to view a larger version of this figure.

Figure 3. Infusion of Hematoxylin Solution in the Submandibular Gland to Confirm the Technique. Gland staining attests to the successful administration of the solution in the submandibular gland. Note that the sublingual gland in the upper left corner is not stained. A non-infused control gland is shown on the left. Please click here to view a larger version of this figure.

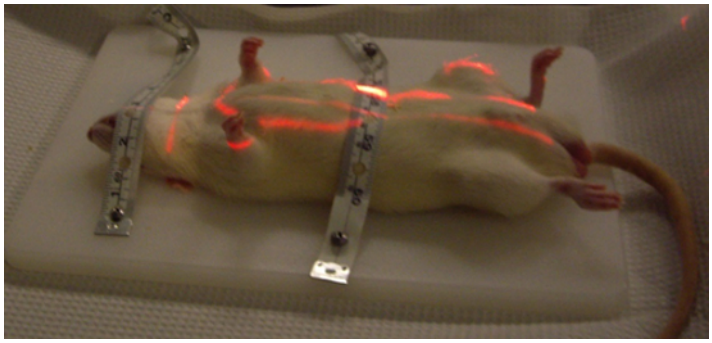

Figure 4. Placement of the Animal for Irradiation. The animal is laid supine on the linear accelerator table, and the head titled back to extend the neck. The collimated slit width was 3 cm. Please click here to view a larger version of this figure.

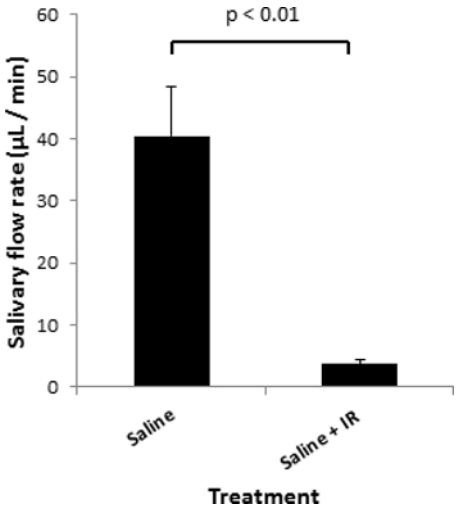

Figure 5. Effect of Fractionated Radiation on Submandibular Gland Function. Saline was instilled in the submandibular glands before radiation. The neck of the animal was irradiated in fractions of 2.5 Gy/day for 8 days. The data shown are mean ± SEM of stimulated submandibular salivary flow rate 8 weeks after completion of radiation scheme. The figure is reproduced with copyright permission from Human Gene Therapy, Mary Ann Liebert, Inc. publisher[15]. Please click here to view a larger version of this figure.

Discussion

Salivary glands often receive radiation doses beyond the threshold of tissue recovery in patients undergoing radiotherapy for head neck cancer, elective ablation of neck nodes, or regional hematologic malignancies. Although the fluid-secreting acinar cells of the gland are terminally differentiated, they are paradoxically sensitive to radiation. The secretory function drops within the first weeks of radiation, and irreversible gland damage results in a chronic low saliva output. To combat poor gland function and oral dryness that ensues, the preservation or restoration of secretory function is crucial. Novel therapies to protect against or repair radiation damage are being investigated[1, 8-11, 16-20].

Systemic administration of a therapeutic carries the inherent risk of unwanted effects in non-targeted tissues. Aimed delivery to the salivary glands diminishes this risk. Retroductal gene therapy is effective at preventing or remedying radiation-associated salivary gland affliction[1, 9-, 11, 16,17,20]. However, using the same technique, salivary glands can also be reengineered to express and secrete proteins, ectopically. Exploiting the basolateral endocrine secretory pathway in salivary cells, transgene expression and hormone secretion into the blood can be achieved[21]. Gene transfer to salivary glands can, therefore, be effectively extended to the correction of certain endocrine disorders.

Although cannulation of salivary gland ducts in small animals can be tricky, practice is central to honing the technique. The tapered end of the cannulae can kink easily during insertion, and make infusion difficult. Increased back-pressure while expelling the solution should forewarn of a bend in the tubing, and the necessity to re-cannulate the duct. Anatomical variations such as aberrant duct orifice or increased duct tortuosity, along with pathologies that limit mouth opening like trismus or submucous fibrosis can make submandibular gland delivery a challenge. Infusion of an impermeant dye when practicing the technique can be a useful indicator of precise delivery. Proper placement of the cannulae and gentle, resistance-free infusion are important factors for the success of the procedure.

Cancer patients are often treated with megavoltage photons generated by a linear accelerator. Radiation is delivered daily in small doses over a course of weeks. Dose fractionation provides periods of recovery between radiations to reduce toxicity on healthy tissues and sensitize tumors. Highly penetrating energy beams of a linear accelerator are useful for the treatment of deep-seated tumors. And, for treatment of tumors closer to the skin, a tissue-equivalent bolus is used to reduce the depth of photon penetration. Similar to clinical practice, a linear accelerator delivered fractionated radiation can be used to locally irradiate submandibular glands of small animals. However, the position of the glands immediately beneath the skin necessitates the placement of a bolus to achieve > 90% radiation dose within the glands. The thickness of the bolus is calculated depending on the energy of photons and the desired depth of tissue penetration.

In this article, we provide experimental guidelines for performing retroductal submandibular gland instillation and local fractionated irradiation of rat submandibular glands. It is our hope that the highlighted methods will aid experimental setup for investigators venturing into the field of salivary gland radiotoxicity.

Disclosures

The authors have nothing to disclose.

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