The effect of radiotherapy in aminoglycoside ototoxicity and noise induced hearing loss

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Table of Contents

List of abbreviations

ABR - Auditory Brainstem Response
C – Celcius
CAP - Compound Action Potential
d - day(s)
dB - decibels
DNA - Deoxyribonucleic Acid
DPOAE - Distortion Products Otoacoustic Emissions
DSB - Double Strand DNA Breaks
FRT - Fractionated Radiotherapy
GP - Guinea pig
Gy - Gray
HC - Hair Cell
HF - High Frequency
Hz – Hertz
IHC – Inner Hair Cell
im - intramuscular
kg - kilograms
kHz – Kilohertz
mg – miligrams
mins - minutes
NIHL - Noise Induced Hearing Loss
NISHL - Noise Induced Sensorineural Hearing Loss
NPC - Nasopharyngeal Carcinoma
OHC – Outer Hair Cell
RISNHL - Radiation Induced Sensorineural Hearing Loss
ROS - Reactive Oxygen Species
RT- Radiotherapy
sc - subcutaneous
SNHL - Sensorineural Hearing Loss
SPL - Sound Pressure Level
TS - Threshold Shift

Abstract

Background: Radiotherapy remains a vital component in treatment protocols for patients with head and neck cancers. While some radiation-induced complications are transient, sensorineural hearing loss is characterized as late and permanent. To date there are no studies assessing the effect of fractionated radiotherapy on sensorineural hearing loss using an animal model. Moreover, the combined effect of radiotherapy and other hearing stressors such as aminoglycoside ototoxicity or acoustic trauma have not been delineated.

Objective: The purpose of the present thesis is to characterize the relationship between radiotherapy dosage and the time course of possible hearing loss, to examine the relationship between radiotherapy and Gentamicin induced ototoxicity and to examine the relationship between radiotherapy and noise induced hearing loss.

Methods: First, a unilateral fractionated radiotherapy scheme was used to examine the effect radiotherapy in an *in vivo* animal model. Second, a 10-day Gentamicin treatment was administered subcutaneously (40 mg/kg/day or 80 mg/kg/day). Third, Animals were exposed to acoustic trauma by a continuous pure tone of 6 kHz at 120 dB SPL for 60 minutes bilaterally. Outcome measures included auditory brainstem responses assessed at baseline, throughout and after treatments, distortion of production of otoacoustic emission and post-mortem cochlear morphology using light and scanning electron microscopy.

Results: Radiation induced sensorineural hearing loss is permanent, dose dependent and caused by an initial sensorial damage followed by an important neural component primarily responsible for its progression. A syngersitic effect was identified with both low and high doses of Gentamicin and radiotherapy. Ears subjected to radiation did not show a delay in recovery after the noise exposure. However, morphology results of cochleae suggested synergistic damage to auditory hair cells that were not detected by auditory testing.

Conclusion: The results of this thesis prove that previous exposure to radiotherapy in the temporal bone region can develop hearing loss long after the end of treatment, therefore long term follow ups in clinical and audiology settings are strongly recommended. Furthermore, cochleae previously exposed to radiotherapy are more susceptible to aminoglycoside ototoxicity and the damages associated with acoustic trauma. This raises an important concern for aminoglycoside treatments of patients with previous radiation exposure and highlights the

importance of protecting the ears of these patients to future potential stressors such as acoustic trauma.

Résumé

Avant-propos: La radiothérapie demeure un composant majeur dans les protocoles de traitements chez les patients atteints de cancers au niveau de la tête et du cou. Alors que la plupart des complications causées par la radiothérapie sont temporaires, la perte d'audition sensorielle elle, est tardive et définitive. À ce jour, aucune étude n'a abordé les effets de la radiothérapie fractionnelle sur la perte de l'ouïe sensorielle sur un modèle animal. De plus, la combinaison des effets qu'engendrent la radiothérapie et d'autres stimulis auditifs tels que l'ototoxicité des aminoglycosides ou des traumatismes acoustiques n'a pas été reportée dans la littérature scientifique.

Objectif : L'objectif de cette thèse vise à définir la relation entre différents dosages radiothérapeutiques et le lapse de temps pouvant s'écouler avant une éventuelle perte d'audition. Aussi, cette thèse examinera la corrélation entre la radiothérapie et l'ototoxicité provoquée par Gentamicine en plus d'examiner le lien entre la radiothérapie et la perte d'ouïe provoquée par quelconque traumatisme sonore.

Méthodologie : D'abord, un traitement unilatéral radiothérapeutique en fractions a été utilisé pour examiner les effets de la radiothérapie sur un model animal. En second lieu, durant 10 jours consécutifs, un traitement sous-cutané de Gentamicine (40 mg/kg/jr or 80 mg/kg/jr) a été administré au cobaye. Enfin, les animaux ont été exposés à un traumatisme sonore entrainé par un son continuel de 6 kHz à 120 dB SPL pour 60 minutes bilatéral. Les mesures d'évaluation des résultats étaient calculées selon les réponses auditives du tronc cérébral mesurées au début, pendant et à la fin des traitements, la distorsion de production d'émission otoacoustique, post-mortem morphologie cochléaire utilisant la microscopie optique ainsi que la microscopie électronique à balayage.

Résultats : La perte auditive causée par la radiothérapie est permanente. Le dégrée de perte auditive varie selon le dosage administré. Elle résulte d'un traumatisme au niveau sensoriel de l'ouïe. Les dommages engendrés par ce traumatisme évoluent ensuite au niveau cérébral, ce dernier étant responsable de sa progression. Un effet de synergie combinant la radiothérapie et la Gentamicine à forte et faible dose a été identifié. Les oreilles exposées à la radiothérapie n'ont pas démontré un délai de reprise des fonctions auditives suite à un traumatisme sonore. Par contre, les résultats histologiques des cochlées révèlent un dommage des cellules ciliées auditives non détecté par les testes auditifs.

Conclusion : Les résultats provenant de cette thèse prouvent qu'avoir été exposé à des traitements de radiothérapie au niveau de la tête et du cou peuvent entrainer des déficiences auditives, et ce, longtemps après la fin des traitements. Pour ce, des suivis en clinique et en audiologie à long terme sont fort recommandés. De plus, les cochlées exposées à la radiothérapie sont plus susceptibles à l'ototoxicité causé par des aminoglycosides ainsi que les traumatismes sonores.

Preface

Contributions of authors

Aren Bezdjian performed the literature review, animal experiments, data collection, and the analysis of all three manuscripts included in this thesis. Aren Bezdjian, Dr. Mario Mujica-Mota and Dr. Sam Daniel contributed to the designing of the experiments cooperatively and participated in the writing of the first two manuscripts. Aren Bezdjian and Dr. Sam Daniel contributed to the designing of the experiments cooperatively and participated in the writing of the third manuscript. Dr. Slobodan Devic conducted the dosimetric evaluation of the radiotherapy model used in this thesis, provided relevance expertise and helped with the writing of the first manuscript. All studies were conducted in animal models at the McGill Auditory Sciences Laboratory located at the Research Institute of the Montreal Children's Hospital of the McGill University Health Centre and were funded by a CIHR operating grant to SJ Daniel and a FRSQ Master's training grant to Aren Bezdjian. Result interpretation and final approval of the manuscript were done by Aren Bezdjian and Dr. Sam Daniel. Dr. Jean Pierre Farmer provided supervision and guidance for the third manuscript. Aren Bezdjian and Dr. Walter Marcantoni conducted the statistical analysis for all three studies. Stephanie Fay Lenhart participated in the data collection and analysis of the third manuscript.

Claims of originality

The present thesis yielded new knowledge by **(1) developing an animal model that allows studying changes in hearing following fractionated radiotherapy protocols simulating those used clinically**. This model served as a basis for the next two studies, which in turn, shed light on the predisposing effects of RT on the hearing structure. The first study described in Chapter 3 illustrated the relationship between RT dosage and the time course that lead to hearing loss. The outcomes revealed (A) that low-dose RT, analogous to clinical low dose therapy does not cause hearing loss. (B) High-dose RT (analogous to clinic high dose therapy) caused both immediate and long term hearing loss. (C) The initial hearing loss observed after RT treatment is likely associated with cochlear sensory cell damage (resulting from auditory hair cells), while the longer term progressive hearing loss observed is likely associated with neural damage (e.g. primary neuron; supporting ganglion cells).

This thesis **(2) showed that aminoglycoside ototoxicity was intensified by radiotherapy at both high and safe low doses**. The second study discussed in Chapter 4 examined the relationship between RT, Gentamicin antibiotic dosage and hearing loss. The experiments showed that (A) low dose Gentamicin therapy (analogous to clinical low dose therapy) did not cause hearing loss, (B) high dose Gentamicin therapy (analogous to clinical high dose therapy) caused hearing loss. The latter two findings were expected as they reported in previous literature. The study revealed (C) a synergistic effect clearly identified by hearing tests and cochlear histology in high dose Gentamicin and RT exposure. Finally, cochlear histology demonstrated the same synergistic effect in the cochleae exposed to low dose Gentamicin and safe dose of RT.

Third, this thesis **(3) showed that acoustic overstimulation following low-dose radiotherapy exposure could lead to hearing-loss.** The last study, presented in Chapter 5, examined the relationship between low doses of RT and acoustic trauma. It primarily looked at the recovery and/or progressivity of hearing abilities following acoustic overstimulation in cochleae exposed to RT as compared to cochleae that were not exposed to RT. The study revealed by the means of cochlear histology that low doses of RT could potentiate auditory hair cell loss post acoustic trauma.

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Dr. Sam Daniel. Without his sustained guidance, my research projects at the McGill Auditory Sciences Laboratory would not have been possible. I thank Dr. Bernard Segal for chairing my thesis committee meeting and for his critical review throughout my time as a master's student in the department of Otolaryngology at McGill University. I am also extremely grateful to my good friend, colleague and mentor Dr. Mario A. Mujica-Mota who has made significant contributions to the work leading to this thesis. I would like to express my gratitude to Dr. Jean-Pierre Farmer and Dr. Slobodan Devic who have acted as my co-supervisors.

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Above all, I want to express my deep gratitude to my parents and siblings, Alex and Sarine, for their infinite support that has helped me overcome many obstacles in life, particularly my father who has always been my source of motivation.

Disclosure

All images used for the purpose of this thesis are either produced by the McGill Auditory Sciences Laboratory or obtained with permission through ELSEVIER's copyright clearing center.

Part of the experiments discussed in this thesis has been presented at the American Society of Pediatric Otolaryngology in Las Vegas, NV in May 2014, at the 4th HBW Pediatric Surgical Research Day in Montreal in June 2014 and at the Montreal Children's Hospital Annual Research Day, Montreal in May 2013. The first manuscript (Chapter 2) included in this thesis was published in the Laryngoscope issue: 124(10) pages 418-424 on December 2014. The second manuscript (Chapter 3) is published in the American Journal of Otolaryngology Head and Neck Surgery on March 2015 currently available online.

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CHAPTER ONE: Introduction

1.1 Rationale

Recent advancements in detection of cancers and improved treatment modalities have contributed to an increased number of Canadian cancer survivors (62 % and 82 % in adults and children, respectively)[1]. Thus, long-term effects of oncology regimens are being evaluated in order to improve overall outcomes. In Canada only, over 4000 patients develop various Head and Neck cancers every year[2]. The Canadian Cancer Society reports hearing loss as one of the major long-term complications resulting from cancer treatments alongside chronic pain, cystitis, fatigue, organ damage and risk of developing other cancers. A recent retrospective chart review from the Montreal Children's and Sainte Justine hospitals evaluated the incidence of platinum induced ototoxicity in over four hundred pediatric patients in Quebec and found ototoxicity to be a major concern even long after completion of chemotherapy[3].

Radiotherapy (RT) remains a vital component in treatment protocols for patients with head and neck cancers. While some radiation-induced complications are transient, sensorineural hearing loss (SNHL) is a late and permanent complication[4]. RT for cancers such as parotid carcinoma, nasopharyngeal carcinoma, medulloblastoma or neuroblastoma often reaches the nearest cochlea[5]. It is estimated that 30% percent of patients whose inner ear is included within the radiation field present with SNHL[4].

Histopathological evidences suggests radiation-induced sensorineural hearing loss (RISNHL) is believed to result from damage to the auditory hair cells of the cochlea by means of apoptosis, more specifically by the generation of reactive oxygen species (ROS) triggering mechanisms. This is concordant with the underlying mechanisms behind sensorineural hearing loss involved in aging, post acoustic trauma and drug toxicity[6].

It is important to highlight that most of the studies conducted thus far on RISNHL are based on single dose animal studies, which do not resemble the fractionated radiation schemes used in clinical scenarios. Only a few authors have performed hearing assessments in animal studies using fractionated radiation over several weeks following similar schemes given to human patients[7,8]. Therefore, the McGill Auditory Sciences Lab alongside the Medical Physics Unit at McGill University and the department of Radiation-Oncology at the Jewish General Hospital established a radiation induction scheme with a radiochromic film dosimetry method

allowing for accurate dosimetric assessment of the radiation field in an animal model of unilateral cochlear irradiation. The RT model is validated and provides important insight on RISNHL giving in small fractions similarly to those seen in clinical cancer treatment protocols[9].

In view of the above there is a need to delineate the effect of fractionated R schemes on hearing abilities over time. This will provide important insights on the progressivity and/or recovery of hearing functions post RT that would in turn advocate adequate follow up timelines in the clinical settings. Furthermore, the potentiating effect of RT on hearing with other hearing stressors such as antibiotic ototoxicity or acoustic overstimulation is yet to be identified. This formed the rationale of this thesis. The resulting objectives of this thesis are given in the next section (1.2).

1.2 Objectives & hypothesis

The overall objective of the present thesis is to elucidate the effect of radiation induced hearing loss after fractionated RT similar to clinical schemes in an *in vivo* animal model.

More precisely, study 1 characterizes the relationship between RT dosage and the time course of possible hearing loss. The objective of the previous study is clearly stated in Chapter 3 of this thesis. Study 2 examines the relationship between RT and Gentamicin induced ototoxicity and study 3 looks at the interaction between radiation exposure and noise induced hearing loss. The previous two study objectives are more explicitly reported in the Chapters 4 and 5 of this thesis, respectfully.

The null hypothesis (N_0) for all three studies discussed in the present thesis was that radiation exposure (radiated ears) would show greater and more permanent hearing loss when compared to the control (non-radiated) ears.

CHAPTER TWO: Background & Literature review

2.1 Anatomy & Physiology of the Human Auditory System

The hearing tests performed in the experiments leading to this thesis will test the auditory pathway. Therefore, an overview of the anatomy and physiology of the auditory system will be presented in this chapter.

2.1.1 Auditory System

The auditory system is broadly divided into two parts: the peripheral auditory system and the central auditory system. The peripheral system includes the external, middle and inner ear, while the central system comprises the auditory brainstem (cochlear nuclei, trapezoid body, superior olivary complex and lateral lemniscus), the midbrain (the inferior colliculi); the thalamus (the medial geniculate nucleus) and the auditory part of the cerebral cortex. Figure 1 show the different parts of the peripheral auditory system. The principle function of the auditory system is to convert acoustic energy into neural stimuli, which are then transmitted to the brain for processing.

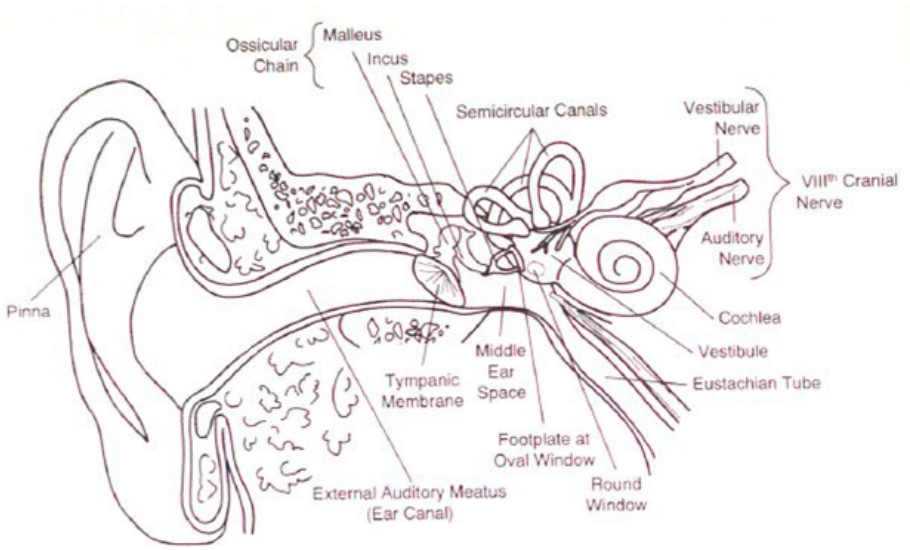

Figure 1. Diagram of the human ear. Adapted from Gelfand, 2009. [10]

2.1.2 External Ear

The external ear consists of the visible part of the ear; the auricle (pinna) and the external auditory canal. The auricle made of elastic cartilage is attached to the head by muscles and ligaments. The deep central portion of the pinna known as the concha, leads into the external

auditory canal, which in turn leads to the tympanic membrane (TM). The external ear is separated from the middle ear by the TM, a thin translucent oval membrane, which itself forms the lateral boundary of the middle ear. In sum, the function of the external ear is to capture acoustic stimuli and direct sound waves towards the TM.

2.1.3 Middle Ear

The human middle ear is also called the tympanic cavity. The tympanic cavity is an air-filled space in the temporal bone. The middle ear houses the three smallest bones of the human body named ossicles; the malleus (hammer), incus (anvil) and stapes (stirrup). The acoustic energy transmitted from the external ear is passed on through the chain formed by the three middle ear ossicles, which in turn, amplify the energy obtained at the tympanic membrane in the footplate of the stapes[11]. The stapes transmits sound to the oval window of the cochlea. Figure 2 shows the middle-ear space of a human.

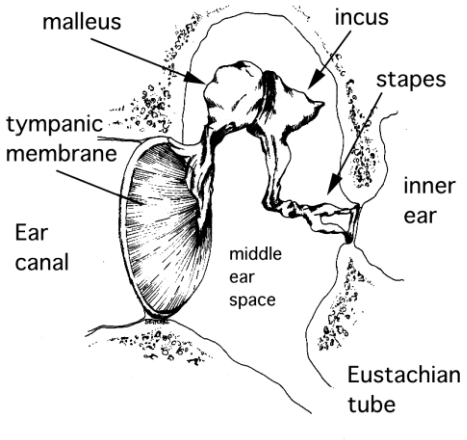

Figure 2. Middle ear showing the tympanic membrane and ossicles
[http://healthfavo.com/inner-ear-bones.html]

2.1.4 Inner Ear

The inner ear serves as the basis of the auditory and vestibular systems. The snail shaped cochlea enclosed in the temporal bone is referred to as the main hearing organ. The cochlea consists of three fluid-filled sections coiled in two and a half turns[11]. The inner duct containing the sensory epithelium is also referred to as the scala media. This later divides the outer duct into the scala vestibuli superiorly and scala tympani inferiorly (Figure 3). The scala vestibuli and scala tympani are filled with perilymph, which resembles extracellular fluid; containing low

concentrations of K+ and high concentrations of Na+. Contrarily, the scala media contains endolymph, which resembles intracellular fluid; containing high concentrations of K+ and low concentrations of Na+[10,12].

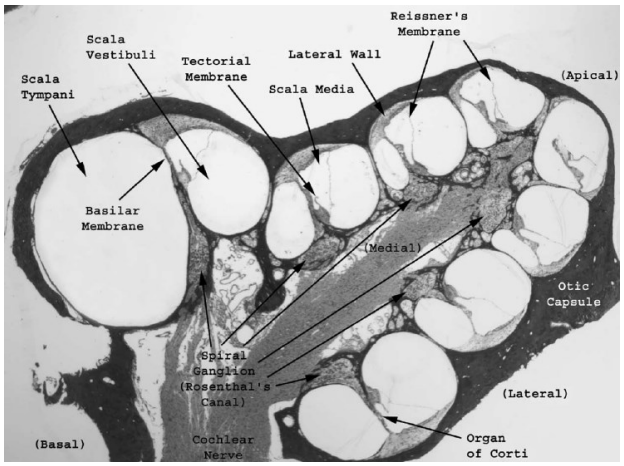

Figure 3. Light micrograph of a cross-section of the guinea pig cochlea. Raphael and Altschuler, 2003[13].

Sound energy enters the cochlea via the stapes bone at the oval window. The scala vestibuli in the basal end of the oval window is the place where the sound-induced vibrations are transmitted to the cochlear fluids. This creates a motion in the basilar membrane, creating a traveling wave that goes from the base of the cochlea to the apex. Each location on the basilar membrane is tuned to a specific frequency. Low frequency stimuli cause more vibration at the apex, while high-frequency stimuli cause more vibration at the base of the basilar membrane. This tonotopy is maintained throughout the auditory pathway[14].

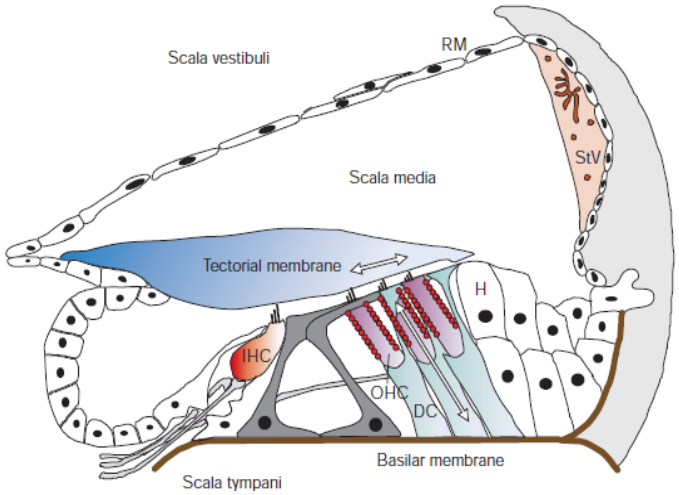

FIGURE 4 A Schematic Diagram of the Organ of Corti [15].

2.1.5 Auditory Hair Cells

Auditory hair cells are classified into two categories; inner hair cells (IHC) arranged as a single row medially and three rows of outer hair cells (OHC) laterally as seen in Figure 5. They are called hair cells because they have tufts of stereocilia (also called hair bundles) projecting from their surfaces. Furthermore, a thin membrane attached over the stereocilia of the hair cells called tectorial membrane follows the movement after the sound-induced vibrations reach the cochlea[11]. This arrangement allows the proper transmission of mechanical energy to hair cells with every acoustically transmitted vibration into the cochlear fluids.

The auditory hair cells located in the organ of Corti act as transducers through their stereocilia, converting the sound-induced vibrations into electrical activity. The mechanical process of the basilar membrane creates a force in the stereocilia of auditory hair cells that allows the opening of sensitive mechanoelectrical transduction channels. This, in turn, promotes depolarization of spiral ganglion neurons (SGN) through the opening of potassium channels[14,16]. This change in the resting membrane potential forms a synapse with a dendrite from a SGN. The axons of the SGNs form the auditory nerve, which exits the cochlea and temporal bone through the internal auditory meatus, transmitting neural stimuli to the auditory cortex of the brain. Finally, there, at the neural level, the stimuli are processed into sound[10,12].

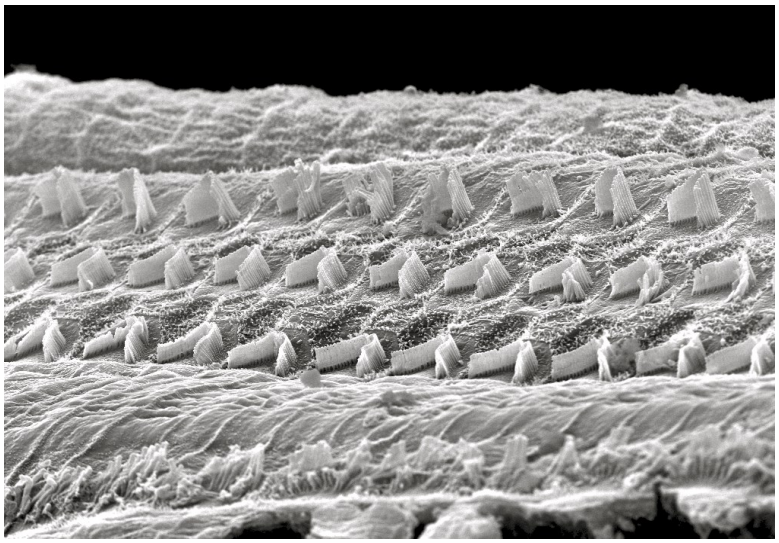

Figure 5. Inner hair cells (IHC) arranged as a single row of inner hair cells medially and three rows of outer hair cells (OHC) laterally. McGill Auditory Sciences Laboratory.

2.1.6 Central Auditory Pathway

The main relays of the pathway serve as an anatomical basis for the Auditory Brainstem Response (ABR), which serves as the primary hearing test of all experiments discussed later in this thesis. The central auditory pathway is demarcated by the cochlear nucleus and the auditory cortex in the temporal lobe and is responsible for the integration of sound stimuli as a whole[17].

While the auditory hair cells in the cochlea are the main signal transducers for sound stimuli, the central auditory pathway integrates the information to elicit a response to sounds. Figure 6 shows the neuroanatomical pathways in the central auditory system, which begins with the auditory nerve fibres travelling from the cochlea to the brain. In sum, neurons of the auditory nerve make the first synaptic connection at the cochlear nucleus located in the dorsolateral side of the brainstem. The axons of neurons from the cochlear nuclei proceed to the superior olivary nuclei complex in the medulla. The neuronal axons proceed to the inferior colliculus in the midbrain, which contains neurons with sharply defined frequency sensitivity, similarly to the cochlea[18]. The outputs are then sent to the medial geniculate body also referred to as the auditory thalamus from where they are finally sent to the auditory cortex[17].

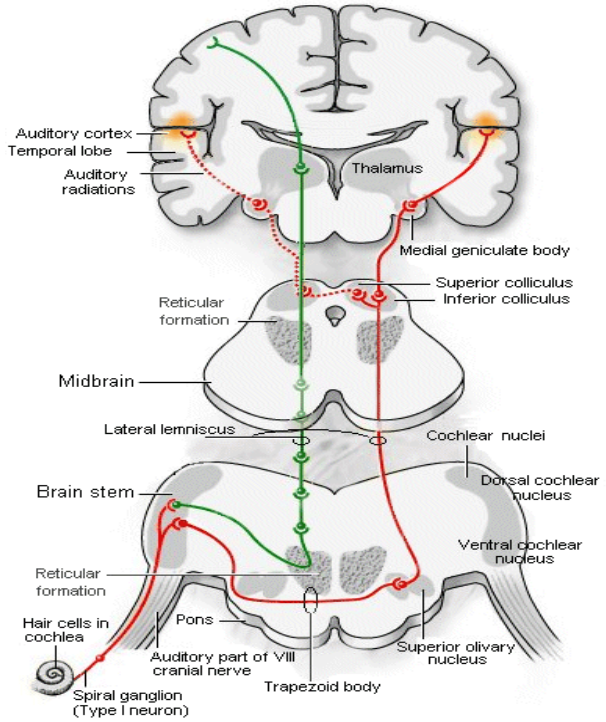

Figure 6. Neuroanatomical pathways in the central auditory system [http://firstyears.org/anatomy/ear.htm]

2.2 Radiotherapy and Radiation Induced Hearing Loss

The principle topic of this thesis revolves around the predisposing effect of RT on hearing loss. Therefore the following chapter will briefly discuss the principles of radiobiology; the concept of fractionation and radiation induced hearing loss in an attempt to give a general overview of how RT works. The unit of dose used for RT is the Gray (Gy), representing the energy absorption of 1 Joule per kilogram (1Gy = 100cGy; 1Gy = 100 rad) (30).

2.2.1 Principles of Radiobiology

RT produces ionizations occurring when the radiation has enough energy to eject electrons from the molecules present in the irradiated tissue or material[19]. X-ray RT is obtained from linear accelerators that increase the energy levels of electrons forming a target resulting in a focused beam of photons[20]. As outlined by Sharma et al., ionizing radiation acts by a complex called "Compton effect". This is where the original ejected electrons in the target tissue also interact with surrounding tissue propagating the ionization until the energy dissipates. The ionization can damage the deoxyribonucleic acid (DNA) by changing the underlying structures within or by the subsequent free radical formation that causes indirect damage to surrounding molecules[20]. This later is supported by studies demonstrating the formation of ROS before DNA damage in cells subjected to radiation[21], suggesting free radicals are initiators of the observed cell damage. An important factor to consider is the attenuation that these energies depending on the density of the tissue irradiated[22]. This concept is the basis of tissue depth-dependent dose absorption used in therapeutic RT.

The most common chromosomal lesions after irradiation are double strand DNA breaks (DSB)[19]. Since single strand DNA breaks can be easily repaired with the opposite DNA strand, DSB attenuates the reproducibility of cells and causes apoptosis, mutation or carcinogenesis[23]. This mechanism explains why highly mitotic cells with greater rates of DNA synthesis such as auditory hair cells are more sensitive to RT[24].

2.2.2 Fractionation of radiotherapy

A groundbreaking research demonstrated that animal sterilization was possible by RT without damage to the skin. This was done by exposing the animal to daily small

doses instead of single large dose of radiation[25]. Hence, the concept of fractionation was born. This innovative RT technique extended the possibilities of dose delivery from a single dose, to one dose a day (fractionated) or twice a day (hyperfractionated) given over several weeks[26]. The main advantage of fractionation RT is the sparing of normal tissues.

The effects of fractionation are explained in terms of radiobiological principles, which have been identified as the four R's of radiobiology[26]. First, the concept of "repair" was illustrated in an experiment of testicular sterilization showing that fractionation provided a decreased skin damage after small fractions were delivered instead of a single total dose[25]. Second, the principle of "reassortment" refers to the radiosensitivity of cells according to their stage and speed in the cell cycle. As mentioned, cells with high mitotic activity are more sensitive than resting cells[27]. Therefore, organs that depend on the reproduction of the cells such as epithelial organs present early outcomes when compared to organs with low cellular reproduction such as auditory hair cells. Third, "repopulation" is a compensatory proliferation occurring after fractionated RT when early reacting tissues start repopulation after 2 to 4 weeks after the beginning of RT, while late-reacting tissues have minimal proliferation, which implies that the approximated time required for observable damage is tissue-dependent[19]. Last, the concept of "reoxygenation" states that tumors have radioresistant compartments of oxygenated and hypoxic cells[27]. Thus, tumors that re-oxygenate efficiently after every fraction are more sensitive to RT due to the damage-enhancer effect of oxygen in these previously hypoxic cells[19].

These four concepts explain the basis of fractionated RT in clinical practice, demonstrating that fractionation is not only effective sparing normal tissue but also enhancing cancer treatment because of the principle of reoxygenation.

2.2.3 Radiation Induced Hearing Loss

RT remains a vital component in treatment protocols for patients with head and neck cancers. Unfortunately, hearing loss is an adverse effect of RT particularly in patients with head and neck cancer, where sometimes large dose delivered to the primary tumor is received by the cochlea. RT can be used in cancer with different purposes such as curative therapy (alone), combined with chemotherapy or following surgical resection of tumors[26]. In general, the radiation dose for early head and neck cancer ranges from 66 to 72 Gy with a 1.8 to 2.0 daily dose[28]. Clinical scenarios where RT delivers high doses

of radiation to the auditory system include stereotactic radiosurgery for vestibular schwanomas and external beam RT for nasopharyngeal carcinomas, paranasal sinus tumors or parotid tumors[4]. Furthermore, brain tumors such as neuroblastomas or medulloblastomas are also treated with focal radiation or whole brain irradiation increase the risk of damage to the ear[29]. About one third of the patients subjected to RT for head and neck cancer present with this adverse effect, which has been characterized as dose-dependent, late, progressive and permanent[30]. It usually affects the high frequencies of the hearing range and progresses toward lower frequencies when it becomes perceptible by the patient. Despite the advancement in medical technology, these clinical scenarios still contain an important risk of hearing loss when patients are subjected to RT.

While complications in the external and middle ear can cause transient hearing loss, RISNHL can be permanent. Total doses delivered to the inner ear starting from 35 to 40 Gy are known to cause RISNHL for fractionated RT[31], affecting initially the high frequency range[32]. RISNHL is believed to be a result of damage of the auditory hair cells of the cochlea. Apoptosis has been demonstrated in cochlear cell systems and is generally accepted as an important mechanism of radiation induced cell death in vivo. RT exerts its effects through the generation of reactive oxygen species mainly at the mitochondria [33,34], which is believed to be a triggering factor in the apoptotic process.

2.3 Noise Induced Hearing Loss

Noise induced hearing loss (NIHL) is an increasingly prevalent disorder that results from exposure to high-intensity sound, especially over a long period of time. Noise is the cause of approximately half of all cases of hearing loss, causing some degree of problems in 5% of the population globally[35]. NIHL is usually permanent. Common sources of harmful noise damaging cochlear hair cells include music, children's toys, transportation, recreational events, equipment, work environment, tools and guns. Given the common exposure to leisure sources of noise in young people, there is an increased concern about NIHL, which has encouraged research on the potential harm it can cause.

When an acoustic stimulus enters into the external auditory canal it is funneled through to the tympanic membrane. The tympanic membrane acts as an elastic diaphragm and drives the ossicular chain of the middle ear system into motion. Then the middle ear

ossicles transfer mechanical energy to the cochlea by way of the stapes footplate hammering against the oval window of the cochlea. This hammering causes fluid within the cochlea (perilymph and endolymph) to push against the stereocilia of the hair cells, which then transmit a signal to the central auditory system within the brain. When the ear is exposed to excessive sound levels or loud sounds over time, the overstimulation of the hair cells leads to heavy production of reactive oxygen species, leading to oxidative cell death[36].

NIHL is therefore the consequence of overstimulation of the hair cells and supporting structures. Structural damage to hair cells (primarily the outer hair cells) will result in hearing loss that can be characterized by an attenuation and distortion of incoming auditory stimuli. The mechanisms involved in hearing loss due to noise overexposure are summarized as followed: a) mechanical trauma to the auditory hair cells and inflammation; b) ischemia–reperfusion injury and c) glutamate excitotoxicity with neuronal degeneration[37-39].

2.4 Aminoglycoside Ototoxicity

It has long been known that the major irreversible complication of aminoglycosides is ototoxicity[40,41]. This finding was first documented shortly after the discovery of streptomycin[40]. Aminoglycosides have variable cochleotoxicity and vestibulotoxicity; streptomycin and gentamicin are primarily vestibulotoxic, whereas amikacin, neomycin, dihydrosterptomycin, and kanamycin are primarily cochleotoxic[42]. Beneficial effects of these antibiotics in Meniere's disease that sometimes outweigh side effects popularized aminoglycosides[43].

The cellular basis for aminoglycoside-induced hearing loss is a destruction of cochlear hair cells. Aminoglycosides appear to generate free radicals within the inner ear, with subsequent permanent damage to sensory cells and neurons, resulting in permanent hearing loss[44]. Histopathologic studies have shown that outer hair cells are more sensitive to ototoxic injury than are inner hair cells. In animal models, histological findings resemble apoptotic cell death rather than necrosis. In cases of aminoglycoside ototoxicity, a variety of free-radical species, including both oxygen and nitrogen free-radical species were detected in the inner ears, which are believed to initiate the apoptotic cascade[45].

Gentamicin, the aminoglycoside of interest for this present thesis causes condensation of the nuclei of outer hair cells followed by the loss of mitochondrial membrane potential and apoptosis[46]. Reactive oxygen species, known to play a role in gentamicin-induced ototoxicity, promote the opening of the mitochondrial permeability pore.

CHAPTER THREE: Manuscript 1

The Effect of Fractionated Radiotherapy in Sensorineural Hearing Loss: an Animal Model

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3.1 Abstract

Objective: The purpose of this study is to assess the effect of fractionated radiotherapy on sensorineural hearing loss using an animal model.

Study Design: In vivo animal study.

Methods: Ears of 25 guinea pigs were divided into three groups: control, irradiated with a total of 48 Gy, and 71 Gy. Unilateral exposure of 48 Gy and 71 Gy fractionated irradiation was given for a four-week period. Auditory brainstem response and distortion products otoacoustic emissions were tested prior to irradiation and 1, 6, 10 and 16 weeks after completion of radiotherapy to assess the hearing threshold shift post radiotherapy over time.

Results: No significant differences in hearing thresholds between the low dose radiation (48 Gy) and the control group (no radiation) underlined that 48 Gy caused no hearing deficits ($p=0.37$). The higher dose (71 Gy) showed progressive deterioration of the hearing function over time. Three-way ANOVA interactions revealed significant group-time effects ($F= 9.261$, $p<0.0001$). DPOAE analysis demonstrated hearing loss at 71 Gy without progression or recovery at all time points, predominantly in the higher frequencies tested.

Conclusion: The present study suggests that in the presence of sensorineural hearing loss due to high dose fractionated radiotherapy, there is an initial sensorial component; however, the neural component is responsible for its progressivity.

Level of evidence: N/A

Keywords: radiotherapy, sensorineural hearing loss, ABR, DPOAE, guinea pig

3.2 Introduction

Recent advancements in medical technologies have led to a drastic improvement in cancer treatments with a subsequent increase of cancer survivors. Consequently, the importance of late effects of RT has been regarded in closer scrutiny. Sensorineural hearing loss (SNHL) is a late and permanent complication observed in 20 to 40% percent of patients whose inner ear is included within the radiation field[30].

While audiometry is the main tool to diagnose SNHL, auditory brainstem responses (ABR) and distortion products otoacoustic emissions (DPOAE) are complementarily used to locate the structures implicated in SNHL such as the eight nerve or the cochlea respectively. However, few clinical studies have comprehensively used these tests showing inconclusive results[48]. The evidence of histopathological studies on human[3] and animals [38,49-52] suggest that damage caused by radiation mainly targets the auditory hair cells. More specifically, radiation-induced sensorineural hearing loss (RISNHL) is believed to result from the auditory hair cells of the cochlea undergoing apoptosis [8,38]. Nonetheless, most evidence is based on single dose animal studies, which do not resemble the fractionated radiation schemes used in clinical scenarios.

Fractionated stereotactic radiation therapy (FSRT) and single-fraction stereotactic radiosurgery (SRS) have often been compared in regards to their efficacy and toxicity in patients with vestibular schwannoma. Outcome differences in local control do not seem to vary[53]. Nevertheless, hearing preservation in patients exposed to FSRT or SRS remains an emerging controversy. Although not resulting in immediate hearing deficits, evidence shows that SRS might cause progressive hearing loss[54]. However, FSRT results in a systematic dose reduction therefore, should reduce toxicity due to smaller fraction sizes per treatment[55]. Few authors have performed hearing assessments in animal studies using fractionated RT over several weeks similarly to schemes given to human patients[7,56,57]. In contrast to single dose experiments, animal[7] and human studies[58] have demonstrated that damage caused by fractionated radiation can also extend to supporting and spiral ganglion cells. Given this evidence, the current study aimed to examine the effects of fractionated RT at the neural and sensorial components of RISNHL assessed by ABR and DPOAE following two schemes of fractionated radiation in an animal model. To our knowledge, this is the first study using DPOAE and ABR together to assess RISNHL in

an animal model of fractionated RT.

3.3 Methods

3.3.1 Experimental Design

Twenty five six-week-old female albino guinea pigs (450 to 500 g) were purchased from Charles River Laboratories (Wilmington, Massachusetts, U.S.A). Only animals with normal ear anatomy were used. The animals were kept in standard housing at 22 ± 4°C ambient temperature and a 12-hour light/dark cycle in the animal care research facilities of The Montreal Children's Hospital Research Institute. The animals had access to food and water ad libitum and were monitored daily for signs of pain, weight loss or head tilt. This study was approved by the McGill University Animal Care Committee.

The animals were divided in two groups depending on the dose of unilateral irradiation received; 48 Gy (n=15) or 71 Gy (n=10). The experimental ears were separated in three groups: control (non-irradiated ears n=25), 48 Gy (n=15) and 71 Gy (n=10). These doses were chosen in accordance to previous studies. Cochlear radiation doses between 45 to 50 Gy have been reported as being the limiting doses in developing SNHL[31,59,60]. Miller et al. performed a study on fractionated RT on guinea pigs confirming that 71 Gy causes SNHL[56]. The auditory tests were performed under general anesthesia with inhaled isofluorane. The tympanic membranes and external auditory canals were inspected before functional evaluation. ABR testing was performed prior to any treatment (baseline measurement) and at 1, 6, 10 and 16 weeks following the end of RT treatment to determine the progressivity of hearing threshold across time.

3.3.2 Irradiation

The radiation setup used in these experiments has been validated through dosimetric experiments conducted in our laboratory[9]. A restrainer from the original design described by Winther, was constructed[61]. An irradiation field size of 6.5 mm x 7.2 mm was placed over the chosen area of radiation; above the inner ear. This setup was positioned inside the Faxitron CP-160 Cabinet XRadiator System (Faxitron X-Ray Corp., Wheeling, IL, USA) on a source to surface distance of 22.9 cm. A 0.5 mm Cu filter was added and the beam parameters were set at maximum (160 kVp at 6.3 mA). For the group receiving 48 Gy, the irradiations were completed at a programmed exposure time of 68

seconds. The fraction size was 2.4 Gy/day given from Monday to Friday for four weeks resulting in a total dose of 48 Gy. For the 71 Gy group, irradiations were completed at a programmed exposure time of 156 seconds with a fraction size of 3.5 Gy/day totaling a dose of 71 Gy.

3.3.3 Dosimetry

Measurement of the irradiator output was performed using EBT Radiochromic films[61]. Briefly, a female albino guinea pig (500 gr) with normal ear anatomy and hearing was euthanized, positioned in the restrainer and kept frozen at -24 °C for 48 hours. The mandible was sectioned and the exposed cranial base superiorly in order to leave enough space to position a piece of film placed in protective plastic bag between the two portions. To quantify the change in optical density (OD) due to the irradiation, a control film was used. The change in OD of the control films was subtracted from the exposed films to obtain the final netOD that was converted to dose using the formalism described by Devic et al.[62]. The obtained output of 1.35 Gy/min was used to calculate the exposure time for the irradiation system when exposing the animals with the same beam quality parameters.

3.3.4 Hearing Assessments

DPOAEs were obtained using the Smart DPOAE system (IHS, Miami, FL). The f2/f1 ratio was 1.2, and the intensity of the stimulus was 65 dB. The frequency of f2 varied between 0.8 and 9.3 kHz for a total of 8 different recording points. Bilateral ABRs were recorded from subdermal electrodes placed at the pinna of the ear tested (active), vertex (reference), and contralateral pinna (ground). Tone burst stimuli (8, 16, 20, and 25 kHz) with Blackman envelope were presented at a rate of 39.1 bursts per second through insert earphones. The stimuli were presented at 80 dB sound pressure level and decreased by 20, 10, and 5 dB to the threshold. Recordings in response to the stimuli were filtered, amplified, and averaged over 1,600 sweeps. The ABR threshold was determined at the lowest intensity where three reproducible waves III and V could be noticed.

3.3.5 Tissue Preparation

Following the final ABR, five randomly chosen cochleae from each group were obtained for histology processing. Each cochlea was fixed with 10% formalin for 48

hours, then the cochlea were decalcified with 10% EDTA dissolved in phosphate buffered saline (0.1 M, pH 7.4) for three weeks at 4°C. Cochleae were dehydrated through a graded series of ethanol (50–100%) and embedded in paraffin to be cut into five-micrometer midmodiolar sections. The slides were stained with hematoxylin and eosin. Images of the Organ of Corti were analyzed with light microscopy and digitally stored (TIFF format) using an AxioCam MR3 camera and the AxioVision 4.7 software (Carl Zeiss, Germany).

3.3.6 Statistical Analysis

Our sample size was calculated using the Chang Bioscience software (Chang Bioscience Inc., CA). A minimum absolute difference was represented by the mean ABR threshold difference of 20 dB, a standard deviation of 13.5 dB and an alpha of .05. This resulted in a calculated sample size of 8 ears per group. The differences between ABR thresholds at each frequency (8, 16, 20, and 25kHz) at the different time points were calculated. For DPOAE measurements, the SNR changes were compared between the 3 groups at every time point. A Multiple Factor ANOVA (frequency x group x time) with a statistical significance set at $p \leq 0.05$ was calculated using JMP 10 Software. (SAS Institute Inc, NC, USA). When significant differences were found, comparisons were assessed with post-hoc Tukey's test.

3.4 Outcomes

3.4.1 Results

Baseline ABR measurements are shown in Figure 1. Two-way analysis (group x frequency) showed no difference between groups (p=0.715). Two animals died due to anesthetic complications during radiation treatment and therefore were excluded from analysis. After the completion of radiation, two animals required tympanocentesis due to effusion and complete resolution was found at subsequent physiological evaluations. These animals were excluded from analysis at the specific time points. No vestibular changes such as head tilt were noticed in the animals monitored up to 16 weeks post RT. The thresholds of the control ears of animals irradiated with 48 Gy and 71 Gy were analyzed in a three way ANOVA interaction showing no significant effects (p=0.955) and therefore were pooled together.

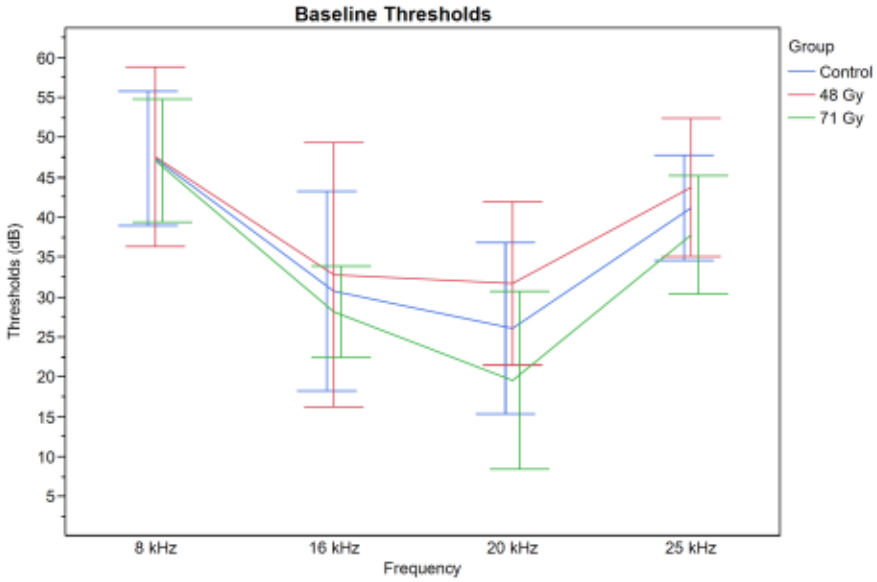

Figure 1. Baseline thresholds of auditory brainstem responses. dB = decibels; Gy = gray; kHz = kilohertz.

Three-way ANOVA interactions (group x time) demonstrated statistically significant effects ($F=9.261$ $p<0.0001$) (Figure 2). The group exposed to 48 Gy had lower threshold shifts in ABRs than the control group in one frequency. However, these groups did not show statistically significant differences during different time points in the ABR testing ($p=0.37$). Contrarily, the group of 71 Gy demonstrated significant differences starting at week six when compared to the controls and the group irradiated with 48 Gy ($p=0.0458$ and $p=0.0449$ respectively). After this time point the group irradiated with 71 Gy displayed significantly greater thresholds than the two other groups ($p< 0.0001$). In the control and 48 Gy group, the maximum hearing threshold shift was found at 10 week of evaluation displaying a 20 dB increase across the frequencies. The group of 71 Gy was found to have a progressive deterioration of the hearing function with highest shift in the 16 kHz frequency displaying a threshold increase of about 60 dB.

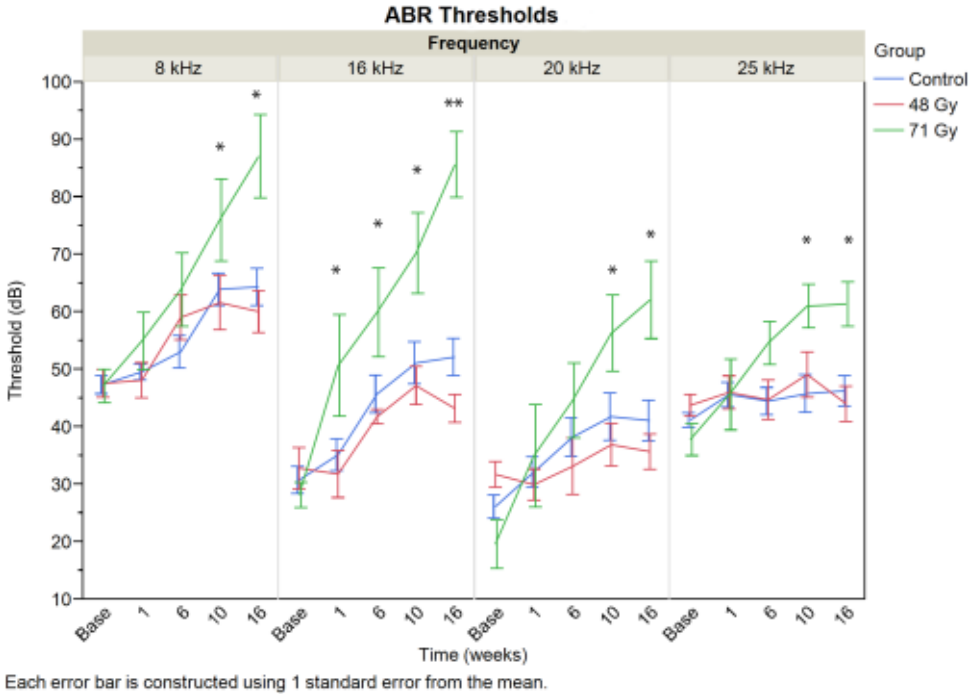

Figure 2. ABR thresholds through the different time points. **P* < 0.05; ***P* < 0.0001. ABR = auditory brainstem responses; dB = decibels; Gy = gray; kHz = kilohertz.

The group factor had a significant effect (F = 39.421, p<.0001) with larger hearing loss in the group of 71 Gy when compared to the control group (p<0.0001). Furthermore, time showed significant effect (F=63.714, p<0.0001) demonstrating the overall progression of hearing loss over time.

As seen in Figure 3, the control and 48 Gy group presented SNR shifts of about 15 dB across all frequencies while the group of 71 Gy presented early shifts particularly at the frequency of 2336 Hz and higher. These shifts were sustained and not progressive. DPOAE analysis of interactions (group x frequency) did not revealed statistically significant effects (F=1.544, p=0.089) but the effect of frequency only showed a statistically significant effect (F=5.332, p<0.0001). Post-hoc analysis showed differences between the group of 71 Gy when compared to the control and 48 Gy group at 3316 Hz of 14.35 dB and 14.34 dB respectively (p=0.0027 and p=0.0418). The groups of 71 Gy and 48 Gy showed statistically significant differences at 4687 Hz and 6633 Hz (p= 0.0242 and p= 0.0103 respectively) and clearly showing a tendency to obtain greater

hearing loss at higher frequencies.

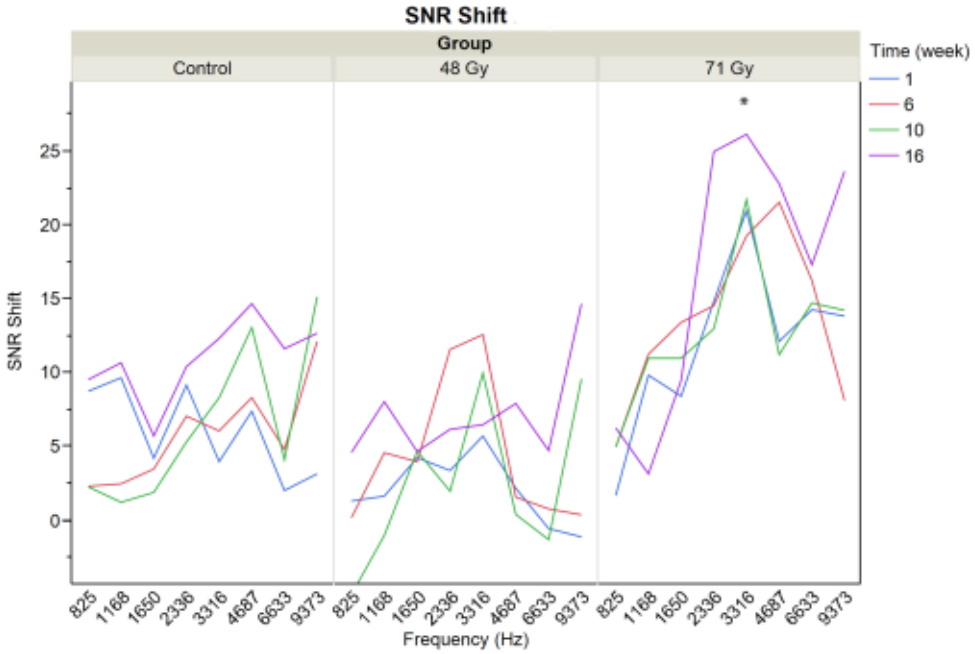

Figure 3. Distortion products otoacoustic emissions signal-to-noise ratio amplitude shifts. *$P < 0.05$. Gy = gray; Hz, hertz; SNR = signal-to-noise ratio.

3.4.2 Histological Analysis

Cross sections of the cochleae showed pathological changes in the irradiated groups. Atrophic stria vascularis was found in the cochleae irradiated with 71 Gy when compared to the controls (Figure 4). Degeneration of the spiral ganglion cells in the cochleae irradiated with 71 Gy was also found. However, the cochleae irradiated with 48 Gy and the controls were similar (Figure 5).

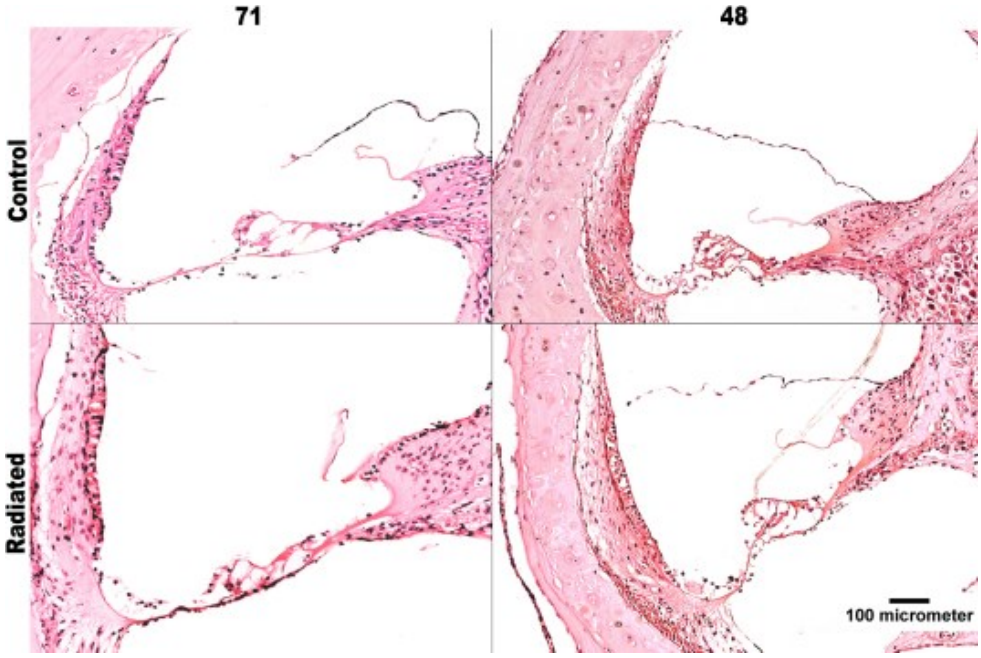

Figure 4. Haematoxylin-eosin cross sections show the pathological changes in the irradiated cochlea. Damage is shown to the stria vascularis and auditory hair cells in the ears irradiated with 71 Gy. However, minimal damage was observed in the ears irradiated with 48 Gy. Gy = gray.

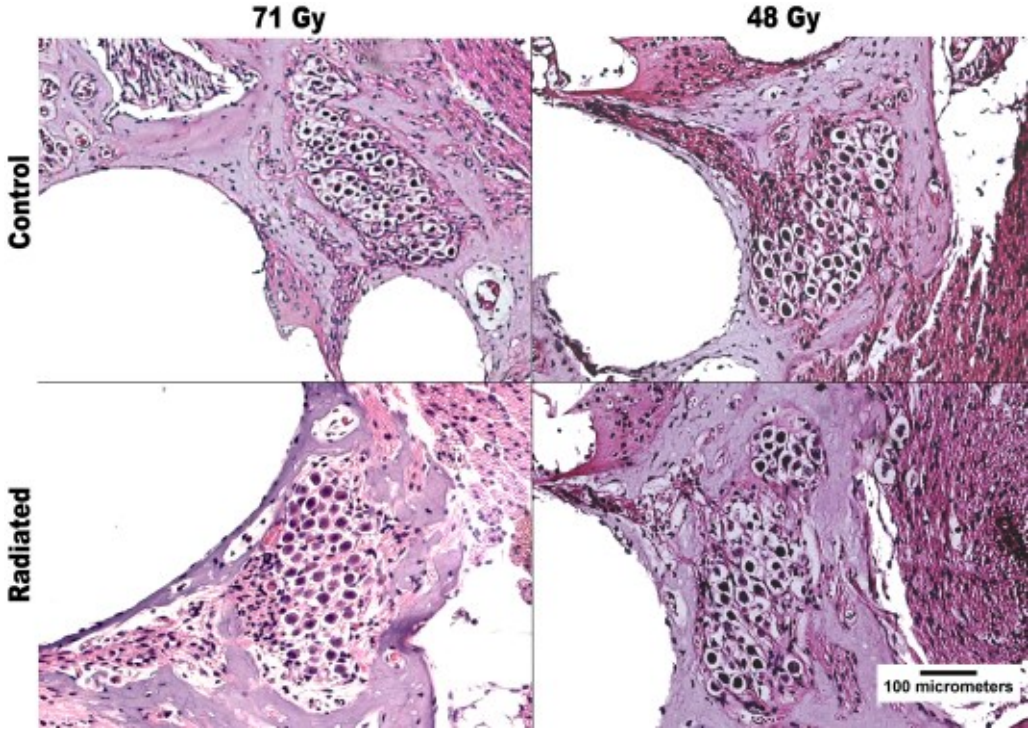

Figure 5. Haematoxylin-eosin cross sections of the spiral ganglion cells. Gy = gray.

3.5 Discussion

The structures involved in fractionated radiation-induced cochlear damage are still subject of debate. Most of the preclinical evidences suggest that the auditory hair cells are mainly affected[8] but physiological and histopathological studies of patients subjected to fractionated RT have suggested the inner hair cells, spiral ganglion cells[58] and stria vascularis[63] to be involved. The animals receiving low dose fractionated RT (48 Gy) had good hearing preservation, which is concordant with clinical studies of patients receiving fractionated radiation (46.8 Gy) for vestibular schwannoma treatment[23]. However, our ABR and DPOAE results revealed a progressive hearing loss in the animals exposed to high dose RT (71 Gy) particularly in the neural component of hearing loss.

Early changes in thresholds were noted at 16 kHz and 25 kHz between control and radiated ears. This finding is consistent with the reports in humans where the high frequencies are initially affected in the evolution of RISNHL[30]. Although not significant, the control ears showed greater threshold shifts in the ABR assessment when compared with the group of 48 Gy, which is similar to what was found by Greene in animal studies using total irradiations ranging from 57 to 70 Gy[57].

Contrasting with the ABR finding, DPOAE test showed more variability. Overall, hearing ability at high frequencies were affected without progression post RT. This might have three implications. First, the damage caused by radiation could have removed cochlear hair cells resulting in an early detection of radiation damage by the DPOAE. Second, progression was only noted in the ABR testing and this might indicate that the hearing loss progression is due to damage to the inner hair cells or spiral ganglion cells. Third, if there is progression in hair cell damage, this might not be detectable with DPOAE. There is still controversy whether DPOAE and ABR assessments should correlate[64,65] especially at high frequencies when standing waves at the ear canal might lead to calibration errors[66]. This might be a limitation of the DPOAE testing itself.

We did not find any recovery in the long term assessment contrary to was observed by Akmansu et al. who assessed DPOAE changes in rats after a single dose of total body irradiation (5.5 Gy)[52]. In their study, hearing tests showed transient decrease in DPOAE amplitudes with partial recovery post eight weeks. The fact that radiation was given as single dose makes the results difficult to extrapolate to a fractionated scheme.

Human studies have presented inconclusive neurophysiological results regarding the structures affected in RISNHL mainly due to its long latency and the mortality rate in cancer patients. There is evidence that hearing loss can be underestimated by having damage to the retrocochlear pathways without any evidence on audiometry[67]. Some studies have identified progressivity in regards to retro-cochlear damage resulting from RISNHL[68], while other authors have noticed transient damage[69]. In a series of unilaterally irradiated patients, Johannesen reported long term SNHL detected by audiometry in the irradiated ears (mean dose of 53 Gy) but with no statistically significant differences in speech audiometry were noted[48]. The latency of the ABR waves was practically unchanged in the irradiated ears, while the OAE demonstrated lower amplitude values. Low et al. showed increased latencies in the irradiated ear of patients receiving cochlear radiation doses of up to 62 Gy [69]. Grau found that about 40% of patients diagnosed with SNHL by audiometry had abnormal ABR[70]. However, the authors noted that these patients had all received doses above 59 Gy. These studies underline that retrocochlear pathways can be damaged in a dose dependent manner.

Animal studies show that outer hair cells and the stria vascularis are mainly affected by RISNHL[49-52,61]. However, most used large single doses of RT that do not resemble actual clinical scenarios. Only few have experimented fractionated radiation over several weeks. Bohne et al. performed studies with fractionated RT and observed damage to spiral ganglion cells, hair cells and supporting cells[7]. However, this study relied solely on histological assessment. Miller et al. demonstrated that doses of 60 to 70 Gy caused progressive hearing loss using ABR assessments without histological confirmation[56]. In our study, we observed minimal histological damage after irradiation with 48 Gy, which correlated with our DPOAE and ABRs results. In addition to the late hair cell damage, ears irradiated with 71 Gy demonstrated degeneration of spiral ganglion cells. These findings can explain the progressive increase of hearing threshold observed in the ABRs and concord with research demonstrating spiral ganglion cells loss in irradiated human temporal bones.3 Moreover, our findings of strial atrophy in the group irradiated with 71 Gy are consistent with findings of other animal models using single dose RT[71]. Furthermore, functional studies of patients subjected to gamma knife surgery for vestibular schwannomas also imply strial damage[72]. These results suggest that

hypofractionation can have a preponderant effect on vascular tissue.

In summary, we found early signs of cochlear damage observed by DPOAE testing (outer hair cells) primarily at the high frequencies. The neural component assessed by ABR (inner hair cells and spiral ganglion cells) showed progression of hearing loss in radiation induced cochlear damage. These findings have potential implications in preclinical studies of prevention of RISNHL and stereotactic radiosurgery. While targeting to the stria vascularis and auditory hair cells can be futile because of their radiosensitivity to high fraction sizes, the spiral ganglion cells could be a functional reserve substrate for radioprotective drugs.

Potential limitations of this study relate to differences in fraction size compared to human protocols. In our study, the animals received fraction sizes greater than what patients receive in current radiation protocols. The animals in the 48 and 71 Gy group did not show serious adverse effects from radiation such as head tilt or facial palsy confirming the reliability of this animal model.

3.5 Conclusion

In the group of ears that received fractionated radiation at ototoxic doses (71 Gy given in 3.5 Gy/fraction), a stable decrease in DPOAE amplitudes was found after one week of completion of radiation mainly in the high frequencies. Progressive ABR shifts were consistently found across all frequencies after six weeks of radiation. These results suggest that in the setting of hearing loss due to high-fractionated RT, there is an initial sensorial damage while the neural component is involved in the progression of radiation-induced SNHL.

3.6 Funding Sources

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3.7 Linking Statement

This study suggests that in the setting of RISNHL, there is an initial sensorial

damage while the neural component is involved in the progression of radiation-induced SNHL. Future studies investigating RISNHL at the neural level by means of histopathological evaluations are needed to determine the exact cellular mechanism leading to RISNHL in the auditory pathway of the brain. Also, future studies investigating the potentiating effect of RISNHL are needed to elucidate synergistic effects that RT could cause. The study, which follows, will look at the factors that may cause hearing loss when patients are treated with radiation therapy followed by an ototoxic drug such as Gentamicin.

CHAPTER FOUR: Manuscript 2

The Effect of Radiation on Gentamicin Ototoxicity; an Animal Model

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Running title: Synergy of radiation and Gentamicin

Part of this study was presented as podium presentation at American Society of Pediatric Otolaryngology in May 2014. This manuscript will serve as part of the Master's thesis of Aren Bezdjian at McGill University.

Dr. Slobodan Devic (Senior Research Scientist) and Aren Bezdjian (Master's Candidate) are supported by the Fonds de Recherche en Santé du Québec (FRSQ).

The authors have no conflicts of interest to disclose

4.1 Abstract

Introduction: Patients undergoing radiotherapy often present serious bacterial infections which require the use of antibiotic treatment. Gentamicin is a commonly used aminoglycoside antibiotic, whose ototoxicity remains a major problem in clinical use. The objective of this study was to determine if radiation (RT) can influence Gentamicin induced ototoxicity.

Methods: Sixteen guinea pigs were exposed to low dose fractioned radiation unilaterally for four weeks (total dose 48 Gy). Animals were then divided to receive low and high doses of Gentamicin (40 mg/kg/day and 80 mg/kg/day) for 10 days. The ears were hence divided in four groups: Gentamicin 40 mg, Gentamicin 80 mg, Gentamicin 40 mg + RT, Gentamicin 80 + RT. Auditory brainstem responses (ABR) and distortion products otoacoustic emissions (DPOAE) were assessed at baseline and before and after Gentamicin treatment. Cochlear morphology using light and scanning electron microscopy were evaluated.

Results: Low dose Gentamicin treatment did not cause hearing loss alone or in combination with radiation exposure. High dose Gentamicin caused significant ABR threshold shifts ($p = .020$), with greater hearing loss in the irradiated ear (difference of 23.6 $\pm$7.5 dB). All animals exposed to high dose Gentamicin had head tilts towards the radiated side. Cochlear morphology revealed the greatest hair cell damage in the Gentamicin 80 + RT group followed by Gentamicin 80.

Conclusion: Results presented in this work suggest that radiation can exacerbate the ototoxicity of Gentamicin at high doses.

4.2 Introduction

Radiation-induced hearing loss has been observed in pediatric patients with brain tumors such as medulloblastoma, receiving high doses of RT in the temporal bone.[74] With the improvement of radiation (RT) techniques such as intensity modulated radiation therapy the doses delivered to the inner ear have been decreased compared to conventional radiation delivery methods.[75] Moreover, it is hypothesized that below certain dose limits, low radiation doses can be considered safe in terms of short and long term adverse effects on the hearing organ.[76] On the other hand, immunosuppression and RT treatments are known to increase the patient's risk of developing serious bacterial infections. Thus, patients with cancer often require treatment with ototoxic antibiotics such as Gentamicin.[77]

While the limiting doses for both RT and Gentamicin ototoxicity have been estimated,[78,79] there is a lack of knowledge about the long term predisposing effects of low dose RT to the inner ear when later exposed to potential ototoxic agents such as Gentamicin.

It is known that RT and Gentamicin increase the reactive oxygen species formation in the cell subjected to their effects as shown in a recent *in vitro* study. The results suggested that RT can potentiate Gentamicin ototoxicity in auditory hair cell lines through the damage of mitochondrial metabolism.[80] However, this hypothesis has not been confirmed *in vivo*. Therefore, the objective of the present study is to determine if low dose radiation exacerbates Gentamicin induced ototoxicity in a guinea pig animal model. Our hypothesis given the results of the previous *in vitro* study is that low dose radiation exposure can predispose to Gentamicin ototoxicity at low and high doses.

4.3 Materials & Methods

4.3.1 Animal Subjects

The study was approved and monitored by the McGill University Health Centre Animal Care Committee in accordance with the Canadian Council of Animal Care Guidelines. Sixteen six-week-old female albino guinea pigs (450 to 500 g) purchased from Charles River Laboratories (Wilmington, MA) were kept in standard housing at 22°C ambient temperature with a 12-hour light/dark cycle. All animals had free access to

food and water and were examined daily for signs of pain, weight loss or permanent head tilt.

4.3.2 Experimental Design

Bilateral baseline auditory brainstem responses (ABR) and distortion products otoacoustic emissions (DPOAE) were performed prior to treatment. All animals were randomly assigned an experimental ear to recieve a total dose of 48 Gy of RT. Bohne et al.[7] delivered fractionated doses of RT in chinchillas and observed that cytocochleograms of animals subjected to doses between 40 and 50 Gy had less than 10% OHC loss and minimal nerve fiber degeneration.

Sixteen weeks post RT treatment, the animals received Gentamicin (Gentocin, Merck Animal Health, Canada) at low (40 mg/kg/day) or high (80 mg/kg/day) doses for ten days. The high dose (80 mg/kg/day) has demonstrated to cause sufficient ototoxicity yet no mortality, while the low dose (40 mg/kg/day) has demonstrated no ototoxicity.[81] The delivery method was subcutaneous in order to cause enough toxicity to observe ototoxicity but not nephrotoxicity. In addition, a single daily dose regimen in our study was used given its pharmacokinetic profile and its outcomes in neonatal sepsis.[82] The ears of the animals were subsequently divided as followed: Gentamicin 40 mg (n=8), Gentamicin 80 mg (n=8), Gentamicin 40 mg + RT (n=8), Gentamicin 80 mg + RT (n=8).

Bilateral ABR and DPOAE were repeated one week and 16 weeks after RT exposure to assess for short and long-term hearing deficits. Final hearing tests were performed after the 10-day Gentamicin treatment to assess for hearing loss caused by Gentamicin alone (control ear) and synergistic effect of Gentamicin + RT (experimental ear) at both doses.

4.3.3 Irradiation System

A customized restrainer was constructed in concordance with Winther's study.[83] A protective lead shield covered the restrainer and properly guided the RT source to the target cochlea. The front teeth of the guinea pig were placed a horizontal wire and lateral screws immobilize its head. The protective lead shielding covered the entire animal leaving the collimator window to create a RT field size of 6.5 mm x 7.2 mm at the level of cochleae.

The animals immobilized with inhalational anesthesia and within the restrainer, were positioned inside a Faxitron CP-160 Cabinet X-Radiator System (Faxitron X-Ray Corp., Wheeling, IL, USA) on tray guide #8. A 0.5 mm Cu filter was added and parameters were set at 160 kVp and 6.3 mA. The fraction size was 2.4 Gy per day given in weekdays for four weeks resulting in a total dose of 48 Gy, which is considered low and non-ototoxic.[84] Using external landmarks (external ear canal and 4 mm from the midline), the beam of the x-ray tube was positioned over the experimental cochlear region. Irradiation time for each fraction was calculated using the output (1.35 Gy/min) measured (for a given setup) using the EBT model radiochromic film based reference dosimetry system as specified in previous study.[85]

4.3.4 Assessment of Hearing

All hearing tests were conducted using inhalational anesthesia with 2 % isofluorane. Once the external ear was inspected, stainless steel needle electrodes were placed subdermally at the pinna, vertex, and contralateral pinna. Bilateral auditory brainstem response (ABR) testing was obtained using SmartEP System (Intelligent Hearing Systems, FL). Tone burst stimuli (8, 16, 20, and 25 kHz) with Blackman envelope were presented through insert earphones at 80 dB sound pressure level, decreasing in steps of 20, 10, and 5 dB to the threshold. Responses to the stimuli were amplified, filtered, and averaged over 1,600 sweeps. The ABR threshold was selected at the last intensity where three reproducible waves III and V could be identified. DPOAEs were obtained using the Smart DPOAE high-frequency software/hardware package (Intelligent Hearing Systems, FL). The otoacoustic emissions were recorded for both ears between 0.8 and 22 kHz for a total of 9 frequencies. Two-tone stimuli at 55 and 65 dB SPL were emitted with a frequency ratio (F1/F2) of 1.22 and averaged 32 times. The signal to noise ratio (SNR) that compares the level of a signal to the level of a constant background noise was used at the F2 amplitude to assess the integrity of the outer hair cells (sensorial component of hearing).

4.3.5 Histological Analysis

The animals were sacrificed 24 hours after the final hearing test and cochleae were obtained. The cochleae were fixed with 10% formalin for 48 hours, decalcified with 10%

EDTA in phosphate buffered saline (0.1 M, pH 7.4) for three weeks at 4°C. The samples were then dehydrated in ethanol and embedded in paraffin and cut into five-micrometer midmodiolar sections. Final slides were stained with hematoxylin and eosin and mounted for light microscopy. Images of the stria vascularis, spiral ganglion cells and hair cells were digitally stored using AxioVision 4.7 microscopy software and a Zeiss AxioCam MR3 camera (Carl Zeiss, Germany).

For scanning electron microscopy, three randomly chosen cochleae from each group were fixed in 2.5% glutaraldehyde for 2 hours then left in 0.1 M phosphate buffer saline for 24 hours at 4°C. The cochleae were then postfixated in osmium tetroxide for 1.5 hours and dehydrated in graded solutions from 35 to 70% ethanol. Once the organ of Corti was dissected under a surgical microscope, the samples were further dehydrated in solutions up to 100% ethanol, critical point dried, mounted and sputter coated with gold. A field emission scanning electron microscope was used for qualitative analysis (Hitachi S4700; Hitachi).

4.3.6 Statistical Analysis

The data was analyzed using a 3x4x4 mixed ANOVA that examined the effects of two within variables, time of measurement (one week post RT, sixteen weeks post RT and post-Gentamicin) and frequency exposure (8, 16, 20 and 25 kHz), and one between variable, treatment (Gentamicin 40 mg, Gentamicin 80 mg, Gentamicin 40 mg + RT, Gentamicin 80 mg + RT). The data was analyzed using SPSS Statistics Software version 20 (IBM Corp.).

4.4 Results

4.4.1 Behavioural Assessment of the Animals

Following the first dose of RT exposure, the animals were examined daily for signs of vestibular toxicity, head tilts, significant weight loss or ear infection. At the completion of RT exposure (total dose of 48 Gy), the animals did not display head tilts, significant reduction in physical activities or weight or ear discharges to suggest that exposure to RT resulted in systemic disturbances. The same observations were noted in the animals after being treated with Gentamicin 40 mg. However, the animals receiving Gentamicin 80 mg displayed systemic changes becoming increasingly apparent

throughout the 10-day treatment. All animals exposed to Gentamicin 80 mg had head tilts towards the radiated ear. The animals also showed signs of reduction in physical activity and weight loss.

4.4.2 Auditory Brainstem Responses

RT dose of 48 Gy did not cause short or long term hearing loss as demonstrated in ABR at all frequencies tested prior to Gentamicin administration (Figure 1). The statistical analysis revealed a significant main effect for the time of measurement, $F = 47.99$, $p < .001$ having greater threshold shifts after receiving Gentamicin. Furthermore, an effect was observed for treatment, $F = 3.11$, $p = .050$ and a significant time x treatment interaction was observed, $F = 5.40$, $p < .001$. No significant differences were detected between the treatments at week one and sixteen, however significant differences were observed after Gentamicin treatment in the animals receiving unilateral RT of 48 Gy.

Post-hoc comparisons of the treatment main effect, using between-groups t-tests, revealed that auditory thresholds shifts were significantly greater for Gentamicin 80 mg compared to Gentamicin 40 mg ($p = .036$), showing a dose dependent effect in the ears treated solely with Gentamicin. A significantly greater threshold shift was observed when comparing baseline to post exposure auditory thresholds in the radiated ears compared to the controls ears of animals receiving Gentamicin 80 mg (14.54 ± 4.32 dB, $p = .018$, Figure 1).

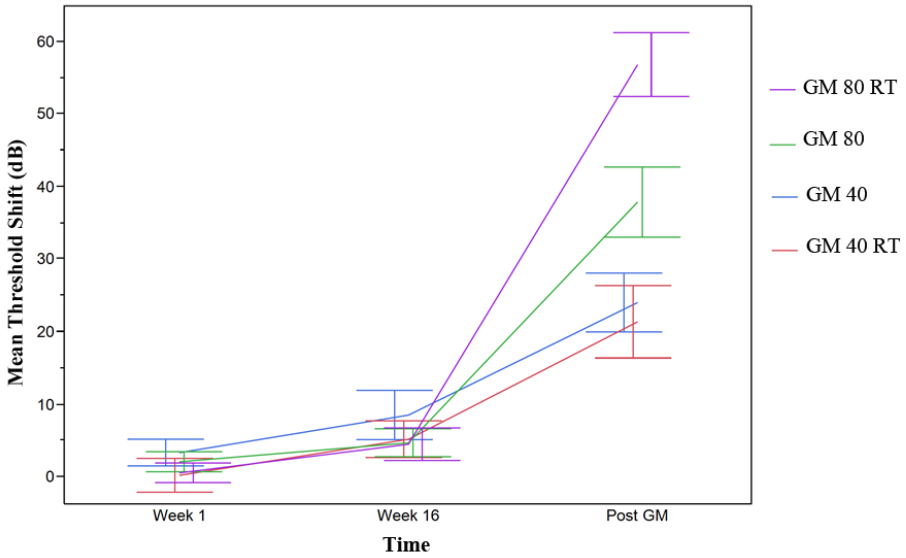

Figure 1. ABR threshold shifts between groups across all time points demonstrated significant difference between Gentamicin 80 + RT compared to Gentamicin 80. Error bars represent Standard Error of the Mean.

The results displayed in Figure 2 compare ABR threshold shifts prior to Gentamicin treatment (week 16 post RT) and post Gentamicin treatment. The radiated ears did not differ from the control ear in the animals subjected to Gentamicin 40 mg at all frequencies tested ($p > .05$). The animals subjected to Gentamicin 80 mg had the greatest hearing threshold shift at 16 kHz when comparing the radiated to control ears (31.07 ± 9.97 dB, $p = .002$). The only other significant threshold shift was found at 8 kHz in the animals subjected to Gentamicin 80 mg when comparing the radiated to control ears (23.50 ± 9.97 dB, $p = .020$). A greater threshold shift was observed at 20 kHz and at 25 kHz; however these results were not significant ($p = 0.15$ and $p = 0.28$; respectively).

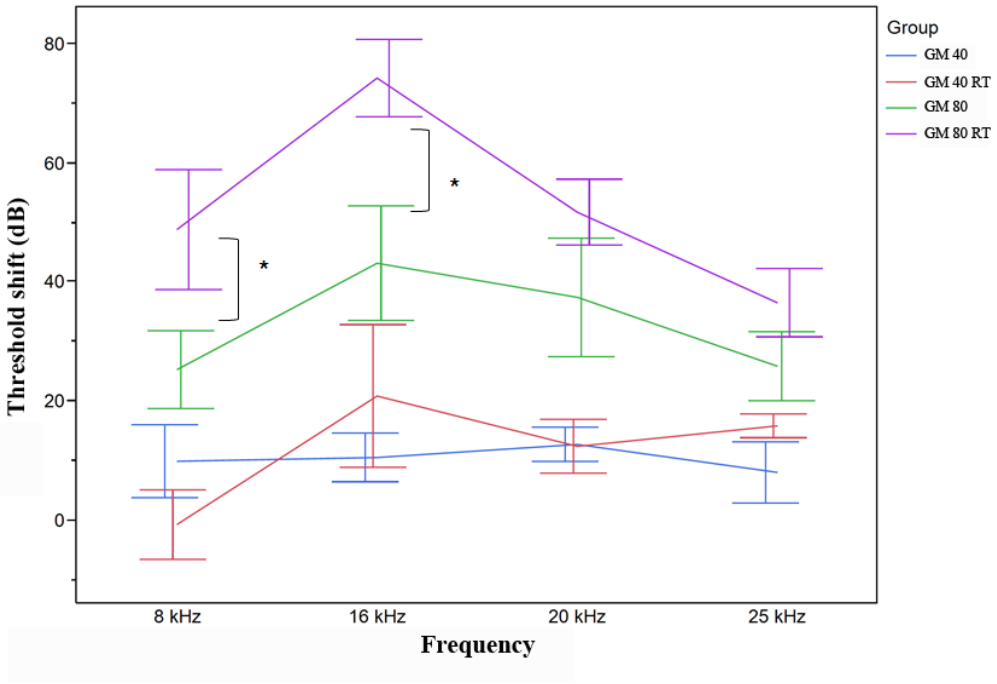

Figure 2. ABR threshold shifts by frequency displayed significant difference at 8 and 16 kHz between the groups of Gentamicin 80 + RT and Gentamicin 80. Error bars represent Standard Error of the Mean.

4.4.3 Distortion Products OtoAcoustic Emissions

DPOAE showed no significant difference between the groups ($p > .05$). However, the ears treated with Gentamicin 80 mg and subjected to RT showed a trend of greater threshold shifts across all frequencies tested, whereas the group of Gentamicin 80 mg displayed a pattern of damage only in the higher frequencies (Figure 3). In the animals subjected to Gentamicin 40 mg there were no significant differences noted between the experimental and control ears.

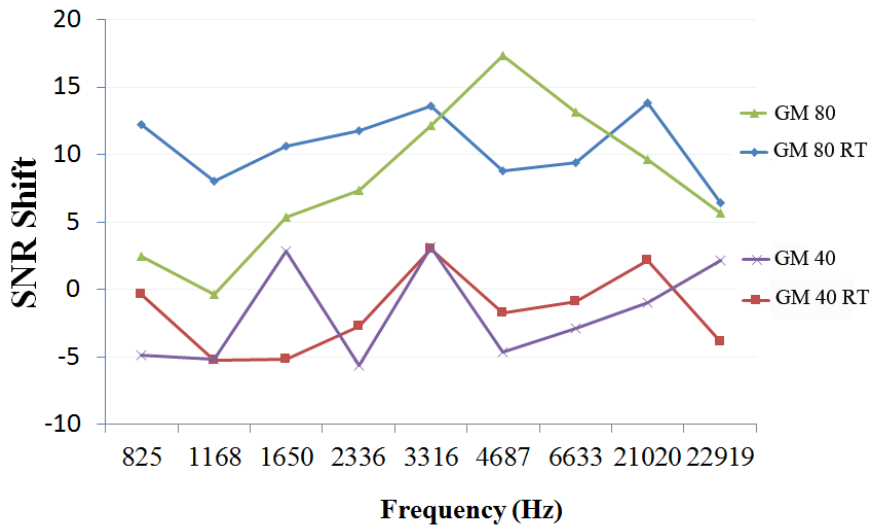

Figure 3. DPOAE showed greater shift across all frequencies tested in Gentamicin 80 + RT, while the Gentamicin 80 had only shifts at the higher frequencies. Error bars were removed for display purposes.

4.4.4 Morphological Analysis of Cochleae

The organ of Corti and stria vascularis of the ears subjected to Gentamicin 40 mg with and without RT exposure demonstrated vacuolization of the stria vascularis and preservation of the hair cell morphology. Contrarily, in the ears subjected to Gentamicin 80 mg, there was preservation of the strial morphology but disarrangement of the inner and outer hair cells structure. In the ears subjected to Gentamicin 80 mg + RT, there was marked damage at both hair cell structures and stria vascularis (Figure 4).

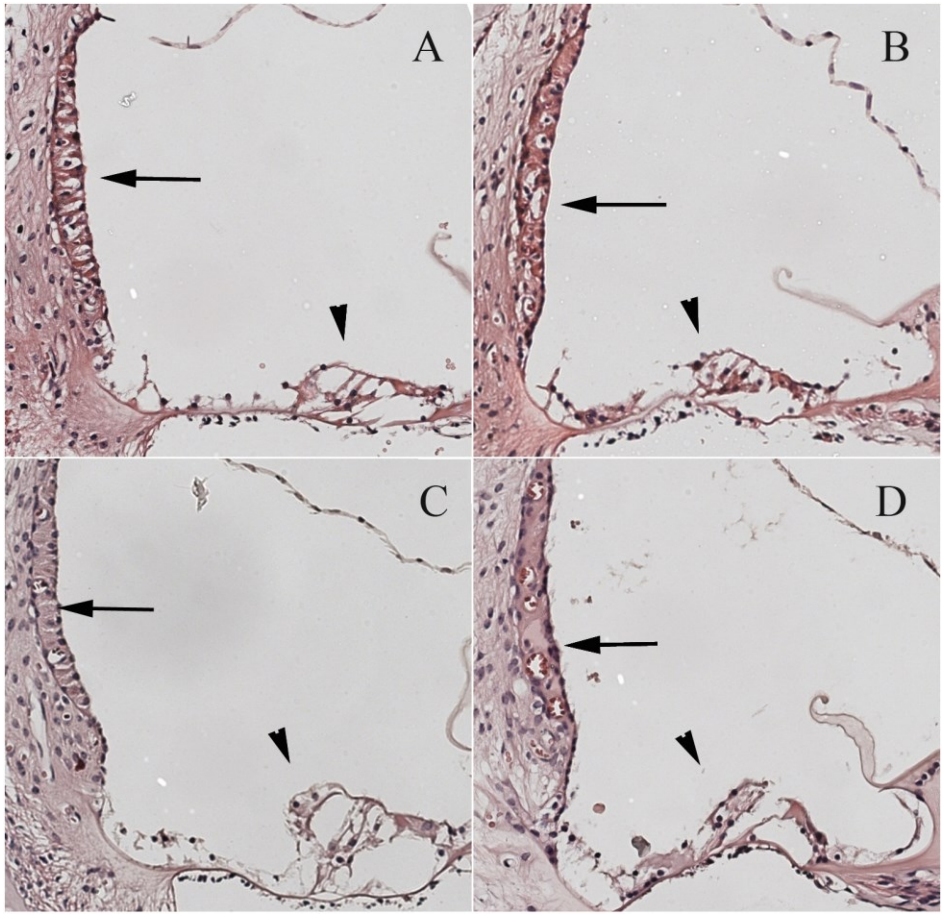

Figure 4. Gentamicin 40 (A) and Gentamicin 40 + RT (B) showed vacuolated stria vascularis (arrows) and preserved morphology (arrowheads). Gentamicin 80 (C) presented abnormal morphology. Gentamicin 80 + RT (D) had dilated vessels and destructed Corti.

Despite the lack of functional changes in the ears treated with Gentamicin 40 mg, qualitative evaluation of spiral ganglion cells evidenced that RT caused morphological changes in the ears subjected Gentamicin 40 mg. Furthermore, the ears exposed solely to Gentamicin 80 mg, evidenced shrinkage of cellular bodies while the ears receiving Gentamicin 80 mg + RT presented marked disarrangement (Figure 5).

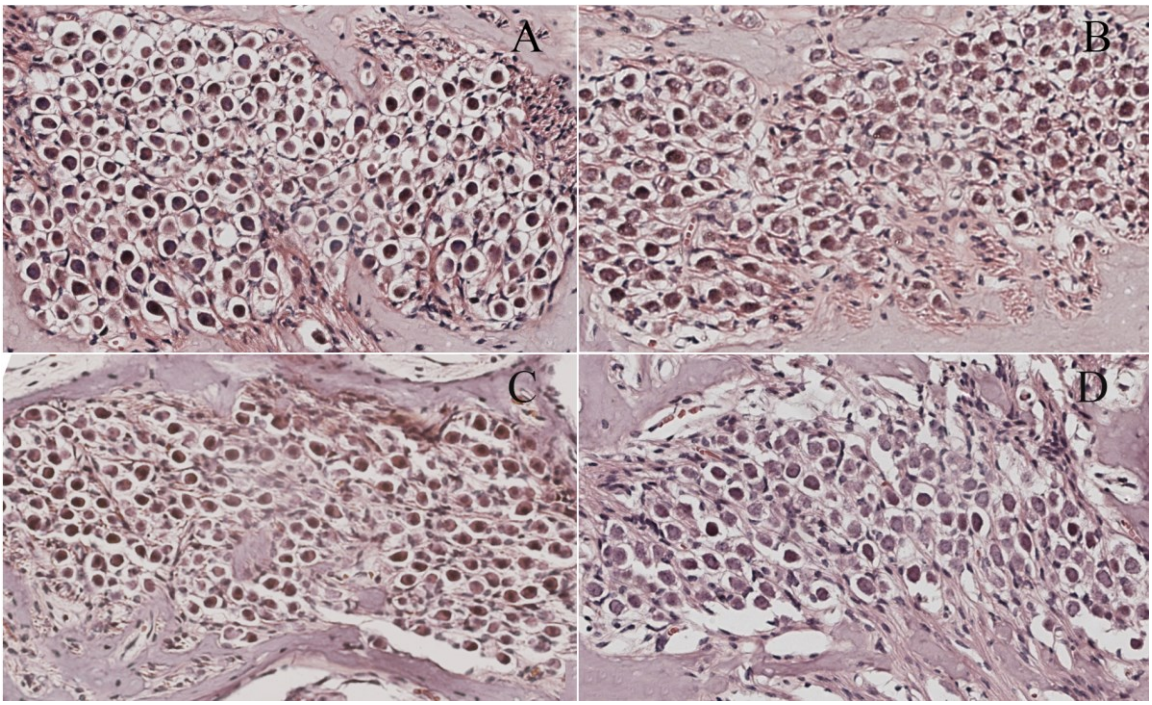

Figure 5. Gentamicin 40 (A) had preserved SGCs. Gentamicin 40 + RT (B) presented changes in cellular bodies of neurons and picnotic nuclei. These changes were marked in Gentamicin 80 (C) and Gentamicin 80 + RT (D).

Scanning electron microscopy revealed a near complete damage in the auditory hair cells of the ears subjected Gentamicin 80 mg + RT. In the ears subjected to Gentamicin 80 mg alone the stereocilia of the hair cells were damaged and absent in some areas. In the Gentamicin 40 mg groups, the stereocilia of the hair cells had remained intact in the ears subjected to Gentamicin 40 mg alone and revealed minimal damage in the samples receiving combined Gentamicin 40 mg + RT (Figure 6).

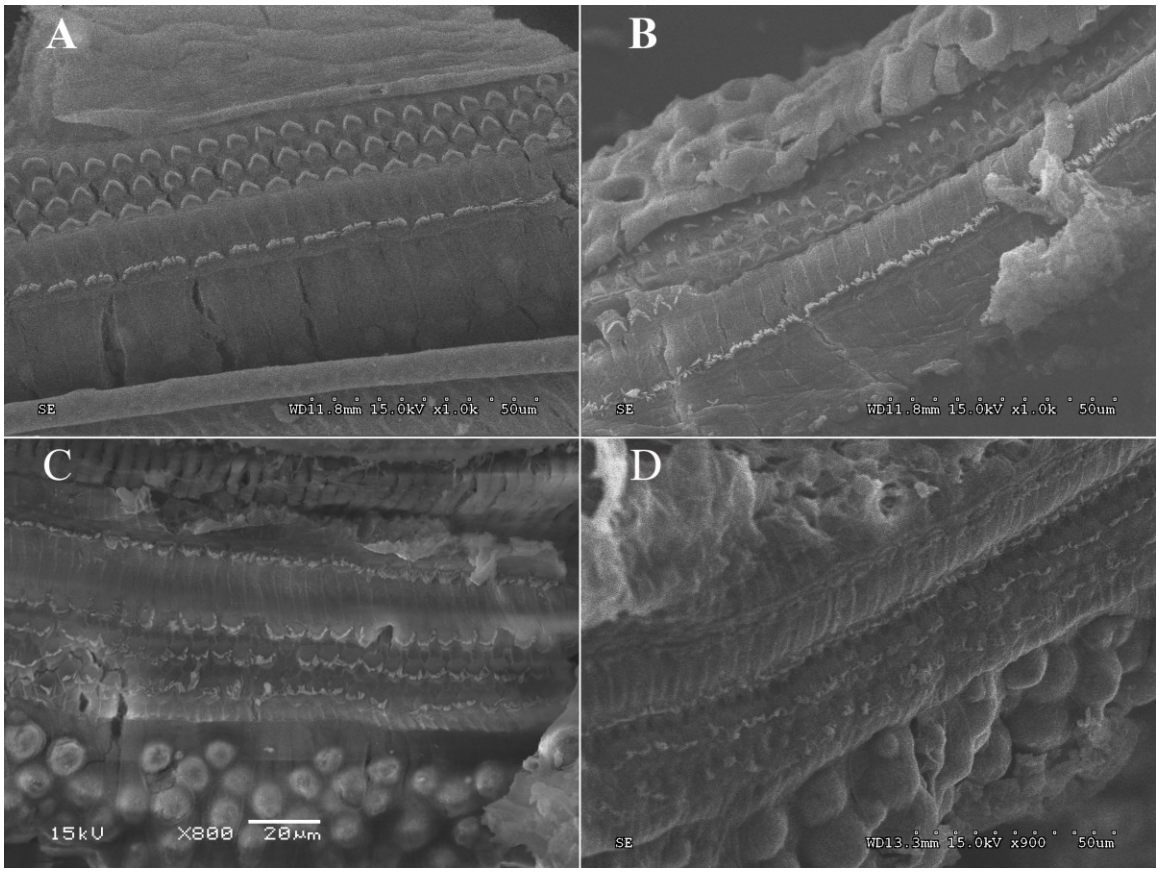

Figure 6. Gentamicin 40 (A): intact inner and outer hair cells. Gentamicin 40 + RT (B): minimal damage. Gentamicin 80 (C): major cell damage and near complete destruction of stereocilia in Gentamicin 80 + RT (D).

4.5 Discussion

The functional outcomes of the present study revealed that RT exposure acted synergistically after treatment with Gentamicin 80 mg as demonstrated by our ABR and DPOAE results. Although not evidenced by the previously mentioned tests, RT showed detrimental effect in the Gentamicin 40 mg group evidenced in the histological findings. Gentamicin 40 mg + RT revealed hair cell and spiral ganglion cell damage while Gentamicin 40 mg showed complete preservation of inner and outer hair cells (Figure 6). To our knowledge this the first study assessing the predisposing effect of RT exposure to Gentamicin ototoxicity *in vivo*.

Fractionated RT to the inner ear of doses above 50 Gy increases the risk of developing hearing deficits in children particularly in the high frequency hearing range.[76] It is believed that this adverse effect is caused by the reactive oxygen species (ROS)

produced after irradiation primarily in the mitochondria,[86] which in the long term predisposes the organ of Corti at risk of damage. However, little is known about the long term potentiating effect of fractionated schemes of lower doses on the hearing structure. Recent studies in infants and children show that hearing loss is now a rare complication of aminoglycoside therapy occurring in 5% to 25% of patients.[87,88] Similarly to RT, its effects are widespread to hair cells and neurons through the generation of ROS,[43,44] and extends to the mitochondria of the cells[89]. Ultimately, this results in the destruction of the OHCs, beginning in the base and progressing to the apex, which leads from high to low frequency hearing deficits[90].

Consistent to our findings, other studies have established the potential predisposing effect of RT exposure to ototoxic agents such as cisplatin. In pediatric patients it has been established that RT predisposes to cisplatin ototoxicity.[91,92] Moreover, as evidenced by Baranak et al.[93] and Miller et al.[94] significantly greater threshold shifts were observed in animals subjected to combined RT and cisplatin in comparison with both therapies alone. Interestingly, Miller et al.[94] showed that fractionated RT enhanced the ototoxicity of cisplatin when this latter was administered at non-ototoxic doses to guinea pigs. While these clinical and animal studies stress the effects of simultaneous RT exposure and other ototoxic agents, our study suggests that RT predisposed the hearing structures to damage even after a long term recovery period.

Similarly, other authors have pointed out synergistic hearing loss of Gentamicin and other ototoxic agents (Table 1). Collins observed that noise and Gentamicin at ototoxic doses had acted synergistically leading to greater hearing loss.[95] Lin et al. evidenced damage to the auditory and spiral ganglion cells in the presence of cochlear ischemia and Gentamicin treatment.[96] In another study, Riggs et al, showed that simultaneous treatment with Gentamicin and cisplatin potentiated cochlear damage.[97] Moreover, studies have demonstrated potentiating effect of Gentamicin ototoxicity by medications such as metronidazole and iron.[98,99] A recent *in vitro* study suggested that RT exposure and Gentamicin had a synergistic effect when cells were exposed to both agents.[80] The authors concluded that the damage was primarily caused through the intrinsic apoptotic pathway, which involves the mitochondrial metabolism. In

concordance, our study proved the synergic effect of combined RT exposure and Gentamicin *in vivo*.

Our DPOAE findings were consistent not only with the dose dependent effect of Gentamicin but also with the predominant high frequency damage.[81] The trend observed in our DPOAE evaluation showed that ears subjected only to Gentamicin 80 mg caused SNR shifts primarily in the high frequency range, while the ears subjected to the combined therapy evidenced SNR shifts across at both low and high frequencies. Despite the lack of statistical significance these findings suggest that low dose RT exposure can potentiate the damage of high doses of Gentamicin at the level of the hair cells.

The ABR testing showed statistical significant difference at 8 and 16 kHz with greater threshold shifts in the group of Gentamicin 80 mg + RT compared to Gentamicin alone. These findings along with head tilt (probably secondary to vestibular toxicity) and damage to the SGC indicate that low dose RT exposure can potentiate the effects of Gentamicin at high doses at the neural component of the hearing loss. Moreover, the results obtained from the ears subjected to Gentamicin 40 mg + RT revealed abnormal morphology of the SGC and auditory hair cells, suggesting that RT's predisposing effect also extends to lower Gentamicin doses, although not evidenced by hearing tests.

Limitations of our study include the high dose per fraction scheme used for our RT exposure model and the absence of a longer follow-up after exposure to Gentamicin. The latter would have been relevant in the ears subjected to Gentamicin 40 mg + RT where progression of functional damage might have been observed at a later period.

Table 1. Selected studies of Gentamicin combinations worsening hearing in guinea pigs compared to gentamicin use alone

Author	Gentamicin Dose	Route	Combined Agent	Dose	Route	Details	Hearing loss compared to GM alone	Cochlear HC loss compared to GM alone
Lin et **al**. (2011)[96]	125 mg/kg single dose	sc	Cochlear ischemia	-	-	For 30 mins prior to GM	60 dB TS More evident in HF	**IHC**: basal turn 42.1%, second turn 42.8% **OHC**: basal & second turn >80%
Marra de Aquino et al. (2008)[100]	10 mg/kg/day x 30 days	im	Amikacin	400 mg/kg x 12d	im	After GM	Reduction in OAE amplitude/intensity	48% more OHC loss
Riggs et al. (1999)[98]	75 mg/kg/day x 14 days	sc	Metronidazole	35 mg/kg/day x 14 days	sc	Same time as GM	10 dB CAP TS	15% OHC loss
Conlom & Smith (1998)[99]	100 mg/kg/day x 30 days	im	Iron	2 mg/kg/day or 6 mg/kg/day x 30d	im	During GM	20 dB CAP TS at HF	-
Riggs et al. (1996)[101]	50 mg/kg/day x 14 days	sc	Cisplatin	6 mg/kg/day x 14 days	sc	Prior, beginning, middle & end of GM	40-50 dB CAP TS	Prior: no difference Beginning: 35% more Middle: 25% more End: 15% more ALL OHC loss
Pye & Collins (1991)[102]	50 mg/kg single dose	sc	Noise	8 kHz at 116 dB SPL	-	1h daily, 20 mins after GM	TS	First row OHC and some IHC
Bhattacharyya & Dayal (1991)[103]	200 mg/kg/day x 7 days	im	Noise	2 kHz at 95 dB SPL	-	2 hrs daily, 30 mins after GM	-	10% more OHC loss 12% more IHC loss
Brummett et al. (1990)[104]	50 mg/kg/day x 16 days	sc	Vancomycin	100 mg/kg or 200 mg/kg x 16d	sc	During GM	CAP reduction uv at 1 kHz	45.9% & 73.8% more OHC loss
Hayashida et al. (1989)[105]	150 mg/kg single dose	im	Ethacrynic acid + noise	30 mg/kg + 60 dB SPL	sc or iv	1.5h after GM	60-80 dB TS More evident in HF	Almost all OHCs destroyed, IHCs were damaged
Collins (1988)[95]	50 mg/kg/day x 10 days	sc	Noise	8 kHz at 116 dB SPL	-	1h at day 1 after GM	HF loss in 33% of GPs	83% more GPs with complete OHC loss
Dodson et al. (1982)[106]	80 mg/kg/day x 5 days	sc	Noise	76 dB SPL x 7 days	-	During GM	-	Apical and basal OHC loss

Abbreviations: GP, guinea pig; kHz, kilohertz; mg, milligrams; kg, kilograms; sc, subcutaneous; im, intramuscular; HF, high frequency; SPL, sound pressure level; d, day(s); mins, minutes; HC, hair cell; OHC, outer hair cell; IHC, inner hair cell; CAP, compound action potential; TS, threshold shit

4.6 Conclusion

The results of the present study show that a previous exposure to low dose RT exposure predisposes to Gentamicin ototoxicity. Hearing loss was greater in the ears subjected to high dose Gentamicin + RT compared to Gentamicin alone. The ears subjected to low dose Gentamicin + RT showed only morphological evidence of cochlear damage. These findings could provide important insight about the risks of prescribing aminoglycosides to pediatric patients with previous RT to the inner ear region.

4.7 Acknowledgments

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4.8 Funding Sources

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4.9 Linking Statement

The present study suggested that RT acts synergistically, making the hearing structure more susceptible to aminoglycoside ototoxicity. Can this however be generalized? The most common form of hearing damage is caused by acoustic trauma. Therefore the authors decided to replicate this study using the same RT exposure but by exposing animals to loud noise instead of aminoglycoside ototoxicity (Chapter 5).

CHAPTER FIVE: Manuscript 3

Synergistic Effect of Radiotherapy and Acoustic Trauma; an Animal Model

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Keywords: guinea pig, radiotherapy, noise, acoustic trauma, synergistic

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The authors have no conflicts of interest to disclose

5.1 Case report that motivated the study

This study was motivated when the primary author learned of a 15-year-old patient diagnosed with a medulloblastoma which was successfully treated with conservative low-dose craniospinal radiotherapy[107], as well as with concurrent cisplatin chemotherapy. Conventional audiometry performed up to 15 month after completion of treatments demonstrated no significant hearing deficits. However 3 years post-treatment, the patient returned with complaints of hearing loss and a disruptive exacerbation of tinnitus. Audiological testing revealed a substantial hearing loss (classified as a Chang Grade 2b loss). Bone conduction audiometry confirmed the presence of SNHL. The patient's history described a sudden hearing loss due to exposure to excessive noise while celebrating his 18th birthday during his first visit to a noisy nightclub. Although his hearing thresholds returned to normal after four weeks, he experienced a progressive hearing loss at higher frequencies, especially in the ear closer to the radiation area when compared to previous audiograms. This was never recovered.

As a result, a study was designed to simulate the radiotherapy typically received by head and neck cancer patients using an animal model in order to determine if such therapy might exacerbate noise-induced hearing loss.

5.2 Introduction

Radiotherapy (RT) is a major component of head and neck cancer therapy. Although recent improvements of radiation techniques, such as intensity-modulated radiation therapy, greatly decreases doses delivered to the inner ear, RT still causes sensorineural hearing loss in 20 to 40% percent of patients whose inner ear is included within the radiation field[30,31]. Radiation induced hearing loss (RIHL) is a late and permanent complication most commonly observed in patients with tumors such as nasopharyngeal carcinomas, medulloblastomas, neuroblastomas and vestibular schwannomas.

Cancer-survivors are amongst individuals exposed to everyday noise. During recreational activities, while at work, or while using personal music devices, exposure to

loud noise is becoming increasingly common. This raises an important concern regarding noise-induced hearing loss (NIHL). NIHL results from exposure to high-intensity sound, especially over a long period of time. Noise is the cause of approximately half of all cases of hearing loss, causing some degree of problems in 5% of the population globally[35].

The mechanisms involved in NIHL are trauma and inflammation of auditory hair, cells, ischemia-reperfusion injury and glutamate excitotoxicity with neuronal degeneration[36,37]. Others have postulated that RIHL results from the formation of free radicals that promote persistent reactive oxygen species (ROS) production and inflammation that in turn, causes functional damage to the auditory hair cells of the cochlea by means of apoptosis. Such damage might also extend to supporting cells and spiral ganglion cells[7,8].

The case report described above might have resulted from such mechanisms. However, there is no clear indication that previous RT exposure can make an ear more vulnerable to stressors such as NIHL. In view of the previous case report, we set out to determine if RT might increase the chances of hearing loss after acoustic trauma[107].

5.3 Materials and Methods

5.3.1 Animal subjects

The study was approved and monitored by the McGill University Health Centre Animal Care Committee in accordance with the Canadian Council of Animal Care Guidelines. Twenty-five two-week-old albino guinea pigs (450 to 500 g; Charles River Laboratories, Wilmington, MA) were kept in a standard housing room at a 22°C ambient temperature with a 12-hour light/dark cycle. All animals had free access to food and water and were examined daily for signs of pain, weight loss or head tilt.

5.3.2 Experimental design

Bilateral baseline auditory brainstem responses (ABR) were performed prior to treatment. One ear of each animal was randomly assigned to receive RT. Based on data from Bohne et al., who delivered fractionated doses of radiation (40 - 50 Gy) and observed less than 10% OHC loss and minimal nerve fiber degeneration[7], it was decided to expose the animals unilaterally for a total dose of 30 Gy (low-dose) or 60 Gy (high-

dose). Thus, animals were divided into 3 groups: (1) low-dose RT + noise (number of animals, $n = 9$); (2) high-dose RT + noise ($n = 9$); and (3) noise ($n = 18$).

Bilateral ABR were repeated one week and 16 weeks after RT to assess short and long-term hearing deficits. All animals were then exposed to acoustic trauma using a continuous pure tone of 6 kHz, at 120 dB SPL for 60 min bilaterally. Hearing tests were performed at 1 day, 7 days and 21 days after noise exposure to assess possible hearing loss, its recovery, or its permanence.

5.3.3 Sample size

A sample size of eight animals was obtained using a power of 80%, an alpha of 0.05 and a minimum absolute difference, which represented ABR amplitude threshold differences of 20 dB with a standard deviation of 20 dB. Therefore, eighteen animals were used for the purpose of this study; 9 receiving low-dose RT and 9 receiving high-dose RT.

5.3.4 Irradiation system

A customized restrainer was constructed in concordance with Winther's study[108]. Briefly, a protective lead shield covered the restrainer and guided the radiation source to the target cochlea. The front teeth of the guinea pig were held with a horizontal wire while lateral screws immobilize the head. The protective lead shielding covered the entire animal leaving the a small collimator window, thereby creating a radiation field size of 6.5 mm x 7.2 mm at the level of cochleae.

Animals were anesthetized with isoflurane and then immobilized using the restrainer, which was positioned inside a Faxitron CP-160 Cabinet X-Radiator System (Faxitron X-Ray Corp., Wheeling, IL, USA) on tray guide #8. A 0.5 mm Cu filter was added and parameters were set at 160 kVp and 6.3 mA. The fraction size for the low-dose group was 3 Gy per day, given on weekdays for two weeks, yielding a total dose of 30 Gy. This low-dose RT was expected to be non-ototoxic. The fraction size for the high-dose RT group was identical, but lasted for 4 weeks instead of 2 weeks, resulting in a total dose of 60 Gy[47].

Using external landmarks (external ear canal and 4 mm from the midline), the beam of the x-ray tube was positioned over the experimental cochlear region. Irradiation

time for each fraction was calculated using the output (1.35 Gy/min) measured (for a given setup) using the EBT model radiochromic film based reference dosimetry system as specified in previous study[109].

5.3.5 Noise exposure

Each animal was anaesthetized with ketamine (50 mg/kg) and xylazine (1 mg/kg) and placed in a sound-proof booth. Acoustic trauma was induced by exposing the animals to a continuous frequency of 6 kHz pure tone through a generator (Intelligent Hearing Systems, Miami, FL) then amplified by an audio amplifier (D-75A, Crown Audio, Inc., Elkhart, IN). The acoustic stimulus was binaurally presented in free field by two loudspeakers (TW 034X0, Audax, France) placed 5 cm in front of the animal's head. The sound levels were monitored by a calibrated Bruel and Kjaer Sound Level Meter.

5.3.6 Assessment of hearing

All hearing tests were conducted using inhalational anesthesia with 2-3 % isofluorane. Once the external ear was inspected, stainless steel needle electrodes were placed subdermally at the pinna, vertex, and contralateral pinna. Bilateral auditory brainstem response (ABR) testing was obtained using SmartEP System (Intelligent Hearing Systems, FL). Tone burst stimuli (8, 16, 20, and 25 kHz) with Blackman envelope were presented through insert earphones at 80 dB sound pressure level, decreasing in steps of 5, 10, and 20 dB to the threshold. Responses to the stimuli were amplified, filtered, and averaged over 1,600 sweeps. The ABR threshold was selected at the last intensity where three reproducible waves III and V could be identified.

5.3.7 Histological analysis

Five randomly chosen cochleae from each group were fixed in 2.5% glutaraldehyde for 2 hours then left in 0.1 M phosphate buffer saline for 24 hours at 4°C. The cochleae were then postfixated in osmium tetroxide for 1.5 hours and dehydrated in graded solutions from 35 to 70% ethanol. Once the organ of Corti was dissected under a surgical microscope, the samples were further dehydrated in solutions up to 100% ethanol, critical point dried, mounted and sputter coated with gold. A field emission scanning electron microscope was used for qualitative analysis (Hitachi S4700; Hitachi).

5.3.8 Statistical Analysis

The data was analyzed using a 4x4x3 mixed ANOVA that examined the effects of two within variables, time of measurement (baseline, day 1, day 7, day 21) and frequency exposure (8, 16, 20 and 25 kHz), and one between variable, treatment (low-dose RT, high-dose RT, and no RT). The data was analyzed using SPSS Statistics Software version 20 (IBM Corp.).

5.4 Results

5.4.1 Auditory brainstem responses

The average volume (dB) that the RT ears could hear at the baseline was 35.44 (SD = 8.84) and for the control ears 33.30 (SD = 5.51). This analyzed using a 2 x 2 between subjects ANOVA, which showed that these averages were similar ($F(1, 21) = 0.007$, $p = 0.93$, $d = 0.00$). Then a 2 x (2) x (2) repeated measures ANOVA was performed to look at the effect of RT on the ears. This showed that the control ($M = 33.30$, $SD = 12.81$) and RT ears ($M = 34.07$, $SD = 12.19$) after RT had a very similar hearing ($F = 0.79$, $p = 0.38$, $d = 0.40$).

Effect of Acoustic Trauma on RT on ABR

A mixed ANOVA analyzed the threshold shift at the four different frequencies (8, 16, 20, 25 kHz) at four different time points (baseline, day 1, day 7, day 21). It was found that there was a significant increase of hearing over time ($F(1,21) = 32.67$, $p = 0.00$, $d = 0.620$). The low and high-dose RT showed no effect ($F(1, 21) = 0.67$, $p = 0.42$, $d = 0.033$). Also, there was no difference in the control and experimental ears ($F(1, 42) = 0.67$, $p = 0.42$, $d = 0.033$) and no significant interactions were found.

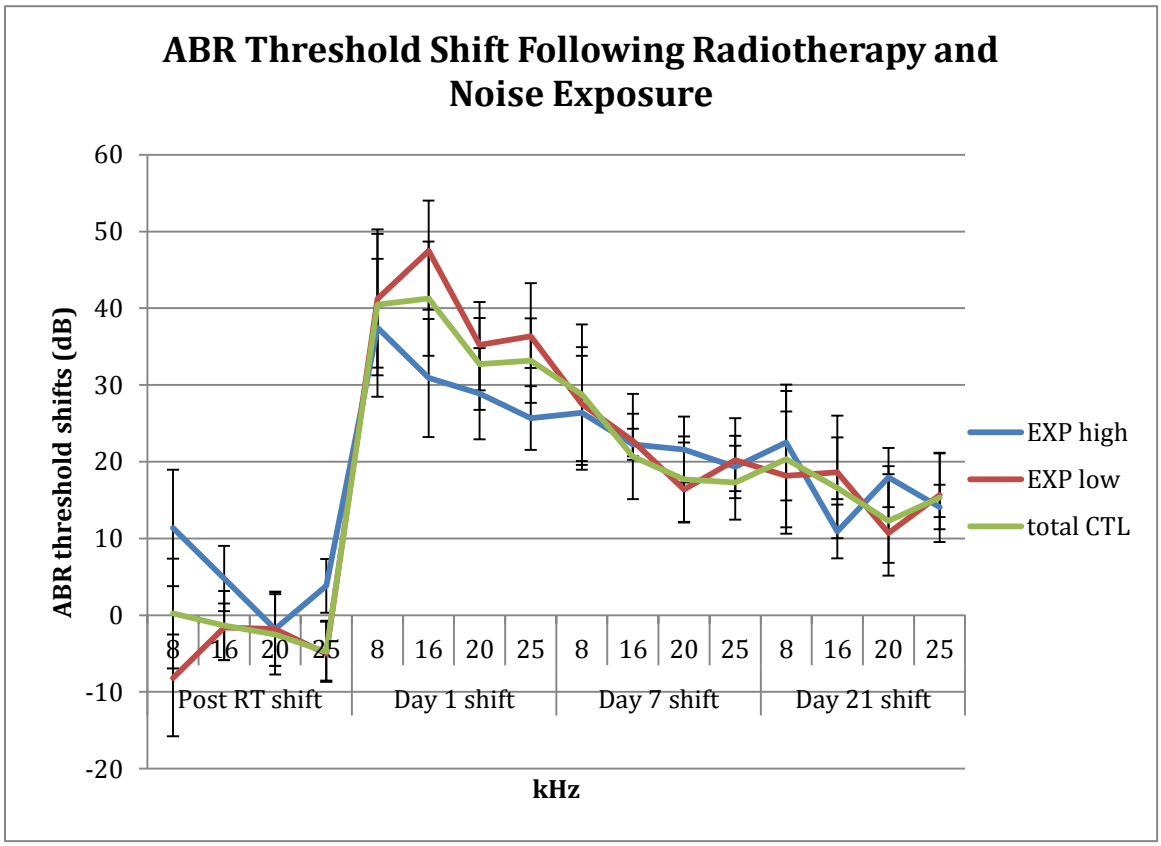

Figure 1. ABR threshold shifts between groups across all time points revealed no significant difference between both experimental and control group. Error bars represent Standard Error of the Mean.

5.4.2 Morphological Analysis of Cochleae

Scanning electron microscopy revealed damage in the auditory hair cells of the ears subjected noise and high-dose RT. However, the ears subjected to low-dose RT exposure and noise did not show any hair cell damage. Similarly, the group receiving only noise did not show any hair cell damage either (Figure 2). Outer auditory hair cells of animals exposed to high-dose RT and noise seemed to exhibit more damage than those of the contralateral ear.

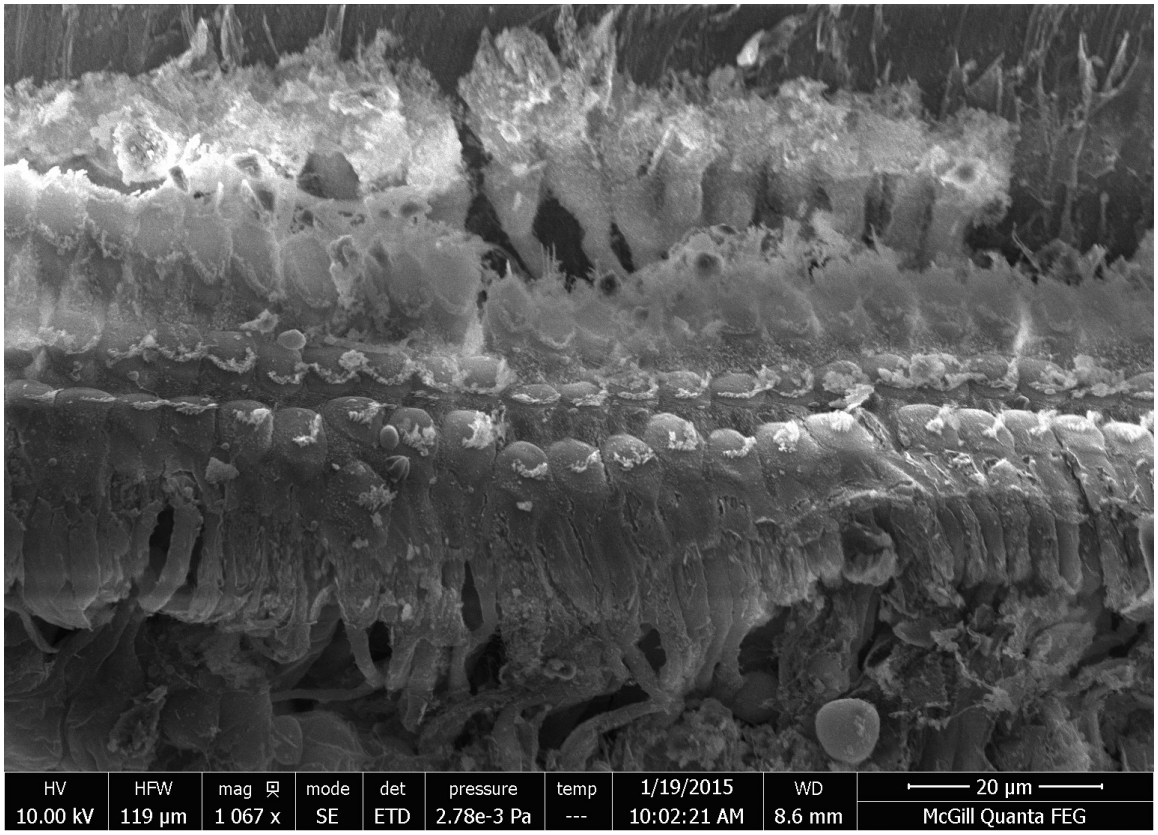

Figure 2. High-dose RT and noise shows clear evidence of outer auditory hair cell damage.

5.5 Discussion

It is well known that chemotherapy agents such as cisplatin and noise exposure can damage the peripheral auditory system and cause hearing loss[110,111]. The combination of cisplatin and noise produced significantly more hair cell loss and hearing loss at the high frequencies than did either the noise or cisplatin alone when the noise level was 85 dB SPL or higher; no interaction was seen when the noise level was at, or below, 70 dB SPL[110,111]. The case described in Section 5.1 suggested that a similar interaction might be present with RT. However, no previous reports in the literature have described peripheral auditory system damage associated with RT and acoustic trauma.

Others have described synergistic ototoxic effects associated with noise and other agents. Table 2 summarizes previous reports on this issue. For example, a recent study by Zawawi et al. from our lab suggested that high-level caffeine ingestion delays hearing

recovery after acoustic trauma[112]. This finding was supported by observations of cochlear histology, which showed evidence of outer hair cell damage in the apex of the cochleae[112]. Others such as Li and Collins reported synergistic hearing loss effects due to simultaneous noise and aminoglycoside exposure[95,113]. Similar conclusions were drawn with agents such as nitric oxide syntase, toluene, nifedipine, styrene and ethyl benzene[114-118].

The present study examined the relationship between low-dose RT and acoustic trauma. It primarily looked at the recovery and/or progressivity of hearing abilities following acoustic overstimulation in cochleae exposed to RT versus cochleae that were not exposed to RT. The study revealed by the means of cochlear histology that low doses of RT could potentiate auditory hair cell loss post acoustic trauma. Auditory brainstem responses did not show any significantly greater hearing loss, or any delay in recovery following acoustic trauma. Increased hearing thresholds were only found at 8 kHz when comparing the experiment high-dose with the experimental low-dose following RT. One day after noise exposure, hearing thresholds were increased to about 30 dB at all frequencies in all experimental groups. The experimental (radiated) and control ears of animals recovered similarly at days 7 and 21 post noise exposure.

However, despite the lack of functional hearing loss changes in the high-dose RT and noise group animals, histological changes were observed in the scanning electron microscopy images when compared to other groups. The presence of histological changes that appeared before functional hearing changes was also described in the experimental group in chapter 4 of this thesis, where low doses of Gentamicin combined with RT yielded cochlear damage without significant changes in ABR threshold. Our own results showed that a synergistic effect caused by high doses of RT and acoustic trauma might be present even when hearing losses were not detected by functional ABR testing.

The present study examined the relationship between low-dose RT and acoustic trauma. It primarily looked at the recovery and/or progressivity of hearing abilities following acoustic overstimulation in cochleae exposed to RT versus cochleae that were not exposed to RT. The study revealed by the means of cochlear histology that low doses of RT could potentiate auditory hair cell loss post acoustic trauma.

The results of this thesis focus on the importance of hearing deficits appearing

long after the end of RT treatments. Therefore, patients with previous RT exposure to the head and neck should routinely be followed in audiology; even years after treatment. Also, documented recommendations must be made bearing in mind the potentiating evidence of RT on hearing stressors as demonstrated by this thesis. For example, clinicians should apprehend the potentiating effect of RT in aminoglycoside ototoxicity. Hence, even when prescribing low and safe doses of these antibiotics, clinicians should be aware about previous RT exposure in patients, warn patients and even follow up hearing abilities, when possible. Furthermore, clinicians should warn patients previously exposed to RT to the head and neck about the potentiating damages associated with acoustic overstimulation. Thus, patients should protect their ears when in loud environments. This will in turn influence work environment and leisure activity choices.

Table 2. Selected studies on synergistic effects of acoustic trauma worsening hearing in animal model

Author	Noise	Route	Combined Agent	Dose	Route	Details	Hearing loss compared to Noise	Pathological findings
Zawawi et al. (2014)[112]	6 kHz pure-tone 110 dB for 60 mins	Free field	Caffeine	25 mg/kg/d	I/P	-	Delay in TS recovery	More OHC loss in apex of cochlea
Inai et al. (2012)[114]	2 kHz pure tone 120dB for 5 hrs	Free field	Nitric oxide syntase	50 mg/kg	I/P	injection 1 hours before noise	TS 1 to 7 days after	increased effect and damage to cochlea
Collins (1988)[95]	8 kHz pure tone 116 dB for 60 mins	Free field	Gentamicin	50 mg/kg/d x 10d	I/P	-	unaffected at 24 and 72 hours after	synergistic effect cause the most HC and PTS
Liu et al. (2012)[116]	1-8kHz white noise 100dB for 2 hrs	Ear-phones	Nifedipine	0.15, 0.5 and 3umol/L x 1 d rest	C/P	1 weeks delay, TS with only nifedipine	-	noise and drug had synergistic effect only in the 0.15 group all other were subtractive
Pryor et al (1983)[115]	4 kHz, 8 kHz and 12 kHz	Free field	Toluene	1200 or 1400ppm, 14hrs/7d/5w	-	-	-	the higher the kHz the higher the hearing loss
Lataye et al. (2005)[117]	band noise 8 kHz 86.2dB 6hrs/5d/4w	Free field	Styrene	300 to 1000 ppm for 6hrs/5d/4w	I/H	2 groups (1 sedentary other with exercised)	damage to subtle to see with cochleogram	increased OHC loss along cochlea but not basal part in both groups
Brandt-Lassen et al. (2000)[119]	4-20 kHz for 2 hr/day x 10	Free field	Toluene	0, 500, 1000, 1500, 2000 ppm 6h/10d	I/H	-	mid-frequency TS to 1500 and 2000 ppm	synergistic effect with high doss of drug
Cappaert et al. (2001)[118]	50 to 25000 kHz broad-band noise 95 or 105 dB 8h/d x 5d	Free field	Ethyl benzene	0, 300 or 400 ppm 8h/d x 5d	I/H	-	cause only minor OHC loss	OHC loss in mid-modiolar region
Li et al. (2008)[113]	20 to 48 kHz pure tone 100dB 3-6 hrs dB or 86 dB 18h/d x 3d	Free field	Gentamicin	exposure to immunofluorescence	incubation	control gentamicin for 30 mins	-	greater OHC loss than control

Abbreviations: GP, guinea pig; kHz, kilohertz; mg, milligrams; kg, kilograms; s/c, subcutaneous; i/m, intramuscular; i/p, intraperitoneal; i/h inhalation; CP, cochlear perfusion HF, high frequency; SPL, sound pressure level; d, day(s); mins, minutes; w, week(s); hrs, hours; HC, hair cell; OHC, outer hair cell; IHC, inner hair cell; CAP, compound action potential; TS, threshold shift, PTS permanent threshold shift.

5.6 Conclusion

Despite the lack of functional changes in the ears treated with high doses of RT and noise, histological changes showing auditory hair cell damage in scanning electron microscopy images revealed a synergistic effect. Future studies are needed to determine if prolonged or repeated noise exposure on previously radiated cochleae damages hair cells. Also, similar studies of human subjects are required.

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CHAPTER SIX: Summary

6.1 Overall Discussion

The present thesis is a preliminary exploration of factors that may lead to hearing loss and ototoxicity. These factors include RT, drug ototoxicity and acoustic overstimulation. Scientific literature on the latter three has delineated specific mechanisms in which these components cause hearing deficits in humans and animal models. Importantly, radiation induced hearing loss, aminoglycoside ototoxicity and noise induced hearing loss share a common underlying mechanism; damage to the auditory hair cells of the cochlea by means of apoptosis that could extend to supporting cells and spiral ganglion cells. Alongside hearing tests, the status of auditory hair cells as well as supporting spiral ganglion cells was examined in the studies encompassed in this thesis.

The first study mentioned in Chapter 3 illustrated the relationship between RT dosage and the time course that lead to hearing loss. The outcomes revealed (A) that low dose RT, analogous to clinical low dose therapy does not cause hearing loss. (B) High dose RT (analogous to clinic high dose therapy) caused both immediate and long term hearing loss. (C) The initial hearing loss observed after RT treatment is likely associated with cochlear sensory cell damage (resulting from auditory hair cells), while the longer term progressive hearing loss observed is likely associated with neural damage (e.g. primary neuron; supporting ganglion cells)

The second study discussed in Chapter 4 examined the relationship between RT, Gentamicin antibiotic dosage and hearing loss. The experiments showed that (A) low dose Gentamicin therapy (analogous to clinical low dose therapy) did not cause hearing loss, (B) high dose Gentamicin therapy (analogous to clinical high dose therapy) caused hearing loss. The latter two findings were expected as they reported in previous literature. The study revealed (C) a synergistic effect clearly identified by hearing tests and cochlear histology in high dose Gentamicin and RT exposure. Finally, cochlear histology demonstrated the same synergistic effect in the cochleae exposed to low dose Gentamicin and safe dose of RT.

The last study mentioned in Chapter 5 of this thesis examined the relationship between low doses of RT and acoustic trauma. It primarily looked at the recovery and/or progressivity of hearing abilities following acoustic overstimulation in cochleae exposed to RT versus cochleae that were not exposed to RT. The study revealed by the means of cochlear histology that low doses of RT could potentiate auditory hair cell loss post acoustic trauma.

6.2 Overall Conclusion

The present thesis yielded new knowledge (1) by developing an animal model to study changes in hearing following fractionated RT protocols simulating those used clinically. Using the same model, (2) aminoglycoside ototoxicity was intensified by RT at both high and safe low doses. Finally, (3) the relationship between RT exposure and hearing ability recovery post acoustic trauma was delineated.

6.2 Future Studies

The outcomes of this thesis shed knowledge on the effect of fractionated RT schemes seen in clinical setting on hearing. The experiments described in this thesis were conducted in animal models. There is a need to translate the outcomes of this thesis into human clinically relevant modalities.

The results of this thesis stresses on importance of hearing deficits appearing long after the end of RT treatments. Therefore, patients with previous RT exposure to the head and neck should routinely be followed in audiology; even years after treatment. Also, documented recommendations must be made bearing in mind the potentiating evidence of RT on hearing stressors as demonstrated by this thesis. For example, clinicians should apprehend the potentiating effect of RT in aminoglycoside ototoxicity. Hence, even when prescribing low and safe doses of these antibiotics, clinicians should be aware about previous RT exposure in patients, warn patients and even follow up hearing abilities, when possible. Furthermore, clinicians should warn patients previously exposed to RT to

the head and neck about the potentiating damages associated with acoustic overstimulation. Thus, patients should protect their ears when in loud environments. This will in turn influence work environment and leisure activity choices.

References

1. Rabik CA, Dolan ME. Molecular mechanisms of resistance and toxicity associated with platinating agents. Cancer treatment reviews 2007; 33:9-23.
2. http://www.entcanada.org/public2/patient14.asp. CSoOHaNSECSoOHaNScNtAf.
3. Peleva E, Emami N, Alzahrani Met al. Incidence of platinum-induced ototoxicity in pediatric patients in Quebec. Pediatric blood & cancer 2014; 61:2012-2017.
4. Fleury B, Lapeyre M. [Tolerance of normal tissues to radiation therapy: ear]. Cancer Radiotherapie 2010; 14:284-289.
5. Raaijmakers E, Engelen AM. Is sensorineural hearing loss a possible side effect of nasopharyngeal and parotid irradiation? A systematic review of the literature. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology 2002; 65:1-7.
6. Ondrey FG, Greig JR, Herscher L. Radiation dose to otologic structures during head and neck cancer radiation therapy. The Laryngoscope 2000; 110:217-221.
7. Bohne BA, Marks JE, Glasgow GP. Delayed effects of ionizing radiation on the ear. The Laryngoscope 1985; 95:818-828.
8. Low WK, Tan MG, Chua AW, Sun L, Wang DY. 12th Yahya Cohen Memorial Lecture: The cellular and molecular basis of radiation-induced sensori-neural hearing loss. Annals of the Academy of Medicine, Singapore 2009; 38:91-94.
9. Mujica-Mota MA, Gasbarrino K, Rappaport JM, Shapiro RS, Daniel SJ. The effect of caffeine on hearing in a guinea pig model of acoustic trauma. American journal of otolaryngology 2014; 35:99-105.
10. Gelfand SA. Essentials of audiology. New York: Thiem 2009.
11. Salvi R, Sun W, Lobarinas E. Anatomy and Physiology of the Peripheral Auditory System., 2007.
12. Bess FH, Humes L. Audiology: the fundamentals. Philadelphia, 2008.
13. Raphael Y, Altschuler RA. Structure and innervation of the cochlea. Brain research bulletin 2003; 60:397-422.
14. Hudspeth AJ. How the ear's works work. Nature 1989; 341:397-404.
15. Ashmore J, Geleoc GS. Cochlear function: hearing in the fast lane. Current biology : CB 1999; 9:R572-574.
16. Hudspeth AJ. Hair-bundle mechanics and a model for mechanoelectrical transduction by hair cells. Society of General Physiologists series 1992; 47:357-370.
17. Musiek FE, Weihing JA, Oxholm VB. Anatomy and physiology of the Central Auditory Nervous System: A Clinical Perspective. New York: Thieme, 2007.
18. Aitkin LM, Webster WR, Veale JL, Crosby DC. Inferior colliculus. I. Comparison of response properties of neurons in central, pericentral, and external nuclei of adult cat. Journal of neurophysiology 1975; 38:1196-1207.
19. Hall EJ, Cox JD. Physical and Biologic Basis of Radiation Therapy. . Mosby Elsevier, 2010.

20. Sharma RA, Vallis KA, McKenna WG. Chapter 29. Basics of Radiation Therapy. In: Abeloff MD, editor. Abeloff´s Clinical Oncology. 4th ed. Philadelphia: Churchill Livingstone; 2008. Philadelphia: Churchill Livingstone, 2008.
21. Ogawa Y, Kobayashi T, Nishioka Aet al. Radiation-induced reactive oxygen species formation prior to oxidative DNA damage in human peripheral T cells. International journal of molecular medicine 2003; 11:149-152.
22. Thwaites DI, Tuohy JB. Back to the future: the history and development of the clinical linear accelerator. Physics in medicine and biology 2006; 51:R343-362.
23. Ward JF. Biochemistry of DNA lesions. Radiation research Supplement 1985; 8:S103-111.
24. Buatti JM, Friedman WA, Meeks SL, Bova FJ. The radiobiology of radiosurgery and stereotactic radiotherapy. Medical dosimetry : official journal of the American Association of Medical Dosimetrists 1998; 23:201-207.
25. Regaud T, Nogier T. Sterilization roentgenienne totale et definitive sans radiodermite, des testicules du belier adulte. Conditions de sa realisation. Compt Rend Soc 1911; 70:202-203.
26. Isaacson SR, Close LG. Chapter 11. Clinical Radiation Biology and Radiotherapy. New York, 2005.
27. Moulder JE, Rockwell S. Hypoxic fractions of solid tumors: experimental techniques, methods of analysis, and a survey of existing data. International journal of radiation oncology, biology, physics 1984; 10:695-712.
28. Kwong DL, Wei WI, Sham JSet al. Sensorineural hearing loss in patients treated for nasopharyngeal carcinoma: a prospective study of the effect of radiation and cisplatin treatment. International journal of radiation oncology, biology, physics 1996; 36:281-289.
29. Stieber VW, Mehta MP. Advances in radiation therapy for brain tumors. Neurologic clinics 2007; 25:1005-1033, ix.
30. Mujica-Mota M, Waissbluth S, Daniel SJ. Characteristics of radiation-induced sensorineural hearing loss in head and neck cancer: a systematic review. Head & neck 2013; 35:1662-1668.
31. Pan CC, Eisbruch A, Lee JS, Snorrason RM, Ten Haken RK, Kileny PR. Prospective study of inner ear radiation dose and hearing loss in head-and-neck cancer patients. International journal of radiation oncology, biology, physics 2005; 61:1393-1402.
32. Honore HB, Bentzen SM, Moller K, Grau C. Sensori-neural hearing loss after radiotherapy for nasopharyngeal carcinoma: individualized risk estimation. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology 2002; 65:9-16.
33. Lyng FM, Seymour CB, Mothersill C. Oxidative stress in cells exposed to low levels of ionizing radiation. Biochem Soc Trans 2001; 29:350-353.
34. Leach JK, Van Tuyle G, Lin PS, Schmidt-Ullrich R, Mikkelsen RB. Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen. Cancer Res 2001; 61:3894-3901.
35. Oishi N, Schacht J. Emerging treatments for noise-induced hearing loss. Expert opinion on emerging drugs 2011; 16:235-245.

36. Hamernik RP, Turrentine G, Roberto M, Salvi R, Henderson D. Anatomical correlates of impulse noise-induced mechanical damage in the cochlea. Hearing research 1984; 13:229-247.
37. Lin HW, Furman AC, Kujawa SG, Liberman MC. Primary neural degeneration in the Guinea pig cochlea after reversible noise-induced threshold shift. Journal of the Association for Research in Otolaryngology : JARO 2011; 12:605-616.
38. Winther FO. X-ray irradiation of the inner ear of the guinea pig. Early degenerative changes in the vestibular sensory epithelia. Acta oto-laryngologica 1969; 68:514-525.
39. Winther FO. X-ray irradiation of the inner ear of the guinea pig. An electron microscopic study of the degenerating vestibular sensory cell. Acta oto-laryngologica 1970; 69:307-319.
40. Haynes DS. Topical antibiotics: strategies for avoiding ototoxicity. Ear, nose, & throat journal 2004; 83:12-14.
41. Strupp M, Arbusow V. Acute vestibulopathy. Current opinion in neurology 2001; 14:11-20.
42. Selimoglu E, Kalkandelen S, Erdogan F. Comparative vestibulotoxicity of different aminoglycosides in the Guinea pigs. Yonsei medical journal 2003; 44:517-522.
43. Kalkandelen S, Selimoglu E, Erdogan F, Ucuncu H, Altas E. Comparative cochlear toxicities of streptomycin, gentamicin, amikacin and netilmicin in guinea-pigs. The Journal of international medical research 2002; 30:406-412.
44. Rybak LP, Kelly T. Ototoxicity: bioprotective mechanisms. Current opinion in otolaryngology & head and neck surgery 2003; 11:328-333.
45. Roland PS. New developments in our understanding of ototoxicity. Ear, nose, & throat journal 2004; 83:15-16; discussion 16-17.
46. Dehne N, Rauen U, de Groot H, Lautermann J. Involvement of the mitochondrial permeability transition in gentamicin ototoxicity. Hearing research 2002; 169:47-55.
47. Mujica-Mota MA, Ibrahim FF, Bezdjian A, Devic S, Daniel SJ. The effect of fractionated radiotherapy in sensorineural hearing loss: an animal model. The Laryngoscope 2014; 124:E418-424.
48. Johannesen TB, Rasmussen K, Winther FO, Halvorsen U, Lote K. Late radiation effects on hearing, vestibular function, and taste in brain tumor patients. International journal of radiation oncology, biology, physics 2002; 53:86-90.
49. Nagel D, Schafer J. Changes in cochlear microphonic response after Y-ray irradiation of the inner ear of the guinea-pig. Archives of oto-rhino-laryngology 1984; 241:17-21.
50. Tokimoto T, Kanagawa K. Effects of X-ray irradiation on hearing in guinea pigs. Acta oto-laryngologica 1985; 100:266-272.
51. Gamble JE, Peterson EA, Chandler JR. Radiation effects on the inner ear. Archives of otolaryngology 1968; 88:156-161.
52. Akmansu H, Eryilmaz A, Korkmaz Het al. Ultrastructural and electrophysiologic changes of rat cochlea after irradiation. The Laryngoscope 2004; 114:1276-1280.
53. Meijer OW, Vandertop WP, Baayen JC, Slotman BJ. Single-fraction vs. fractionated linac-based stereotactic radiosurgery for vestibular schwannoma: a

single-institution study. International journal of radiation oncology, biology, physics 2003; 56:1390-1396.
54. Murphy ES, Suh JH. Radiotherapy for vestibular schwannomas: a critical review. International journal of radiation oncology, biology, physics 2011; 79:985-997.
55. Hall EJ, Brenner DJ. The radiobiology of radiosurgery: rationale for different treatment regimes for AVMs and malignancies. International journal of radiation oncology, biology, physics 1993; 25:381-385.
56. Miller MW, Riedel G, Hoistad Det al. Ototoxicity after combined platinum and fractionated radiation in a novel guinea pig model. American journal of otolaryngology 2009; 30:1-7.
57. Greene JS, Giddings NA, Jacobson JT. Effect of irradiation on guinea pig ABR thresholds. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery 1992; 107:763-768.
58. Grau C, Moller K, Overgaard M, Overgaard J, Elbrond O. Auditory brain stem responses in patients after radiation therapy for nasopharyngeal carcinoma. Cancer 1992; 70:2396-2401.
59. van der Putten L, de Bree R, Plukker JTet al. Permanent unilateral hearing loss after radiotherapy for parotid gland tumors. Head & neck 2006; 28:902-908.
60. Schot LJ, Hilgers FJ, Keus RB, Schouwenburg PF, Dreschler WA. Late effects of radiotherapy on hearing. European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies 1992; 249:305-308.
61. Winther FO. X-ray irradiation of the inner ear of the guinea pig. Early degenerative changes in the cochlea. Acta oto-laryngologica 1969; 68:98-117.
62. Devic S, Seuntjens J, Sham Eet al. Precise radiochromic film dosimetry using a flat-bed document scanner. Medical physics 2005; 32:2245-2253.
63. Hoistad DL, Ondrey FG, Mutlu C, Schachern PA, Paparella MM, Adams GL. Histopathology of human temporal bone after cis-platinum, radiation, or both. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery 1998; 118:825-832.
64. Kakigi A, Hirakawa H, Harel N, Mount RJ, Harrison RV. Comparison of distortion-product and transient evoked otoacoustic emissions with ABR threshold shift in chinchillas with ototoxic damage. Auris, nasus, larynx 1998; 25:223-232.
65. Gehr DD, Janssen T, Michaelis CE, Deingruber K, Lamm K. Middle ear and cochlear disorders result in different DPOAE growth behaviour: implications for the differentiation of sound conductive and cochlear hearing loss. Hearing research 2004; 193:9-19.
66. Siegel JH, Hirohata ET. Sound calibration and distortion product otoacoustic emissions at high frequencies. Hearing research 1994; 80:146-152.
67. Anteunis LJ, Wanders SL, Hendriks JJ, Langendijk JA, Manni JJ, de Jong JM. A prospective longitudinal study on radiation-induced hearing loss. American journal of surgery 1994; 168:408-411.
68. Li JJ, Guo YK, Tang QLet al. Prospective study of sensorineural hearing loss following radiotherapy for nasopharyngeal carcinoma. The Journal of laryngology and otology 2010; 124:32-36.

69. Low WK, Burgess R, Fong KW, Wang DY. Effect of radiotherapy on retro-cochlear auditory pathways. The Laryngoscope 2005; 115:1823-1826.
70. Grau C, Moller K, Overgaard M, Overgaard J, Elbrond O. Sensori-neural hearing loss in patients treated with irradiation for nasopharyngeal carcinoma. International journal of radiation oncology, biology, physics 1991; 21:723-728.
71. Kim CS, Shin SO. Ultrastructural changes in the cochlea of the guinea pig after fast neutron irradiation. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery 1994; 110:419-427.
72. Wackym PA, Runge-Samuelson CL, Nash JJet al. Gamma knife surgery of vestibular schwannomas: volumetric dosimetry correlations to hearing loss suggest stria vascularis devascularization as the mechanism of early hearing loss. Otology & neurotology : official publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology 2010; 31:1480-1487.
73. Bezdjian A, Mujica-Mota MA, Devic S, Daniel SJ. The Effect of Radiotherapy on Gentamicin Ototoxicity: An Animal Model. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery 2015.
74. Williams GB, Kun LE, Thompson JW, Gould HJ, Stocks RM. Hearing loss as a late complication of radiotherapy in children with brain tumors. Ann Otol Rhinol Laryngol 2005; 114:328-331.
75. Huang E, Teh BS, Strother DRet al. Intensity-modulated radiation therapy for pediatric medulloblastoma: early report on the reduction of ototoxicity. Int J Radiat Oncol Biol Phys 2002; 52:599-605.
76. Hua C, Bass JK, Khan R, Kun LE, Merchant TE. Hearing loss after radiotherapy for pediatric brain tumors: effect of cochlear dose. Int J Radiat Oncol Biol Phys 2008; 72:892-899.
77. Selimoglu E. Aminoglycoside-induced ototoxicity. Current pharmaceutical design 2007; 13:119-126.
78. Paulino AC, Lobo M, Teh BSet al. Ototoxicity after intensity-modulated radiation therapy and cisplatin-based chemotherapy in children with medulloblastoma. Int J Radiat Oncol Biol Phys 2010; 78:1445-1450.
79. Naeimi M, Maamouri G, Boskabadi Het al. Assessment of aminoglycoside-induced hearing impairment in hospitalized neonates by TEOAE. Indian journal of otolaryngology and head and neck surgery : official publication of the Association of Otolaryngologists of India 2009; 61:256-261.
80. Chung M. Combined radiation and gentamicin induced ototoxicity *School of Biological Sciences*. China: Nanyang Technological University, 2012:30.
81. Collins PW, Twine JM. The ototoxic effects of different doses of gentamicin on the cochlea of pigmented guinea pigs. British journal of audiology 1985; 19:257-264.
82. Rao SC, Srinivasjois R, Hagan R, Ahmed M. One dose per day compared to multiple doses per day of gentamicin for treatment of suspected or proven sepsis in neonates. Cochrane database of systematic reviews 2011:CD005091.

83. Winther FO. X-ray irradiation of the inner ear of the guinea pig. Early degenerative changes in the cochlea. Acta Otolaryngol 1969; 68:98-117.
84. Mujica-Mota MA, Ibrahim F, Bezdjian A, Devic S, Daniel SJ. The Effect of Fractionated Radiotherapy in Sensorineural Hearing Loss: An Animal Model. . The Laryngoscope (Under review) 2014.
85. Mujica-Mota MA, Devic S, Daniel SJ. The Relevance of Dosimetry in Animal Models of Cochlear Irradiation. Otology & Neurotology 2013; (In press).
86. Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. Free radical biology & medicine 2000; 29:222-230.
87. Fischel-Ghodsian N. Genetic factors in aminoglycoside toxicity. Pharmacogenomics 2005; 6:27-36.
88. McCracken GH, Jr. Aminoglycoside toxicity in infants and children. The American journal of medicine 1986; 80:172-178.
89. Jensen-Smith HC, Hallworth R, Nichols MG. Gentamicin rapidly inhibits mitochondrial metabolism in high-frequency cochlear outer hair cells. PloS one 2012; 7:e38471.
90. Forge A, Schacht J. Aminoglycoside antibiotics. Audiology & neuro-otology 2000; 5:3-22.
91. Miettinen S, Laurikainen E, Johansson R, Minn H, Laurell G, Salmi TT. Radiotherapy enhanced ototoxicity of cisplatin in children. Acta oto-laryngologica Supplementum 1997; 529:90-94.
92. Warrier R, Chauhan A, Davluri M, Tedesco SL, Nadell J, Craver R. Cisplatin and cranial irradiation-related hearing loss in children. The Ochsner journal 2012; 12:191-196.
93. Baranak CC, Wetmore RF, Packer RJ. Cis-platinum ototoxicity after radiation treatment: an animal model. Journal of neuro-oncology 1988; 6:261-267.
94. Miller MW, Riedel G, Hoistad Det al. Ototoxicity after combined platinum and fractionated radiation in a novel guinea pig model. Am J Otolaryngol 2009; 30:1-7.
95. Collins PW. Synergistic interactions of gentamicin and pure tones causing cochlear hair cell loss in pigmented guinea pigs. Hearing research 1988; 36:249-259.
96. Lin CD, Kao MC, Tsai MHet al. Transient ischemia/hypoxia enhances gentamicin ototoxicity via caspase-dependent cell death pathway. Laboratory investigation; a journal of technical methods and pathology 2011; 91:1092-1106.
97. Riggs LC, Brummett RE, Guitjens SK, Matz GJ. Ototoxicity resulting from combined administration of cisplatin and gentamicin. Laryngoscope 1996; 106:401-406.
98. Riggs LC, Shofner WP, Shah AR, Young MR, Hain TC, Matz GJ. Ototoxicity resulting from combined administration of metronidazole and gentamicin. The American journal of otology 1999; 20:430-434.
99. Conlon BJ, Smith DW. Supplemental iron exacerbates aminoglycoside ototoxicity in vivo. Hearing research 1998; 115:1-5.
100. Aquino TJ, Oliveira JA, Rossato M. Ototoxicity and otoprotection in the inner ear of guinea pigs using gentamicin and amikacin: ultrastructural and functional aspects. Brazilian journal of otorhinolaryngology 2008; 74:843-852.

101. Riggs LC, Brummett RE, Guitjens SK, Matz GJ. Ototoxicity resulting from combined administration of cisplatin and gentamicin. The Laryngoscope 1996; 106:401-406.
102. Pye A, Collins P. Interaction between sound and gentamicin: immediate threshold and stereociliary changes. British journal of audiology 1991; 25:381-390.
103. Bhattacharyya TK, Dayal VS. Potentiation of cochlear hair cell loss by acoustic stimulus and gentamicin in the guinea pig. The Anatomical record 1991; 230:136-145.
104. Brummett RE, Fox KE, Jacobs F, Kempton JB, Stokes Z, Richmond AB. Augmented gentamicin ototoxicity induced by vancomycin in guinea pigs. Archives of otolaryngology--head & neck surgery 1990; 116:61-64.
105. Hayashida T, Hiel H, Dulon D, Erre JP, Guilhaume A, Aran JM. Dynamic changes following combined treatment with gentamicin and ethacrynic acid with and without acoustic stimulation. Cellular uptake and functional correlates. Acta oto-laryngologica 1989; 108:404-413.
106. Dodson HC, Bannister LH, Douek EE. The effects of combined gentamicin and white noise on the spiral organ of young guinea pigs. A structural study. Acta oto-laryngologica 1982; 94:193-202.
107. Peleva E, Aloy E, Carret AS, Daniel SJ. Hearing loss in a pediatric patient following cisplatin chemotherapy and subsequent exposure to excessive noise. International journal of pediatric otorhinolaryngology 2014; 78:2301-2304.
108. Winther FO. X-ray irradiation of the inner ear of the guinea pig. An electron microscopic study of the degenerating outer hair cells of the organ of Corti. Acta oto-laryngologica 1970; 69:61-76.
109. Mujica-Mota MA, Devic S, Daniel SJ. The relevance of dosimetry in animal models of cochlear irradiation. Otology & neurotology : official publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology 2014; 35:704-711.
110. Gratton MA, Salvi RJ, Kamen BA, Saunders SS. Interaction of cisplatin and noise on the peripheral auditory system. Hearing research 1990; 50:211-223.
111. Gratton MA, Kamen BA. Potentiation of cisplatin ototoxicity by noise. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 1990; 8:2091-2092.
112. Zawawi F, Bezdjian A, Mujica-Mota M, Daniel SJ. The effect of caffein on acoustic overstimulation in a guinea pig model. unpliblished manuscript, McGill Auditory Sciences Laboratory 2015.
113. Li S, Zhang SQ, Liu SWet al. Assessment criteria for rotated stereociliary bundles in the guinea pig cochlea. Otology & neurotology : official publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology 2008; 29:86-92.
114. Inai S, Watanabe K, Okubo K. Inducible nitric oxide synthase participates in cochlear damage after acoustic stimulation in Guinea pigs. Journal of Nippon Medical School = Nippon Ika Daigaku zasshi 2012; 79:121-128.
115. Pryor GT, Dickinson J, Howd RA, Rebert CS. Transient cognitive deficits and high-frequency hearing loss in weanling rats exposed to toluene. Neurobehavioral toxicology and teratology 1983; 5:53-57.

116. Liu J, Niu YG, Li WXet al. Interaction of a calcium channel blocker with noise in cochlear function in guinea pig. Acta oto-laryngologica 2012; 132:1140-1144.
117. Lataye R, Campo P, Loquet G, Morel G. Combined effects of noise and styrene on hearing: comparison between active and sedentary rats. Noise & health 2005; 7:49-64.
118. Cappaert NL, Klis SF, Muijser H, Kulig BM, Smoorenburg GF. Simultaneous exposure to ethyl benzene and noise: synergistic effects on outer hair cells. Hearing research 2001; 162:67-79.
119. Brandt-Lassen R, Lund SP, Jepsen GB. Rats exposed to Toluene and Noise may develop Loss of Auditory Sensitivity due to Synergistic Interaction. Noise & health 2000; 3:33-44.