Novel strategies for the prevention of cisplatin-induced ototoxicity

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This thesis is dedicated to my family, to my brothers Ariel and Diego and to my mother Marcela. Their endless support and encouragement provided me with inner strength and have molded me into who I am today.

Abstract

Cisplatin is a commonly used chemotherapeutic agent. Unfortunately, serious side effects limit its clinical use such as ototoxicity, which presents as bilateral and progressive sensorineural hearing loss. Regrettably, there is currently no treatment for cisplatin-induced ototoxicity. The pathophysiology remains unclear, however, it is believed that inflammation and oxidative stress are the main mechanisms leading to cell death. In the present thesis, various aspects of cisplatin-induced ototoxicity and potential treatment strategies are evaluated. We begin with a review of the literature in what concerns the entrance and egress of cisplatin from cochlear cells. Cisplatin has a predilection for the inner ear tissues and the reason for such an occurrence is unknown. We describe the receptors that may play a role in cisplatin-induced ototoxicity and that are present in cochlear cells. Understanding the circulating pathways of cisplatin within the inner ear can provide some insight into the mechanisms of cisplatin-induced ototoxicity. Once inside the cell, cisplatin can elicit an inflammatory response. For this reason, we decided to evaluate the potential of dexamethasone as a protective agent against cisplatin's toxic effects *in vivo*. It was observed that a central regulator of inflammation was decreased as a result of the therapy; however, the hearing was not preserved. An anti-inflammatory did not provide sufficient protection to preserve hearing following the cisplatin treatment. ROS have also been implicated in cisplatin-induced cytotoxicity. It appears that cisplatin can lead to an increased expression of ROS that can overwhelm the natural antioxidant response of the cochlea. Thus, the potential of an exogenous antioxidant as a protective agent was evaluated *in vivo*. Erdosteine, a derivative of methionine, provided protection against cisplatin-induced ototoxicity at high frequencies of hearing as well as partially prevented OHC loss. Because these two compounds provided only but partial benefits, we decided to evaluate a more specific and targeted approach, gene therapy for cisplatin-induced ototoxicity. We performed a systematic review of the literature in order to evaluate the potential of genetic manipulation in experimental animal and *in vitro* studies. Interestingly, a variety of genes have

been evaluated as potential targets for inhibiting cisplatin-induced cytotoxicity such as apoptotic suppressors, copper transporters, regulators of the antioxidant response and neural growth factors. Consequences of genetic manipulation in the inner ear tissues remain to be assessed in order for gene therapy to become a conventional therapeutic option. Because the cochlea is embedded in bone, is fluid filled and contains various cell types, it has been a challenge to detect the expression of manipulated genes in a particular cell type of interest. While homogenization of a whole cochlea and posterior RNA extraction can provide us with the general expression levels of a certain gene, it does not allow for the determination in a cellular subpopulation of the cochlea. One possibility is the use of laser capture microdissection of cells of interest from a histological section. With this approach, the cells of interest are obtained and RNA can then be extracted and gene expression levels determined. However, the process of obtaining histological sections from cochlear samples requires fixation and decalcification steps which are known to cause RNA degradation. Hence, we decided to evaluate combinations of fixatives and decalcifying agents in order to determine which protocol would yield the greatest quantity of RNA from the cochlea and also preserve the morphology. The resulting protocol with methacarn fixation and decalcification in Morse's solution can therefore be used in future studies that aim to determine genetic expression, a regularly performed experiment, in a specific cellular subtype of interest in the cochlea.

Résumé

Le cisplatine est un agent chimiothérapeutique couramment utilisé. Malheureusement, de graves effets secondaires limitent son utilisation clinique telle que l'ototoxicité qui se présente comme une perte auditive neurosensorielle bilatérale et progressive. Il n'existe actuellement aucun traitement pour l'ototoxicité du cisplatine. La physiopathologie reste obscure, cependant, on croit que l'inflammation et le stress oxydant sont les principaux mécanismes conduisant à la mort des cellules. Dans cette thèse, différents aspects de l'ototoxicité du cisplatine et des stratégies thérapeutiques potentielles sont évaluées. Nous commençons par une revue générale de la littérature en ce qui concerne l'entrée et la sortie du cisplatine des cellules cochléaires. Nous décrivons les récepteurs qui peuvent jouer un rôle dans l'ototoxicité du cisplatine et qui sont présents dans les cellules cochléaires. Une fois à l'intérieur de la cellule, le cisplatine peut provoquer une réaction inflammatoire. Pour cette raison, nous avons décidé d'évaluer le potentiel de la dexaméthasone comme agent protecteur contre les effets toxiques du cisplatine *in vivo*. Il a été observé qu'un régulateur important de l'inflammation a été réduit, cependant, l'audition n'a pas été préservée. Un anti-inflammatoire n'a pas fourni une protection suffisante pour préserver l'audition après le traitement avec le cisplatine. Les dérivés réactifs de l'oxygène ont également été impliqués dans la cytotoxicité induite par le cisplatine. Le cisplatine peut conduire à une expression accrue des dérivés réactifs de l'oxygène et peut compromettre la réponse antioxydante naturelle de la cochlée. Ainsi, le potentiel d'un antioxydant exogène comme agent de protection a été évalué *in vivo*. Erdosteine, un dérivé de la méthionine, a diminué la perte d'audition au niveau des hautes fréquences ainsi qu'empêché partiellement la perte de cellules ciliées externes. Étant donné que ces deux produits ont générés uniquement une protection partielle, nous avons décidé d'évaluer une approche plus spécifique et ciblée, la thérapie génique. Puisque la thérapie génique est en phase expérimentale et n'est pas encore disponible en tant que modalité de traitement traditionnel, nous avons effectué une revue systématique de la littérature afin

d'évaluer le potentiel de la manipulation génétique chez les animaux de laboratoire et les expériences *in vitro*. Fait intéressant, plusieurs gènes ont été évalués comme des cibles potentielles pour inhiber la cytotoxicité induite par le cisplatine. Les conséquences des manipulations génétiques doivent encore être évaluées dans le but que la thérapie génique puisse devenir une option thérapeutique conventionnelle. Parce que la cochlée est ancrée dans l'os, est remplie de fluide et contient divers types de cellules, il a été difficile de détecter l'expression des gènes manipulés dans un type cellulaire d'intérêt. Alors que l'homogénéisation de la cochlée entière et l'extraction subséquente de l'ARN peut nous fournir une idée générale des niveaux d'expression d'un gène, ceci ne permet pas la détermination dans une sous-population cellulaire de la cochlée. Une possibilité est l'utilisation de la microdissection par capture laser des cellules d'intérêt à partir d'une coupe histologique. Avec cette approche, les cellules d'intérêt sont obtenues et l'ARN peut ensuite être extrait. Cependant, le processus d'obtention de coupes histologiques à partir d'un échantillon cochléaire nécessite des étapes de fixation et de décalcification qui sont connues pour causer une dégradation de l'ARN. Donc, nous avons décidé d'évaluer des combinaisons de fixateurs et réactifs de décalcification afin de déterminer quel protocole engendrerait la quantité d'ARN la plus élevée et qui, également, préserverait la morphologie. Le protocole établi peut donc être utilisé dans de futures études visant à déterminer l'expression génétique dans un sous-type cellulaire spécifique d'intérêt dans la cochlée.

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List of Abbreviations

ABR	Auditory brainstem response
Adv	Adenovirus
Atox1	Anti-oxidant 1
Bcl-xL	B-cell lymphoma-extra large
BDNF	Brain-derived neurotrophic factor
cDNA	Complementary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
Ctr	Copper transporter
dB	Decibel
DPOAE	Distortion product otoacoustic emission
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FGF2	Basic fibroblast growth factor
GAPDH	Glyceraldehyde phosphate dehydrogenase
GDNF	Glial cell-derived neurotrophic factor
GJB2	Gap junction protein beta-2
HGF	Hepatocyte growth factor
4-HNE	4-hydroxynonenal
HSV	Herpes simplex virus
HO-1	Heme oxygenase-1
IHC	Inner hair cell
IκB-α	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IP	Intraperitoneal
IP3R	Inositol trisphosphate receptor
KIM-1	Kidney injury molecule-1
MAPK	Mitogen-activated protein kinase
MET	Mechanotransduction
mRNA	Messenger RNA
NF-kB	Nuclear transcription factor-kappa B
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2
NOX	NADPH oxidase
NT-3	Neurotrophin-3
OCT	Organic cation transporter
OHC	Outer hair cell
OTOS	Otospiralin

PBS	Phosphate buffered saline
rAAV	Recombinant adeno-associated virus
rAd	Recombinant adenovirus
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RT-PCR	Real-time reverse transcriptase polymerase chain reaction
RWM	Round window membrane
S-D	Sprague-Dawley
siRNA	Short interfering RNA
SLC	Solute carrier SLC
SLF	Spiral ligament fibrocyte
SOD1	Cu/Zn superoxide dismutase
SOD2	Mn superoxide dismutase
TNF-α	Tumour necrosis factor-alpha
TRP	Transient receptor potential
TRPA	Transient receptor potential ankyrin
TRPC	Transient receptor potential canonical
TRPM	Transient receptor potential melastatin
TRPML	Transient receptor potential mucolipin
TRPP	Transient receptor potential polycystin
TRPV	Transient receptor potential vanilloid
TRPV1	Transient receptor potential cation channel subfamily V member 1
VSOR	Volume-sensitive outwardly rectifying chloride channel
XIAP	X-linked inhibitor of apoptosis protein

Statement of Original Contribution to Knowledge

The research studies included in this thesis provide an original contribution to knowledge with regard to potential strategies in preventing and/or treating hearing loss as a consequence of chemotherapy treatments with cisplatin. To date, there is no treatment available for cisplatin-induced ototoxicity. Previous research addressing this issue is slowly deciphering its pathophysiology. In the second chapter, a review of the channels and receptors that may play a role in cisplatin influx and efflux from inner ear cells was completed. As new receptors are being found on cells of the inner ear, their possible implication in cisplatin toxicity is being considered. Receptors and channels that allow for the passage of cisplatin into or out of the cell directly affect the concentration of cisplatin within the cell, hence the level of toxicity. To our knowledge, this is the first review evaluating the different receptors found in the inner ear cells that may play a role in cisplatin-induced ototoxicity. As the pathophysiology of this condition is still unclear, determining the possible influx and efflux pathways of cisplatin is critical in developing protective strategies.

The study described in chapter 3 was undertaken to explore the role of a systemic administration of a glucocorticosteroid, dexamethasone in this case, in preventing damage to the inner ear structures caused by cisplatin. Cisplatin is known to increase inflammatory cytokines. To our knowledge, this is the first study investigating the potential protective effect of a systemic administration of a steroid for this condition. As a result, it was observed that a steroid treatment, even at high doses, provided partial protection against cisplatin as evidenced by histological analysis and measurements. Yet, the morphological findings did not translate into functional protection; the hearing was not preserved.

As reactive oxygen species appear to play an important role in this condition, the study described in chapter 4 was completed in order to evaluate the potential protective effect of an antioxidant, erdosteine, against the oxidative stress generated by cisplatin. A high dose of erdosteine was found to be protective in an animal model as evidenced by hearing testing and morphological analysis.

As a result of the previous two studies, the potential of a more specific and precise treatment led to the completion of a systematic review exploring the possibility of gene therapy as a possible future treatment strategy (chapter 5). As gene therapy is not yet available as a mainstream treatment modality, the review included *in vitro* and experimental animal studies. This is the first systematic review published to assess this possibility. Following an extensive analysis, the data demonstrated the efficacy of gene therapy for cisplatin-induced ototoxicity. However, further investigation regarding safety, immunogenicity, and consequences of genetic manipulation in the inner ear tissues must be completed.

Following this research, it was observed that determining the genetic expression of a specific gene in cochlear tissues is essential in determining whether the manipulation of a gene of interest was successful. Such a course of action is exceptionally complicated in inner ear tissues as the cochlear sample presents various limitations including being enclosed within bone. As a result, a study was undertaken to determine which cochlear processing protocol (fixation and decalcification) would yield the greatest relative expression of genes (i.e. messenger RNA) (chapter 6). Cochlear samples were processed in various combinations of fixatives and decalcifying solutions, messenger RNA was then quantified. Morphological analysis of the tissues was also completed. Such a study had not been undertaken previously.

Contribution of Authors

Chapter 2:

Waissbluth S, Daniel SJ. **Cisplatin-Induced Ototoxicity: Transporters Playing a Role in Cisplatin Toxicity.** Hear Res 2013 Mar 1;299C:37-45. Reprinted with permission.

- *Conceptualized the review and searched current literature*: Sofia Waissbluth.
- *Analyzed literature*: Sofia Waissbluth.
- *Drafted and revised the manuscript:* Sofia Waissbluth, Sam J. Daniel.

Chapter 3:

Waissbluth S, Salehi P, He X, Daniel SJ. **Systemic Dexamethasone for the Prevention of Cisplatin-Induced Ototoxicity.** Eur Arch Otorhinolaryngol 2012 Aug 21. Reprinted with permission.

- *Conceptualized and designed experiments*: Sofia Waissbluth, Sam J. Daniel.
- *Performed the experiments*: Sofia Waissbluth, Pezhman Salehi.
- *Analyzed experimental data*: Sofia Waissbluth, Xinying He.
- *Drafted and revised the manuscript*: Sofia Waissbluth, Sam J. Daniel.

Chapter 4:

Waissbluth S, Dupuis I, Daniel SJ. **Protective Effect of Erdosteine against Cisplatin-Induced Ototoxicity in a Guinea Pig Model.** Otolaryngol Head Neck Surg. 2012 Apr;146(4):627-32. Reprinted with permission.

- *Conceptualized and designed experiments*: Sofia Waissbluth, Sam J. Daniel.
- *Performed the experiments*: Sofia Waissbluth, Isabelle Dupuis.
- *Analyzed experimental data*: Sofia Waissbluth, Sam J. Daniel.
- *Drafted and revised the manuscript:* Sofia Waissbluth, Sam J. Daniel.

Chapter 5:

Waissbluth S, Pitaro K, Daniel SJ. **Gene Therapy for Cisplatin-Induced Ototoxicity: A Systematic Review of In Vitro and Experimental Animal Studies**. Otol Neurotol. 2012 Apr;33(3):302-10. Reprinted with permission.

- *Conceptualized and designed search*: Sofia Waissbluth.
- *Analyzed experimental data*: Sofia Waissbluth, Koby Pitaro.
- *Drafted and revised the manuscript*: Sofia Waissbluth, Sam J. Daniel.

Chapter 6:

Waissbluth S, Chan SW, Chen JZ, McIntosh M, Daniel SJ. **RNA Preservation in Decalcified Cochlear Samples**. Otol Neurotol. 2013 Feb;34(2):331-7. Reprinted with permission.

- *Conceptualized and designed experiments*: Sofia Waissbluth, Sam W. Chan, Junjian Z. Chen, Sam J. Daniel.
- *Performed the experiments*: Sofia Waissbluth, Sam W. Chan
- *Analyzed experimental data*: Sofia Waissbluth, Sam W. Chan, Junjian Z. Chen
- *Drafted and revised the manuscript*: Sofia Waissbluth, Sam J. Daniel.
- *Provided equipment and reagents*: Matthew McIntosh, Junjian Z. Chen

Chapter 1

Introduction

1.1. Principles of the auditory system

Hearing is an essential part of interacting with our environment. It is necessary for sound detection, localisation and discrimination of location, pitch, loudness and quality of sounds [1]. Consequently, hearing allows us to communicate and to develop speech, language and literacy skills [2]. It also impacts on cognitive development and psychosocial adaptation [3].

The auditory system is made up of peripheral and central components. The peripheral components include the outer, middle and inner ear while the central components include the auditory brainstem (cochlear nuclei, trapezoid body, superior olivary complex, and lateral lemniscus), midbrain (inferior colliculi), thalamus (medial geniculate nucleus) and cerebral cortex [4]. The outer ear consists of the pinna, which is made up of elastic cartilage and provides sound localisation, and the external ear canal, which is approximately 2.5 cm long in adults and transmits sound to the tympanic membrane (Fig 1.1). The outer ear not only directs sound towards the tympanic membrane but also increases sound pressure levels by a phenomenon known as outer ear resonance [5]. The outer ear resonance generates a gain of approximately of 10 to15 decibels (dB) at the 3 to 5 kHz frequency range [6].

The sound reaching the tympanic membrane will cause it to vibrate. The major function of the tympanic membrane vibrations is to transfer the sound energy (sound waves) into mechanical energy. The tympanic membrane is around 85 mm^2 in area and has a thickness of 40 to 120 um [7]. The tympanic membrane is located on the lateral wall of the middle ear cavity while the oval and round windows are on the medial wall of the middle ear cavity. The tympanic membrane and the oval window are connected by the ossicles, the smallest bones in the body: the malleus (hammer), incus (anvil), and stapes (stirrup). The tympanic membrane and the ossicles therefore act as a transducer, changing energy forms. Because the tympanic membrane is larger than the oval window, the effective vibratory area of the tympanic membrane is up to twenty times greater than that of the stapes footplate. Also, the oval window is a barrier between an air filled cavity (middle ear) and a fluid filled cavity (inner ear). As a result, the middle ear acts as

an impedance matching device transmitting energy from a low pressure, high displacement vibration area to a high pressure, low displacement vibration area [8] (Fig 1.2). In addition, the manubrium of the malleus is 1.3 times longer than the length of the long process of the incus. As a result, there is a sound pressure gain of approximately 25 to 30 dB at the entrance of the cochlea [6,9]. All of these processes are essential in order to maximize the sound energy arriving to the fluids of the inner ear.

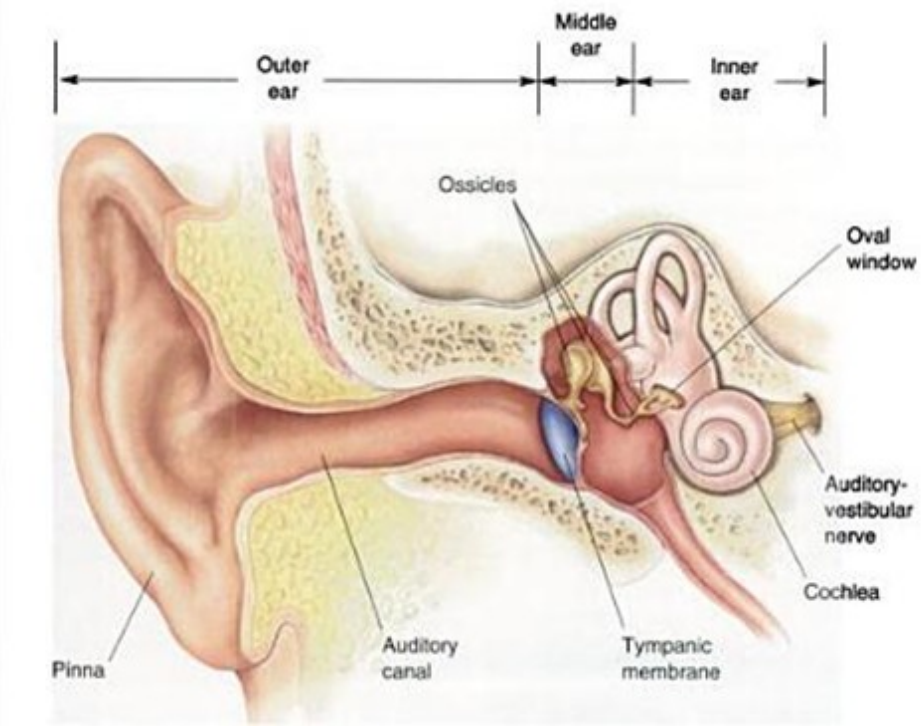

Figure 1.1. The outer, middle and inner ear. Sound waves travels through the auditory canal until they reach the tympanic membrane. The vibrations of the tympanic membrane consequently cause ossicle movement which transmit their energy to the oval window of the cochlea. Mark F. Bear, Barry W. Connors, Michael A. Paradiso, "Neuroscience: Exploring the Brain", Lippincott Williams & Wilkins, 2007, (reprinted with permission).

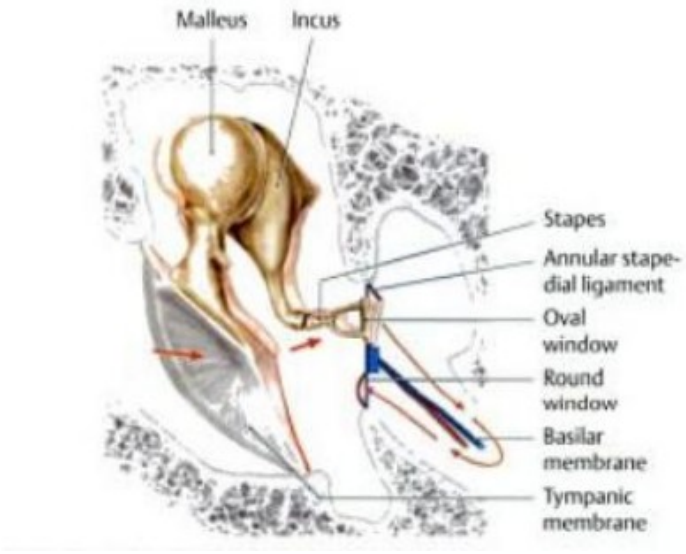

Figure 1.2. Ossicular chain in the middle ear. The malleus is bound to the tympanic membrane laterally and the incus medially while the stapes is bound to the incus laterally and the oval window medially. Anne M. Gilroy, Brian R. MacPherson, Lawrence M. Ross, "Atlas of Anatomy", Thieme Medical Publishers Inc, 2008 (reprinted with permission).

The inner ear contains the sensory organs for hearing and balance, the cochlea and the vestibular system respectively, and is embedded in the petrous part of the temporal bone. It is a fluid filled cavity and contains the sensory cells for sound transduction [4]. The cochlea is a snail-shaped structure surrounding a bony axis, the modiolus. Uncoiled, the human cochlea is approximately 3 to 3.5 cm in length and contains three fluid filled chambers: the scala vestibuli, scala media and scala tympani. The first two are separated by Reissner's membrane while the last two are separated by the basilar membrane [8]. These divisions allow for varying concentrations of electrolytes between the chambers. The scala vestibuli and tympani are filled with perilymph, which has a high Na^+ and low K^+ concentration, and are connected at the apex by the helicotrema. The scala media, on the other hand, is filled with endolymph which has a high K^+ and low Na^+

concentration [10]. The different ionic composition of both fluids is essential for the hearing process. The scala media filled with endolymph is the ideal environment for the organ of Corti hair cells that sit upon the basilar membrane because the high K^+ concentration is necessary for the generation of the endocochlear potential, the driving force for sensory transduction [11] (Fig 1.3).

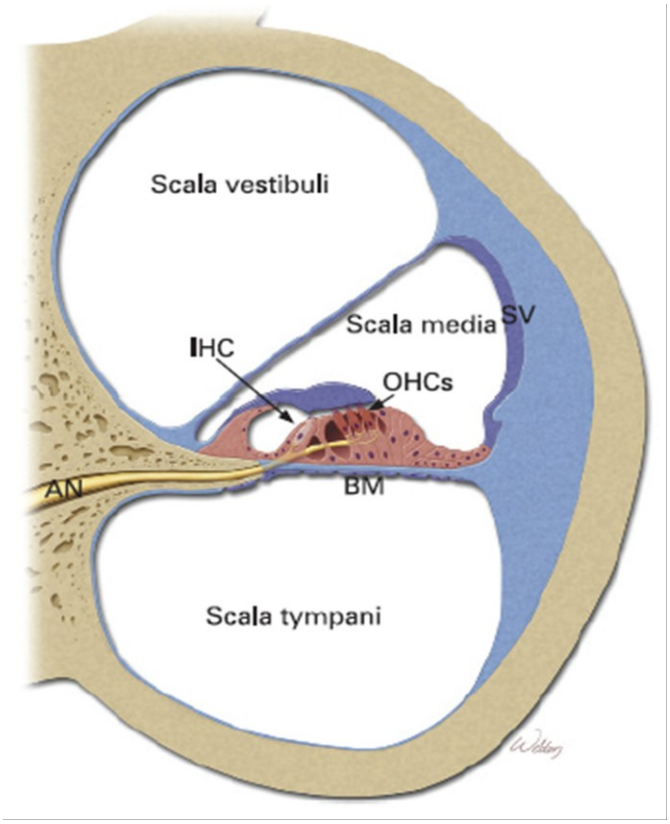

Figure 1.3. Cross section of the cochlea. The scala vestibuli and scala tympani are filled with perilymph while the scala media is filled with endolymph. SV: stria vascularis, OHCs: outer hair cells, IHC: inner hair cell, BM: basilar membrane, AN: auditory nerve. Reproduced from "Hearing loss in children with very low birth weight: current review of epidemiology and pathophysiology", R Cristobal, J S Oghalai, 93;(6):F462-8, 2008, with permission from BMJ Publishing Group Ltd.

The organ of Corti is made up of hair cells and supporting cells. There are two types of hair cells in the cochlea, the inner (IHCs) and outer hair cells (OHCs), and each type has specific characteristics. There are around 3500 IHCs and 12000 OHCs for each ear and they respond to mechanical stimulation [5]. They are considered neuroepithelial cells with an apical membrane suited for mechanotransduction and a basal membrane suited for the release of neurotransmitter (Fig 1.4). These cells are arranged in parallel rows along the basilar membrane with one row of IHCs and three to four rows of OHCs [12]. At the apical membrane, bundles of actin filaments covered in plasma membrane known as stereocilia, are seen arranged in a "W" shaped pattern and project upwards [13] (Fig 1.5). It is believed that as these stereocilia are displaced by mechanical stimulation, the mechanotransduction channels (MET) found on the stereocilia open allowing ion influx (K^+, Ca^{++}) and consequently causes cell depolarization. As a result, the change of the intracellular potential in the hair cell triggers the influx of Ca^{++} which then activates the release of the neurotransmitter, believed to be glutamate, towards its associated neuron [14].

The spiral ganglion, containing the cell bodies of the auditory nerve, synapses with the hair cells [5]. IHCs synapse with type I neurons and are considered the sensory cells of the auditory system while OHCs synapse with type II neurons and are thought to be responsible for the IHC sensitivity and fine-tuning of the cochlear frequency response [5,10,15,16].

The stria vascularis is found on the lateral wall of the cochlea. This is the vascularized epithelium responsible for the generation of the endocochlear potential [17]. The stria vascularis cells secrete K^+ into the endolymphatic space (scala media) following the recycling of K^+ from the scalae containing perilymph [11]. As such, a potential of +80 mV is generated as compared to the perilymph, and is essential for the transduction process [10].

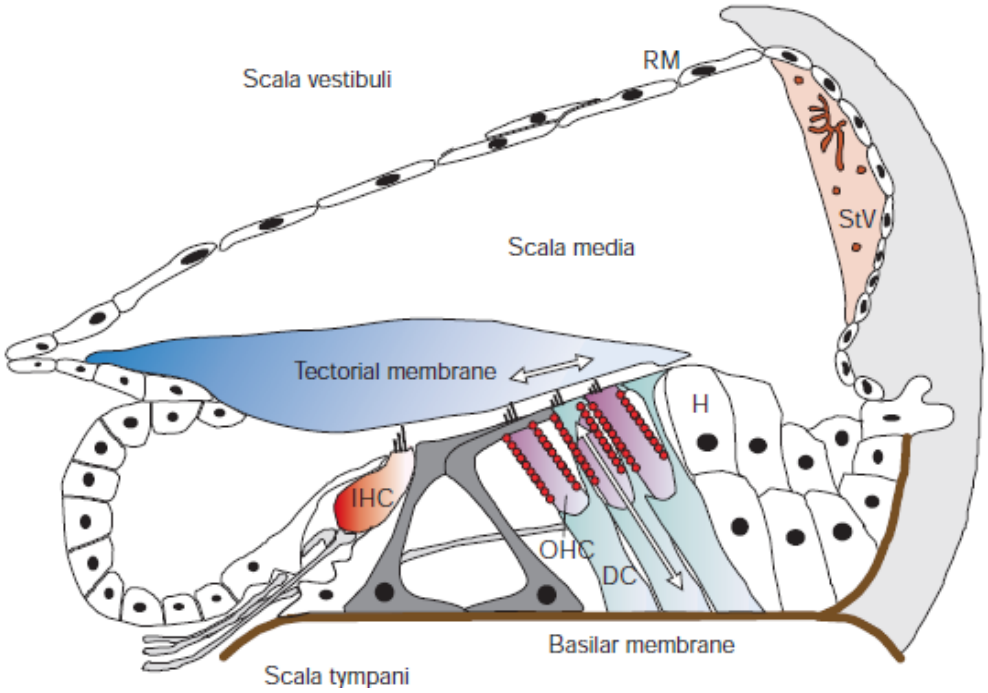

Figure 1.4. Schematic diagram of the scala media. The outer hair cells (OHC) aligned in three rows and inner hair cells (IHC) (1 row) sit upon the basilar membrane. The stereocilia on their apical surface are associated with the tectorial membrane which allows for their adequate deflection. StV: stria vascularis, DC: Deiter cell (cochlear supporting cell), H: Hensen cell (cochlear supporting cell), RM: Resissner's membrane. Reprinted from "Cochlear function: Hearing in the fast lane", 12;9(15):R572-4, Ashmore J, Géléoc GS, 1999 with permission from Elsevier.

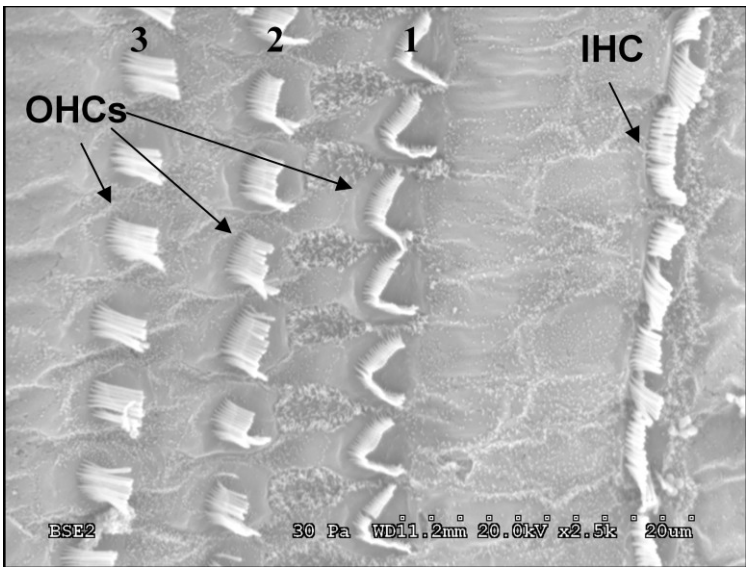

Figure 1.5. Scanning electron microscopy of hair cells; superior view (guinea pig). The stereocilia of the three rows of outer hair cells (OHCs) and one row of inner hair cells (IHC) can be seen.

The central components of the auditory system include the cochlear nuclei, the trapezoid bodies, the superior olivary complexes, the lateral lemnisci and the inferior colliculi of the brainstem; the medial geniculate nuclei of the thalamus and the auditory cortex. As the cochlear nerve fibers enter the brainstem, they synapse with cells in the cochlear nuclei. Up to 95% of the afferent fibers innervate the IHCs while the remaining 5% synapse with the OHCs. From the nuclei, fibers project towards the superior olivary complexes, the nuclei of the lateral lemnisci and the inferior colliculi. The superior olivary complex is notable since it is the first level at which bilateral information is received. Projections from the inferior colliculi then proceed to the thalamus (mostly the medial geniculate nuclei) where all of the fibers synapse. From here, the auditory radiations then project towards the auditory cortex [4] (Fig 1.6). Efferent pathways have also been detected, yet their functions remain unclear. They are believed to mirror the afferent pathways and to play a role in speech discrimination and in protecting from acoustic trauma [6,18].

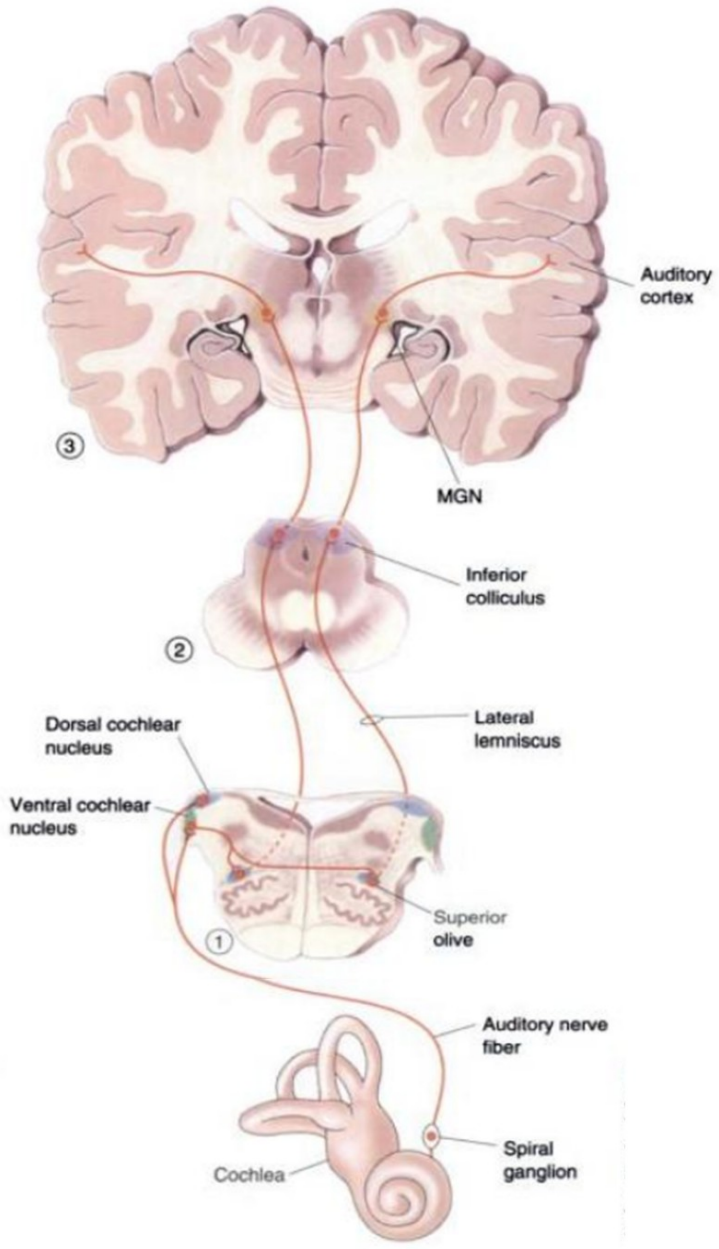

Figure 1.6. The afferent auditory pathway. The sensory information from the cochlea travels by the auditory nerve and ascends the central nervous system by synapsing at the cochlear nuclei. Projections then synapse at the superior olivary complexes and ascend by the lateral lemnisci until they reach the inferior colliculi in the midbrain. All sensory information arrives at the medial geniculate bodies (MGB) of the thalamus, and from there neurons project to the auditory cortex. Mark F. Bear, Barry W. Connors, Michael A. Paradiso, "Neuroscience: Exploring the Brain", Lippincott Williams & Wilkins, 2007, (reprinted with permission).

1.2. Hearing and deafness

The human auditory system allows for the detection of frequencies in the range of 20 to 20000 Hz with a greater sensitivity for the 500 to 4000 Hz range, which corresponds to the frequencies for the understanding of human speech [10,19]. The auditory system is structured in a way that each frequency is systematically organized within all of the structures of the auditory system, starting at the cochlear level and along the auditory pathway, to the auditory cortex. Such an arrangement is known as tonotopy and is a characteristic of the auditory system [20,21].

At the cochlear level, the basilar membrane is a key component for tonotopy. Sound waves entering the external ear are transformed into mechanical energy by the middle ear. On the medial wall of the middle ear, we find the oval window which is attached to the stapes bone (the smallest ossicle), as previously mentioned. As a consequence of the vibrations of the ossicles, the oval window oscillates and a fluid wave is created within the fluid filled cochlea. Consequently, the traveling wave in the basilar membrane will reach its corresponding frequency area, where most of the energy will dissipate. The maximal deformation of the basilar membrane will occur at the base of the cochlea when responding to high frequency sounds and at the apex when responding to low frequency sounds [21] (Fig 1.7). Such a distribution influences the clinical presentations of a variety of pathologies affecting the inner ear.

Hearing loss is a prevalent condition and can be categorized based on the area of the auditory system affected. It can be classified into three types: conductive, sensorineural, and mixed hearing loss. Conductive hearing loss results from damage to the outer and/or middle ear. Any condition impeding the traveling sound wave from reaching the inner ear is considered a cause of conductive hearing loss and some examples are otitis externa, cerumen impaction, otitis media, a perforated tympanic membrane and otosclerosis [5,22]. When the disorder affects the inner ear and/or the auditory nerve, the resulting hearing loss is termed as sensorineural (*sensory* for the cochlea, *neural* for the cochlear nerve). Cortical deafness is also a type of sensorineural hearing loss; however, it is extremely

rare[23]. Examples of conditions leading to sensorineural hearing loss are presbycusis, noise-induced hearing loss, drug-induced hearing loss and autoimmune disease [5]. Mixed hearing loss is a combination of both sensorineural and conductive hearing loss and examples of causes are inner ear malformations, genetic alterations, chronic ear infections, otosclerosis, head injuries and tumors [24].

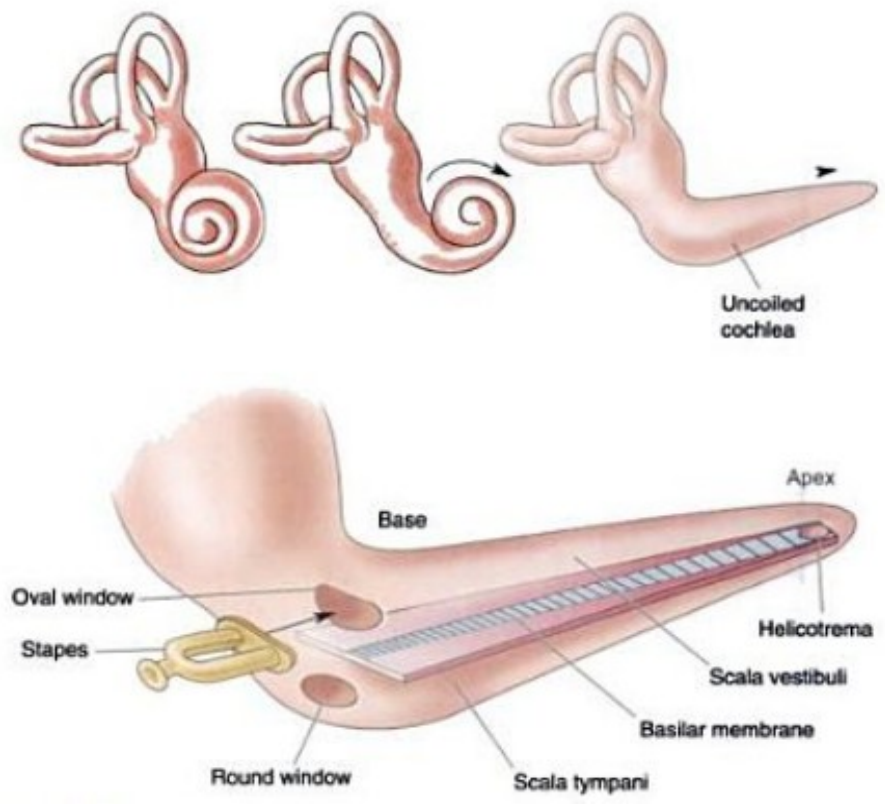

Figure 1.7. Schematic diagram of the uncoiled basilar membrane. The movement of the stapes on the oval window creates fluid displacement within the cochlear scalae. As a result, the basilar membrane moves. The tonotopic organization of the basilar membrane is such that high frequency sounds correspond to the base of the cochlea while low frequency sounds correspond to the apex of the cochlea. Mark F. Bear, Barry W. Connors, Michael A. Paradiso, "Neuroscience: Exploring the Brain", Lippincott Williams & Wilkins, 2007, (reprinted with permission).

The impact of hearing loss on quality of life will depend greatly on the type and degree of hearing loss, and clinically, it is possible to determine these characteristics by performing audiology testing. The most common preliminary testing procedure, following history taking and clinical hearing tests, is called audiometry. Pure tone audiometry is a subjective test in which the patient responds to sound stimuli of varying frequencies and loudness, and so, requires a cooperative patient. The hearing sensitivities at each frequency (usually tested from 250 to 8000 Hz) are plotted on a chart known as an audiogram [25]. Another way of screening for hearing loss is the auditory brainstem response (ABR) test. Because this test will detect the ascending auditory pathway's response to sound, no response from the patient is necessary, and it can be performed when lying still or while sleeping. This test allows the detection of retrocochlear pathologies (i.e. the cochlear nerve and ascending auditory pathway) and is widely used in auditory research as experimental animals can be anesthetized for the procedure. The auditory stimuli are presented by earphones and the evoked potentials are then measured by electrodes (on the mid-scalp and pinna). As the nervous system responds to the stimuli, signals corresponding to the different structures of the auditory pathway generate waveform peaks. The amplitude of the response (microvoltage [μV]) is plotted against time (milliseconds [msec]). The waveform peaks are labeled I to VII and are believed to represent auditory structures as follows: (I) distal VIIIth nerve, (II) proximal VIIIth nerve, (III) cochlear nucleus, (IV) superior olivary complex, (V) lateral lemniscus and (VI-VII) thalamus (uncertain origin) [26] (Fig 1.8). The aforementioned procedure was employed for the experiments performed in studies described in chapters 3 and 4.

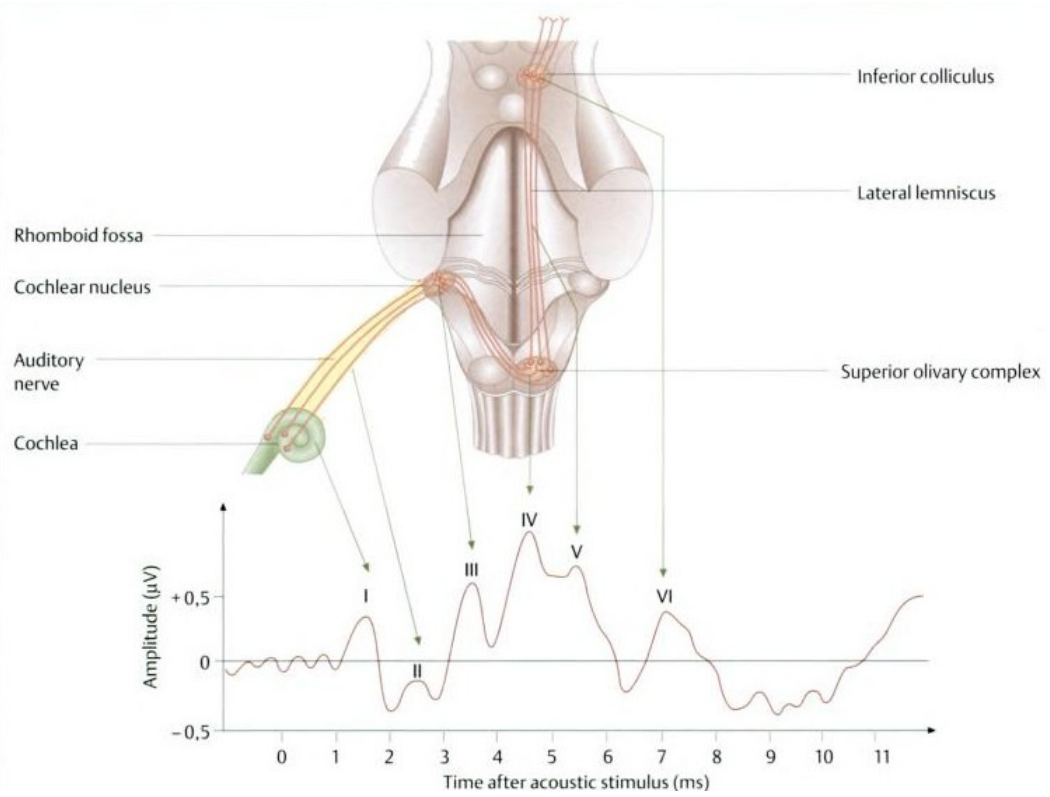

Figure 1.8. Schematic diagram of an auditory brainstem response output. The areas of the auditory pathway are correlated to their corresponding generated waves on the ABR output. Rudolf Probst, Gerhard Grevers, Heinrich Iro, **"**Basic Otorhinolaryngology: A Step-by-Step Learning Guide**",** Thieme Medical Publishers Inc, 2005 (reprinted with permission).

1.3. Cisplatin and ototoxicity

Cisplatin (cisplatinum; *cis*-diamminedichloroplatinum(II); *cis*-[$PtCl_2(NH_3)_2$]) is a chemotherapeutic agent and is the first member of the platinum-based antineoplastic drugs [27]. The molecule was first produced in 1844 by Michele Peyrone and was known as Peyrone's chloride. The actual structure of the molecule was later described in 1893 by Alfred Werner [28]. Several years later, in 1965, Barnett Rosenberg and colleagues discovered the cytostatic effect of cisplatin through good fortune as they observed that cisplatin inhibited cell division in bacteria (*Escherichia coli*) [29]. Shortly thereafter, it was observed that cisplatin had an antineoplastic effect *in vivo* as it inhibited tumor growth of

implanted sarcoma cells in mice [30], and since 1978, cisplatin has been approved for clinical use [31].

Cisplatin is currently part of various treatment protocols, alone or in combination therapy, for germ cell tumors, head and neck carcinoma, lung cancer, ovarian cancer, endometrial cancer, testicular cancer and bladder cancer [32-36], [37], [38], [39]. It is consequently a widely used chemotherapeutic agent. Unfortunately, cisplatin can cause important side effects which can limit its clinical use such as nephrotoxicity [40], ototoxicity [41] and emesis [42]; and less commonly, neurotoxicity [43], hypersensitivity reactions [44], ocular toxicity [45-47], syndrome of inappropriate antidiuretic hormone [48] and vascular toxicities [39,49]. Efforts to synthesize an equally potent yet less toxic chemotherapeutic agent have not been successful. Consequently, the cancer surviving patients are presenting with various toxicities arising from cisplatin chemotherapy, which can lead to devastating consequences on their quality of life.

While supportive measures exist for several of these side effects, cisplatin-induced ototoxicity remains a major medical problem with no approved treatment to date. For instance, cisplatin-induced nephrotoxicity can be prevented by administering sufficient hydration, by fractionating or prolonging the administration time of cisplatin or by adding a nephroprotective agent such as amifostine [50,51]. Another very common side effect is acute and delayed emesis as cisplatin is categorized as a highly emetogenic agent, being categorized as such because the proportion of patients who experience emesis in the absence of antiemetic prophylaxis, is greater than 90% [52]. The American Society of Clinical Oncology (ASCO) recommends a three drug combination treatment with a 5-HT_3 serotonin receptor antagonist, dexamethasone and aprepitant, an antiemetic drug [53].

Cisplatin-induced ototoxicity is a prevailing side effect of cisplatin chemotherapy; however, its prevalence varies tremendously, ranging from 20 to 90% [54]. This variation is due to various issues such as the cumulative dose [55], the rate of administration [56], genetic predisposition [57], the criteria used to grade hearing loss, the grades of hearing loss reported in the literature, the timing of

hearing testing and the presence of risk factors [58]. Risk factors that have been shown to be implicated are age (< 5 years old) [55], renal insufficiency [59], cranial irradiation [60], history of noise exposure [59] and use of other ototoxic drug [61]. Clinically, cisplatin-induced ototoxicity presents as an irreversible and bilateral sensorineural hearing loss that begins initially in the high frequency range of hearing, and progresses towards the lower frequencies [41]. It is a dose related complication and the severity of the hearing loss seems to be associated to the cumulative dose administered [59]. Patients can present with hearing loss days or weeks following the oncology regimen, or many years after completion of the treatment with cisplatin [62]. Tinnitus, also commonly referred to as ringing or buzzing in the ear [63], has also been described as a consequence of cisplatin chemotherapy [64].

The resulting hearing loss can significantly impact on the patient's quality of life. They can experience psychological distress, anxiety and discouragement as a result of the inability to communicate with others. Consequently, they can feel withdrawn from society and this could lead to depression [65]. In children, especially in the prelingual stage, hearing loss can also impact on the development of speech and language skills and can delay psychosocial development [66,67]. Thus, hearing loss impacts greatly at the individual and societal level, and there is no treatment thus far for hearing loss resulting from cisplatin chemotherapy.

1.3.1. Mechanisms of cisplatin-induced ototoxicity

Cisplatin is a small and highly reactive molecule. It has a platinum core with two chloride groups and two ammonia groups in the *cis* configuration. As such, the molecule is not reactive. As it is exposed to water, cisplatin becomes active by an aquation reaction where the chloride ions are replaced by water molecules. This reaction occurs in tissues where a high concentration of water and a low concentration of chloride ions are present [68]. This newly charged molecule is now capable of binding to a variety of molecules such as DNA, RNA and proteins (Fig 1.9).

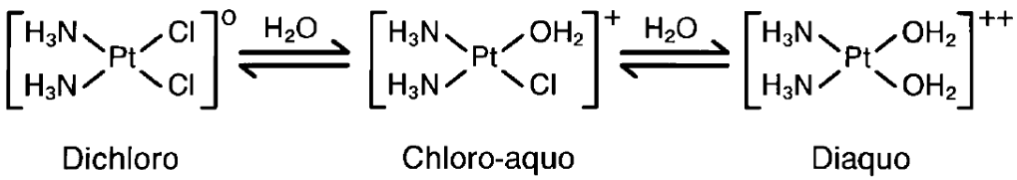

Figure 1.9. Aquation reactions for cisplatin. Cisplatin (*cis*-[PtCl2(NH3)2] or *cis*-diamminedichloroplatinum(II)) possesses two chloride groups that are subsequently replaced by two H_2O molecules. The diaquated molecule is now reactive. Reprinted with permission © (1999) American Society of Clinical Oncology. All rights reserved. Go, R et al: J Clin Oncol 17(1), 1999: 409-422.

Cisplatin appears to enter cells by passive diffusion and by facilitated transport; arguments for both processes have been described. It is believed that cisplatin can enter the cell, in part, by passive diffusion since the uptake of platinum by cells is linear with time, concentration-dependent and non-saturable [69-71]. However, investigations relating to the resistance of certain tumor cells to platinum containing compounds has led to the hypothesis that cisplatin may enter cells by facilitated transport [72]. Various mechanisms have been suggested for cellular resistance to cisplatin and include interference from cisplatin binding to its main target, DNA, and interference with the cellular signals indicating DNA damage [73]. Many studies have aimed at determining the transporters that allow for cisplatin influx and efflux. Resistance to cisplatin cytotoxicity has been detected in cells presenting a decrease in cellular uptake of cisplatin and also an increased efflux of cisplatin [74,75]. Decreasing the concentration and time of intracellular cisplatin can lead to a decrease in the cytotoxic potential of this platinum containing compound. In the eighties, interestingly, an association between copper and cisplatin was observed. The administration of copper to mice injected with tumor cells reduced the nephrotoxic effect of cisplatin [76] while the administration of cisplatin to rats inhibited the normal accumulation of copper in the kidneys [77,78]. Consequently, it appears that the metabolism of copper and cisplatin are somewhat associated. Komatsu *et al*. observed that a cancerous cell line transfected with the copper-transporting P-type ATPase (ATP7B), a copper

trafficking protein, led to the cells becoming resistant to cisplatin and copper. The intracellular platinum content was decreased and the efflux of cisplatin was increased [79]. The ATPases, ATP7A and ATP7B, redistribute copper within the cells, and also are responsible for the excretion of excess copper out of the cell. Various studies have associated cisplatin resistance to the ATPases in cancer cell lines [80-82]. Nakayama *et al*. suggested that the expression of ATPases may be used as a predictive marker for cisplatin resistance [83]. Because of the interest in ATPases and copper metabolism being a potential pathway for cisplatin efflux, interest in copper importers increased. Cisplatin appears to enter cells through the copper transporter 1 (CTR1) as demonstrated by Ishida *et al.* where yeast cells lacking the *Ctr1* gene exhibited increased resistance and decreased intracellular cisplatin concentrations. They suggest CTR1 to be an important component for cisplatin uptake [84]. Another research group found similar results [85]. Furthermore, overexpression of CTR1 in cultured cells led to an increase in intracellular cisplatin concentration [75,86]. Cisplatin uptake may also be attributed to another transporter, the organic cation transporter 2 (OCT2). It was observed that a cancer cell line transfected with OCT2 caused an increase in cisplatin uptake, and that the addition of OCT2 inhibitors repressed the uptake and cytotoxicity of cisplatin [87]. Furthermore, the administration of cisplatin to OCT2 double knock-out mice only caused mild nephrotoxicity, and no ototoxicity as compared to the wild type mice [88]. Because cisplatin is among the most active antineoplastic agents, it is of great interest to determine which channels or receptors are responsible for its entry and export from cells. The majority of the studies describing potential influx and efflux transporters are performed on cultured cells and aim to direct new research into determining methods to reduce resistance of cancer cells to cisplatin. As part of this dissertation, the objective of the article described in chapter 2 was to determine which transporters may play a role in cisplatin-induced ototoxicity specifically. In this article, I describe which transporters are located in the cochlea, and that appear to play a role in cisplatin cytotoxicity thus far.

Once inside the cell, and following the aquation reactions, cisplatin can bind to various molecules. While DNA appears to be its main target for

cytotoxicity [89], there is evidence that cisplatin can bind to a variety of proteins [90] [91-95] as well as RNA [96] [97]. Consequently, the molecules are distorted and their functions, altered. Because the diaquated cisplatin molecule possesses two potential H_2O leaving groups, it can form cross-links by binding two areas of the DNA molecule. Various researchers have aimed to determine how cisplatin binds to DNA; it appears that intrastrand as well as interstrand cross-links are formed, however, 1,2-intrastrand cross-links are the most frequently observed [98]. These DNA adducts (covalent binding of carcinogens to DNA) cause structural changes in the DNA molecules and consequently can disrupt replication and transcription. Cells can then activate their repair mechanisms [99], and if the DNA damage is too great, the cell undergoes cell death. In such, it has been suggested that DNA adducts are an important factor in the cytotoxic action of cisplatin [100]. However, it appears that only about 1% of intracellular cisplatin is bound to DNA [101], and it has been described that cisplatin cytotoxicity can be independent of DNA damage [102,103].

In the inner ear, cisplatin has been shown to target the stria vascularis, the spiral ligament, the organ of Corti and the spiral ganglion neurons [104,105]. Although cisplatin uptake has been detected in inner ear tissues, the uptake appears to be considerable in the stria vascularis [106-108]. In addition, adduct formation does not completely account for the damage elicited in the inner ear. Evidence has shown that cisplatin can elicit the local inflammatory response as well as generate oxidative stress, two processes known to initiate apoptotic pathways (Fig 1.10). Cisplatin can stimulate the production and release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) which can activate nuclear transcription factor-kappa B (NF-κB) [109]. NF-κB, a transcription factor known to regulate the expression of inflammatory genes can, conversely, regulate the expression of IL-1β and TNF-α thus resulting in a positive feedback loop and an increase in the inflammatory cascade [110]. TNF-α also recruits inflammatory cells into the organ of Corti thus increasing the proinflammatory environment in the cochlea [111]. As a result, multiple studies have been completed with the objective of determining the

protective effect of an anti-inflammatory agent against cisplatin-induced ototoxicity yielding inconsistent results [112-119]. All of these studies evaluated the potential of an intratympanic administration (i.e. depositing the drug directly in the middle ear) of a steroid, most frequently dexamethasone, to protect the cochlea from cisplatin cytotoxicity. Intratympanic injections can circumvent systemic side effects; however, the procedure itself can cause local complications [120], and the concentrations reached in the inner ear fluids can be erratic [121]. A systemic administration is simple to perform and has the advantage of being conveniently controlled. As part of this dissertation, the study described in chapter 3 had the objective of evaluating the potential of a systemic administration of dexamethasone, a potent anti-inflammatory, against cisplatin-induced ototoxicity *in vivo*. See chapter 3 for further details on materials, methods and results.

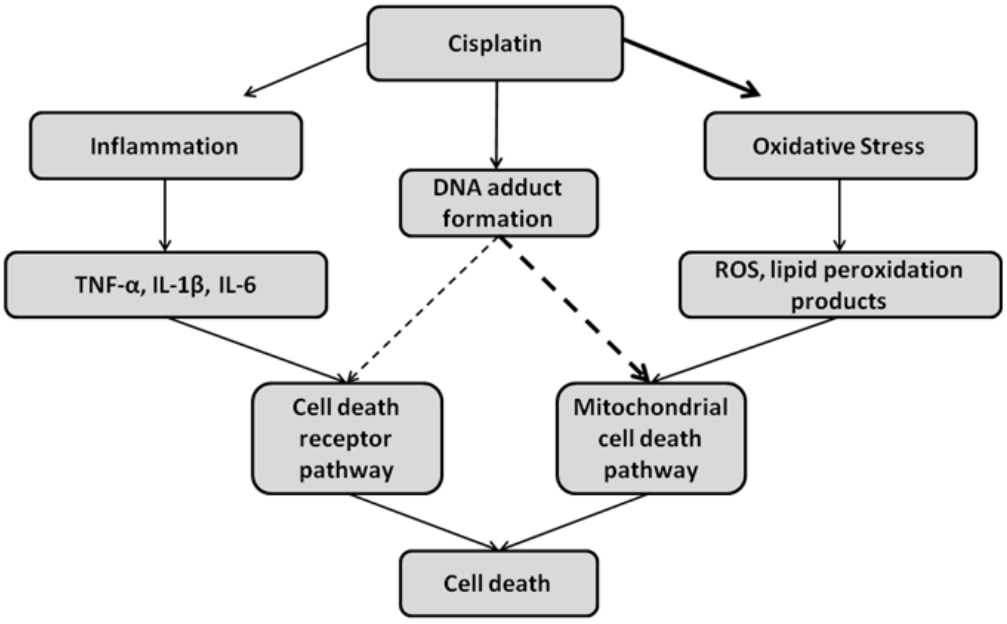

Figure 1.10. Overview of mechanisms for cisplatin-induced ototoxicity. Cisplatin can bind DNA creating adducts which can elicit the apoptotic cascade, more significantly by activating the intrinsic, or mitochondrial pathway. Cisplatin causes local inflammation as well as oxidative stress in the cochlea. Both processes can lead to cell death by apoptosis.

As previously mentioned, cisplatin can generate oxidative stress within the cochlea [122-124]. It induces the upregulation of NOX-3, a NADPH oxidase highly expressed in the inner ear, and produces an increase in local superoxide production [125,126]. The reactive oxygen species (ROS) form hydrogen peroxide or react with nitric oxide resulting in peroxynitrite formation. Peroxynitrite can then interact with proteins and form nitrotyrosine while hydrogen peroxide can lead to the production of 4-hydroxynonenal (4-HNE) [127]. These molecules are pro-apoptotic and cytotoxic and they can contribute to the degenerative process of cochlear cells [128]. Through the production of ROS, cisplatin can, in addition, activate NF-κB which has been shown to be pro-apoptotic in an auditory cell line [129]. Furthermore, cisplatin can deplete cochlear cells of glutathione, an endogenous antioxidant, and can reduce the activity of antioxidant enzymes in the inner ear, specifically superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase [130]. As a result, the cell can become overwhelmed by the oxidative environment and undergo apoptosis [128,131]. In order to protect the cells of the inner ear against the oxidative stress caused by cisplatin, researchers have studied various products aimed at reducing the ROS production and action [114,116,132-149]. In this dissertation, chapter 4 describes a study undertaken at our laboratory evaluating the potential of erdosteine to protect the cochlea from cisplatin-induced ototoxicity. Erdosteine is a thiol derivative exhibiting antioxidant effects. It is a synthetic derivative of methionine, a naturally occurring amino acid, and is considered a prodrug with two blocked sulfhydryl groups. Following first-pass metabolism, the sulfhydryl groups are liberated and active metabolites are released into the blood stream [150]. A systemic administration of erdosteine provided protection against cisplatin-induced ototoxicity in the high frequency ranges of hearing. Evidence was provided by decreased hearing threshold shifts on ABR testing and increased outer hair cell viability (see chapter 4).

Following the extensive literature search on otoprotective agents for cisplatin-induced ototoxicity and the studies described in chapters 3 and 4, a pharmacological agent is not necessarily the most favorable approach.

Pharmaceutical drugs can affect organs other than the target and lead to side effects. Another goal of this dissertation was to determine the potential of a more specific treatment modality for cisplatin-induced ototoxicity. As described in chapter 5, the potential of gene therapy as an otoprotective strategy was evaluated. Because gene therapy products have not yet been approved for human subjects, a systematic review of experimental animal studies and *in vitro* experiments regarding gene therapy and cisplatin-induced cytotoxicity was performed. Interestingly, various target genes and diverse modalities for gene manipulation resulted in promising alternatives for this condition.

In order for gene therapy to be successful, the expression of the genes upregulated or silenced must be quantified in the cells of interest. Because the cochlea is small, fluid-filled and embedded in the temporal bone, it has been a challenge to determine RNA, or gene expression, in specific cells of the cochlea. Currently, the most common method of determining RNA in cochlear samples is to homogenize the entire excised cochlea and in doing so, a general idea of gene expression in the cochlea as a whole is obtained. Specifics for the different cell types cannot be obtained. Another possible method is to process the cochlea for histology (fixation, decalcification, embedding and microtomy) and with the use of laser capture microdissection, capture the areas of interest from histological sections and extract their corresponding RNA. Nevertheless, the processes of fixation and decalcification have been shown to cause RNA degradation [151,152]. In the study described in chapter 6, a new protocol for processing cochlear samples is described by which the greatest quantity of RNA is conserved and the cochlear morphology is preserved. The objective of this study was to determine the adequate combination of fixative and decalcifying agent that would yield the greatest RNA quantity while maintaining the cochlear morphology, with the notion that laser capture could be successful.

This dissertation includes five original studies completed during the doctoral program. In the following chapters, details concerning rationale, materials and methods, results and discussion for each study are described.

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Preface – Chapter 2

The entry of cisplatin into cancer cells has been of considerable interest given that cisplatin is one of the most potent antineoplastic agents currently used. The mechanisms by which cisplatin can elicit tumor cell death are slowly being revealed, and cellular uptake of cisplatin is an important factor in the consequent cytotoxicity. A large number of patients receiving cisplatin chemotherapy develop progressive and irreversible hearing loss as a consequence of their treatment yet no strategy is currently available to prevent or treat the resulting hearing loss. The cochlea is particularly susceptible to cisplatin and the specific basis for such a phenomenon is unclear. Some literature has shed light on potential channels and receptors that may allow cisplatin to enter cells; however, most of the studies published focus on chemosensitivity and chemoresistance. As such, the focus of their work is to potentiate cisplatin's cytotoxicity.

In the following chapter, an extensive review of the literature was performed in order to determine which channels and receptors allow the influx and efflux of cisplatin and which of these transporters are found in cochlear cells. Increasing our understanding of cisplatin's transcellular movement is an important aspect of developing otoprotective strategies. To our knowledge, this is the first review to focus on transporters that may play a role in cisplatin cytotoxicity and that are found in the cochlea.

Chapter 2

Cisplatin-Induced Ototoxicity: Transporters Playing a Role in Cisplatin Toxicity

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Abstract

Cisplatin is a potent antineoplastic agent widely used for a variety of cancer types. Unfortunately, its use leads to dose limiting side effects such as ototoxicity. Up to 93% of patients receiving cisplatin chemotherapy will develop progressive and irreversible sensorineural hearing loss which leads to a decreased quality of life in cancer survivors. No treatment is currently available for cisplatin-induced ototoxicity. It appears that cisplatin causes apoptosis by binding DNA, activating the inflammatory cascade as well as generating oxidative stress in the cell. Various studies have aimed to assess the potential protective effects of compounds such as antioxidants, anti-inflammatories, caspase inhibitors, anti-apoptotic agents and calcium channel blockers against the toxicity caused by cisplatin in the inner ear with variable degrees of protection. Nevertheless, the pathophysiology of cisplatin-induced ototoxicity remains unclear. This review summarizes all of the known transporters that could play a role in cisplatin influx, leading to cisplatin-induced ototoxicity. The following were evaluated: copper transporters, organic cation transporters, the transient receptor potential channel family, calcium channels, multidrug resistance associated proteins, mechanotransduction channels and chloride channels.

Keywords: cisplatin, hearing loss, ototoxicity, protection, channels, receptors, transporters.

2.1. Introduction

Cisplatin is a potent antineoplastic agent widely used for a variety of cancer types including germ cell tumors [1], nasopharyngeal carcinoma [2], lung cancer [3], ovarian cancer [4], endometrial cancer [5] and testicular cancer [6]. Unfortunately, its use leads to dose limiting side effects such as nephrotoxicity, neurotoxicity and ototoxicity [7]. Up to 93% of patients receiving cisplatin chemotherapy will develop progressive and irreversible sensorineural hearing loss which leads to a decreased quality of life in cancer survivors [8]. No treatment is currently available for cisplatin-induced ototoxicity. It appears that cisplatin causes apoptosis by binding DNA, activating the inflammatory cascade as well as generating oxidative stress in the cell [9,10].

Cisplatin (*cis*-diamminedichloroplatinum(II)) is a highly reactive molecule. Once inside the cell, it is transformed to its more active form as water molecules replace the chloride groups by an aquation reaction [11,12].The aquated form can then bind a variety of macromolecules including RNA, proteins (such as superoxide dismutase [13], membrane phospholipids, microfilaments and DNA, which is its primary target [12]. Consequently, the cell responds by cell cycle arrest and DNA repair or activation of the caspase cascade which leads to apoptosis [9]; although a caspase-independent pathway has also been detected [14-16]. The interaction between cisplatin and DNA leads to the generation of intrastrand and extrastrand crosslinks; 85 to 90% of bound platinum corresponds to intrastrand adducts [17]. The modified DNA molecules are repaired by the human excinuclease or they are recognized by DNA damage recognition proteins which transmit signals of DNA injury and subsequently lead to apoptosis [18]. As such, it is hypothesized that the resulting DNA damage is the main source of cytotoxicity for cisplatin.

Cisplatin also induces oxidative stress. The ROS form hydrogen peroxide or react with nitric oxide resulting in peroxynitrite formation. Peroxynitrite can interact with proteins and form nitrotyrosine while hydrogen peroxide can lead to the production of 4-HNE as a result of catalysis by iron and posterior interaction with polyunsaturated fatty acids in cell membranes. These molecules are pro-

apoptotic and cytotoxic [9]. In addition, the oxidative environment results in cytochrome c release from the mitochondria which leads to an increase in calcium release from the endoplasmic reticulum [9]. The calcium release then causes a massive cytochrome c release activating the apoptotic cascade [19]. Furthermore, cisplatin can, through the production of ROS, activate NF-κB [20] which can regulate the expression of proinflammatory cytokines such as IL-1β and TNF-α. TNF-α can, in addition, activate NF-κB resulting in a positive feedback loop and an increase in the inflammatory cascade [21].

Various studies have aimed to assess the potential protective effects of compounds such as antioxidants [22-32], anti-inflammatories [33-37], caspase inhibitors [38], anti-apoptotic agents [39,40] and calcium channel blockers [41] against the toxicity caused by cisplatin in the inner ear with variable degrees of protection. The greatest level of otoprotection seems to arise from antioxidant treatments. On the other hand, there is great controversy regarding antioxidants being administered concomitantly with oncology regimens [42]. The pathophysiology of cisplatin-induced ototoxicity remains unclear and further studies are needed to shed light on this condition. The goal of this manuscript is to review all the known transporters that could play a role in cisplatin influx/efflux to the cochlear cells leading to cisplatin-induced ototoxicity.

2.2. Copper transporters

It is believed that cisplatin can enter the cell through passive diffusion or by transporters [43]. There is evidence that copper transporters may be involved in cellular influx and efflux of cisplatin [44-49]. Copper transporter 1 (Ctr1) is a major copper influx transporter and has been shown to mediate the uptake of cisplatin, carboplatin and oxaliplatin [47]. The Ctr1 transporter has been found to be expressed in the OHCs, the IHCs, the spiral ganglion neurons as well as the stria vascularis [49]. Deletion of the CTR1 gene in yeast resulted in increased cisplatin resistance and a reduction in intracellular cisplatin content. In addition, cisplatin, like copper, can cause degradation and delocalization of the Ctr1 transporter [44,50]. Furthermore, knockdown of the CTR1 gene by small interfering RNA in the HEI-

OC1 cell line, derived from immortomouse cochleae, resulted in a decreased cisplatin uptake [49]. Thus, cisplatin and copper metabolisms are intertwined. When the CTR1 gene expression was enhanced in ovarian cancer cells, an increase in cellular copper and cisplatin content was observed. However, the over-expression of CTR1 caused a proportion of copper and cisplatin to be delivered into other cellular compartments and therefore causing only a slight increase in the sensitivity to the cytotoxic effect of both molecules [50]. On the other hand, Berreta *et al*. observed that the over-expression of CTR1 in the cancerous cell line A431 and its cisplatin-resistant variant A431/Pt did not cause an increase in cellular cisplatin accumulation and they suggest that Ctr1 does not play a central role in cellular cisplatin resistance [51].

The copper transporter Ctr2 also seems to be involved in copper uptake [52,53]. To the best of our knowledge, there is no data on whether the Ctr2 transporter is expressed in the cochlea. The deletion of the CTR2 gene in yeast cells did not affect cisplatin resistance [44]. Blair *et al* observed that the knockdown of CTR2 expression increased cellular cisplatin accumulation as well as cytotoxicity in mouse embryo fibroblasts [54]. The same group also demonstrated that cisplatin causes an increase in the expression of CTR2 [55]. As a result, Lee *et al* decided to evaluate the expression of CTR1 and CTR2 in forty women with ovarian carcinoma. They observed that a high CTR1 expression was significantly associated with sensitivity to platinum-based chemotherapy and a longer progression-free survival. On the contrary, a low CTR1 and high CTR2 expression were significantly associated with resistance to platinum-based chemotherapy and shortest survival [56].

The copper transporting P-type ATPases ATP7A and ATP7B also seem to be involved in cisplatin cytotoxicity. The ATPases receive copper delivered by Atox1 (anti-oxidant 1), a copper-responsive transcription factor [57], at the trans-Golgi network [58,59]. It has been suggested that Atox1 can function as a copper chaperone delivering copper to ATP7A and ATP7B and also as a copper-dependent transcription factor [57]. They regulate intracellular copper levels by trafficking excess copper into vesicles which can fuse with the basal membrane

and release the copper into the extracellular space by exocytosis (ATP7A) or by excretion at the apical membrane of the cell (ATP7B) [58]. It has been suggested that ATP7A may bind and sequester platinum-containing compounds and consequently prevent them from reaching their site of action and consequent cytotoxicity [60,61]. Over-expressing the ATPases resulted in resistance to platinating agents [62]. Both ATP7A and ATP7B have been detected in the organ of Corti, the stria vascularis and the spiral ganglion neurons [63]. No study, to our knowledge, has evaluated the possibility of altering ATPases and their functions to prevent cisplatin-induced ototoxicity. While developing resistance to cisplatin may be deleterious for cancer cells, it may be beneficial for cochlear cells. Further knowledge regarding the mechanisms of copper metabolism and cisplatin are needed if protective strategies involving the copper pathway are to be considered for cisplatin-induced ototoxicity.

Table 2.1 describes the localization of the transporters and figure 2.1 is a schematic diagram of the possible pathways taken by cisplatin in the cell.

2.3. Organic cation transporters

The organic cation transporter (OCT)2 may also be involved in cisplatin-induced ototoxicity [64,65]. OCTs are part of the solute carrier (SLC) 22A family and are electrogenic [66]. Their driving force is the electrochemical gradient of the organic cation being transported and can be reversible with respect to direction [67]. OCT2 expression has been detected in the OHCs, the IHCs and the stria vascularis of the mouse cochlea [65]. However, in another study, it was found to be expressed in the spiral ganglion neurons and the stria vascularis of the mouse cochlea and absent in the hair cells [49]. Of the three isoforms (OCT1, 2 and 3), OCT2 seems to be the important transporter for cisplatin. OCT1 does not seem to be involved in cisplatin uptake as demonstrated in kidney cells [68,69]. It has been suggested that since various cancer cell lines do not express OCT2, it may be that OCT2 does not mediate cisplatin uptake in cancer cells as in normal cells and as a result may be an interesting target for otoprotective strategies [67] as demonstrated by the use of an OCT2 inhibitor, cimetidine [63,65]. Katsuda *et al* evaluated whether

a systemic administration of cimetidine influenced the antitumor effect of cisplatin *in vitro* and *in vivo*. They concluded that cimetidine did not inhibit the cytotoxicity of cisplatin on osteosarcoma cells [70]. Additional evidence regarding OCT2 expression in cancer tissue is required in order to insure that administration of competitive inhibitors of OCT2 do not diminish the cytotoxic effect of cisplatin on cancer cells when trying to prevent cisplatin-induced ototoxicity.

2.4. Transient receptor potential channel family

The transient receptor potential (TRP) superfamily is composed of integral membrane proteins functioning as ion channels which are expressed in just about every cell type [71]. They conduct cations and are, in their majority, non-selective channels [72]. Six of the seven discovered subfamilies have been detected in the human species: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPML (mucolipin) and TRPP (polycystin) [71]. The majority are located in the plasma membrane and their functions seem to be modulated by associated proteins or by forming signaling complexes [71]. In the inner ear, all six subfamilies have been detected [73-78]. TRPA1 appears to be involved in the development of neurotoxic effects resulting from platinum-based chemotherapy [79,80]. TRPA1 has been detected in hair cells; however, its function is still unclear [81-83]. Trigeminal ganglia from mice exposed to cisplatin had an increased expression of TRPA1 and TRPV1 [79]. Furthermore, mouse organ of Corti explants exposed to 4-hydroxynonenal, a product of lipid peroxidation, activated the TRPA1 channel and produced an increase in gentamycin-Texas Red conjugate uptake [84]. The pore size of TRPA1 is thought to be approximately 11 to 13.8 Å (1.1-1.38 nm) [85] and gentamycin molecules, with a diameter of less than 1 nm, could then permeate through TRPA1 [84]. Cisplatin molecules have a size of about 0.5 nm [86] and because TRPA1 is a non-selective cation channel, the possibility that aquated cisplatin may enter the cell, through TRPA1, exists [87] and needs to be assessed.

TRPV1 expression is also increased when cells of the inner ear are exposed to cisplatin and is found in the IHCs and OHCs, the supporting cells, the

spiral ganglion neurons and the stria vascularis [73]. More interestingly, post-transcriptional gene silencing of TRPV1 in the presence of cisplatin resulted in reduced cellular calcium influx *in vitro*, decreased auditory brainstem response threshold shifts, and greater OHC counts *in vivo*. It is suggested that the intracellular calcium rise is a result of TRPV1 activation and that TRPV1 may play a part in cisplatin-induced apoptosis [73]. In addition, TRPV1-mediated apoptosis by induction of maintained calcium influx has been shown to be blocked by inhibiting the TRPV1 channels [88]. It remains unclear whether cisplatin cytotoxicity results from cisplatin influx through TRPV1 or solely by inducing a maintained calcium influx leading to apoptosis. Furthermore, the TRPV1 pore size is 10.1Å [20] and may be functionally coupled to TRPA1 [89].

TRPV4 is also expressed in the IHCs and OHCs, the stria vascularis and the spiral ganglion neurons [90] and it is thought to be implicated in rare forms of hereditary hearing loss [71,91]. It has also been suggested that TRPV4 may be implicated in the uptake and retention of aminoglycosides [92]. No studies are yet available regarding cisplatin uptake and TRPV4.

Other members of the TRPV family have been found to be expressed in the inner ear. Both TRPV5 and TRPV6 are calcium selective channels and are part of the calcium absorptive system [93]. They are active at low calcium concentrations and inactivate to prevent a calcium overload [72]. They have been detected in the organ of Corti, the spiral ganglion neurons, the supporting cells, the spiral ligament and the spiral limbus (Table 1). The stria vascularis seems to express TRPV5 channels; however, the expression of TRPV6 channels in the stria is still unclear [74,93]. The precise functions of these newly discovered channels are uncertain yet TRPV5(-/-) and TRPV6(-/-) mice develop abnormalities in calcium reabsorption in the kidney and intestine respectively as compared to wild-type mice [72,94]. Because they are active at low calcium concentrations and since they prevent excess calcium influx [95], their role seems to be in calcium homeostasis and they would unlikely be part of the apoptotic cascade. Since there is a lack of knowledge regarding these channels and their potential functions, further research needs to be completed in order to suggest any relation to cisplatin ototoxicity.

The channels TRPML3 [78,96], TRPC3 [76,77], TRPP2 and TRPP3 [75] have also been detected in the cochlea. TRPML3 may play a role in membrane trafficking and in the endocytic pathway [97] while TRPC3 may act as redox sensor [98]. The function of these channels in the inner ear is not clear; mutations in TRPML3 may be implicated in hearing disorders [91]. Further research is required in order to establish whether these channels are implicated in cisplatin uptake and cytotoxicity in the inner ear.

2.5. Calcium channels

Calcium homeostasis is crucial for the regulation of a variety of physiological responses including the hearing process [99,100]. Calcium channels can be voltage-gated or ligand-gated and both classes are found in the inner ear cells [101-105]. Ligand-gated calcium channels include the inositol trisphosphate receptor (IP3R) and the ryanodine receptor [99] and they participate in calcium release from intracellular stores [106]. The IP3R has been detected in the cochlear sensory epithelium, the stria vascularis, the spiral ligament, the spiral ganglion neurons and the spiral limbus [103,107] while the ryanodine receptor isoforms have been detected in the IHCs, the OHCs, the supporting cells, the spiral ganglion neurons and the stria vascularis [101,106,108]. While it is known that calcium release is involved in auditory neurotransmission [101], it has been suggested that the IP3R and the ryanodine receptor may play a role in the mitochondrial apoptosis pathway [109]. Furthermore, it has been observed that ROS can open these channels hence leading to an increase in cytosolic calcium levels [110]. HeLa cells exposed to cisplatin exhibited an increase in calcium concentration which was reduced by an IP3R antagonist [111]. The authors also observed an activation of calpain following the calcium increase and suggest a caspase-independent apoptosis pathway [112] that is dependent on IP3R activation as a result of cisplatin exposure [111]. Further studies are required to assess the involvement of ryanodine receptors in cisplatin-induced cytotoxicity since they may also play a role in the apoptotic cascade.

The contribution of voltage-gated calcium channels in cisplatin-induced cytotoxicity has also been considered. L-type calcium channels, gated by high

voltage, are expressed in the lateral wall, the organ of Corti and the spiral ganglion neurons [113-115]. Cisplatin-induced hyperalgesia is a known side effect of cisplatin administration. Rats injected with cisplatin develop painful peripheral neuropathy. When carbamazepine is also injected along with cisplatin, there is a significant attenuation of cisplatin-induced pain [116,117]. Carbamazepine is a sodium channel blocker as well as an L-type calcium channel blocker [118]. Perhaps the protective effect of carbamazepine on cisplatin-induced hyperalgesia is in part due to its capacity to block calcium channels. On the other hand, T-type calcium channels are gated by low voltage and are expressed in the organ of Corti, the stria vascularis and the spiral ganglion neurons [41,104,105]. Its involvement in cisplatin-induced ototoxicity was evaluated by So *et al* [41]. It was observed that an administration of a T-type calcium channel antagonist (flunarazine), to cells exposed to cisplatin, inhibited mitochondrial dysfunction, decreased lipid peroxidation and decreased apoptosis as compared to cisplatin-treated cells [41]. Therefore, a T-type calcium channel antagonist protected cells from cisplatin-induced cytotoxicity. Additional studies would be warranted to evaluate the mechanisms underlying these protective effects. Although there is no evidence to suggest cisplatin may enter cells through calcium channels, the importance of the calcium pathway in cisplatin-induced ototoxicity and the relationship between calcium and the transient receptor potential channel family [110] is the rationale for discussing calcium channels in this review (see Fig 2.2).

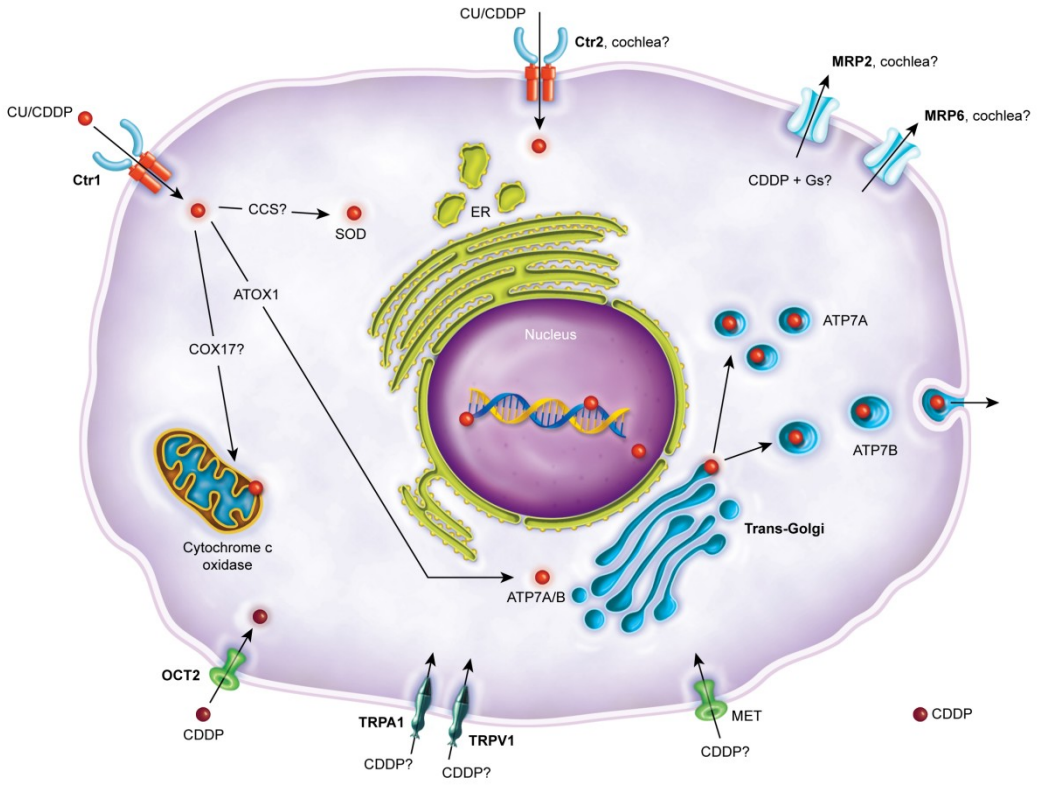

Figure 2.1. Schematic diagram of possible influx and efflux pathways for cisplatin. Cisplatin (CDDP) can enter the cells through passive diffusion or by transporters. CDDP can enter the cell through copper transporter Ctr1. Once inside the cell, CDDP can follow the copper pathway by binding the copper chaperone ATOX1. ATOX1 then interacts with ATP7A/B. CDDP can then be sequestered in cytoplasmic vesicles and can be trafficked towards the cell surface. Other copper chaperones (COX17 (cytochrome c oxidase copper chaperone), CCS (copper chaperone for SOD1)) have not been described to bind CDDP, yet CDDP has been detected in their delivery sites (cytochrome c oxidase, SOD). There is no evidence to date that the copper transporter Ctr2 is present in cochlear cells. CDDP can also enter the cell through OCT2 and possibly TRPV1. No evidence is yet available to demonstrate that CDDP can enter the cell through TRPA1 or the MET channel. Interestingly, recent research has suggested that CDDP can exit the cell through the MRP2 transporter. No studies have evaluated the presence of MRP2 in the cochlea. Calcium also plays an important role in cisplatin toxicity as it is a main component of the apoptotic cascade. Calcium can enter the cytoplasm through TRPV1, L-type and T-type calcium channels as well as through IP3R and RyR, all found in cochlear cells.

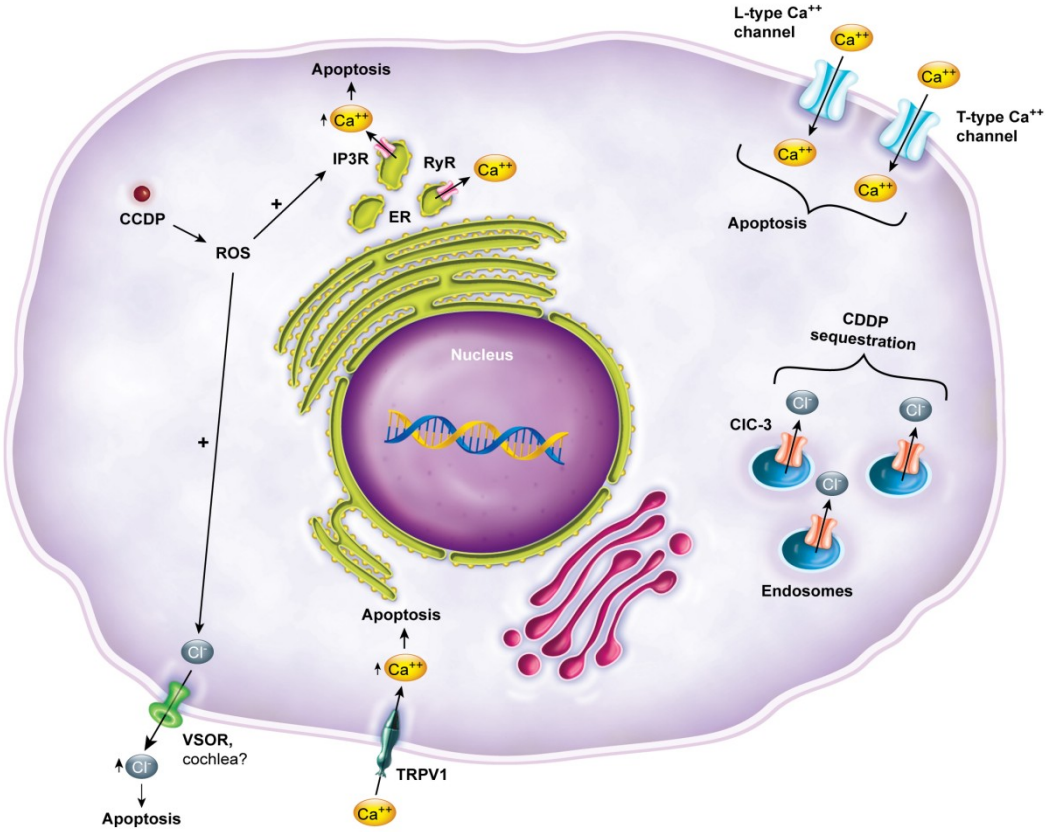

Figure 2.2. Schematic diagram of calcium and chloride transporters related to cisplatin toxicity. Once cisplatin (CDDP) is inside the cell, it can cause an increase in reactive oxygen species (ROS) which have been shown to open the inositol trisphosphate receptor (IP3R) and ryanodine receptor (RyR) and participate in calcium release from intracellular stores. Channels that cause calcium influx (L-type, T-type calcium channels, TRPV1) also seem to be involved in cisplatin toxicity by increasing the intracellular calcium stores that may lead to apoptosis. ROS appears to also open volume-sensitive outwardly rectifying chloride channel (VSOR) which can release chloride and lead to apoptosis. The exact mechanism for this occurrence is unclear. Another chloride channel of interest is ClC-3 that seems to be involved in cisplatin-induced cytotoxicity as it may cause intracellular CDDP sequestration.

2.6. Mechanotransduction channel

The mechanotransduction (MET) channel is a non-selective cation channel with a preference for calcium. Because of technical issues and experimental challenges, clear characteristics for the MET channel have been difficult to determine [119]. These channels are found in the apical portion of the stereocilia of the hair cells [120], are believed to have a pore size of 12.5 ± 0.8 Å and a highly electronegative outer surface [121]. Interestingly, it has been observed that MET channels allow uptake of gentamycin-Texas Red conjugate in IHCs and OHCs of rat cochleae hence participating in aminoglycoside ototoxicity [120].

Amiloride, a potassium-sparing diuretic, blocks MET channels [122] as well as macropinocytosis though the inhibition of the Na^+/H^+ exchanger [123]. We previously discussed that the knockdown of the CTR2 gene causes an increase in cisplatin uptake, it also increases macropinocytosis. Amiloride blocked the increase in macropinocytosis and Ctr2 was found to control the rate of macropinocytosis [124]. However, since amiloride can also block MET channels, it cannot be discarded that part of the increase in cisplatin uptake is due, in part, to the MET channels. The cisplatin molecule is smaller than the pore size estimated for the MET channel and to our knowledge; no study has yet evaluated the possibility of MET channels participating in cisplatin uptake by hair cells. Although cisplatin targets various cell types in the inner ear that do not possess the MET channel, there is no evidence yet available to demonstrate that the MET channel does not participate in cisplatin influx in hair cells.

Table 2.1. Localization of transporters and channels found in cochlear cells that may be related to cisplatin toxicity

	Organ of Corti	SGN	Stria vascularis	Spiral ligament	Supporting cells	Spiral limbus	Reissner's membrane	References
Ctr1	+ IHC, OHC	+	+					More et al. 2010
Atp7a	+	+	+					Ding et al. 2011
Atp7b	+	+	+					Ding et al. 2011
Oct2	+IHC, OHC	+	+					Ciarimboli et al. 2010; More et al. 2010
Trpa1	+							Corey et al. 2004; Nagata et al. 2005; Kwan et al. 2006
Trpv1	+IHC, OHC	+	+		+			Mukherjea et al. 2008
Trpv4	+IHC, OHC	+	+					Takumida et al. 2005
Trpv5	+IHC, OHC	+	+	+	+	+		Takumida M et al. 2009; Yamauchi D et al. 2010
Trpv6	+IHC, OHC	+	+/-	+	+	+		Takumida M et al. 2009; Yamauchi D et al. 2010
Trpml3	+IHC, OHC		+	+	+	+	+	Nagata et al. 2008; Castiglioni et al. 2011
Trpc3	+IHC, OHC	+		+	+	+		Phan et al. 2010; Tadros et al. 2010
Trpp2		+	+	+	+			Takumida and Anniko 2010
Trpp3	+IHC, OHC	+	+					Takumida and Anniko 2010
Ip3r	+	+	+	+		+		Imamura and Adams 2003; Gossman and Zhao 2008
Ryr	+IHC, OHC	+	+		+			Lioudyno et al. 2004; Morton-Jones et al. 2006; Liang et al. 2009
L-type ca^{++} channel	+IHC, OHC	+	+	+	+	+		Hafidi and Dulon 2004; Layton et al. 2005; Chen et al. 2011
T-type ca^{++} channel	+IHC, OHC	+	+		+			So et al. 2005; Uemaetomari et al. 2009; Lei et al. 2011
Cftr	+IHC, OHC							Homma et al. 2010
Clc-3	+ OHC		+*	+*		+*		Oshima et al. 1997; Kawasaki et al 1999

(+) = present, IHC = inner hair cell, OHC = outer hair cell, * the tissue was processed by RT-PCR and was a mixture of spiral ligament, stria vascularis, spiral prominence epithelial cells and vascular elements.

2.7. Multi-drug resistance proteins

MRP2 is part of the subfamily C of the human ABC (ATP-binding cassette) superfamily [125], one of the largest families of transmembrane proteins. Most of these proteins are transporters and use the energy generated by ATP hydrolysis to drive the transport of various molecules across cell membranes, including antineoplastic agents [126]. MDR1, also known as ABCB1 or P-glycoprotein, is one of the most studied of the members of this family. It was demonstrated that it functions as an efflux pump and has been involved in cellular resistance to chemotherapeutic agents [127]. MDR1 expression is increased when cells are exposed to cisplatin; however, cisplatin is not a substrate for MDR1 [128,129]. ABCG2 (MXR/BCRP), also involved in drug resistance, does not transport cisplatin either [126,127,130]. MRP1 (ABCC1) was detected in the rat cochlea [131] and seems to be involved in drug resistance [126]. Nevertheless, cisplatin has not been shown to be a substrate for MRP1 [132]. On the other hand, MRP2 seems to mediate cisplatin resistance. An over-expression of MRP2 has been associated with cisplatin resistance while a decreased expression resulting from genetic manipulation decreased the resistance to cisplatin [133,134]. Cisplatin, once inside the cell, can bind glutathione and form a complex [135]. This complex is thought to be toxic and it has been suggested that this complex may exit the cell through MRP2 [136]. Another member of the ATP-binding cassette superfamily, MRP6 (ABCC6), has also been shown to confer low levels of resistance to cisplatin, possibly by its capacity to transport glutathione conjugates [137,138]. To the best of our knowledge, there is no evidence so far demonstrating the presence of MRP2 or MRP6 in cochlear tissues. Further research is necessary to evaluate the different members of the ABC superfamily and cisplatin efflux. Other families such as the major facilitator superfamily (MFS), the small multidrug-resistance family (SMR), the resistance-nodulation-cell division family (RND) and the multidrug and toxic compounds extrusion family (MATE) are also multi-drug resistance proteins [139]. There association to cisplatin influx or efflux would be interesting to investigate.

2.8. Chloride channels (CFTR, VSOR, ClC)

CFTR (cystic fibrosis transmembrane conductance regulator), also known as ABCC7, is a chloride channel yet it can also regulate other ion channels such as the outwardly rectifying chloride channel (ORCC) or the epithelial sodium channel (ENaC) [140]. It is also a member of the ATP-binding cassette superfamily [126] and has been detected in IHCs and OHCs [141]. It has been suggested that CFTR may participate in apoptosis as it may mediate glutathione efflux. This may correlate with an increase in intracellular ROS as the cell becomes depleted of glutathione, when under stress [142,143]. Interestingly, there is recent evidence that cisplatin does not inhibit CFTR; it seems to be insensitive to cisplatin [144]. On the other hand, another chloride channel, VSOR (volume-sensitive outwardly rectifying chloride channel), seems to be inhibited by cisplatin [144]. Other studies report contradictory results, demonstrating that the VSOR channel is activated by cisplatin administration [145,146]. VSOR, also known as volume-regulated anion channels (VRAC) or volume-sensitive organic anion channels (VSOAC) can be activated by ROS and lead to apoptosis; however, the exact mechanism for this occurrence is unclear [145,147,148]. In contrast, VSOR dysfunction can cause cisplatin resistance [146,149] and it has been suggested that VSOR activity may be a prerequisite for cisplatin-induced apoptosis [149]. There is no literature to date examining the existence of VSOR in cochlear cells. There is no evidence so far that either CFTR or VSOR channels mediate cisplatin transport, however, they seem to participate in cisplatin resistance.

Another chloride channel of interest is ClC-3, a member of the CLC family of the chloride channels in mammals present in endosomes and synaptic vesicles [140]. ClC-3 is an intracellular voltage-dependent chloride channel and is expressed in many tissues including the OHCs [150] and the lateral wall of the cochlea [151]. Most recently, it has been suggested that ClC-3 may be involved in cisplatin resistance [152]. Cultured cells exposed to cisplatin and the chloride channel blocker NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid) induced resistance to cisplatin and avoided apoptosis as NPPB upregulated ClC-3. These results indicate that the chloride channel ClC-3 may be involved in cisplatin-

induced cytotoxicity. The authors of this study suggest that since there is a correlation between ClC-3 activation and intracellular acidification (mechanism unclear) and that an acid environment can cause cisplatin sequestration, ClC-3 may cause intracellular cisplatin sequestration [152]. Therefore, they propose ClC-3 as a potential pharmacological target to improve cisplatins' efficacy whereas we suggest ClC-3 can also be a target for otoprotection.

2.9. Conclusions

Because cisplatin is a small and highly reactive molecule, various transporters have been suggested to be involved in cisplatin uptake by cells. Our current review illustrates evidence that copper transporters Ctr1 and Ctr2 and organic cation transporter OCT2 are involved in cisplatin transport through plasma membranes. We also suggest other possible channels that may play a role in cisplatin uptake such as the transient receptor potential channel family and the mechanotransduction channels. Further studies are needed in order to determine which channel is responsible for the greatest uptake of cisplatin into cells and thereby identify a suitable protective strategy to prevent cisplatin-induced ototoxicity. As different transporters may be accountable for varying degrees of toxicity, elucidating the definite pathways for cisplatin and understanding the contribution of transporters in depth, is a key component in the search for otoprotective approaches.

2.10. References

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Preface - Chapter 3

As discussed in the introduction of this thesis, it is believed that cisplatin can elicit an inflammatory response in the inner ear. It has been demonstrated that cisplatin can cause an increase in pro-inflammatory cytokines and consequently lead to cell death. Various researchers have aimed to assess the benefit of an anti-inflammatory agent as a protective strategy against cisplatin-induced ototoxicity. Most of the studies previously undertaken have evaluated the potential of a local administration (transtympanic) of dexamethasone, a potent corticosteroid, against the toxicity caused by cisplatin in an animal model, including guinea pig, mouse and rat. The current work is the first study evaluating the protective effect of a systemic administration of dexamethasone against cisplatin-induced ototoxicity.

A transtympanic administration of a compound can lead to inconsistent concentrations reaching the inner ear fluids, and consequently the cells of interest. For this reason, the objective of the following study was to evaluate the effects of a systemic administration of a corticosteroid (dexamethasone) on cisplatin-induced ototoxicity. Furthermore, low and high doses of dexamethasone were assessed. The majority of the previously published articles report only on hearing test outcomes to support their conclusions. The current study evaluated the impact of dexamethasone by evaluating hearing test outcomes, cochlear morphological analyses and measurements as well as immunohistochemistry.

Chapter 3

Systemic Dexamethasone for the Prevention of Cisplatin-Induced Ototoxicity

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Abstract

Objective: Ototoxicity is a common side effect of cisplatin chemotherapy. This study was undertaken to determine the potential protective effects of a systemic administration of dexamethasone against cisplatin-induced ototoxicity.

Study design: A prospective controlled trial conducted in an animal model.

Setting: Animal care research facilities of the Montreal Children's Hospital Research Institute.

Subjects and Methods: An experimental guinea pig model was used. The animals were divided as follows: group 1 (n = 10): 12 mg/kg intraperitoneal (IP) cisplatin, group 2 (n = 14): 15 mg/kg/day dexamethasone IP for 2 days followed by cisplatin 12 mg/kg IP, group 3 (n = 14): 10 mg/kg/day dexamethasone IP for 2 days. On day 3, they received cisplatin 12 mg/kg IP followed by 20 mg/kg/day dexamethasone for 2 days and group 4 (n = 5): 10 ml of saline IP twice a day for 3 days. Auditory brainstem response (ABR) threshold shifts were measured at 4 frequencies (8, 16, 20 and 25 kHz) for groups 1, 2 and 3. Histological changes in the organ of Corti, the stria vascularis, the spiral ligament and the spiral ganglion neurons as well as scanning electron microscopy for outer hair cells were completed. Immunohistochemistry for tumour necrosis factor-alpha (TNF-α) was performed.

Results: ABR threshold shifts were similar in all groups. Histological and scanning electron findings demonstrate that dexamethasone has greater protective effect on the stria vascularis.

Conclusion: Systemic dexamethasone administration in a guinea pig model did not provide significant protection against cisplatin-induced ototoxicity. Dexamethasone may be useful in future applications as a complementary treatment.

3.1. Introduction

Cisplatin is a widely used chemotherapeutic agent in pediatric and adult oncology protocols. Unfortunately, hearing loss is a major dose-limiting side effect presenting as a bilateral, irreversible and progressive sensorineural hearing loss leading to a decrease in quality of life of cancer patients [1]. In the inner ear, cisplatin targets the organ of Corti, the spiral ganglion neurons (SGNs), the stria vascularis and the spiral ligament [2,3]. Once cisplatin enters the cell, it induces cell death mainly by apoptosis resulting from two main processes: oxidative stress and inflammation [4].

Cisplatin stimulates the inner ear local inflammatory response. Through the production of ROS, cisplatin activates NF-κB which can regulate the expression of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 [4-6]. TNF-α can activate NF-κB resulting in a positive feedback loop and increase the inflammatory response [7]. TNF-α also recruits inflammatory cells into the inner ear [8].

Glucocorticosteroids have a history of use for inner ear conditions and have been proven non toxic for the cochlear structures [9,10]. Glucocorticosteroids inhibit mitogen-activated protein kinases, important regulators of pro-inflammatory transcription factors [11], by activating mitogen-activated protein kinase phosphatase-1 [12]. They can also induce nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκB-α) expression which suppresses NF-κB inhibiting the inflammatory signaling cascade [13]. Transtympanic injections of glucocorticosteroids have been evaluated as potential treatment strategies for cisplatin-induced ototoxicity with variable degrees of protection [10, 14-16]. We hypothesized that the variability in the effectiveness of transtympanic dexamethasone might be caused, in part, by a variable degree of penetration of the dexamethasone reaching the inner ear from the middle ear space.

This led us to conduct the current study investigating the potential protective effect of a systemic administration of dexamethasone against the toxicity caused by cisplatin in the inner ear. This is the first publication, to our

knowledge, addressing the systemic administration of dexamethasone for cisplatin-induced ototoxicity.

3.2. Materials and Methods

3.2.1. Animals

Forty-three female albino Hartley guinea pigs weighing 500 to 800 g (Charles River Laboratory, Senneville, Quebec) were used in the current study. The animals had free access to water and food. The animals were kept in the animal care research facilities of the Montreal Children's Hospital Research Institute under standard laboratory conditions; housed in a room at 20 ± 4 °C ambient temperature and a 12 hour light/dark cycle. The study was approved and monitored by the Animal Care Committee in accordance with the Canadian Council of Animal Care guidelines.

3.2.2. Experimental Design

The guinea pigs (43) were assigned to 4 groups: group 1 (n = 10) received 12 mg/kg of intraperitoneal (IP) cisplatin; group 2 (n = 14) received 12 mg/kg cisplatin IP followed by 15 mg/kg/day of dexamethasone IP for 3 days; group 3 (n = 14) received 10 mg/kg/day of dexamethasone IP for 2 days, on day 3, 12 mg/kg of cisplatin IP was administered followed by 20 mg/kg/day of dexamethasone IP for 3 days; group 4 (n = 5) received 10 ml of isotonic saline IP for 3 days. Dexamethasone injections for groups 2 and 3 were dissolved in saline therefore the negative control group (group 4) received saline only and were employed as normal controls.

Since dexamethasone reaches a maximal concentration in perilymph 2 hours following an IP injection [17], it was decided to administer cisplatin an hour and a half following the dexamethasone injections in order to obtain the greater concentrations of both products simultaneously in the inner ear.

The dosage of cisplatin has been determined by previous research at our laboratory [15]. It was demonstrated that this dose causes sufficient ototoxicity as a model yet very low mortality rates.

3.2.3. Cisplatin and Dexamethasone Treatment

Animals were anesthetized with isoflurane. The animals received 12 mg/kg of cisplatin IP under anesthesia. A subcutaneous bolus of 10 ml of sterile isotonic saline was injected following the cisplatin administration for hydration. Once treatment with cisplatin (1mg/ml, Hospira, Canada) began, animals received two subcutaneous injections of sterile saline (10 ml) per day for hydration. The dexamethasone injections were prepared by dissolving the dexamethasone 21-phosphate disodium salt (Cat. No. D1159, Sigma-Aldrich Canada) in 0.9% NaCl in order to obtain a concentration of 5 mg/ml. Animals were euthanized after 72 hours following the cisplatin administration.

3.2.4. Auditory Brainstem Response

The auditory brainstem response (ABR) was performed prior to any injection (baseline measurement) and 72 hours following the cisplatin IP administration (post measurement) in order to determine the ABR threshold shifts (SPL dB). Hearing threshold was defined as the lowest intensity of stimulation that resulted in a clear reproducible waveform. The tympanic membranes and external auditory canals were inspected prior to the ABR measurement using an operating microscope. Animals with preexisting hearing loss and/or any abnormality in the external or middle ear were excluded from the study. The active electrode was placed subcutaneously within the pinna of the tested ear, the reference electrode at the vertex and the ground electrode on the pinna of the contralateral pinna. The ABR was measured at four frequencies (8, 16, 20 and 25 kHz) on the Smart EP device (Intelligent Hearing Systems) using tone burst stimulus with a rate of 39.1 bursts/second and alternating polarity. The response to the stimulus was averaged from 1600 sweeps. The measurements began at 80 dB and subsequently being decreased or increased by 20 dB and then 10 dB until the last three clear reproducible waveforms were obtained. Threshold shifts were calculated by comparing the pre- and post-cisplatin hearing threshold values. Two animals in group 3 died before completing the post measurement ABR.

3.2.5. Histological Evaluation

Immediately following the post-ABR measurement, the animals were euthanized and the cochleae were dissected. The cochleae were fixed in 10% neutral buffered formalin for 48 hours at room temperature. Subsequently, decalcification was achieved by submerging the samples in 10% EDTA at room temperature for seven days with daily change of the solution. The specimens were then processed for an hour in 10% neutral buffered formalin, next in 50% alcohol and were maintained in 70% alcohol until preparation for paraffin embedding. Once embedded in paraffin, the specimens were then mounted in order to obtain midmodiolar plane cuts. Sections of 5μm of thickness were collected on glass slides and stained with haematoxylin and eosin staining. Sections were examined with a Zeiss Axiophot light microscope equipped with a Zeiss AxioCam MRc camera with which digitalized images were obtained.

3.2.5.1. Strial Cross Sectional Area

The strial cross sectional area analysis was performed using the public domain NIH ImageJ program (U.S. National Institutes of Health; http://rsb.info.nih.gov/ij/). The periphery of the stria vascularis was delineated in every half turn of the cochlea in three segments: apex, middle and base and the cross-sectional area was determined (μm^2) (Fig. 3.1). Six different measurements were made and averaged for every region. Subsequently, the areas from the groups were compared.

3.2.5.2. Spiral Ganglion Cell Densities

Spiral ganglion cell densities were determined with the use of the NIH ImageJ program. Images of the cochleae (TIFF) were obtained, the boundaries of Rosenthal's canal were outlined and the areas of the outlined spaces were calculated in mm^2 (Fig. 3.1). The number of perikarya within the outlined spaces was counted and densities (number of perikarya/area in mm^2) were determined as previously described [18].

3.2.6. Scanning Electron Microscopy

Cochlear samples were processed as previously described [19]. The samples were analyzed under a field emission scanning electron microscope (Hitachi S4700, Hitachi LTD., Tokyo, Japan) in order to visualize and evaluate OHC morphology.

3.2.7. Immunohistochemistry: TNF-αlpha

Microtome sections were evaluated by immunohistochemical staining for the detection of TNF-α using a biotin-free detection system. Sections were deparaffinized with xylene and ethanol baths. Slides were then heated in a microwave oven with 0.01M citrate buffer for 10 min followed by 3% H_2O_2 in ethanol for 10 min at room temperature. Slides were washed three times with 0.1 M phosphate buffered saline (PBS) at pH 7.4 for 5 min and were then incubated overnight with the primary antibody at room temperature. Slides were washed with PBS for 15 min and then incubated with the enhancer reagent for 30 min at room temperature. After washing with PBS, slides were incubated with polymer–horseradish peroxidase for 45 min. After a final wash in PBS, slides were treated with diaminobenzidine in order to visualize the immunoreaction, counterstained with Mayer's hematoxylin for 2 min, dehydrated in ethanol, cleared in xylene and mounted in Eukitt. All slides were evaluated in a blinded fashion, without knowledge of the treatment administered. The expression of TNF-α was graded as faint, moderate or strong.

3.2.8. Statistical Analysis

The data were analyzed using analysis of variance (one way ANOVA). Post-hoc comparisons were made with Tukey's multiple comparison test. Statistical significance was set at p-value ≤ 0.05. Sample size for the experimental groups were calculated using the sample size calculator from the Department of Statistics of the University of British Columbia using the following criteria: power = 0.80, alpha = 0.05, sigma = 15, mu1 = 0 and mu 2 = 25. (http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html).

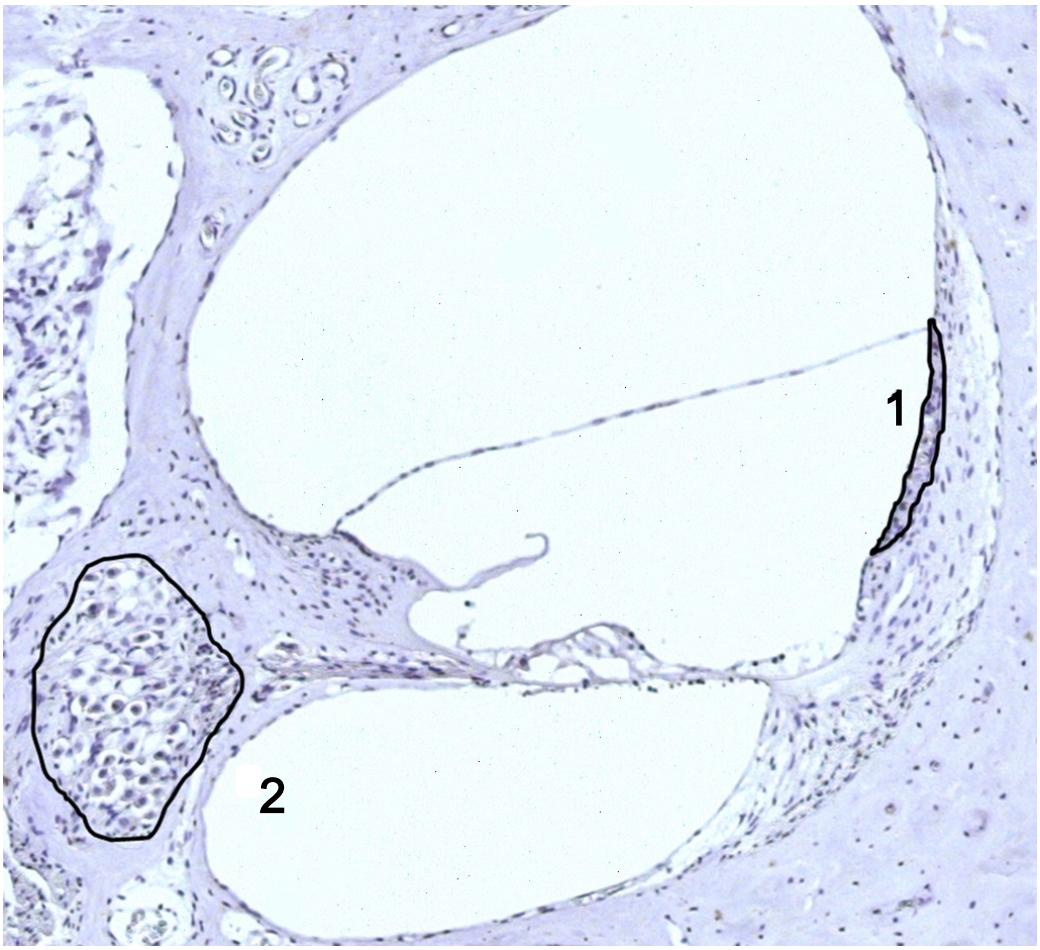

Figure 3.1. Section of a cochlear sample. Dark outlined areas demonstrate the spaces delineated in order to calculate the strial cross sectional areas (1) and the SGN densities (2). Hematoxylin stain.

3.3. Results

3.3.1. Auditory Brainstem Response

IP cisplatin administration caused a marked hearing loss. The average threshold shifts in the group receiving cisplatin only are 46 ± 9 dB at 8 kHz, 47 ± 7.1 dB at 16 kHz, 38 ± 4.3 dB at 20 kHz and 47 ± 4.4 dB at 25 kHz (Fig. 3.2). Therefore, a single injection of 12 mg/kg IP resulted in intense hearing loss throughout all of the frequencies tested.

Guinea pigs receiving cisplatin and dexamethasone (group 2) also presented marked hearing loss with threshold shifts of 48 ± 5.4 dB at 8 kHz, 51 ± 4.8 dB at 16 kHz, 51 ± 3.7 dB at 20 kHz and 41± 4.3 dB at 25 kHz (Fig. 3.2). There was no otoprotection following this scheme of treatment.

Guinea pigs treated with dexamethasone in a greater dose (group 3) also demonstrated a marked hearing loss in the ABR measurements. The threshold shifts were 61 ± 16.5 dB at 8 kHz, 52 ± 10.2 dB at 16 kHz, 43 ± 9.5 dB at 20 kHz and 48 ± 9.1 dB at 25 kHz (Fig.3.2). There was no otoprotection observed following a 2 day prophylaxis and a high dose dexamethasone treatment.

No statistically significant difference in ABR threshold shifts was observed between the groups ($p > 0.05$ for all frequencies tested). No significant otoprotection on ABR measurements was conferred by the use of systemic dexamethasone on cisplatin-induced ototoxicity.

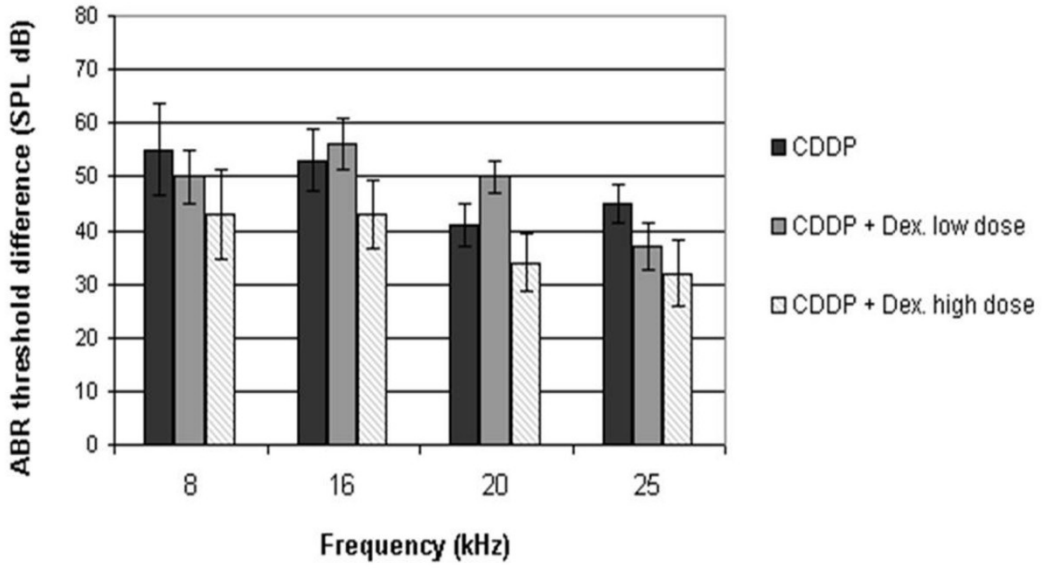

Figure 3.2. Auditory brainstem response (ABR) threshold shifts in decibels (mean ± SEM) for cisplatin (CDDP) and CDDP + dexamethasone treated guinea pigs. ABR measurements were taken before and 72 hours following the CDDP injection. No statistically significant difference was observed between the group receiving CDDP only and the groups receiving CDDP + dexamethasone. * = $P < 0.05$.

3.3.2. Light Microscopy Examination

Light microscopy of cochlear samples was performed in order to obtain general histological characteristics. Cochlear samples obtained from animals receiving only cisplatin demonstrated partial loss of OHCs with collapse of the tunnel of Corti and Nuel's space. The lateral wall findings consisted of protrusion of marginal cells into the endolymphatic space as well as strial edema. SGNs displayed partial detachment of the myelin sheath (Fig. 3.3B). These results were observed in the three cochlear segments (apex, middle, base). A very slight detachment was also observed in animals receiving saline only (Fig. 3.3A).

Samples obtained from animals receiving cisplatin and dexamethasone (group 2) revealed disruption of the microarchitecture of the organ of Corti similar to the samples obtained from the animals receiving cisplatin only (Fig. 3.3C). The lateral wall and SGN findings were also similar. On the other hand, animals treated with cisplatin and a greater dose of dexamethasone (group 3) exhibited preserved morphology of the tunnel of Corti and Nuel's space (Fig. 3.3D). Also, strial edema was decreased as compared to the cisplatin animal samples (Fig. 3.4). However, the SGN myelin sheath detachment findings did not differ from the animals receiving cisplatin only.

3.3.3. Strial Cross Sectional Area

The strial cross sectional areas were calculated for all of the groups. Saline treated animal samples were used to establish the area of the half sections for the apex, middle and base segments of the cochlea in normal guinea pigs. The cross sections observed in the saline treated group demonstrated dense striae and clearly defined nuclei with no bulging of cells into the endolymphatic space (Fig. 3.4A).

The cross sections pertaining to the animals treated only with cisplatin revealed decreased strial density, increased cellular vacuolization (Fig. 3.4B) and greater cross sectional areas as compared to the saline treated animals (Fig. 3.5).

The areas calculated for samples obtained from the animals treated with cisplatin and dexamethasone (group 2) were greater compared to the saline treated group and slightly lower compared to the cisplatin treated group (Fig 3.5); the

striae were denser and exhibited less vacuolization (Fig. 3.4C). Samples from group 3 also demonstrated reduced areas as compared to the cisplatin treated group yet no statistically significant difference was observed. We can appreciate dense striae, minimal bulging into the endolymph and a rather conserved morphology (Fig. 3.4D). No statistically significant difference was detected between the groups in terms of strial areas ($p>0.05$ for all segments of the cochlea).

3.3.4. Spiral Ganglion Neuron Densities

SGN densities were calculated for all groups. No statistically significant difference was detected between the groups in terms of SGN densities ($p>0.05$) (Fig. 3.6).

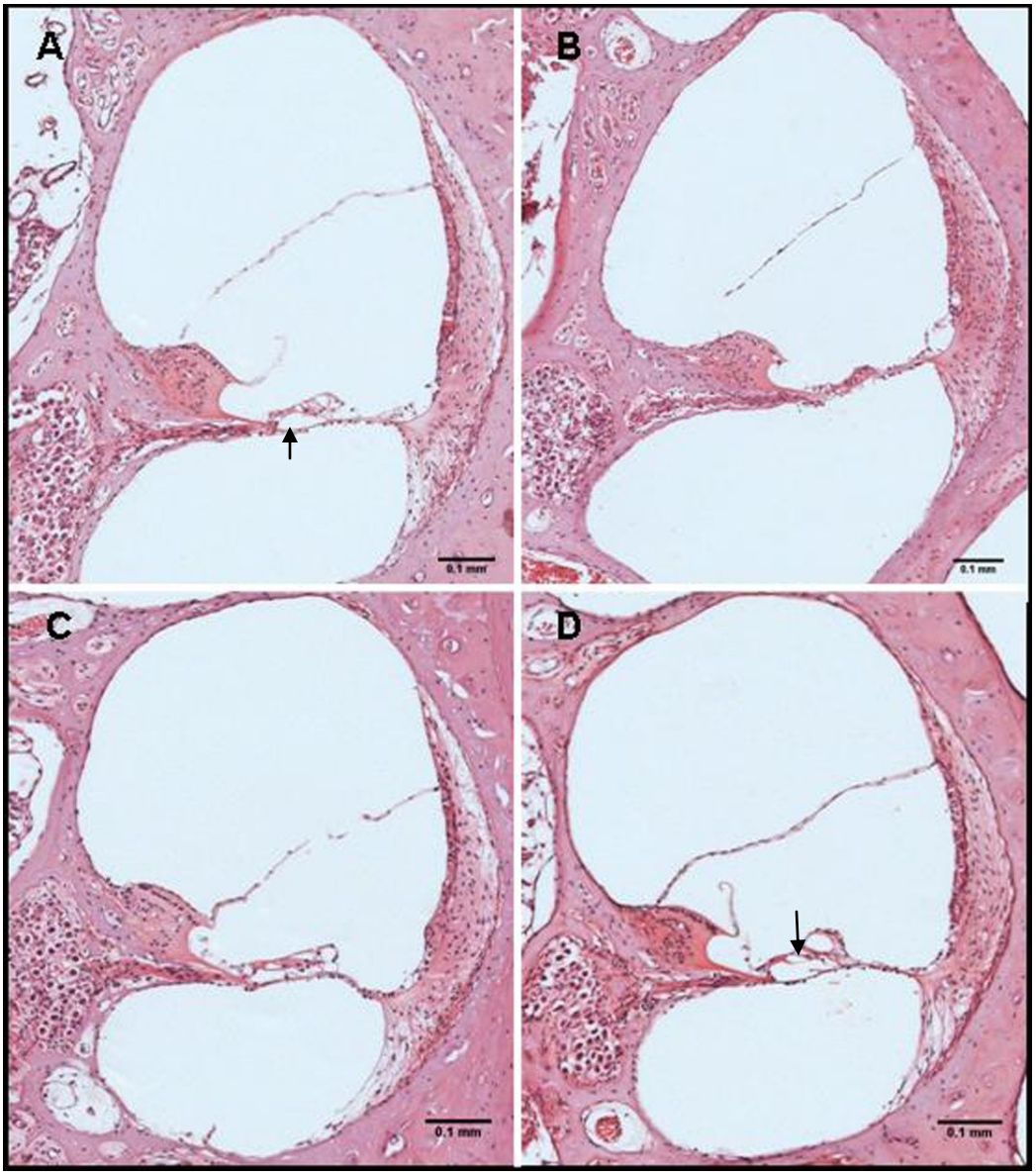

Figure 3.3. Sections of cochlear samples: base of cochlea: A) saline (negative control), B) cisplatin (positive control), C) cisplatin + dexamethasone (group 2) and D) cisplatin + greater dose of dexamethasone (group 3). Hematoxylin and eosin stain. Collapse of the tunnel of Corti and Nuel's space (indicated by arrows) is observed in cisplatin treated animals as well as protrusion of marginal cells into the perilymphatic space and strial edema. Similar changes are visible in C.

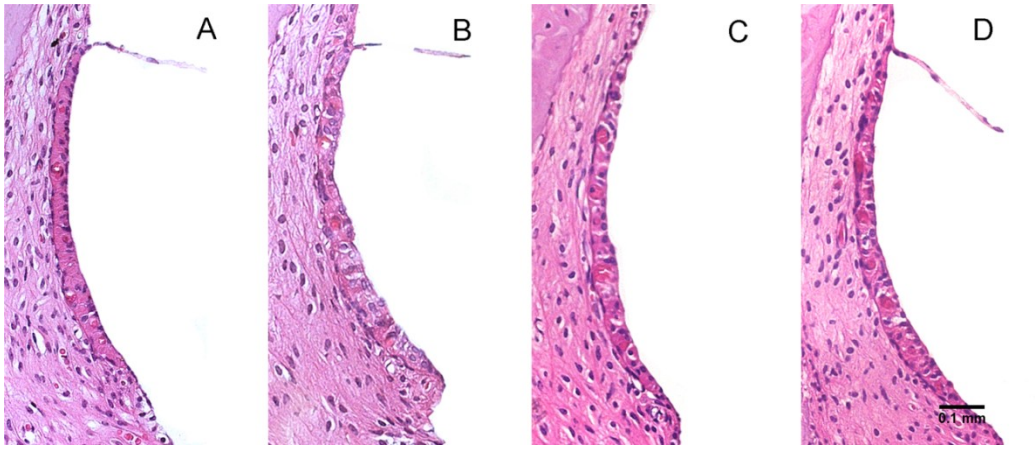

Figure 3.4. Strial cross sections. A) saline (negative control), B) cisplatin (positive control), C) cisplatin + dexamethasone (group 2) and D) cisplatin + greater dose of dexamethasone (group 3). Hematoxylin and eosin stain. Greatest strial edema and increased cross sectional areas were observed in cisplatin treated animals (B).

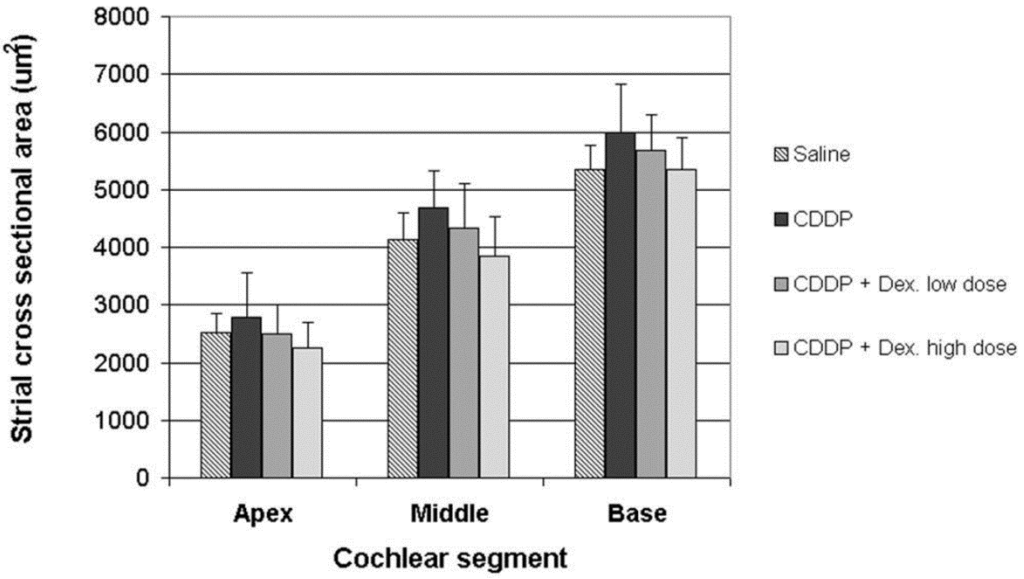

Figure 3.5. Cross sectional area (um^2) of the apex, middle and base of the cochleae. Measurements for the right and left cochleae were averaged. Six different measurements were assessed for every segment of every cochlea in order to increase the precision of the measurements. The cisplatin (CDDP) treated group demonstrated the greatest areas. No statistically significant difference was observed between CDDP treated animals and animals receiving CDDP and dexamethasone in low or high dosage (Dex.).

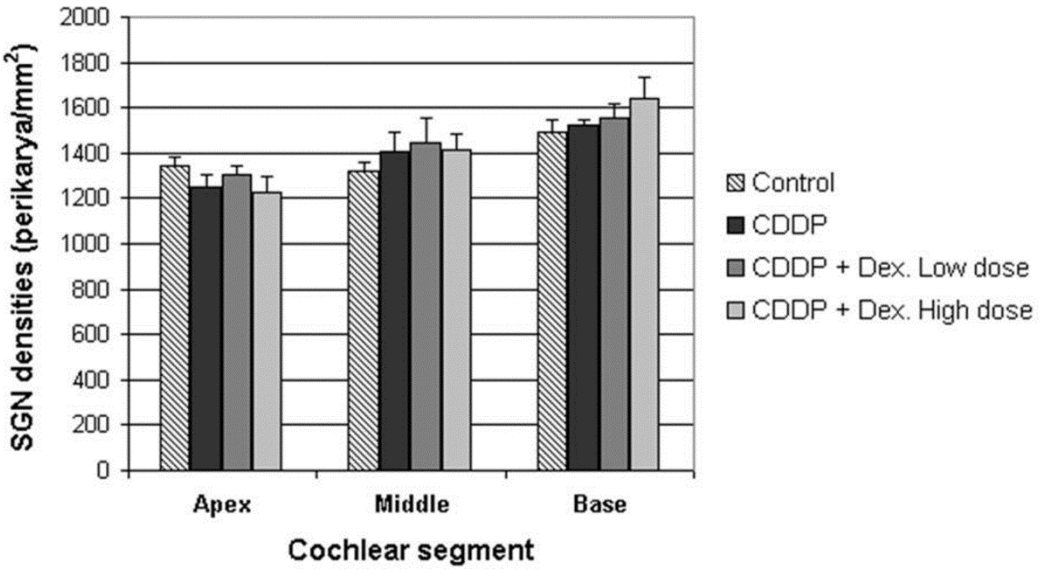

Figure 3.6. Spiral ganglion cell densities. The control group consisted of the saline treated animals. Treatment with cisplatin (CDDP) did not significantly decrease the SGN densities as compared to the control group. No statistically significant difference was observed between any of the groups and for any segment of the cochlea. * = $P < 0.05$.

3.3.5. Scanning Electron Microscopy

Animals receiving saline did not present with OHC loss or irregularities of stereocilia (Fig 3.7a). Damage and loss of stereocilia as well as rupture of the cuticular plate were clearly visible in cisplatin-treated animals (Fig 3.7b) and in animals receiving cisplatin and the lower dose of dexamethasone (Fig 3.7c). Greater preservation of OHCs was detected in the group receiving cisplatin and the greater dose of dexamethasone (Fig 3.7d).

3.3.6. Immunohistochemistry

Immunohistochemical detection of TNF-α in the normal cochlea was faint; the OHCs, stria vascularis, spiral ligament and SGNs stained weakly (Fig. 3.8A) whereas strong immunostaining was observed in the cisplatin treated animal samples for the above mentioned cochlear areas (Fig.3.8B). Strong immunostaining was also observed for the samples obtained from group 2 (Fig. 3.8C). Most interesting was the staining observed for the last group, receiving cisplatin and the greater dose of dexamethasone (group 3). Here, moderate immunostaining was observed (Fig. 3.8D).

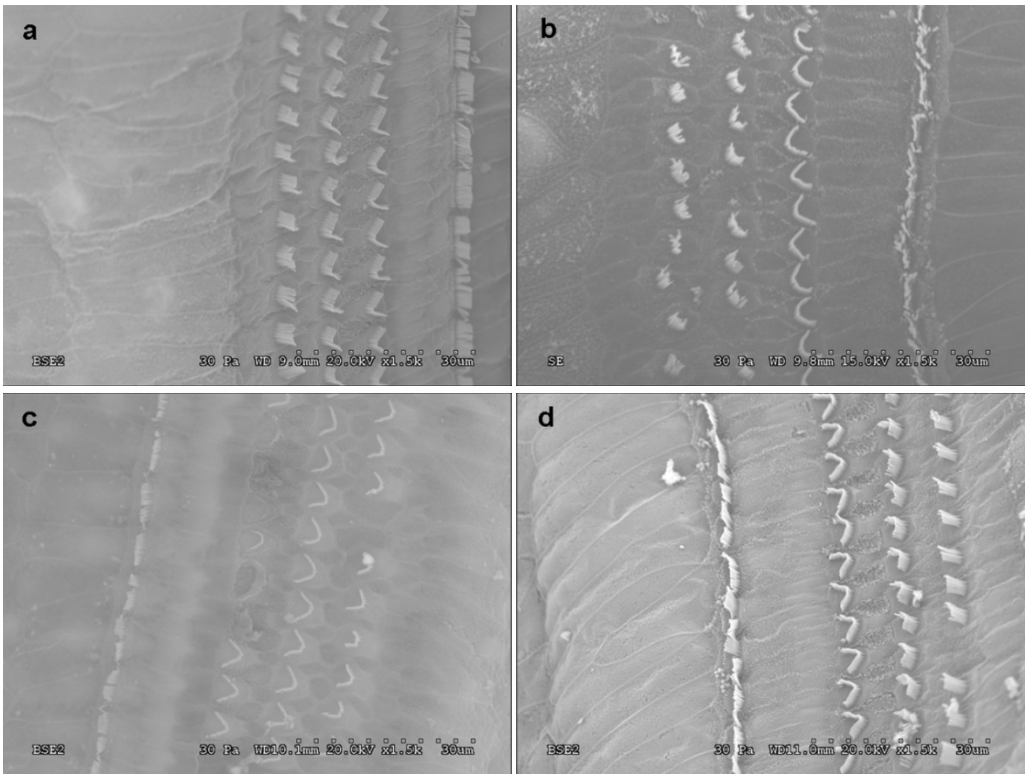

Figure 3.7. Scanning electron microscopy of outer hair cells (OHCs). a) Saline b) cisplatin, c) cisplatin + lower dose of dexamethasone (group 2) and d) cisplatin + greater dose of dexamethasone (group 3). Greater loss of OHCs is observed in b and c. Animals receiving saline did not present with OHC loss. Some OHC loss was detected in d.

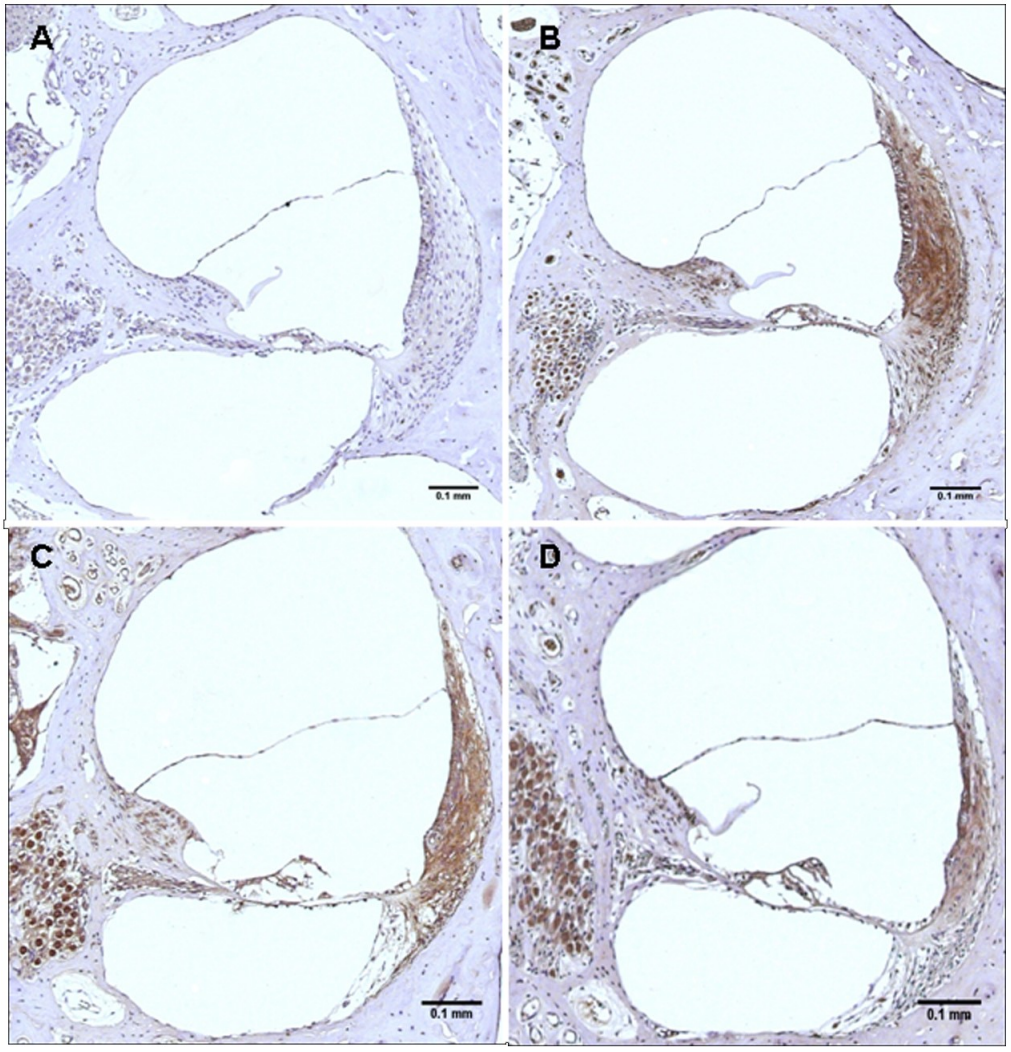

Figure 3.8. TNF-α immunoexpression in cochlear samples: A) saline B) cisplatin C) cisplatin + lower dose of dexamethasone (group 2) and D) cisplatin + greater dose of dexamethasone (group 3). Counterstained with Mayer's haematoxylin. Strong immunostaining is observed in B and C, moderate immunostaining is observed in D and very faint immunostaining is observed in A.

3.4. Discussion

Cisplatin is a commonly used chemotherapeutic agent. It has potent antineoplastic activity and can cause important adverse effects such as nephrotoxicity, ototoxicity and neurotoxicity which limit its clinical use [20]. Cisplatin ototoxicity leads to a bilateral and irreversible sensorineural hearing loss that is progressive from higher to the lower frequencies [1]. It quickly binds DNA and proteins and thereby inhibits their functions. Once bound, cisplatin induces the generation of reactive oxygen species and initiates the inflammatory cascade. These events can lead to apoptosis and therefore to a decrease in the number of cells in the cochlea necessary for an adequate function of the inner ear [4].

To date, literature on transtympanic administration of dexamethasone against cisplatin-induced ototoxicity presents different magnitudes of protective effects. Hill *et al* [10], Daldal *et al* [14] and Murphy *et al* [15] previously reported that intratympanic dexamethasone injections may provide some protection against cisplatin-induced ototoxicity in a mouse [10] and guinea pig model respectfully [14,15]. Paksoy *et al* observed a significant protective effect from intratympanic dexamethasone injections in a rat model with decreased threshold shifts on ABR testing [16]. The previously mentioned studies only reported hearing test results (ABR or distortion product otoacoustic emissions) as evidence. A transtympanic administration can avoid systemic side effects nevertheless the technique can cause local complications [21]. Also, concentrations reached in cochlear fluids are unpredictable [22]. To our knowledge, no previous study has evaluated the protective effect of a systemic administration of glucocorticosteroids on cisplatin-induced ototoxicity. It has been demonstrated that an intraabdominal or intratympanic injection of dexamethasone provide similar concentrations in perilymph in 30 minutes [17]. A systematic approach is the easiest route of administration and is easily controlled. In addition, dexamethasone does not interfere with the cytotoxic action of cisplatin [23], a frequently cited concern.

In our study, ABR outcomes did not demonstrate a functional protection from dexamethasone on cisplatin-induced ototoxicity. No difference was detected comparing animals treated with cisplatin alone or with cisplatin and

dexamethasone. All experimental animals presented significant hearing loss at all frequencies tested (Fig.3.2).

Cisplatin is known to target the organ of Corti, the OHCs, the SGNs, the stria vascularis and the spiral ligament [2,3]. Morphological alterations were observed following cisplatin administration. Partial loss of OHCs with collapse of the tunnel of Corti and Nuel's space, protrusion of marginal cells into the endolymphatic space, strial edema as well as partial detachment of the myelin sheath of SGN was observed. Similar findings have been previously reported [3, 18, 24]. OHC loss was further demonstrated with scanning electron microscopy which allowed detection of missing OHCs as well as rupture of the cuticular plates. A high dose of dexamethasone preserved the morphology of the tunnel of Corti, Nuel's space and the stria vascularis and decreased the strial edema.

Various authors [3, 24-27] have aimed to assess the histological pattern of injury to the stria vascularis caused by cisplatin. We observed decreased strial density, increased cellular vacuolization and increased cross sectional areas in cisplatin-treated animals. The cross sectional area measurements coincide with previous studies [24, 25]. The group that received the highest dose of dexamethasone in addition to cisplatin demonstrated similar area measurements to the saline treated group (Fig. 3.5). Statistical analysis of the strial measurements are limited by the number of animals in each group seen as cochleae that did not meet histological criteria were discarded i.e. section not in midmodiolar plane, broken cochleae from dissection. We can observe a tendency but further studies with a greater number of animals are required.

The reason for greater morphological preservation of the stria vascularis is unclear. Glucocorticosteroid receptors are highly expressed in the spiral ligament, stria vascularis and OHCs [28, 29]. Yet, our results demonstrate the lack of protection from dexamethasone on OHCs. Dexamethasone has also been shown to increase cochlear blood flow [9]. It may be that since the stria vascularis is highly vascularised and that cochlear blood flow is increased, the quantity of dexamethasone reaching the lateral wall may be greater as compared to other areas of the cochlea.

The SGNs displayed partial detachment of the myelin sheath when exposed to cisplatin. Findings did not differ from the animals receiving cisplatin and dexamethasone at any dosage. As for SGN densities, no statistically significant difference was detected between the groups. As previously reported, our results also suggest that cisplatin administration may not result in SGN loss (12 mg/kg IP) [18].

TNF-α has been shown to be a key pro-inflammatory cytokine in cisplatin-induced ototoxicity [6]. Immunohistochemistry for TNF-α was performed in order to detect whether dexamethasone administration decreases the expression of this cytokine. Cisplatin administration led to a strong immunoexpression of TNF-α in OHCs, the stria vascularis, the spiral ligament and in SGNs. Concomitant administration of dexamethasone in a high dose provided only a slight reduction in staining. It seems that the inflammatory component of cisplatin-induced ototoxicity may play a small role in the pathophysiology given that when administering a high dose of dexamethasone, cochlear morphological and functional alterations are still observed. A limitation of the study is the lack of long term follow-up. The guinea pig model used allows post-ABR testing to be performed on day 3, a situation not occurring in a clinical setting. Further studies employing different animal models allowing for long term treatment and follow-up would be required.

3.5. Conclusion

We studied the protective effect of a systemic dexamethasone administration against cisplatin-induced ototoxicity in a guinea pig model. We did not observe significant protection against cisplatin-induced ototoxicity. Dexamethasone seems to decrease TNF-α expression slightly as well as protect the stria vascularis from morphological alterations. Dexamethasone may be useful in future applications as a complementary treatment for cisplatin-induced ototoxicity.

3.6. Acknowledgments

The authors wish to thank Rujuan Huo from the Centre for Bone and Periodontal Research for their invaluable technical assistance.

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Preface - Chapter 4

In the previous chapter, the potential protective effect of a glucocorticoid, dexamethasone, against cisplatin-induced ototoxicity was assessed. As cisplatin causes an increase in the inflammatory mediators of the inner ear, a potent anti-inflammatory such as dexamethasone was believed to provide protection against the toxicity caused by cisplatin. The systemic administration of dexamethasone in a guinea pig model led to a partial protective effect as observed in the morphological analyses and measurements as well as the decrease in TNF-α immunostaining of the cochlear cells. However, hearing as tested by ABR was not conserved.

Cisplatin also causes the production of ROS within the cochlea. Cells exposed to cisplatin show an increase in superoxide and hydrogen peroxide, and this toxic environment leads to the activation of the intrinsic apoptotic pathway. In order to evaluate the potential of an antioxidant to protect against the toxicity caused by cisplatin, the following study was undertaken. Guinea pigs exposed to cisplatin were concomitantly administered a strong antioxidant: erdosteine. The antioxidant was given systemically. The hearing of the guinea pigs as well as morphological analysis of the OHCs were assessed.

Chapter 4

Protective Effect of Erdosteine against Cisplatin-Induced Ototoxicity in a Guinea Pig Model

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Abstract

Objective: Cisplatin is a commonly used chemotherapeutic agent. One of its major dose limiting side effects is ototoxicity. No treatment has yet been approved for this condition. The objective of this study was to determine the potential protective effect of a systemic administration of erdosteine against cisplatin-induced ototoxicity.

Study design: A prospective controlled trial conducted in an animal model.

Setting: Animal care research facilities of The Montreal Children's Hospital Research Institute.

Subjects and Methods: A total of 27 guinea pigs were assigned to 4 groups, each receiving a different concentration of intraperitoneal erdosteine: group 1 (control group) (n = 9) did not receive erdosteine, group 2 (n = 6) received 100 mg/kg/day, group 3 (n = 6) received 200 mg/kg/day and group 4 (n = 6) received 500 mg/kg/day. The animals in the experimental groups received the erdosteine injection daily for 4 days. All of the animals received 12 mg/kg of intraperitoneal cisplatin. Auditory brainstem response (ABR) threshold shifts were measured at 4 frequencies (8, 16, 20 and 25 kHz) for all groups. Scanning electron microscopy and outer hair cell counts were performed to assess the protective effect of erdosteine.

Results: Significant protection is observed in groups 3 and 4 at 25 kHz. These findings are supported by outer hair cell counts by scanning electron microscopy.

Conclusion: A systemic administration of erdosteine appears to provide an otoprotective effect at high frequencies for cisplatin-induced ototoxicity.

4.1. Introduction

Cisplatin is a commonly used chemotherapeutic agent for various forms of cancers. Its clinical use has been limited by a variety of side effects including ototoxicity. Cisplatin causes dose related, cumulative, progressive and irreversible sensorineural hearing loss which leads to a decreased quality of life [1]. Damage to inner ear structures begins at the basal turn of the cochlea hence affecting high frequency hearing primarily which then progresses to the low frequency areas [2].

ROS have been shown to be implicated in the process of apoptosis caused by cisplatin in cochlear structures [3]. Cisplatin administration *in vivo* has been shown to deplete cochleae of glutathione, an endogenous antioxidant, and decrease the activity of antioxidant enzymes in the inner ear such as superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase [4]. As a result, superoxide and hydrogen peroxide, are increased in the cochlear tissues generating a toxic environment for the cells. Superoxide can react with nitric oxide and generate peroxynitrite. It can also be transformed into hydrogen peroxide which can form, through catalysis by iron, the hydroxyl radical. Peroxynitrite and hydroxyl radical contribute to the degenerative process of cochlear structures [5].

Erdosteine is a thiol derivative containing two sulfhydryl groups (-SH) which are released after hepatic first-pass metabolism. The 3 resulting metabolites possess pharmacologically active sulfhydryl groups hence can act as free radical scavengers and antioxidants [6-7]. As a result of its antioxidant properties, erdosteine has been evaluated *in vivo* for various conditions such as drug-induced nephrotoxicity [8] [9] [10] [11], drug-induced hepatotoxicity [12], ischemia/reperfusion injury [13], drug-induced cardiotoxicity [14] and is currently being employed as a mucolytic and antioxidant for chronic obstructive pulmonary disease [5,15].

The following study was designed to establish the protective effect of an IP administration of erdosteine on cisplatin-induced ototoxicity in a guinea pig model.

4.2. Materials and Methods

4.2.1. Animals

Healthy female albino Hartley guinea pigs (500 - 900 g) were used in the current study (Charles River Laboratory, Senneville, Quebec). The animals had free access to commercial food and water. They were kept under standard laboratory conditions; housed in a room at 20 ± 4 °C ambient temperature with a relative humidity of 50 ± 5% and a 12 hour light/dark cycle. The animals were kept in the animal care research facilities of the Montreal Children's Hospital Research Institute. The study was approved and monitored by the Animal Care Committee in accordance with the Canadian Council of Animal Care guidelines.

4.2.2. Experimental Design

The animals (27) were assigned to 4 groups, each receiving a different concentration of IP erdosteine: group 1 (control group) (n = 9) did not receive erdosteine, group 2 (n = 6) received 100mg/kg/day, group 3 (n = 6) received 200 mg/kg/day and group 4 (n = 6) received 500 mg/kg/day. The animals in the experimental groups received the erdosteine injection daily for 4 days. All of the animals received 12 mg/kg of IP cisplatin on day 1. The dosage of cisplatin has been determined by previous research at our laboratory [16]. It was demonstrated that this dose causes sufficient ototoxicity as a model yet very low mortality rates.

4.2.3. Erdosteine and Cisplatin Treatment

The erdosteine injections were prepared by dissolving erdosteine (Haohua Industry, China) in sodium bicarbonate solution. 0.9% NaCl was then added to the mixture in order to obtain a total volume of 10 ml. Cisplatin (1mg/ml, Hospira) was administered under anesthesia with isoflurane. A subcutaneous bolus of 10 ml sterile isotonic saline was injected daily for hydration and supplements of hay were given for the duration of the experiment. The animals were monitored daily for signs of pain and weight loss. Animals were euthanized 72 hours following the cisplatin injection. Treatments and pre-ABR measurements were performed by

the main author and post-measurements were evaluated by an external audiology reviewer blinded to the treatments administered.

4.2.4. Auditory Brainstem Response

The auditory brainstem response (ABR) was performed prior to any injection (baseline measurement) and 72 hours following the cisplatin IP administration (post measurement) in order to determine the ABR threshold shifts (SPL dB). Hearing threshold was defined as the lowest intensity of stimulation that resulted in a clear reproducible waveform. The tympanic membranes and external auditory canals were inspected prior to the ABR measurement using an operating microscope. Animals with preexisting hearing loss and/or any abnormality in the external or middle ear were excluded from the study. The active electrode was placed subcutaneously within the pinna of the tested ear, the reference electrode at the vertex and the ground electrode on the pinna of the contralateral pinna. The ABR was measured at four frequencies (8, 16, 20 and 25 kHz) on the Smart EP device (Intelligent Hearing Systems) using tone burst stimulus with a rate of 39.1 bursts/second and alternating polarity. The response to the stimulus was averaged from 1600 sweeps. The measurements began at 80 dB and subsequently being decreased or increased by 20 dB and then 10 dB until the last three clear reproducible waveforms were obtained. Threshold shifts were calculated by comparing the pre- and post-cisplatin hearing threshold values.

4.2.5. Scanning Electron Microscopy

Immediately following the post-ABR measurement, animals were euthanized and cochlear dissection was completed; cochleae were removed, dissected and fixed in 2.5% glutaraldehyde for 2 hours. The cochleae were then left in 0.1 M phosphate buffer solution for 24 hours at 4°C. The specimens were post-fixed in osmium tetroxide for 1 ½ hours and later dehydrated in a series of ethanol solutions for 15 minutes each: 35 percent, 50 percent and 70 percent. The cochleae were drilled until the covering bone became thin. The bone covering was then removed and the organ of Corti was dissected. These samples were dehydrated in a series of

ethanol solutions for 15 minutes each: 90 percent, 95 percent and absolute ethanol followed by critical point drying, mounting on pin stubs and sputter coated with gold. The samples were analyzed under a field emission scanning electron microscope (Hitachi S4700, Hitachi LTD., Tokyo, Japan).

4.2.6. Outer Hair Cell Counts

OHC counts were performed for scanning electron microscopy samples of groups 1, 2 and 4. A modified version of a 4-grade scale for qualitative and quantitative evaluation of cochlear damage described previously [17] was used: Normal (N): OHC with normal stereocilia; Grade 1: OHC with 10% to 50% damage or loss of stereocilia; Grade 2: OHC with less than 50% of stereocilia remaining; Grade 3: rupture of the cuticular plate and missing hair cells. The results are presented as the percent of OHCs in each grade category for the apical, middle and basal turns.

4.2.7. Statistical Analysis

Differences between the four groups were evaluated with the use of the Kruskal-Wallis one-way analysis, and Wilcoxon rank-sum test was subsequently performed for further analysis between two groups. Statistical significance was set at p-value ≤ 0.05. Sample size for the experimental groups were calculated using the sample size calculator from the Department of Statistics of the University of British Columbia using the following criteria: power = 0.80, alpha = 0.05, sigma = 15, mu1 = 0 and mu 2 = 25. (http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html).

4.3. Results

4.3.1. Auditory Brainstem Response

Animals in the control group demonstrated a large hearing loss presenting mean ABR threshold shifts of 55 dB at 8 kHz, 51 dB at 16 kHz, 42 dB at 20 kHz and 46 dB at 25 kHz (Fig 4.1). A dose of 12 mg/kg of IP cisplatin caused evident ototoxicity in the guinea pigs. Animals exposed to cisplatin and the lowest concentration of erdosteine (100mg/kg) too showed marked ototoxicity and no

data to support otoprotection. On the other hand, animals exposed to cisplatin and higher concentrations of erdosteine, 200 mg/kg and 500 mg/kg, demonstrated significant otoprotection at 25 kHz with a p-value of 0.004, median value of 27.5 and confidence interval (10.95, 42.38) for group 3 and p-value of 0.003, median of 23 and confidence interval (10.07, 34.10) for group 4. The highest dose also showed significant difference as compared to the control group at 20 kHz with a p-value of 0.036, median of 26.0 and confidence interval (-0.16, 41.59) (Table 4.1).

4.3.2. Scanning Electron Microscopy: Outer Hair Cells Counts

Animals receiving cisplatin IP only (group 1) presented damage throughout the cochlea with a greater percentage of cells being assigned to grade 3. Greater injury was observed in the basal turn of the cochlea. Few cells were included in grades 1 and 2. Animals which received 100 mg/kg of erdosteine IP presented hair cell counts in a similar pattern to group 1 and no statistically significant difference in hair cell counts were observed in this group. Cochleae dissected from group 4, receiving 500 mg/kg of erdosteine IP, presented a greater number of cells assigned to grade 1 and a decrease in percentage of cells in grade 3 (Fig. 4.2). Statistically significant decreases in OHC counts assigned to grade 3 were observed for the middle and basal turn of the cochleae in this group (Table 4.2).

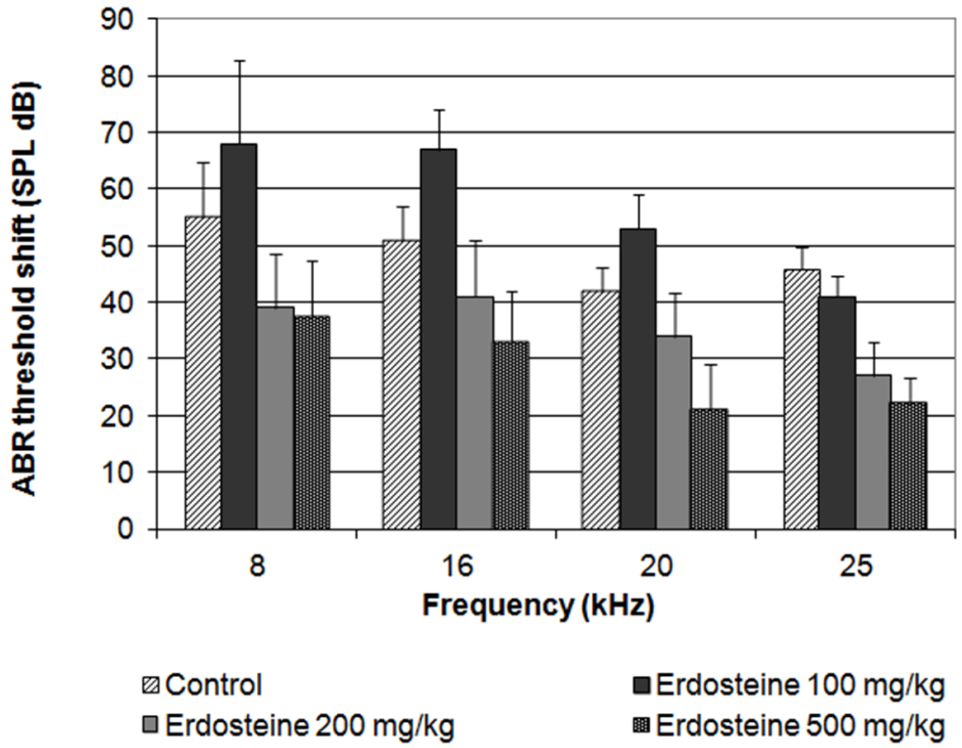

Figure 4.1. Auditory brainstem response shifts in decibels (mean ± SEM) for cisplatin and cisplatin plus erdosteine treated guinea pigs. Threshold shifts were calculated by subtracting baseline measurements from post-measurements. Statistical significant differences were observed at the 20 kHz frequency with 500 mg/kg of erdosteine and at 25 kHz frequency for the groups treated with 200 mg/kg and 500 mg/kg, * = $p < 0.05$.

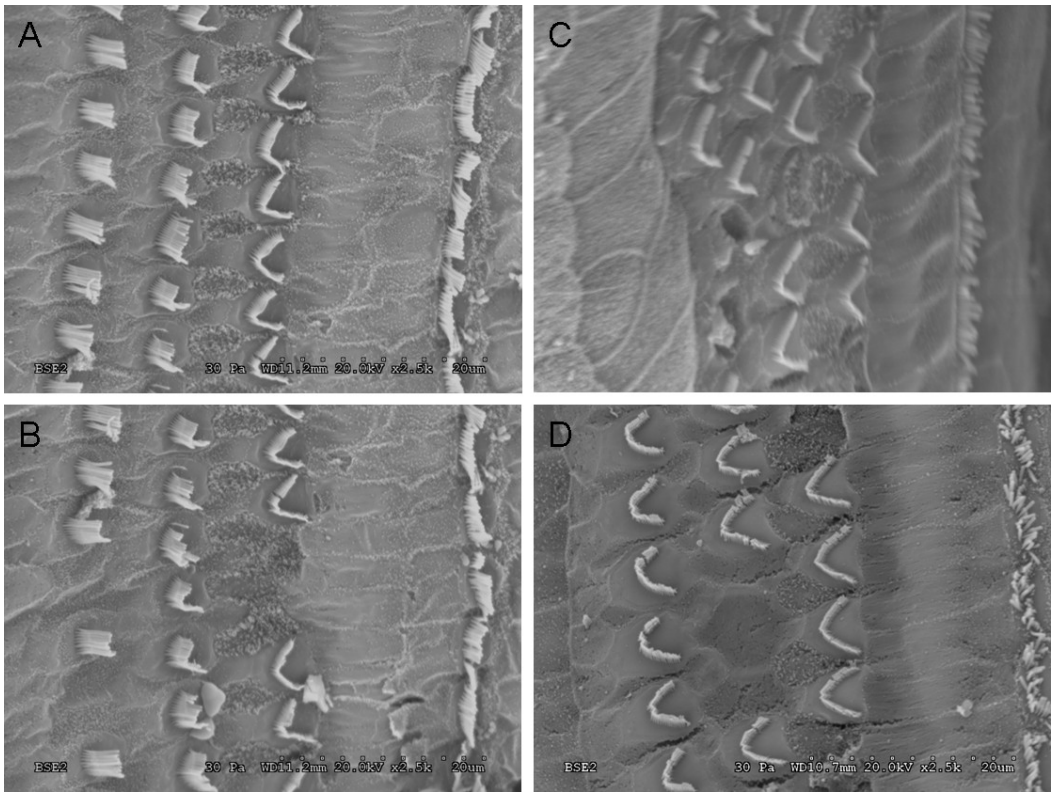

Figure 4.2. Scanning electron microscopy of outer hair cells in basal turn of the cochlea. A. normal, B. cisplatin (group 1), C. cisplatin + erdosteine 100 mg/kg (group 2) and D. cisplatin + erdosteine 500 mg/kg (group 3). OHCs observed in group 4 present a greater preservation of stereocilia as compared to cisplatin treated animals.

Table 4.1. Statistical analysis

Kruskal -Wallis test

Freq.	p-value	ABR Threshold shifts[1] (95% confidence interval) Group 1	Group 2	Group 3	Group 4
8 kHz	0.245	55 (31.99, 77.34)	68 (29.12, 105.90)	39 (14.52, 63.82)	37.5 (12.06, 62.94)
16 kHz	0.067	51 (37.26, 64.74)	67 (48.72, 85.44)	41 (15.31, 66.36)	32.75 (8.91, 56.60)
20 kHz	0.026*	42 (31.89, 51.78)	53 (37.58, 68.75)	34 (13.65, 53.83)	20.9 (-0.16, 41.59)
25 kHz	0.011*	46 (37.79, 54.66)	41 (31.06, 50.60)	27 (10.95, 42.38)	22.1 (10.07, 34.10)

Wilcoxon Rank Sum test

20 kHz	p-value	Median	25 kHz	p-value	Median
Groups 1-2	0.114	Group 1: 42.0	Groups 1-2	0.456	Group 1: 49.0
Groups 1-3	0.456	Group 2: 58.5	Groups 1-3	0.004*	Group 2: 36.3
Groups 1-4	0.036*	Group 3: 36.3	Groups 1-4	0.003*	Group 3: 28.0
		Group 4: 26.0			Group 4: 23.0

[1] values in SPL dB. * p-value < 0.05

Table 4.2. Outer hair cell counts and grading

Grade	N	1	2	3
Normal				
Apex	100 ± 0	0	0	0
Middle	100 ± 0	0	0	0
Base	100 ± 0	0	0	0
Cisplatin				
Apex	84.4 ± 2.9	0.5 ± 0.7	1.9 ± 2.7	13.2 ± 1.0
Middle	71.7 ± 9.8	3.0 ± 4.2	4.0 ± 4.2	22.5 ± 1.5
Base	63.3 ± 7.6	0	8.1 ± 7.9	28.6 ± 0.3
Cisplatin + Erdosteine 100 mg/kg				
Apex	91.0 ± 4.8	0	0	9.1 ± 4.9
Middle	78.3 ± 1.7	1.1 ± 1.5	0	20.7 ± 0.2
Base	70.8 ± 5.6	1.9 ± 3.9	0.5 ± 1.1	26.8 ± 8.4
Cisplatin + Erdosteine 500 mg/kg				
Apex	87.9 ± 9.7	1.0 ± 2.3	0	11.1 ± 8.6
Middle	90.9 ± 10.1	0.6 ± 1.2	0.3 ± 1.0	8.1 ± 9.3*
Base	89.7 ± 8.7	0.8 ± 2.3	0.8 ± 2.4	8.6 ± 6.9*

N: normal, *P-value ≤ 0.001.

4.4. Discussion

These findings support the fact that thiol derivatives such as erdosteine provide protection against oxidative stress caused by cisplatin. Cisplatin induces superoxide production in cochlear tissues which react with nitric oxide to form peroxynitrite, a toxic radical capable of activating the apoptotic cascade through caspase-3 [2]. Erdosteine is activated by first pass metabolism generating Met I, the pharmacologically active metabolite containing an active sulfhydryl group [7]. It has been observed that Met I is capable of reacting with ROS, nitric oxide and nitric oxide-derived peroxynitrite and furthermore has both antioxidant and scavenging activities [18]. Erdosteine has shown to provide protection against cisplatin-induced nephrotoxicity [11,19-21] and cisplatin-induced hepatic oxidant injury [22] when given orally.

One previous study evaluated the potential protective effect of erdosteine on cisplatin-induced ototoxicity in a rat model. Erdosteine was administered orally and hearing loss was evaluated with distortion product otoacoustic emissions (DPOAEs). The authors observed significant protection on cochlear function as measured by DPOAEs [23]. In this study, we demonstrate that an IP administration of erdosteine provides otoprotection as observed by auditory brainstem response measurements and OHC counts by scanning electron microscopy. Limitations of the study include the lack of enzymatic activity detection for the main antioxidant enzymes of the inner ear. Catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase are important modulators of the oxidative stress response of the cochlea [2]. Furthermore, the number of animals per experimental group was minimal. A larger study would provide greater insight. Additional studies are required in order to determine dosage and interference with cisplatin oncology regimens.

4.5. Conclusion

A systemic administration of erdosteine seems to be a promising future therapeutic strategy for cisplatin-induced ototoxicity as observed by the changes in hearing thresholds and OHC counts.

4.6. Acknowledgments

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Preface - Chapter 5

In the two previous studies, pharmacological approaches were evaluated as potential protective strategies against cisplatin-induced ototoxicity. A potent steroid as well as an antioxidant were evaluated. Unfortunately, both products provided partial protection as demonstrated in chapters 3 and 4. As a result, the potential of a more specific and precise treatment led to the completion of a systematic review exploring the possibility of gene therapy as a possible future treatment strategy for this condition.

Gene therapy can offer long-term outcomes as well as cellular selectivity. Seeing that gene therapy is not yet available as a mainstream treatment modality, the review included *in vitro* and experimental animal studies in which genetic manipulation was the method of choice with the aim of protecting cells against the toxicity caused by cisplatin. This is the first systematic review published to assess this possibility and is described in detail in the following chapter.

Chapter 5

Gene Therapy for Cisplatin-Induced Ototoxicity: A Systematic Review of In Vitro and Experimental Animal Studies

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Abstract

Objective: Ototoxicity is a frequent side effect of cisplatin treatment. No therapy is currently available for cisplatin-induced ototoxicity. A systematic review of experimental animal studies and *in vitro* experiments was conducted to evaluate gene therapy as a potential future therapeutic option.

Data sources: Eligible studies were identified through searches of electronic databases Ovid MEDLINE, Ovid MEDLINE In-Process, Embase, PubMed, Biosis Previews, Scopus, ISI Web of Science and The Cochrane Library.

Study selection: Articles obtained from the search were independently reviewed by 2 authors using specific criteria in order to identify experimental animal studies and *in vitro* experiments conducted to evaluate gene therapy for cisplatin-induced ototoxicity. No restriction was applied to publication dates or languages.

Data extraction: Data extracted included experiment type, cell type, species, targeted gene, gene expression, method, administration, inner ear site evaluated, outcome measures for cytotoxicity and significant results.

Results: 14 articles were included in this review. *In vitro* and *in vivo* experiments have been performed to evaluate the potential of gene expression manipulation for cisplatin-induced ototoxicity. Twelve different genes were targeted including *NTF3, GDNF, HO-1, XIAP, Trpv1, BCL2, Otos, Nfe2l2, Nox1, Nox3, Nox4* and *Ctr1*. All of the included articles demonstrated a benefit of gene therapy on cytotoxicity caused by cisplatin.

Conclusion: Experimental animal studies and *in vitro* experiments have demonstrated the efficacy of gene therapy for cisplatin-induced ototoxicity. However, further investigation regarding safety, immunogenicity and consequences of genetic manipulation in the inner ear tissues must be completed in order to develop future therapeutic options.

5.1. Introduction

Cisplatin is a commonly used platinum-based chemotherapeutic agent. It is a potent antineoplastic compound and can lead to side effects such as ototoxicity and nephrotoxicity limiting its clinical use [1]. Ototoxicity is expressed as a bilateral, progressive, cumulative and dose related sensorineural hearing loss [2]. Cisplatin enters the cell through copper transporters Ctr1 [3-4] and OCT2 [5] and by passive diffusion [6]. Inside the cell, cisplatin binds DNA and proteins creating irreversible adducts [7]. Furthermore, it generates ROS [8-9], initiates the inflammatory cascade [10] and induces mitochondrial dysfunction [11] resulting in apoptosis [12]. Systemic and local deliveries of various pharmaceuticals have been investigated for this condition such as anti-apoptotic molecules, antioxidants and anti-inflammatories. No treatment is currently available for cisplatin-induced ototoxicity.

Human gene therapy is a new and innovative process arising from molecular biology and biotechnology by which nucleic acids are transferred to patients cells in order to obtain a therapeutic effect. It offers advantages over current treatment modalities such as long-term outcomes following a single application, cellular selectivity and correction of genetic disorders [13]. Various obstacles have prevented gene therapy from being successful. The choice of therapeutic gene, administration route, delivery system, target, stable and tissue-specific expression and the host's immune response are some of the issues that must be carefully evaluated in order to obtain therapeutic benefits from gene therapy [14]. The current systematic review aims to analyze the literature regarding gene therapy for cisplatin-induced ototoxicity in experimental animal studies and *in vitro* experiments.

5.2. Methods

A systematic review was performed in order to retrieve all articles discussing gene therapy in experimental animal studies and *in vitro* experiments relating to cisplatin-induced ototoxicity. All articles published before January 2011 (search date: January 18, 2011) were eligible for evaluation. No restriction was applied to

publication dates or languages. Non-English articles were translated for assessment. Databases searched are Ovid MEDLINE, Ovid MEDLINE In-Process, Embase, PubMed, Biosis Previews, Scopus, ISI Web of Science and The Cochrane Library. Search terms included Medical Subject Headings (MeSH) and key words such as: *cisplatin, cisplatinum, ototoxicity, hearing loss, deafness, cochlea, gene, gene therapy, vector, RNA, virus, DNA, retrovirus, adenovirus, herpes simplex virus, adeno-associated virus, lentivirus and liposome*. Full search strategies can be viewed in the addendum following chapter 7.

Studies were included for data extraction and evaluation when they involved *in vitro* and/or experimental animal experiments regarding cisplatin-induced ototoxicity and gene therapy. Articles presenting clear outcome measures such as cell survival, cell count, cell morphology or hearing assessment compared to a control group were included. When articles consisted of various experiments or steps unrelated to ototoxicity, specific data relating to genetic manipulation, cytotoxicity and hearing were extracted from the articles. Experiments including gene therapy in combination with other treatment modalities were excluded. Reviews, letters and editorials were not included. Bibliographies of selected studies were searched as well for any supplementary articles of relevance. Two authors independently reviewed the articles obtained from the search and extracted the following data: experiment type, cell type, species, targeted gene, gene expression, method, administration, inner ear site evaluated, outcome measures for cytotoxicity and significant results. A comparison was performed and discrepancies were resolved by dialogue.

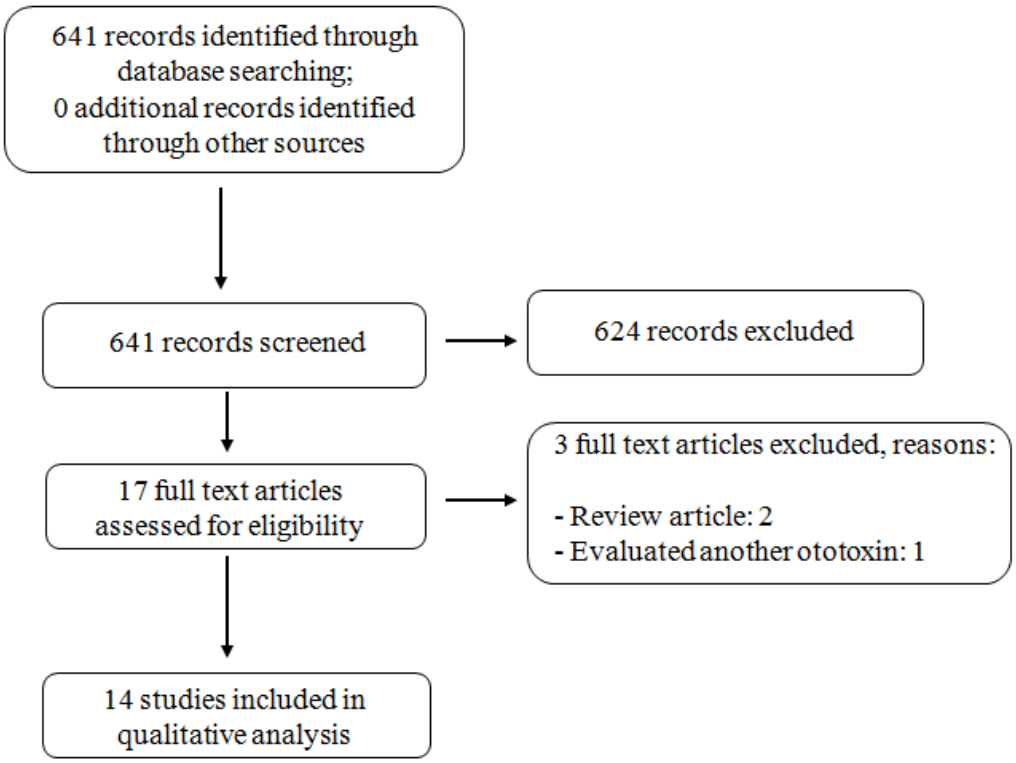

Figure 5.1. Flow diagram for eligible articles

5.3. Results

A total of 641 articles were identified from the databases searched, 17 met the inclusion criteria. These articles were obtained and carefully reviewed. Three studies were excluded as two were review articles [15-16] and one evaluated ototoxicity induced by a different ototoxin [17]. Out of the 14 remaining articles, two were written in Chinese and twelve were in English.

Eight of fourteen articles employed viral vectors including herpes simplex virus (HSV) amplicon, recombinant adeno-associated virus (rAAV), recombinant adenovirus (rAd) and adenovirus (Adv) to upregulate the expression of the desired gene. HSV vectors have a broad cell type range with preference for cells of neuronal origin. Their use results in high levels of expression however the duration of the expression is limited and cytopathic effects of the virus have been identified [18]. HSV transgene expression has been detected in cochlear supporting cells, auditory neurons, Reissner's membrane and spiral ligament but not in the stria vascularis or hair cells (HCs) [19]. AAV is a small single-stranded DNA virus that can infect a variety of cell types. It can integrate into the host genome and generate a stable and long-term expression of the selected gene. However, the packaging of the vector is complex and presents limitations for the size of the inserted gene [20]. AAV transgene expression has been detected in cochlear HCs, supporting cells, Reissner's membrane, auditory neurons and spiral ligament but not in the stria vascularis [19]. One of the most common viruses used for gene therapy is Adv. It is capable of infecting a wide variety of post-mitotic cells and its ease of production and high transduction efficiency are attractive characteristics for a gene therapy vector [19-20]. Also, Adv has been a successful vector for all the inner ear structures previously mentioned, including the stria vascularis. Still, a major disadvantage is that it elicits the host inflammatory response against the cell receiving the viral vector which prevents a possible subsequent administration [20].

Two authors used a plasmid vector for chemical transfection. Three used RNA interference to silence the target genes with short interfering RNA (siRNA) through chemical transfection while one study delivered naked siRNA

successfully (Table 5.1). siRNAs are small in size and show great specificity and efficacy nevertheless the silencing effect is transient, lasting weeks [21-22].

Three of the included articles performed *in vitro* and *in vivo* experiments regarding cytotoxicity induced by cisplatin while eight evaluated cultured cells only and three performed *in vivo* experiments exclusively. Although many of the articles presented multiple experiments, only those related directly to cytotoxicity and/or hearing loss in relation to gene manipulation were evaluated. The most common type of cell line used was HEI-OC1, a conditionally immortalized cell line derived from the Immortomouse organ of Corti [23]. The cell line was used for diverse analyses such as cell viability determination, inflammatory and apoptotic cascade evaluation, ROS production and cellular accumulation of platinum. Hence, it is a valuable cell line with multiple uses. Other cell cultures used are: Sprague-Dawley (S-D) rat spiral ganglion neuron (SGN) and spiral ligament fibrocyte (SLF) cultures, Wistar rat HC and SGN cultures, C57BL/6 mouse SGN, African green monkey kidney fibroblast-like cells (COS-7) [24] and UB/OC-1 cells which are also derived from Immortomouse organ of Corti [25].

In terms of *in vivo* experiments, both mice (CBA/CaJ) and rats (S-D and Wistar) were used. The experimental animals received the treatment solutions by different administration procedures, mostly by injecting directly through the round window membrane (RWM) (3); also by RWM application (1), by transtympanic approach (1) and by injecting directly into the scala vestibuli (1). Of the six articles presenting *in vivo* experiments, three [26-28] used one or more methods such as real-time reverse transcriptase polymerase chain reaction (RT-PCR), western blot, enzyme-linked immunosorbent assay (ELISA), immunocytochemistry or fluorescence microscopy to detect expression of the targeted gene in the *in vitro* section of the research whereas two [29-30] integrated immunofluorescence, immunohistochemistry, western blot or RT-PCR methods to detect expression of the targeted gene in tissues obtained from the experimental animals. The gene must be transcribed in the cell, messenger RNA (mRNA) and the targeted protein must be synthesized and the latter must then exhibit biological functions [13]. In RNA interference, the double-stranded RNA introduced in the

cell yields small RNA duplexes by Dicer-mediated cleavage. One of the strands, the passenger strand, is destroyed while the other strand, the guide strand, is assembled into the RNA-induced silencing complex (RISC) and directs cleavage of mRNAs containing sequence homologous to the single stranded RNA [31]. Hence, detection of mRNA and/or protein expression is necessary to evaluate RNA interference success.

In terms of targeted genes (Table 5.2), a diversity of different categories of proteins were aimed for gene therapy including neurotrophic factors (*NTF3, GDNF*), apoptosis mediators (*XIAP, BCL2*), NADPH oxidases (*Nox1, Nox3, Nox4*), an antioxidant response regulator (*Nfe2l2*), a cytoprotective enzyme (*HO-1*), a copper transporter (*Ctr1*), a non selective cation channel (*Trpv1*) and a newly discovered protein with unknown functions (*Otos*). Up-regulation of neurotrophin-3 (NT-3) *in vitro* and *in vivo* with concomitant cisplatin administration demonstrated improved SGN survival and enhanced neurite outgrowth as compared to the control vector [26, 32]. Co-transduction of neurotrophin-3 and GDNF seems to provide an enhanced protection against cisplatin-induced ototoxicity as compared to NT-3 up-regulation solely as observed by protein levels secreted into the culture media and SGN cell count following injection of the HSV amplicon through the round window membrane of mice [27]. The up-regulation of the apoptotic suppressor XIAP by recombinant AAV vector *in vivo* two months prior to cisplatin treatment resulted in decreased auditory brainstem response (ABR) threshold shifts and greater OHC counts as compared to controls [28-29]. A decrease in apoptotic cell count was also determined *in vitro* [28]. As for Bcl-2, up-regulation by viral vectors in HC and SGN cell cultures treated with cisplatin demonstrated improved cell survival and greater axonal lengths. Unfortunately, no *in vivo* experiments were performed [33-34]. NADPH oxidases present multiple functions including ROS generation. RNA interference was employed to silence Nox1, Nox3 and Nox4. When silencing Nox1 or Nox4 by siRNA *in vitro* with subsequent cisplatin treatment, increased cell viability was detected, intracellular ROS levels were decreased and apoptosis was inhibited as detected by decreased effector caspase activation (caspase-3) [35].

Cisplatin-mediated ROS generation was also reduced by Nox3 siRNA *in vitro* [30]. The same research team later evaluated *in vivo* impacts of Nox3 siRNA transtympanic application followed by cisplatin administration. As a result, decreased ABR threshold shifts and greater OHC count were detected. The expression of Bax, a pro-apoptotic protein, was decreased while Bcl-2 expression was restored. Furthermore, expression of Trpv1 and kidney injury molecule-1 (KIM-1), considered ototoxicity markers, was also decreased as measured by RT-PCR [36]. It has been demonstrated that Trpv1 is up-regulated in dorsal root ganglion neurons following platinum drug treatment [37]. In particular, ROS generation by the NADPH oxidase pathway also induces Trpv1 expression [38]. Reducing Trpv1 expression by siRNA while administering cisplatin has been shown to decrease cellular calcium influx *in vitro* and decrease ABR threshold shifts and OHC count [30]. TRPV1 is a non selective cation channel with an increased permeability for calcium [39]; increased cytosolic calcium influx through TRPV1 may lead to apoptosis [40-42].

Another channel involved in platinum toxicity is Ctr1, a copper transporter which has been shown to mediate cellular cisplatin uptake [43]. Ctr1 has been localized in outer and inner HCs, stria vascularis and SGN of the mouse cochlea. Down-regulating Ctr1 expression *in vitro* followed by cisplatin treatment led to a decrease in cisplatin cellular uptake [4]. Cisplatin toxicity is significantly determined by the cellular accumulation of cisplatin-derived metabolites [44-45]. No *in vivo* experiment was performed [4].

Seen as cisplatin generates ROS, Nrf2, a transcription factor involved in the antioxidant response, was up-regulated *in vitro* in order to determine its effect on the inflammatory cascade in cisplatin-induced cytotoxicity. By the use of western blot and ELISA methods, it was observed that increased expression of Nrf2 decreased cytosolic IkB-α degradation and Nf-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) nuclear translocation after cisplatin exposure. Cisplatin-induced MAPKs (mitogen-activated protein kinase) activation was also abolished. In addition, Nrf2 overexpression led to a decrease of pro-inflammatory cytokine secretion induced by cisplatin (TNFα, IL-1β and IL-6) [46].

Nrf2 regulates various genes including *HO-1*, a stress response protein [47]. Cisplatin exposure causes a decrease in the levels of Nrf2 and HO-1 [48]. An *in vitro* experiment was performed to up-regulate the expression of HO-1. Cells were transfected and exposed to cisplatin. As a result, increased cellular viability and decreased ROS production were observed as compared to the control [49].

Otospiralin, a novel protein with unknown function that seems to be necessary for neurosensory epithelium survival, [50] was also up-regulated in a SLF culture and followed by cisplatin exposure. Decreased apoptosis, increased viability and reduction of the intrinsic apoptotic pathway activation were detected [51].

Table 5.1. Extracted data from included studies.

Author/Year	Experiment	Cell type	Species	Target gene	Expression	Method	Administration	Measurement tool for cytotoxicity	Results
Chen X *et al*. 2001	Cell culture	Cochlear explant	Mouse (C57BL/6)	*NTF3*	Up-regulated	Viral vector (HSV amplicon)	-	SGN cell count and neurite outgrowth	Improved SGN survival and enhanced neurite outgrowth
Bowers WJ Chen W *et al.* 2002	Cell culture	SGN	Rat (S-D†)	*NTF3*	Up-regulated	Viral vector (HSV amplicon)	-	SGN cell count and apoptotic nuclei count	Improved SGN survival
	in vivo	-	Mouse (CBA/CaJ)	*NTF3*	Up-regulated	Viral vector (HSV amplicon)	Injection into the scala vestibuli	Cell loss visualization, DNA fragmentation and SGN cell count	Improved SGN survival
Chen XW *et al.* 2003	*in vivo*	-	Mouse (CBA/CaJ)	*NTF3/ GDNF*	Up-regulated	Viral vector (HSV amplicon)	Injection through the RWM	SGN cell count	Co-expression of NT-3 and GDNF increased SGN survival
Kim H *et al*. 2006	*in vitro*	HEI-OC1 cells[1]	Immorto-mouse	*HO-1*	Up-regulated	Plasmid vector Chemical transfection	-	Cell viability and ROS production	Increased cell viability and decreased ROS production
Cooper LB *et al.* 2006	*in vivo*	-	Rat (S-D)	*XIAP*	Up-regulated	Viral vector (rAAV)	Injection through the RWM	OHC count and ABR	Decreased ABR threshold shifts and greater OHC count
Staecker H *et al*. 2007	Cell culture	Hair cells	Rat (Wistar)	*BCL2*	Up-regulated	Viral vector (Adv, HSV)	-	Hair cell count	Improved hair cell survival

[1]House Ear Institute-organ of Corti 1, derived from Immortomouse cochlea.

Table 5.1.Cont.

Author/Year	Experiment	Cell type	Species	Target gene	Expression	Method	Administration	Measurement tool for cytotoxicity	Results
Staecker H *et al*. 2007	Cell culture	SGN	Rat (Wistar)	*BCL2*	Up-regulated	Viral vector (HSV)	-	SGN cell count	Improved SGN survival
Chan DK *et al*. 2007	Cell culture	COS7 cells[2]	Monkey	*XIAP*	Up-regulated	Viral vector (rAAV)	-	Cell count	Decreased apoptotic cell count
	in vivo	-	Rat (S-D)	*XIAP*	Up-regulated	Viral vector (rAAV)	Injection through the RWM	OHC count and ABR	Decreased ABR threshold shifts and greater OHC count
Zhuo XL *et al*. 2008	Cell culture	SLF[3]	Rat (S-D)	*OTOS*	Up-regulated	Viral vector (rAd)	-	SLF cell count, viability and apoptosis cascade	Decreased apoptotic cell count, increased viability and reduced apoptosis pathway activation
So H *et al*. 2008	Cell culture	HEI-OC1 cells	Immorto -mouse	*NFE2L2*	Up-regulated	Plasmid vector Chemical transfection	-	Inflammatory cascade	Decreased pro-inflammatory cytokines, kinases and transcription factors
Mukherjea D *et al.* 2008	Cell culture	UB/OC-1 cells[4]	Immorto -mouse	*TRPV1*	Silenced	siRNA Chemical transfection	-	Calcium influx	Reduced cellular calcium influx
	Cell culture	UB/OC-1 cells	Immorto -mouse	*NOX3*	Silenced	siRNA Chemical transfection	-	ROS production	Decreased ROS production

[2]African Green Monkey Kidney fibroblast-like cells (*Cercopithecus aethiops*). † Sprague-Dawley [3] Spiral ligament fibrocytes. [4] Cochlear epithelium derived from Immortomouse organ of Corti.

Table 5.1.Cont.

Author/Year	Experiment	Cell type	Species	Target gene	Expression	Method	Administration	Measurement tool for cytotoxicity	Results
Mukherjea D *et al.* 2008	*in vivo*	-	Rat (Wistar)	*TRPV1*	Silenced	siRNA Chemical transfection	RWM application	ABR, OHC morphology & count	Decreased ABR threshold shifts and greater OHC count
Wang GP *et al.* 2009	Cell culture	SGN	Rat (S-D)	*BCL2*	Up-regulated	Viral vector (Adv)	-	SGN count and neurite length	Improved SGN survival and greater axonal lengths
Kim HJ *et al.* 2010	Cell culture	HEI-OC1 cells	Immorto-mouse	*NOX1* and *NOX4*	Silenced	siRNA Chemical transfection	-	ROS production, cell viability and apoptosis cascade	Increased cell viability, decreased ROS production and pro-apoptosis activation
Mukherjea D, Jajoo S *et al*. 2010	*in vivo*	-	Rat (Wistar)	*NOX3*	Silenced	Naked siRNA	Transtympanic application	ABR, OHC count, apoptosis cascade and ototoxicity markers	Decreased ABR threshold shifts and Bax, TRPV1 and KIM-1 expression, greater OHC count and Bcl-2 expression
More SS *et al*. 2010	Cell culture	HEI-OC1 cells	Immorto-mouse	*CTR1*	Silenced	siRNA Chemical transfection	-	Cellular accumulation of platinum	Decreased cellular accumulation of platinum

Table 5.2. Targeted genes and their main known functions.

Gene	Encodes	Main functions
BCL2	B-cell CLL/lymphoma 2	Apoptotic suppressor [90]
CTR1	Copper transporter 1	Copper transport and homeostasis [104, 105]
GDNF	Glial cell-derived neurotrophic factor	Development and maintenance of neural tissues [80-82]
HO-1	Heme oxygenase-1	Heme degradation; stress response protein [95-97]
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)	Regulator of the antioxidant response [94, 95]
NOX1	NADPH oxidase 1	Host defense response; stimulation of cell division [98, 99]
NOX3	NADPH oxidase 3	ROS generation; biogenesis of otoconia/otolith [99, 103]
NOX4	NADPH oxidase 4	Unknown: possible role in ROS generation, kidney oxygen sensor [100- 102]
NTF3	Neurotrophin-3 (NT-3)	Growth factor in CNS and PNS [70, 71, 73]
OTOS	Otospiralin	Unknown: Possible role in cell repair mechanism [50, 106, 107]
TRPV1	Transient receptor potential cation channel subfamily V member 1	Non selective cation channel involved in nociception [91, 92]
XIAP	X-linked inhibitor of apoptosis protein	Apoptotic suppressor [88, 89]

5.4. Discussion

Ototoxicity is a common side effect of cisplatin treatment presenting as a bilateral, progressive and irreversible sensorineural hearing loss that may lead to a poor quality of life [52]. Hence, gene therapy to restore hearing in these patients seems interesting particularly when current approaches as hearing aids and cochlear implants cannot provide perfect hearing [19]. Various pharmacological strategies have been investigated in order to protect cochlear structures against cisplatin such as anti-inflammatories, antioxidants, calcium channel blockers, caspase inhibitors, etc. A transtympanic approach is often chosen as to avoid systemic side effects and potential interactions with cisplatin's tumoricidal effect. However, no treatment for cisplatin-induced ototoxicity has been approved to date [2]. The cochlea presents certain advantages for genetic manipulation in comparison to other tissues. Anatomically, it is a relatively well isolated organ and thus decreases the possibility of reaching additional tissues and generating nonessential or deleterious effects [53]. Targeting the specific cells to be treated is one of the major difficulties of gene therapy, especially when the tissue is as complex as the cochlea [54]. Seen as cisplatin targets prominent areas in the cochlea [55] and considering that it is a fluid filled organ, local delivery seems an attractive alternative to reach the implicated areas and be effective. In addition, a variety of monitoring methods are available in order to establish efficacy and safety in the different sections of the cochlea. Furthermore, the choice of vector is determinant. AAV and lentiviral vectors have the potential for long term transgene expression and may be an interesting approach not only for cisplatin-induced ototoxicity which is progressive and irreversible but also for treating noise-induced hearing loss [53].

A diversity of genes have been targeted in inner ear gene therapy studies for example *ATOH1* (*Math1*) [56], *CAT* (catalase), *SOD1* (Cu/Zn superoxide dismutase), *SOD2* (Mn superoxide dismutase) [57], *BDNF* (brain-derived neurotrophic factor) [58], *HGF* (hepatocyte growth factor) [59], *GJB2* (gap junction protein beta-2) [60], *Bcl-xL* (B-cell lymphoma-extra large) [61], *FGF2* (basic fibroblast growth factor)[62], *NTF3* [58, 63-64], *GDNF* [65-66], *XIAP* [67], *TGF-beta1*

(transforming growth factor beta1) [68] and *BCL2* [69]. In this review, we observed multiple gene targets for cisplatin-induced ototoxicity in experimental animal studies and *in vitro* experiments with promising results.

5.4.1. Neurotrophic Factors (NT-3, GDNF)

Neurotrophin-3, encoded by the gene *NTF3* [70], is a neurotrophic factor corresponding to the neurotrophin family which regulate neuronal survival, differentiation and synaptic plasticity [71]. NT-3 has been detected in the cochlea [72] and seems to be key in cochlear sensory neuron survival [73]. It is believe that NT-3 possesses different functions depending on the period of development [74-76]. An experiment performed on NT-3 deficient neonatal mice demonstrated that the lack of NT-3 resulted in differential loss of sensory neurons without selectivity of ganglion cell type. The resulting altered pattern of afferent innervation seemed to be responsible for the altered pattern of efferent innervation [77]. NT-3 and its receptor seem to be essential in preserving appropriate developing inner ear nerve fiber distribution [78].

Glial cell-derived neurotrophic factor encoded by the *GDNF* gene on chromosome 5 [79] has been demonstrated to protect and maintain survival of dopaminergic neurons of the midbrain [80] and also structural and functional development of postnatal myenteric [81] and peripheral neurons [82]. Local application of GDNF to the round window membrane has demonstrated protection against noise-induced hearing loss [83-84].

The up-regulation of NT-3 [26, 32] and NT-3/GDNF [27] *in vitro* and *in vivo* presents promising options for cisplatin-induced ototoxicity.

5.4.2. Apoptosis

5.4.2.1. Apoptosis Regulators (Bcl-2, XIAP)

Cisplatin, as a result of ROS generation and increased calcium influx, activates the caspase cascade [85]. Caspases are components of both the extrinsic (cell death receptor) and intrinsic (mitochondrial) apoptosis pathways [86]. Cisplatin seems to preferentially activate the intrinsic pathway with cytochrome c release from the

mitochondria and activation of initiator caspase-9 [87]. Cytosolic cytochrome c binds to apoptotic protease activating factor-1, dATP and procaspase-9 and forms the apoptosome which will cleave procaspase-9 and activate caspase-9 [86]. Caspase-9 then activates effector caspases (caspase-3, -6 and -7) which lead to apoptosis. One of the targets of effector caspases is XIAP, a potent inhibitor of apoptosis [88]. XIAP is unique in that it can inhibit both initiator (caspase-9) and effector (caspase-3 and -7) caspases [89].

Following a stressful event, the Bcl-2 family members are activated which consist of pro-apoptotic (e.g. Bax, Bad) and anti-apoptotic (e.g. Bcl-2, Bcl-xL) proteins. Interaction between the two groups will determine the cells fate depending on the magnitude of the stress [90].

Gene therapy with viral vectors to up-regulate the expression of Bcl-2 or XIAP have proven effective for cisplatin-induced cytotoxicity *in vitro* and *in vivo* as observed by improved cell survival, neurite lengths and improved ABR measurements [28-29, 33-34].

5.4.2.2. Transient receptor potential cation channel

TRPV1 is a non selective cation channel belonging to the TRPV subfamily of the large TRP (transient receptor potential) ion channel super family. It can be activated by capsaicin, heat, acid and various lipids and is involved in peripheral nociception [91];[92]. Interestingly, TRPV1 is also involved in apoptosis by inducing maintained calcium influx [42]. Post-transcriptional gene silencing of Trpv1 in presence of cisplatin resulted in reduced cellular calcium influx, decreased ABR threshold shifts and greater OHC count [30]. Seen as the role of TRPV1 is not clearly identified, further studies need to be conducted in order to evaluate consequences of gene therapy using this target gene.

5.4.3. Oxidative stress

5.4.3.1. Antioxidant Response (Nrf2, HO-1)

Cisplatin administration results in generation of ROS such as hydrogen peroxide, superoxide anion and the hydroxyl radical which react with membrane

phospholipids and generate 4-HNE, a highly toxic molecule or nitric oxide generating peroxynitrite radical [12]. Consequently, caspases can be activated and lead to apoptosis. Cochlear tissues exposed to cisplatin also demonstrated depletion of glutathione and antioxidant enzyme activity [93]. Nrf2, a transcription factor encoded by the NFE2L2 gene, is localized in the cytoplasm when inactive yet as ROS are generated in the cell, Nrf2 translocates to the nucleus and generates and antioxidant response [94]. Various proteins are then activated for example catalase, superoxide dismutase and HO-1 [95]. HO-1 is best known for catalyzing the degradation of heme. The bile pigments generated during the heme degradation process possess antioxidant properties [96]. It has been demonstrated that HO-1 also presents cytoprotective qualities [97]. Experiments performed to upregulate the expression of Nrf2 and HO-1 in HEI-OC1 cell cultures while exposed to cisplatin have demonstrated decreased ROS production, pro-inflammatory cytokines, kinases and transcription factors and increased cell viability [46, 49]. These *in vitro* studies are interesting as they show benefits for cisplatin-induced cytotoxicity and are encouraging for future *in vivo* experiments in this field.

5.4.3.2. NADPH oxidases

NADPH oxidase (NOX) proteins consist of enzyme complexes in charge of catalysing the reduction of oxygen with NADPH as an electron donor and consequently generating superoxide; a ROS [98]. Seven members are currently known to be part of this family and are found in a variety of tissues [99]. The articles included in this review assessed the interference of Nox1, Nox3 and Nox4 gene expression along with cisplatin exposure. NOX1 has been demonstrated to be involved in the host defense response and stimulation of cell division [99] whereas NOX4 does not have clear known functions. It has been hypothesized to be involved in ROS generation and as an oxygen sensor in the kidney [100-102]. Nox3, on the other hand, is highly expressed in the inner ear [103] and seems to be implicated in ROS generation and otoconia biogenesis [99]. As of result of RNA interference, ROS generation was decreased, apoptosis was inhibited and cell

viability increased [30, 35-36]. These results are promising, yet more interesting is NOX3 seen as it is expressed throughout the inner ear.

5.4.3.3. Copper transporter 1

Ctr1 is a high affinity copper transporter of great importance seen as copper is an essential cofactor involved in ROS elimination, connective tissue formation, iron metabolism and much more [104]. However, a delicate balance in copper levels must be maintained in order to avoid deficiency or toxicity [105]. This transporter is of particular interest considering it mediates cellular cisplatin uptake [3]. RNA interference of Ctr1 in a cell culture administered cisplatin demonstrates decreased cellular accumulation of cisplatin [4]. Although the main objective of the study was to determine the role of Ctr1 in cisplatin-induced ototoxicity, the possibility of silencer RNA as a future approach is mentioned.

5.4.4. Otospiralin

Otospiralin is a novel protein discovered in the inner ear [106]. It is found in the spiral limbus fibrocytes, spiral ligament and subepithelial regions of the vestibule [50] however it is not inner ear specific [107]. Despite its unknown functions, Otos seems to be required by HCs and supporting cells of the cochlea as its down-regulation leads to organ of Corti degeneration and irreversible hearing loss [50]. Cultured spiral ligament fibrocytes with exposure to cisplatin were transfected in order to up-regulate Otos expression. Interestingly, decreased apoptosis and increased viability were observed in comparison to the control group [51]. While these outcomes are intriguing, further knowledge of this protein and its functions is mandatory to consider gene therapy.

5.5. Conclusion

Gene therapy for inner ear conditions is progressing significantly. The use of viral and non-viral vectors was efficient in manipulating genetic expression for cisplatin-induced cytotoxicity. Outcome measures were adequate and rationales for choosing the targeted genes were logical. Experimental animal studies and *in*

vitro experiments have demonstrated the efficacy of gene therapy for cisplatin-induced ototoxicity. However, further investigation regarding safety, immunogenicity and consequences of genetic manipulation in the inner ear tissues must be completed in order to evolve into an actual clinical option.

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Preface - Chapter 6

Following the research completed for the study detailed in chapter 5, it was observed that determining the genetic expression of a specific gene in cochlear tissues is exceedingly complicated as the cochlea is a difficult sample to process. The cochlea possesses a variety of cell types, is fluid-filled and is embedded in bone. Manual extraction of varying cell types from the cochlea is therefore complex, imprecise and time consuming. In addition, it is nearly impossible to isolate one cell type by this method. In order to confirm the expression level of the targeted gene modified by gene therapy in the cells of interest, it is essential to quantify RNA from those cells.

Various authors have aimed at extracting a specific cell type from the cochlea for posterior gene expression analysis using laser capture microdissection, a dissection performed from histological sections. However, in order for the cochlea to be microdissected from a histological section, it must initially undergo fixation and decalcification. Both of these processes are known to cause damage to nucleic acids and therefore would hinder the posterior genetic expression analysis. As a result, the following study was undertaken to determine which cochlear processing protocol (fixation and decalcification) would yield the greatest relative expression of RNA without destroying histological features of the processed cochleae. Cochlear samples were processed in various combinations of fixatives and decalcifying solutions, and RNA was then quantified. Morphological analysis of the tissues was also completed. Such a study had not been undertaken previously.

Chapter 6

RNA Preservation in Decalcified Cochlear Samples

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Abstract

Hypothesis: Decalcification of cochlear samples in Morse's solution following methacarn fixation provides greater RNA quantification and morphological preservation of cochlear structures as compared to EDTA and formic acid decalcifying solutions following methacarn fixation.

Background: A variety of fixatives and decalcifying agents can fragment or chemically alter RNA in samples inhibiting their isolation and quantification. Morphological alterations can also be observed in light microscopy analyses. The cochlea is embedded in bone, hence fixation and decalcification steps are mandatory in order to obtain histological sections and preserve the cochlea for morphological evaluation.

Methods: Cochlear samples obtained in a RNase free environment were processed in four combinations of decalcifying agents in combination with methacarn fixation. Samples in protocols 1, 2 and 3 were fixed in methacarn for 4 hours at 4°C followed by decalcification at 4°C with Morse's solution, 10% EDTA and 5% formic acid solution respectively. Samples processed with protocol 4 were decalcified in Morse's solution at 4°C followed by fixation for 4 hours at 4°C. Real-time PCR analysis was performed on total RNA extracted. Histology sections were evaluated for morphology preservation of cochlear structures.

Results: RNA was isolated in all samples. Relative expression levels were greatest with protocol 1 and lowest with protocol 3. Morphology preservation was adequate with protocols 1, 2 and 3.

Conclusion: Of the four protocols evaluated, methacarn fixation followed by decalcification in Morse's solution provided the greatest genetic expression levels as well as the best tissue morphology preservation in the cochlea.

6.1. Introduction

Genetic expression profiling is becoming a significant tool in characterizing and evaluating cellular functions [1]. Messenger RNA (mRNA) resulting from the transcription of genomic DNA is a determining factor in posterior protein synthesis [2]. As the cell reacts to its environment, mRNA expression levels will vary accordingly hence there is interest in determining the expression profiles of cells under specific stresses and also under physiological conditions [1, 3]. Unfixed frozen tissue is the norm when preserving RNA integrity and posterior gene expression detection is the goal [4]. Unfortunately, depending on the nature of the sample and procurement conditions, freezing the sample may be impossible. Samples obtained from the operating room cannot always be frozen immediately and delays in freezing and storage of samples may lead to RNA degradation [5].

Formaldehyde fixation and subsequent paraffin embedding is common practice for storage of biological tissues since the paraffin blocks are easy to handle and good preservation of morphology is obtained [6]. Routine formaldehyde fixation (generally as 10% neutral buffered formaldehyde) is an adequate tissue preservative however it also generates fragmented and chemically altered RNA [7-8]. Previous studies have demonstrated methacarn to be one of the optimal fixatives to be used when RNA quality and quantity are desired [6-7, 9-11].

Samples containing bone such as the cochlea require a supplementary decalcification step. Various studies have attempted to evaluate the effect of decalcification on RNA integrity with varying results. Ethylenediaminetetraacetic acid (EDTA) has been shown to have mild or no effect on RNA quantity and provided good morphological detail as compared to other decalcifying agents yet requires a long period of time to achieve decalcification [12-14]. Another study assessing decalcifying protocols for detection of specific RNA demonstrated no significant difference between 5% formic acid, Morse's solution [15] and EDTA-based solutions when evaluating ribosomal RNA quantities [16].

Because the cochlea is small in size and is embedded in bone, it has been a challenge to detect RNA levels in cochlear samples in order to assess gene expression [17]. In this study, various combinations of decalcifying agents in

combination with methacarn fixation were evaluated in order to determine the most effective protocol for RNA preservation in cochlear samples while maintaining morphological detail.

6.2. Materials and Methods

6.2.1. Tissue preparation

Sixteen male Sprague-Dawley rats (450-550 g) were kindly provided by Dr. Charles V. Rohlicek's laboratory at The Montreal Children's Hospital. Following anesthesia with sodium pentobarbital, cochlear samples were quickly obtained while maintaining an RNAse free environment.

6.2.2. Processing protocols

Four protocols were evaluated in this study (Table 6.1). Cochlear samples in groups 1, 2 and 3 were fixed in methacarn for 4 hours at 4°C followed by decalcification at 4°C with Morse's solution, 10% EDTA (containing 10% RNAlater (Ambion cat. AM7024)) and 5% formic acid solution respectively. The samples were immersed in fixative following the dissection. The Morse solution was prepared by mixing equal amounts of solution A and B right before use (solution A: 90% formic acid (50%), RNAse free water (50%); solution B: sodium citrate dihydrate (20 g), RNAse free water (100 ml))[15]. Group 4 samples were decalcified in Morse's solution at 4°C followed by fixation for 4 hours at 4°C. The reversed order of fixation and decalcification has shown to have an effect of RNA preservation [18]. All decalcifying solutions were freshly prepared with RNAse free water and stored at 4°C. The methacarn solution was prepared with methanol 60%, chloroform 30% and glacial acetic acid 10% [18]. Eight cochleae were processed in each of the four different protocols. Decalcification times varied between samples and protocols. On average, samples processed with protocols 1 and 4 reached decalcification in 2 days, with protocol 2, 3 days and with protocol 3, 1 day.

Table 6.1. Protocols evaluated for processing cochlear samples

	Protocols	
1	Fixation: Methacarn for 4 hours at 4°C	Decalcification: Morse's solution at 4°C
2	Fixation: Methacarn for 4 hours at 4°C	Decalcification: 10% EDTA at 4°C
3	Fixation: Methacarn for 4 hours at 4°C	Decalcification: 5% formic acid at 4°C
4	Decalcification: Morse's solution at 4°C	Fixation: Methacarn for 4 hours at 4°C

Decalcification times varied from sample to sample based on size of the sample.

6.2.3. Histology

Following fixation and decalcification, cochlear samples were dehydrated in a series of ethanol solutions and paraffin embedding was performed. Once embedded, the specimens were then mounted in order to obtain midmodiolar plane cuts. Sections of 5µm of thickness were collected on glass slides and stained with haematoxylin and eosin for histological evaluation. Sections were examined with a Zeiss Axiophot light microscope equipped with a Zeiss AxioCam MRc camera with which digitalized images were obtained (TIFF images).

6.2.4. RNA preparation

Total RNA was extracted from whole cochleae. Once the fixation and decalcification steps were completed, the cochlear samples were dehydrated in a series of ethanol solutions (50%, 60%, 70%, 80%, 90%, and 100%) for 10 minutes while chilled on ice. The ethanol solutions were prepared with RNAse free water. The samples were incubated overnight in 100% ethanol. After centrifuging the samples at low speed, the ethanol was completely removed and 400 ul of RecoverAll digestion buffer and 4ul of protease (Ambion Cat. 1975) were added to the sample and incubation for 3 hours at 50 °C was completed. The homogenized lysate was then collected and RNA isolation was accomplished according to the manufacturer's instructions (RecoverAll Total Nucleic Acid Isolation kit by Ambion Cat. 1975). The RNA yield and quality were then evaluated using the NanoDrop 1000 spectophotometer which gives precise measurements. The ratio of absorbance at 260 and 280 nm (A_{260}/A_{280} ratio) and concentration (ng/ul) were obtained. The RNA quality was also assessed with the use of the Agilent Bioanalyzer 2100 which can determine RNA integrity[19].

6.2.5. Reverse transcription and Real-Time PCR

The RNA samples were reverse transcribed with the use of the QuantiTect Reverse Transcription Kit by Qiagen (Qiagen Cat. 205311) obtaining complementary DNA (cDNA). Samples were stored at -20°C until real time-polymerase chain reaction was performed. Real-time PCR reactions consisted of

0.6 ul of each primer (10 uM), 0.4 ul of $MgCl_2$ (Bio-Rad Cat. 170-8872EDU), 0.8 ul of sample cDNA (10 ng), 10 ul of the iQ SYBR Green Supermix reagent (Bio-Rad Cat. 170-8880) and adjusted to a total volume of 20 ul with DNase/RNase free water. Negative control reactions were set up as above excluding any cDNA. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used for normalization. Real-time PCR was performed on the MyiQ™ Single-Color Real-Time PCR Detection. Cycling conditions were as follows: 95°C for 1 min 30 sec followed by 50 cycles at 95°C for 15 s; and 60°C for 30 s; a melt curve program was added as follows: of 95° for 1 min; 55°C for 1 min; and 40 cycles beginning at 55°C for 15 sec with a 1°C/cycle increment.

Genes evaluated were a) *Pmp22*, encoding peripheral myelin protein 22, a component of myelin found in the spiral ganglion [20-23]; b) *Slc26a5,* encoding Prestin, a protein in charge of the electromechanical properties of the OHC of the inner ear [24-26] and c) *Atp1a1*, encoding Na,K ATPase subunit alpha-1 found in the sprial ganglion, organ of Corti [27] and lateral wall [28-29] of the cochlea. The sequences of primers used are shown in table 6.2. Relative gene expression was determined using the Relative Expression Software Tool [30]. The control samples to which the gene analysis was normalized are the samples from protocol 1.

Possible breakdowns or mutations in the RNA obtained from the rat samples were evaluated by Sanger sequencing. Sequencing primers were designed for *Pmp22* as greater expression of this gene was detected. Two primer sets were designed to amplify two different sizes of PCR products; 152 bp and 210 bp. The sequences of primers were as follows:

152 bp forward: 5′-TCCTTCACATCGCGGTGCT-3′,

152 bp reverse: 5′- GGAGTAGCAGTGCTGGAC-3′,

210 bp forward: 5′- TCTACTCTTGTTGGGGATCC-3′,

210 bp reverse: 5′- CCTGGACAGACTGAAGCCATT-3′.

Table 6.2. Primer sequences for RT-PCR

Gene	Primer sequence (5′–3′)	Annealing Tm (°C)	Size (bp*)
Gapdh	(F) CATAGACAAGATGGTGAAGGT	54.20	131
	(R) CCTTCATTGACCTCAACTACA	55.31	
Pmp22	(F) GGATCCTGTTCCTTCACAT	54.72	96
	(R) ATGGACACAGGACTGATCTCT	56.11	
Slc26a5	(F) TGCCCATCACTAAGTGGT	54.72	180
	(R) TACCCCGTTATCATGTACTGTT	56.13	
Atp1a1	(F) TCTCCTCAACAGAAGCTCAT	55.05	94
	(R) TGTCAATGACTCTCCAGCTT	55.90	

All primers are designed to *rattus norvegicus* sequence. Size of amplified fragment. * bp: base pairs

6.2.6. Statistical analysis

The data were analyzed using the Kruskal–Wallis one-way analysis of variance test. Post-hoc comparisons were made with the Bonferroni-Dunn test. Statistical significance was set at p-value ≤ 0.05. All statistical analyses were performed with the aid of Graphpad Prism version 4 software (Graphpad, San Diego, CA).

6.3. Results

6.3.1. General morphological analysis

Light microscopic evaluation was performed for all gross structures of the cochlea in order to obtain a general perspective of morphology preservation. Particular emphasis was placed on the morphology of the organ of Corti, the stria vascularis, the spiral ligament and the spiral ganglion neurons (SGNs).

Samples fixed with methacarn and decalcified with Morse's solution (protocol 1) demonstrated the best preservation of cochlear morphology. The inner spiral tunnel (inner spiral sulcus), the tunnel of Corti and Nuel's space were not collapsed and hair cells and supporting cells had clearly defined nuclei (Fig.6.1a). The stria vascularis was not atrophied or edematous, presented adequate strial density and had no cellular vacuolization. The spiral ligament also demonstrated clearly defined nuclei as well as a normal structure (Fig. 6.2a). The SGNs demonstrated clear nuclei yet exhibited slight myelin sheath detachment (Fig. 6.3a).

Samples fixed with methacarn and decalcified with EDTA (protocol 2) presented similar findings in regards to hair cells, the inner spiral tunnel, the stria vascularis, the spiral ligament, the tunnel of Corti and Nuel's space. The supporting cells were slightly atrophied (Fig. 6.1b, 6.2b). The SGNs nuclei were not clearly defined however the myelin sheath detachment was not observed in these samples (Fig. 6.3b).

Samples processed with protocol 3 (methacarn fixation followed by 5% formic acid decalcification) exhibited very similar morphological details to samples processed with protocol 1 (Fig. 6.1c, 6.2c, 6.3c). The main concern was

the presence of shattered or cracked areas. This may be due to overdecalcifying the specimens.

The poorest morphology preservation was seen in samples processed with protocol 4 (i.e., decalcification before fixation). As can be seen in Fig.6.1d and 6.2d, the structures best preserved are the stria vascularis, the spiral ligament and the hair cells, although detachment of the hair cells and supporting cells occurred at the base of the organ of Corti. The inner spiral tunnel, the tunnel of Corti and Nuel's space could not be differentiated and the SGNs also exhibited detachment of their myelin sheath (Fig. 6.3d).

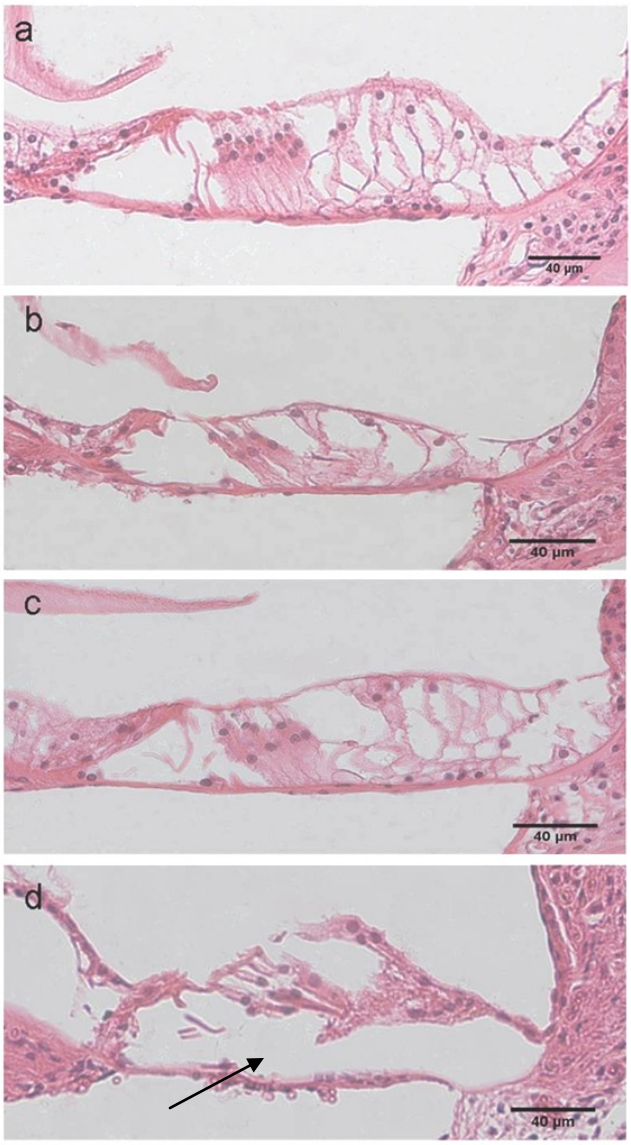

Figure 6.1. Light microscopy: organ of Corti. Morphological appearance of samples processed with: a) protocol 1: methacarn + Morse' solution b) protocol 2: methacarn + EDTA c) protocol 3: methacarn + 5% formic acid and d) protocol 4: Morse's solution decalcification followed by methacarn fixation. Samples processed with protocol 1 demonstrate best conserved morphology. Fig 6.1c exhibits shattered or cracked areas while in Fig 6.1d, detachment of the hair cells and supporting cells at the base of the organ of Corti were observed. The inner spiral tunnel, the tunnel of Corti and Nuel's space could not be differentiated. Hematoxylin and eosin staining.

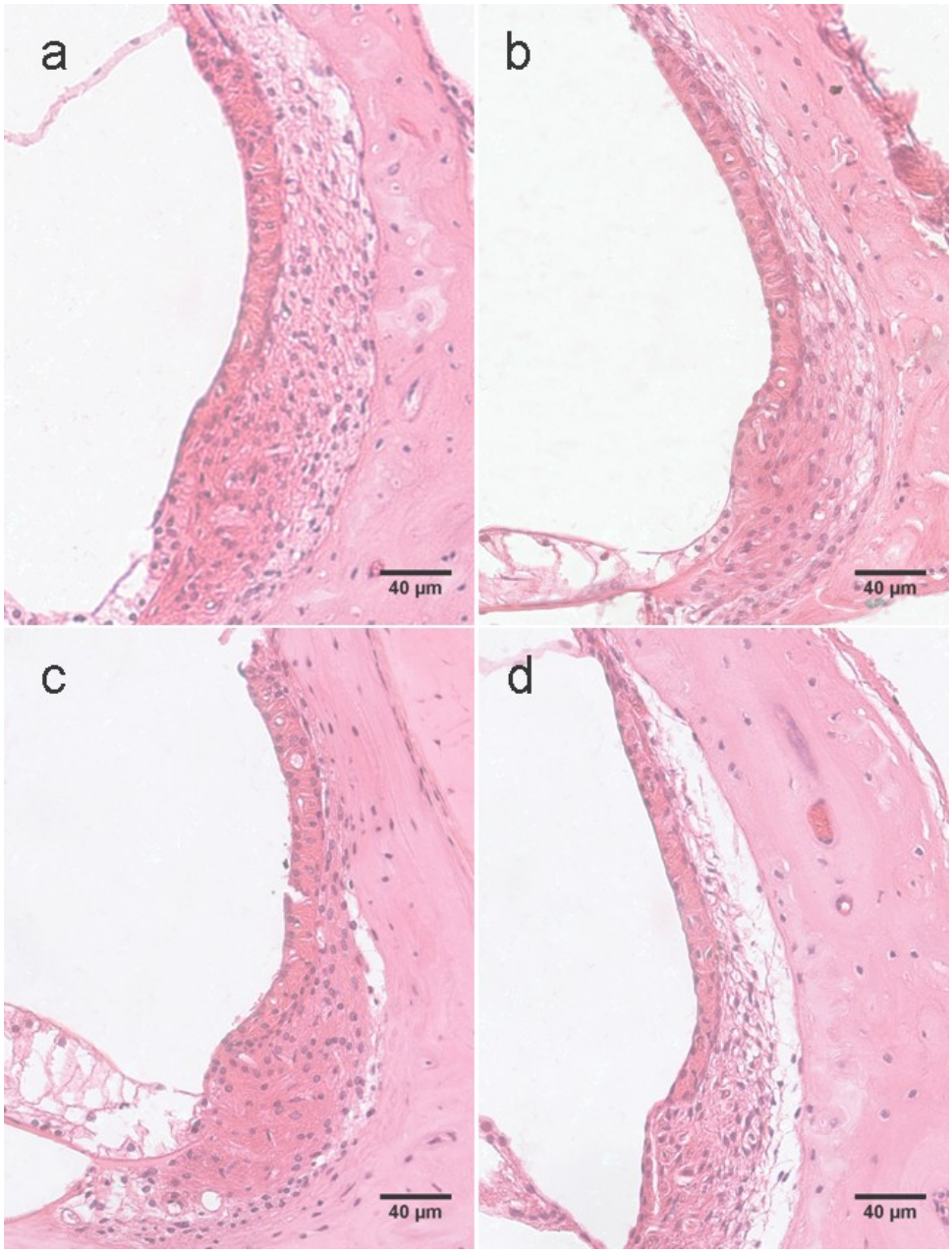

Figure 6.2. Light microscopy: lateral wall. Morphological appearance of samples processed with: a) protocol 1: methacarn + Morse' solution b) protocol 2: methacarn + EDTA c) protocol 3: methacarn + 5% formic acid and d) protocol 4: Morse's solution decalcification followed by methacarn fixation. The stria vascularis seems to be the area best preserved in all protocols. Hematoxylin and eosin staining.

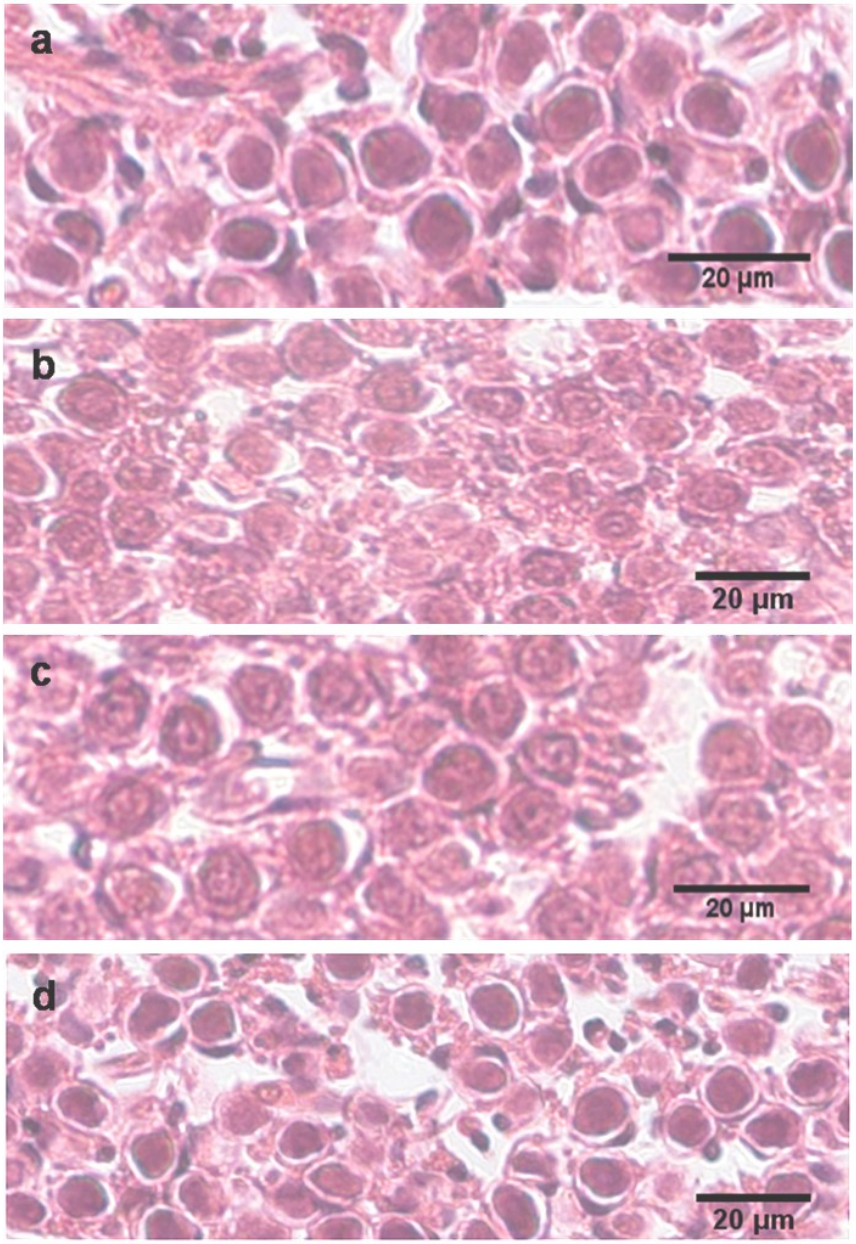

Figure 6.3. Light microscopy: spiral ganglion neurons. Morphological appearance of samples processed with: a) protocol 1: methacarn + Morse' solution b) protocol 2: methacarn + EDTA c) protocol 3: methacarn + 5% formic acid and d) protocol 4: Morse's solution decalcification followed by methacarn fixation. Spiral ganglion neuron myelin sheath detachment can be observed in Fig 3a, 3c and 3d while this phenomenon was not observed in samples processed with protocol 3 (Fig 6.3b). Hematoxylin and eosin staining.

6.3.2. Gene expression

The relative expressions of *Pmp22, Slc26a5* and *Atp1a1* were determined. Genes representing proteins found in inner ear cells were selected in order to verify the expression levels of inner ear cells and not surrounding tissues such as bone or circulating blood cells. Relative expression levels of *Pmp22, Slc26a5* and *Atp1a1* were greatest when cochlear samples were processed with protocol 1 (methacarn fixation + Morse solution decalcification) (Fig. 6.4).

The relative expression of *Slc26a5* was significantly different between all groups ($P = 0.0241$). When samples were processed with protocol 3 (methacarn fixation followed by decalcification with 5% formic acid), *Slc26a5* was not detected. Particularly, a significant difference was observed between protocols 1 and 2 ($P < 0.05$).

The relative expression of *Pmp22* was more robust, being able to detect and quantify its expression within all of the samples and with no significant difference observed between all of the groups ($P = 0.2889$). As for *Atp1a1*, significant differences were observed between all groups ($P = 0.0228$). The greatest relative expression was detected when samples were treated with protocol 1 and the lowest when samples were processed with protocol 3 (Fig. 6.4).

6.3.3. Proof of concept: quality of the preserved RNA

Sanger sequencing was performed on samples obtained from protocol 1, the protocol which demonstrated greater relative expressions, in order to determine breakdowns or mutations in the RNA obtained from the rat cochlear samples. Primers were designed in order to obtain two PCR products of varying sizes (152 and 210 bp) for *Pmp22*. Following the sequencing procedure, the yielded sequences were BLASTed against the rat genome database. The resulting sequences obtained were of 152 bp and 210 bp in length and did not exhibit any breakdowns or mutations. They corresponded to the *Pmp22* gene of the *Rattus norvegicus* strain.

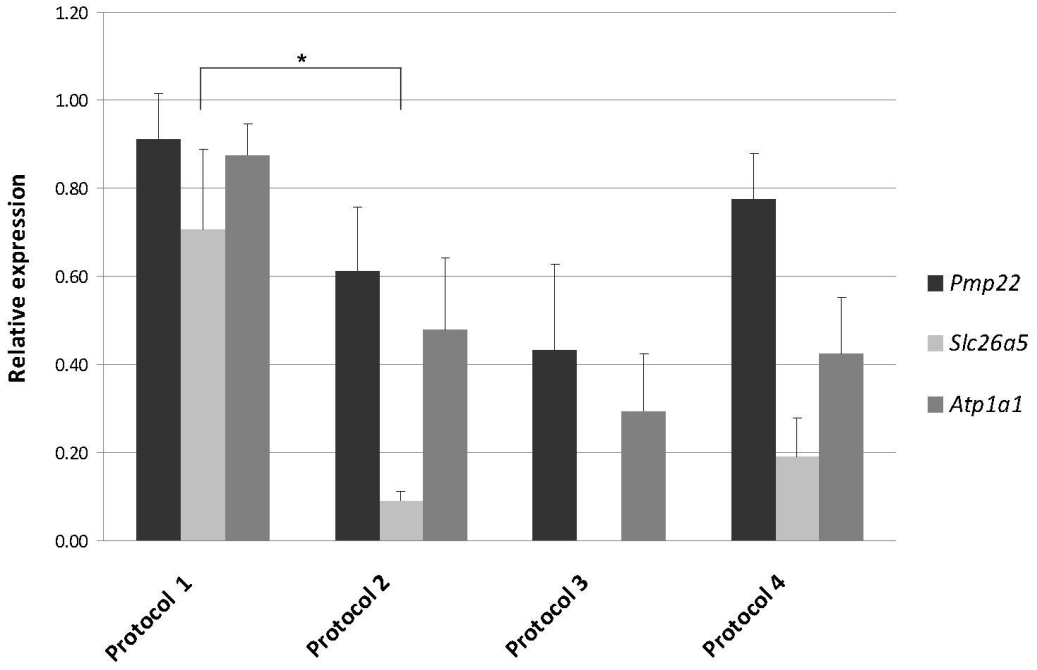

Figure 6.4. Relative expression levels for *Pmp22, Slc26a5* and *Atp1a1* following fixation and decalcification of cochlear samples. Relative expressions were greatest when cochlear samples were processed with protocol 1 (methacarn + Morse's solution). *Slc26a5* and *Atp1a1* expression levels were significantly different in all groups. Particularly, *Slc26a5* expression was significantly different between protocols 1 and 2 and was not detected with protocol 3. * $P \leq 0.05$.

6.4. Discussion

As technological advances are made, greater interest is focused on molecules such as DNA, RNA and their associated proteins. This phenomenon is especially true in regards to cochlear samples seen as various conditions of the inner ear seem to have a genetic component [31-33] and the literature is lacking in regards to genetic expression profiling. The soft tissue of the cochlea is surrounded by bone and therefore requires fixation and a decalcification step in order to obtain histological sections. It is well known that fixatives [7-8] and decalcifying agents [12-14] can destroy or inhibit the quantification of gene expression. As a result, frozen sections are usually obtained in order to avoid alterations induced by fixatives. Unfortunately, this procedure is not possible for tissues such as the cochlea which contain bone, a structure too solid to be cut by a cryostat. Consequently, fixatives and decalcifying agents are needed to process cochlear samples in order to obtain histological sections. Histological sections are commonly necessary in research studies for analysis of morphological alterations.

Methacarn was the chosen fixative in this study since previous articles have demonstrated methacarn to be the fixative of choice for RNA detection and quantification [6-7, 9-11]. The combination of methacarn followed by decalcification with Morse's solution, EDTA and 5% formic acid were evaluated. When samples were processed with methacarn and decalcified with Morse's solution or with 5% formic acid, morphological analysis was very similar, with preservation of cochlear structures. These two protocols provided the fastest decalcifying times, approximately 1-2 days. Unfortunately, samples processed with methacarn and 5% formic acid (protocol 3) generated the lowest relative expressions for all genes tested. Walsh et al observed loss of tissue morphology and reduced amounts of detectable mRNA in bone samples when processed with 10% neutral buffered formalin fixation and decalcification with a buffered formic acid solution [13].

Samples decalcified in EDTA (protocol 2) demonstrated slightly longer decalcification times, approximately 3 to 4 days. Morphological analysis was adequate and relative genetic expression was decreased as compared to protocol 1, specifically regarding *Slc26a5*. Ryan et al described that decalcification with

EDTA (with 4% paraformaldehyde) following fixation with 4% paraformaldehyde did not produce a decrease of mRNA detection by in situ hybridization [14]. Another study reported no significant difference in rRNA retention between 5% formic acid, Morse's solution and EDTA-based solutions when processing mouse mandibles [16]. Shao et al observed that optimal RNA quality and a slight decrease in RNA quantity was detected when rat femurs were processed with 20% EDTA for decalcification followed by fixation in methacarn [18]; the order of fixation and decalcification were reversed.

Because a reversal of the fixation and decalcifications steps seem to have an effect on RNA preservation, we included protocol 4, decalcifying in Morse's solution followed by fixation in methacarn. Morphological structure was relatively conserved but relative expression of *Pmp22, Slc26a5* and *Atp1a1* was poor as compared to the reversed order (protocol 1) and EDTA decalcification.

Methacarn is composed mainly of methanol and chloroform, with a small amount of glacial acetic acid. Methanol is a dehydrant fixative leaving nucleic acids unchanged [8], chloroform is commonly used in nucleic acid experiments since it can dissociate proteins from RNA [34] and glacial acetic acid is used to acidify the solutions as it is known that a slightly acidic environment prevents RNA degradation [35]. As a result, methacarn as a fixative seems ideal for nucleic acid research.

Morse's solution was made with formic acid, RNAse free water and sodium citrate. Citrate is known to inactivate DNAses and hence avoid the breakdown of DNA[36]. The same process occurs with RNA base hydrolysis. Various storage solutions for RNA preservation contain sodium citrate for this reason. This could explain why the highest yield of RNA was obtained with protocol 1. RNA can be hydrolyzed by ribonucleases which require divalent cations, preferably magnesium [37-39]. Decalcification with EDTA is achieved by chelating the calcium ions from the samples [40, 41]. Perhaps the RNA preservation was decreased as compared to decalcification with Morse's solution because EDTA must chelate two ions simultaneously, calcium and magnesium. Therefore, the chelating capacity for magnesium may be decreased and RNA hydrolysis may

occur. As for formic acid, on the other hand, it can increase the rate of apurinic/apyrimidinic (AP) site formation that itself eventually decomposes into a nick (discontinuity in a double-stranded DNA molecule) and subsequently into nucleic acid fragmentation. It can depurinate nucleic acids and lead to its degradation [42]. Consequently, this may explain why the lowest RNA yields were obtained following decalcification with formic acid.

As a conclusion, we hereby demonstrate the greatest gene expression detection by real-time PCR and preserved morphological characteristics when cochlear samples are processed with methacarn as a fixative followed by decalcification in Morse's solution.

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Chapter 7

Summary and Future Work

Various aspects of cisplatin-induced ototoxicity have been presented in this thesis. Although the pathophysiology of this condition remains unclear, it was the objective of this current work to determine what receptors and transporters could play a role in cisplatin's influx and efflux pathways within cochlear cells and to determine the potential of various protective approaches against cisplatin-induced ototoxicity. It was found that cisplatin appears to enter the cochlear cells by the copper transporter CTR1 and the organic cation transporter OCT2 and potentially through the copper transporter CTR2 and the TRPV1 ion channel. Although the TRPA1 ion channel and the MET channel express characteristics that may allow for cisplatin's passage, sufficient evidence is lacking to confirm this possibility. Cisplatin appears to exit the cochlear cells by the copper transporting ATPases and although it has been suggested that the multi-drug resistance proteins MRP2 and MRP6 may play a role in cisplatin efflux, there is no evidence so far to demonstrate the presence of these proteins in cochlear cells. Furthermore, the roles of calcium (IP3R, RyR, TRPV1, L-type and T-type calcium channels) and chloride channels (VSOR, ClC-3) are discussed as participating members of the apoptotic cascade. This is the first review to assess the different types of channels and transporters that may be involved in cisplatin-induced ototoxicity; nevertheless, as novel channels are being identified in cochlear cells, the potential for cisplatin as a substrate will generate new potential channels to be explored. Further research is required in order to determine which transporter or channel is responsible for the greatest amount of cisplatin transit in and out of cells.

It was demonstrated that the administration of a potent anti-inflammatory *in vivo*, dexamethasone, decreased the expression of TNF-α in all of the cochlear targets of cisplatin. Unfortunately, the hearing was not preserved despite a partially preserved cochlear morphology as well as outer hair cells. A high dose of dexamethasone provided partial protection to the cochlear structures, yet it did not translate into a functional benefit for hearing preservation. The animal model used was a guinea pig model, receiving 12 mg/kg of cisplatin IP with post measurements being performed at 72 hrs. Limitations of this study are the lack of

a long term administration of cisplatin and the lack of quantitative measurements for inflammatory cytokines. A high dose of dexamethasone in an acute setting did not protect from hearing loss. A future project using an animal model that can withstand a long term treatment with cisplatin would surely provide insight into a chronic model response. Quantitative measurements were performed from histological sections, however, quantitative analysis of the inflammatory cytokines such as IL-1β, IL-6 and TNF-α would be of interest in future work.

It was also demonstrated that the administration of an antioxidant provided partial protection against cisplatin-induced ototoxicity as evidenced by the preservation of hearing at high frequencies and greater OHC counts. Three doses of erdosteine were tested, with the greatest concentration providing the greatest protection. The animal model was also a guinea pig model receiving cisplatin 12 mg/kg IP and post measurements being performed at 72 hrs. As a future work, it would be of interest to evaluate a long term treatment with an antioxidant such as erdosteine and to evaluate the impact on the antioxidant enzymes of the cochlea in order to establish the mechanisms of protection. Also of interest would be the administration of a combination treatment with an antioxidant and an anti-inflammatory since both products provided partial benefits. Perhaps a combination treatment could have a synergistic beneficial effect. However, the effect of erdosteine of the anti-tumorigenic properties of cisplatin must be addressed. Further research is required for these treatments to be evaluated in human clinical trials.

The potential of gene therapy was also evaluated in this thesis. Although gene therapy is not a conventional treatment modality so far, clinical trials are being conducted worldwide for a variety of conditions. The manipulation of genes to provide a clinical benefit has various advantages as compared to other pharmacological therapies. Depending on the gene of interest, the cell of interest and the vectors used, an acute or chronic expression can be obtained as well as cellular specificity in terms of the cells targeted. Another benefit of using gene therapy for cisplatin-induced ototoxicity is the fact that the inner ear is somewhat separated from other organs and allows for a local administration; a transtympanic

approach. In this way, systemic side effects can be prevented. A systematic review was performed since it is a type of study that can provide a high level of evidence. Experimental animal studies and *in vitro* experiments demonstrated the potential of gene therapy as a protective modality against cisplatin-induced ototoxicity. Of all the articles evaluated, fourteen met the pre-established inclusion and exclusion criteria and were analyzed in detail. Interestingly, in the articles that evaluated *in vivo* models, all of them described a local administration. Furthermore, a variety of genes were chosen as targets including apoptotic suppressors, oxidative stress regulators, copper transporters, neural growth factors, stress response proteins and non selective cation channels. The results described included improved spiral ganglion neurons and hair cell survival, decreased ROS production, decreased platinum accumulation, reduced calcium influx and decreased ABR thresholds. Gene therapy is still under study and although, to our knowledge, no clinical trials are being conducted for hearing loss, the results from this systematic review are promising. Further studies evaluating the long term consequences, safety and immunogenicity of genetic manipulation in the inner ear should be completed in order for gene therapy to be considered a therapeutic option for cisplatin-induced hearing loss. Perhaps downregulating OCT2 would provide insight into how much this particular transporter is responsible for cisplatin influx and whether its deficiency prevents hearing loss. Also, upregulating proteins responsible for the natural antioxidant response in the cochlea such as glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase would be of interest to evaluate the impact of oxidative stress in cisplatin-induced ototoxicity.

Lastly, we established a protocol of fixation and decalcification for the processing of cochlear samples in molecular biology. Four different protocols were evaluated. Fixation in methacarn followed by decalcification in Morse's solution allowed for the preservation of cochlear structures as seen with light microscopy as well as yielded the greatest quantity of RNA. The identification and processing of gene expression from one particular cell type of interest in the cochlea is of high interest in the scientific community and can now be achieved

by processing cochlear samples with this new protocol. Samples are fixed, decalcified, embedded in paraffin and finally cut by microtomy. Once histological sections are obtained, the cells of interest can be obtained with laser capture microdissection. Consequently, the RNA of a specific cell type can be obtained and analyzed. Because fixation and decalcification steps are known to destroy nucleic acids, this new protocol will provide an opportunity to analyze gene expression in various cell types of the cochlea. Further work of interest would be the analysis of gene expression of the cells of the stria vascularis, the OHCs and the spiral ganglion neurons of experimental animals exposed to cisplatin. The impact of cisplatin in these different cell types is unclear. Also of interest is the evaluation of gene expression of a specific gene manipulated by gene therapy. As a result, non-specific cellular targeting could be identified. Moreover, this protocol could be used to analyze unknown causes of hearing loss, genetic causes of hearing loss and other conditions in which the pathophysiology remains unclear such as noise-induced hearing loss, sudden sensorineural hearing loss or Ménière's disease.

Addendum for chapter 5: Search strategy

Inclusion criteria:

- No language restriction
- Cell cultures
- Experimental animal studies
- Demonstrate benefit/no benefit, specifically for cisplatin-induced ototoxicity
- Gene therapy alone, no combinations with other treatments

Exclusion criteria:

- Combined treatments

Search strategy:

Research launched in Ovid Medline, Ovid Medline in Process, Embase, PubMed, Biosis Previews, Scopus, ISI Web of Knowledge, Cochrane library Tuesday January 18 2011

Ovid Medline, Medline in process, Embase, Biosis Previews

1 - Cisplatin/

2 - (Abiplatin* OR Biocisplatin* OR Biocysplatin* OR briplatin* OR cddp ti OR cis ddp OR cis diamine dichloroplatin* OR cis diaminechloroplatin* OR cis diaminedichloroplatin* OR cis diammine dichloroplatin* OR cis diamminedichloroplatin* OR cis dichloridiammineplatin* OR cis dichloroadiamine platin* OR cis dichlorodiamine platin* OR cis dichlorodiamineplatin* OR cis dichlorodiammine platin* OR cis dichlorodiammineplatin* OR cis-diamine dichloroplatin* OR cis-diaminechloroplatin* OR cis-diaminedichloroplatin* OR cis-diammine dichloroplatin* OR cis-diamminedichloroplatin* OR cis-dichloridiammineplatin* OR cis-dichloroadiamine platin* OR cis-dichlorodiamine platin* OR cis-dichlorodiamineplatin* OR cis-dichlorodiammine platin* OR cis-dichlorodiammineplatin* OR cis platin* OR Cisplat* OR cis-platin* OR cisplatyl OR Diamine dichloroplatin* OR diaminedichloroplatin* OR diammine dichloroplatin* OR diamminedichloroplatin* OR diaminodichloroplatin* OR dichlorodiamineplatin* OR dichlorodiamine platin* OR dichlorodiammineplatin* OR dichlorodiammine platin* OR mpi 5010 OR mpi5010 OR neoplatin* OR nk 801 OR nsc 119875 OR NSC-119875 OR platamine OR platiblastin* OR

Platidiam OR platimine OR platinex OR platinol OR platinum diamine dichloride* OR platinum diammine dichloride* OR platinum diaminedichloride* OR platinum diaminodichloride* OR platinum diamminedichloride* OR Platinum Diamminodichloride* OR platiran* OR platistin* OR platosin* OR randa OR romcis OR spi 077).tw.

3 - exp Hearing Loss/ OR ototoxicity/

4 - (Hypoacusis OR Hypoacuses OR Deaf* OR ototox* OR oto-tox* OR Hearing OR ear OR ears OR hear OR hears OR aural OR auditor* OR cochl*).tw.

5 - Exp Gene Therapy/ OR exp viruses/ OR exp virus/ OR exp RNA/ OR exp DNA/ OR exp liposomes/ OR exp liposome/ OR exp genes/ OR exp gene/

6 - (Gene OR Genes OR Vector* OR Virus* OR Viral OR RNA OR Ribonucleic acid* OR DNA OR Deoxyribonucleic acid* OR Retrovir* OR Adeno* OR Lentivir* OR Liposom*).tw.

9 – 1 or 2
10 – 3 or 4
11 – 5 or 6
12 – 9 and 10 and 11

PubMed

1 – Cisplatin[mh]

2 - Abiplatin*[tiab] OR Biocisplatin*[tiab] OR Biocysplatin*[tiab] OR briplatin*[tiab] OR "cddp ti"[tiab] OR "cis ddp"[tiab] OR cis diamine dichloroplatin*[tiab] OR cis diaminechloroplatin*[tiab] OR cis diaminedichloroplatin*[tiab] OR cis diammine dichloroplatin*[tiab] OR cis diamminedichloroplatin*[TIAB] OR cis dichloridiammineplatin*[TIAB] OR cis dichloroadiamine platin*[TIAB] OR cis dichlorodiamine platin*[TIAB] OR cis dichlorodiamineplatin*[TIAB] OR cis dichlorodiammine platin*[TIAB] OR cis dichlorodiammineplatin*[TIAB] OR cis-diamine dichloroplatin*[TIAB] OR cis-diaminechloroplatin*[TIAB] OR cis-diaminedichloroplatin*[TIAB] OR cis-diammine dichloroplatin*[TIAB] OR cis-diamminedichloroplatin*[TIAB] OR cis-dichloridiammineplatin*[TIAB] OR cis-dichloroadiamine platin*[TIAB] OR cis-dichlorodiamine platin*[TIAB] OR cis-dichlorodiamineplatin*[TIAB] OR cis-dichlorodiammine platin* OR cis-dichlorodiammineplatin*[tiab] OR cis platin*[tiab] OR Cisplat*[tiab] OR cis-platin*[tiab] OR cisplatyl[tiab] OR Diamine dichloroplatin*[tiab] OR diaminedichloroplatin*[tiab] OR diammine dichloroplatin*[tiab] OR diamminedichloroplatin*[tiab] OR diaminodichloroplatin*[tiab] OR dichlorodiamineplatin*[tiab] OR dichlorodiamine platin*[tiab] OR dichlorodiammineplatin*[tiab] OR dichlorodiammine platin*[tiab] OR mpi 5010[tiab] OR mpi5010[tiab] OR

neoplatin*[tiab] OR nk 801[tiab] OR nsc 119875[tiab] OR NSC-119875[tiab] OR platamine[tiab] OR platiblastin*[tiab] OR Platidiam[tiab] OR platimine[tiab] OR platinex[tiab] OR platinol[tiab] OR platinum diamine dichloride*[tiab] OR platinum diammine dichloride*[tiab] OR platinum diaminedichloride*[tiab] OR platinum diaminodichloride*[tiab] OR platinum diamminedichloride*[tiab] OR Platinum Diamminodichloride*[tiab] OR platiran*[tiab] OR platistin*[tiab] OR platosin*[tiab] OR randa[tiab] OR romcis[tiab] OR spi 077[tiab]

3 - Hearing Loss[mh]

4 - Hypoacusis[tiab] OR Hypoacuses[tiab] OR Deaf*[tiab] OR ototox*[tiab] OR oto-tox*[tiab] OR Hearing[tiab] OR ear[tiab] OR ears[tiab] OR hear[tiab] OR hears[tiab] OR aural[tiab] OR auditor*[tiab] OR cochl*[tiab]

5 - Gene Therapy[mh] OR viruses[mh] OR virus[mh] OR RNA[mh] OR DNA[mh] OR liposomes[mh] OR liposome[mh] OR genes[mh] OR gene[mh]

6 - Gene[tiab] OR Genes[tiab] OR Vector*[tiab] OR Virus*[tiab] OR Viruses[tiab] OR Viral[tiab] OR RNA[tiab] OR Ribonucleic acid*[tiab] OR DNA[tiab] OR Deoxyribonucleic acid*[tiab] OR Retrovir*[tiab] OR Adenoc*[tiab] OR Adenor*[tiab] OR Adenoi*[tiab] OR Adenov*[tiab] OR Adenos*[tiab] OR Adeno-*[tiab] OR Adeno[tiab] OR Lentivir*[tiab] OR Liposom*[tiab]

9 – 1 or 2
10 – 3 or 4
11 – 5 or 6
12 – 9 and 10 and 11

Scopus

((TITLE-ABS-KEY(**abiplatin***) OR TITLE-ABS-KEY(**biocisplatin***) OR TITLE-ABS-KEY(**biocysplatin***) OR TITLE-ABS-KEY(**briplatin***) OR TITLE-ABS-KEY(**cddp ti**) OR TITLE-ABS-KEY(**cis ddp**) OR TITLE-ABS-KEY(**cis diamine dichloroplatin***) OR TITLE-ABS-KEY(**cis diaminechloroplatin***) OR TITLE-ABS-KEY(**cis diaminedichloroplatin***) OR TITLE-ABS-KEY(**cis diammine dichloroplatin***) OR TITLE-ABS-KEY(**cis diamminedichloroplatin***) OR TITLE-ABS-KEY(**cis dichloridiammineplatin***) OR TITLE-ABS-KEY(**cis dichloroadiamine platin***) OR TITLE-ABS-KEY(**cis dichlorodiamine platin***) OR TITLE-ABS-KEY(**cis dichlorodiamineplatin***) OR TITLE-ABS-KEY(**cis dichlorodiammine platin***) OR TITLE-ABS-KEY(**cis dichlorodiammineplatin***) OR TITLE-ABS-KEY(**cis-diamine dichloroplatin***) OR TITLE-ABS-KEY(**cis-diaminechloroplatin***) OR TITLE-ABS-KEY(**cis-diaminedichloroplatin***) OR TITLE-ABS-KEY(**cis-diammine dichloroplatin***) OR TITLE-ABS-KEY(**cis-diamminedichloroplatin***) OR TITLE-ABS-KEY(**cis-dichloridiammineplatin***) OR TITLE-ABS-KEY(**cis-dichloroadiamine**

platin*) OR TITLE-ABS-KEY(**cis-dichlorodiamine platin***) OR TITLE-ABS-KEY(**cis-dichlorodiamineplatin***) OR TITLE-ABS-KEY(**cis-dichlorodiammine platin***) OR TITLE-ABS-KEY(**cis-dichlorodiammineplatin***) OR TITLE-ABS-KEY(**cis platin***) OR TITLE-ABS-KEY(**cisplat***) OR TITLE-ABS-KEY(**cis-platin***) OR TITLE-ABS-KEY(**diamine dichloroplatin***) OR TITLE-ABS-KEY(**diaminedichloroplatin***) OR TITLE-ABS-KEY(**diammine dichloroplatin***) OR TITLE-ABS-KEY(**diamminedichloroplatin***) OR TITLE-ABS-KEY(**diaminodichloroplatin***) OR TITLE-ABS-KEY(**dichlorodiamineplatin***) OR TITLE-ABS-KEY(**dichlorodiamine platin***) OR TITLE-ABS-KEY(**dichlorodiammineplatin***) OR TITLE-ABS-KEY(**dichlorodiammine platin***) OR TITLE-ABS-KEY(**mpi 5010**) OR TITLE-ABS-KEY(**mpi5010**) OR TITLE-ABS-KEY(**neoplatin***) OR TITLE-ABS-KEY(**nk 801**) OR TITLE-ABS-KEY(**nsc 119875**) OR TITLE-ABS-KEY(**nsc-119875**) OR TITLE-ABS-KEY(**platamine**) OR TITLE-ABS-KEY(**platiblastin***) OR TITLE-ABS-KEY(**platidiam**) OR TITLE-ABS-KEY(**platimine**) OR TITLE-ABS-KEY(**platinex**) OR TITLE-ABS-KEY(**platinol**) OR TITLE-ABS-KEY(**platinum diamine dichloride***) OR TITLE-ABS-KEY(**platinum diammine dichloride***) OR TITLE-ABS-KEY(**platinum diaminedichloride***) OR TITLE-ABS-KEY(**platinum diaminodichloride***) OR TITLE-ABS-KEY(**platinum diamminedichloride***) OR TITLE-ABS-KEY(**platinum diamminodichloride***) OR TITLE-ABS-KEY(**platiran***) OR TITLE-ABS-KEY(**platistin***) OR TITLE-ABS-KEY(**platosin***) OR TITLE-ABS-KEY(**randa**) OR TITLE-ABS-KEY(**romcis**) OR TITLE-ABS-KEY(**spi 077**))) AND ((TITLE-ABS-KEY(**hypoacusis**) OR TITLE-ABS-KEY(**hypoacuses**) OR TITLE-ABS-KEY(**deaf***) OR TITLE-ABS-KEY(**ototox***) OR TITLE-ABS-KEY(**oto-tox***) OR TITLE-ABS-KEY(**hearing**) OR TITLE-ABS-KEY(**ear**) OR TITLE-ABS-KEY(**ears**) OR TITLE-ABS-KEY(**hear**) OR TITLE-ABS-KEY(**hears**) OR TITLE-ABS-KEY(**aural**) OR TITLE-ABS-KEY(**auditor***) OR TITLE-ABS-KEY(**cochl***))) AND ((TITLE-ABS-KEY(**gene**) OR TITLE-ABS-KEY(**gene therapy**) OR TITLE-ABS-KEY(**genes**) OR TITLE-ABS-KEY(**vector***) OR TITLE-ABS-KEY(**virus***) OR TITLE-ABS-KEY(**viral**) OR TITLE-ABS-KEY(**rna**) OR TITLE-ABS-KEY(**ribonucleic acid***) OR TITLE-ABS-KEY(**dna**) OR TITLE-ABS-KEY(**deoxyribonucleic acid***) OR TITLE-ABS-KEY(**retrovir***) OR TITLE-ABS-KEY(**adeno***) OR TITLE-ABS-KEY(**lentivir***) OR TITLE-ABS-KEY(**liposom***)))

ISI Web of Knowledge

13 #12 AND #11
12 #8 AND #5
11 #10 OR #9
10 TI=(Gene OR Genes OR Vector* OR Virus* OR Viral OR RNA OR Ribonucleic acid* OR DNA OR Deoxyribonucleic acid* OR Retrovir* OR Adeno* OR Lentivir* OR Liposom*)

9 TS=(Gene OR Genes OR Vector* OR Virus* OR Viral OR RNA OR Ribonucleic acid* OR DNA OR Deoxyribonucleic acid* OR Retrovir* OR Adeno* OR Lentivir* OR Liposom*)

8 #7 OR #6

7 TI=(Hypoacusis OR Hypoacuses OR Deaf* OR ototox* OR oto-tox* OR Hearing OR ear OR ears OR hear OR hears OR aural OR auditor* OR cochl*)

6 TS=(Hypoacusis OR Hypoacuses OR Deaf* OR ototox* OR oto-tox* OR Hearing OR ear OR ears OR hear OR hears OR aural OR auditor* OR cochl*)

5 #4 OR #3 OR #2 OR #1

4 TI=(Cisplat* OR cis-platin* OR cisplatyl OR Diamine dichloroplatin* OR diaminedichloroplatin* OR diammine dichloroplatin* OR diamminedichloroplatin* OR diaminodichloroplatin* OR dichlorodiamineplatin* OR dichlorodiamine platin* OR dichlorodiammineplatin* OR dichlorodiammine platin* OR mpi 5010 OR mpi5010 OR neoplatin* OR nk 801 OR nsc 119875 OR NSC-119875 OR platamine OR platiblastin* OR Platidiam OR platimine OR platinex OR platinol OR platinum diamine dichloride* OR platinum diammine dichloride* OR platinum diaminedichloride* OR platinum diaminodichloride* OR platinum diamminedichloride* OR Platinum Diamminodichloride* OR platiran* OR platistin* OR platosin* OR randa OR romcis OR spi 077)

3 TS=(Cisplat* OR cis-platin* OR cisplatyl OR Diamine dichloroplatin* OR diaminedichloroplatin* OR diammine dichloroplatin* OR diamminedichloroplatin* OR diaminodichloroplatin* OR dichlorodiamineplatin* OR dichlorodiamine platin* OR dichlorodiammineplatin* OR dichlorodiammine platin* OR mpi 5010 OR mpi5010 OR neoplatin* OR nk 801 OR nsc 119875 OR NSC-119875 OR platamine OR platiblastin* OR Platidiam OR platimine OR platinex OR platinol OR platinum diamine dichloride* OR platinum diammine dichloride* OR platinum diaminedichloride* OR platinum diaminodichloride* OR platinum diamminedichloride* OR Platinum Diamminodichloride* OR platiran* OR platistin* OR platosin* OR randa OR romcis OR spi 077)

2 TI=(Abiplatin* OR Biocisplatin* OR Biocysplatin* OR briplatin* OR cddp ti OR cis ddp OR cis diamine dichloroplatin* OR cis diaminechloroplatin* OR cis diaminedichloroplatin* OR cis diammine dichloroplatin* OR cis diamminedichloroplatin* OR cis dichloridiammineplatin* OR cis dichloroadiamine platin* OR cis dichlorodiamine platin* OR cis dichlorodiamineplatin* OR cis dichlorodiammine platin* OR cis dichlorodiammineplatin* OR cis-diamine dichloroplatin* OR cis-diaminechloroplatin* OR cis-diaminedichloroplatin* OR cis-diammine dichloroplatin* OR cis-diamminedichloroplatin* OR cis-dichloridiammineplatin* OR cis-dichloroadiamine platin* OR cis-dichlorodiamine platin* OR cis-

dichlorodiamineplatin* OR cis-dichlorodiammine platin* OR cis-dichlorodiammineplatin* OR cis platin*)

1 TS=(Abiplatin* OR Biocisplatin* OR Biocysplatin* OR briplatin* OR cddp ti OR cis ddp OR cis diamine dichloroplatin* OR cis diaminechloroplatin* OR cis diaminedichloroplatin* OR cis diammine dichloroplatin* OR cis diamminedichloroplatin* OR cis dichloridiammineplatin* OR cis dichloroadiamine platin* OR cis dichlorodiamine platin* OR cis dichlorodiamineplatin* OR cis dichlorodiammine platin* OR cis dichlorodiammineplatin* OR cis-diamine dichloroplatin* OR cis-diaminechloroplatin* OR cis-diaminedichloroplatin* OR cis-diammine dichloroplatin* OR cis-diamminedichloroplatin* OR cis-dichloridiammineplatin* OR cis-dichloroadiamine platin* OR cis-dichlorodiamine platin* OR cis-dichlorodiamineplatin* OR cis-dichlorodiammine platin* OR cis-dichlorodiammineplatin* OR cis platin*)

Cochrane Library

#1 MeSH descriptor Cisplatin explode all trees

#2 Abiplatin* OR Biocisplatin* OR Biocysplatin* OR briplatin* OR cddp ti OR cis ddp OR cis diamine dichloroplatin* OR cis diaminechloroplatin* OR cis diaminedichloroplatin* OR cis diammine dichloroplatin* OR cis diamminedichloroplatin* OR cis dichloridiammineplatin* OR cis dichloroadiamine platin* OR cis dichlorodiamine platin* OR cis dichlorodiamineplatin* OR cis dichlorodiammine platin* OR cis dichlorodiammineplatin* OR cis-diamine dichloroplatin* OR cis-diaminechloroplatin* OR cis-diaminedichloroplatin* OR cis-diammine dichloroplatin* OR cis-diamminedichloroplatin* OR cis-dichloridiammineplatin* OR cis-dichloroadiamine platin* OR cis-dichlorodiamine platin* OR cis-dichlorodiamineplatin* OR cis-dichlorodiammine platin* OR cis-dichlorodiammineplatin* OR cis platin* OR Cisplat* OR cis-platin* OR cisplatyl OR Diamine dichloroplatin* OR diaminedichloroplatin* OR diammine dichloroplatin* OR diamminedichloroplatin* OR diaminodichloroplatin* OR dichlorodiamineplatin* OR dichlorodiamine platin* OR dichlorodiammineplatin* OR dichlorodiammine platin* OR mpi 5010 OR mpi5010 OR neoplatin* OR nk 801 OR nsc 119875 OR NSC-119875 OR platamine OR platiblastin* OR Platidiam OR platimine OR platinex OR platinol OR platinum diamine dichloride* OR platinum diammine dichloride* OR platinum diaminedichloride* OR platinum diaminodichloride* OR platinum diamminedichloride* OR Platinum Diamminodichloride* OR platiran* OR platistin* OR platosin* OR randa OR romcis OR spi 077

#3 MeSH descriptor Hearing Loss explode all trees

#4 Hypoacusis OR Hypoacuses OR Deaf* OR ototox* OR oto-tox* OR Hearing OR ear OR ears OR hear OR hears OR aural OR auditor* OR cochl*

#5 MeSH descriptor Gene Therapy explode all trees
#6 MeSH descriptor Gene explode all trees
#7 MeSH descriptor Virus Therapy explode all trees
#8 MeSH descriptor RNA Therapy explode all trees
#9 MeSH descriptor DNA Therapy explode all trees
#10 MeSH descriptor Liposome Therapy explode all trees
#11 Gene OR Genes OR Vector* OR Virus* OR Viral OR RNA OR Ribonucleic acid* OR DNA OR Deoxyribonucleic acid* OR Retrovir* OR Adeno* OR Lentivir* OR Liposom*
#12 (#1 OR #2)
#13 (#3 OR #4)
#14 (#5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11)
#15 (#12 AND #13 AND #14)