Noise-induced Hearing Loss:

treatment and prevention

Thesis submitted for the degree of

Doctor of Medicine

to the University of Leicester

United Kingdom.

Mr Raguwinder Singh Sahota - MBChB MRCS (Eng) DoHNS (Eng)

Department of Medicine, Biological Sciences and Psychology

University of Leicester

United Kingdom

Submitted May 2016

Revised manuscript submitted June 2017

Noise-Induced Hearing Loss: treatment and prevention

Mr Raguwinder Singh Sahota MBChB MRCS (Eng) DoHNS (ENG)

Abstract:

Noise-induced hearing loss (NIHL) is one of the most common sensory disabilities in humans. NIHL is ranked as the world's top industrial injury and a significant cause of hearing loss in teenagers, thus affecting many age groups. This research is timely since a recent study has demonstrated that NIHL has increased dramatically in adolescents, with a 30% increase in this group in the past 10 years, thus coining the term “MP3” generation. The implications of the "MP3" generation will lead to increasing long-term health costs and life-long hearing problems necessitating the use of hearing aids from a young age. Research has shown that exposure to acoustic trauma causes an increase in metabolic activity within the inner ear, with a initial peak in free radical production during the acoustic trauma (within hours) followed by a secondary peak at seven to 10 days after the trauma. Free radicals cause decreased cochlear blood flow, excitotoxic neuronal swelling, and induction of cell death within the inner ear. Recent studies have demonstrated that antioxidant treatments can scavenge free radicals and thus attenuate downstream effects of free radical production. While this has been a major breakthrough for NIHL research, the specific roles played by the different classes of free radicals (reactive nitrogen/oxygen species) remains unclear.

The aims of this project were:
AIM 1: To determine whether inhibition of reactive nitrogen species can prevent NIHL, using taurine as a nitric oxide scavenger.
AIM 2: To establish if there was a dose-dependant response to any effect observed.

The research directly addresses a prominent and recognised otolaryngological disease that affects mental and social health. The long-term goal is to develop a pharmaceutical therapy for NIHL to ultimately prevent a debilitating disease and improve human health. Our work also looks at early use of stem cell therapies to repair after acoustic trauma.

Acknowledgements

With the guidance and relentless support of Dr Sharon Oleskevich and Professor Henry Pau my work occurred. I will be forever grateful and will never be able to thank you enough. Along with further guidance from Professor David R Ryugo who was there when circumstances changed and acted as a rock to help me.

I would also like to thank my colleagues in Garvan institute for their support and advice during this work; in particular members of the hearing department- Mr. Alex A Borecki and Mr Joshua Allen. I would also like to express my sincerest appreciation to Mr. Chris Jasieniecki of the Operations Department at the Garvan whose technical support and skills allowed us to modify our equipment for our experiments.

I would like to dedicate this thesis to my family (Gurmail, Rani, Gurminder, Jagpreet and Asif) who have always supported me in every endeavor I have taken and to Mr Andrew A Moir who has been my inspiration for my career in Ear, Nose & Throat/Head & Neck surgery. Lastly and certainly not least Kate, who made me write up my thesis when it could have easily been forgotten, thank you for always being there with me as my pillar, my greatest supporter, and the mother to our Sonny.

Thank you.

Academic Output

Poster Presentations (4):

- **Free radical scavengers to mitigate Noise-Induced Hearing Loss: Is there a role for Red Bull?**
  Sahota RS, Borecki AA, Allen JAM, Hoehn KL, Pau H, Ryugo D, Oleskevich S.
  British Academic Conference in Otolaryngology (BACO)(15th conference), Liverpool, UK, *International, July 2015*

- **Free Radical Scavengers to Mitigates Noise-Induced Hearing Loss**
  Sahota RS, Borecki AA, Allen JAM, Hoehn K, Pau H, Oleskevich S
  Clinical Academic Training Forum, University of Nottingham, *Regional, November 2014*

- **Free Radical Scavengers to Mitigates Noise-Induced Hearing Loss: is there a role for Red Bull?**
  Sahota RS, Borecki AA, Allen JAM, Hoehn K, Pau H, Oleskevich S
  Association of Research Otolaryngology MidWinter Meeting 2012, San Diego, *International, February 2012*

- **Taurine: A Free Radical Scavenger that Mitigates Noise-Induced Hearing Loss**
  Sahota RS, Borecki AA, Allen JAM, Hoehn K, Pau H, Oleskevich S
  Australian Neuroscience Conference 2012 (32nd meeting), Gold Coast, Australia, *International, January 2012*

Oral Presentations (8):

- **Free radical scavengers to mitigate Noise-Induced Hearing Loss: Is there a role for Red Bull?**
  Sahota RS, Borecki AA, Allen JAM, Hoehn KL, Pau H, Ryugo D, Oleskevich S.
  British Academic Conference in Otolaryngology (BACO)(15th conference), Liverpool, UK, *International, July 2015*

- **Overview of Otoprotection to mitigate the effects of Noise-Induced Hearing Loss: a panacea or fallacy?**
  Sahota RS.
  National Hearing Biomedical Research Unit (NHBRU), ***Invited Speaker***, *Local, April 2015*

- **Otoprotection to mitigate the effects of Noise-Induced Hearing Loss: a panacea or fallacy?**
  Sahota RS.
  Royal Society of Medicine (RSM)- Novel interventions for the human cochlea: emerging research and therapies, ***Invited Speaker***, *International, Febuary 2015*

- **The role of taurine, a free radical scavenger, that mitigates noise-induced hearing loss in mice**
  Sahota RS, Borecki AA, Allen JAM, Hoehn KL, Pau H, Ryugo D, Oleskevich S.
  SWEAM, *National, May 2013*

- **Taurine- A free radical scavenger that mitigates noise-induced hearing loss: Is there a role for Red Bull?**
  Sahota RS, Borecki AA, Allen JAM, Hoehn KL, Pau H, Ryugo D, Oleskevich S.
  ENT Masterclass, Registrars Gold medal section, at the Royal College of Surgeons (England), *National **(winner)**, January 2013*

- **Taurine: A free radical scavenger that mitigates noise-induced hearing loss**
  Sahota RS, Borecki AA, Allen JAM, Hoehn KL, Pau H, Oleskevich S.

Australian Neuroscience Conference 2012 (32nd meeting)- for Australasian Auditory Workshop, *International, January 2012*

- **Free Radical Scavengers To Mitigate Noise-Induced Hearing Loss**
  Sahota RS, Borecki AA, Allen JAM, Hoehn KL, Pau H, Oleskevich S.
  University of Western Sydney Neuroscience Sensory Symposium (2nd), ***Invited Speaker***, *National, December 2011*

- **Reactive oxygen species versus reactive nitrogen species: which causes more damage in noise-induced hearing loss?**
  Sahota RS, Sullivan JM, Hoehn KL, Pau H, Oleskevich S.
  Australian Neuroscience Conference 2011 (31st meeting)- for Australasian Auditory Workshop, *International, January 2011*

Abstracts published (2):

- **Free radical scavenger to mitigates noise-induced hearing loss: Is there a role for Red Bull?**
  Journal of ENT Masterclass. 2014; 5 (1).
  Sahota,R.S., Allen, J.A., Borecki,A.A., Hoehn, K.L., Pau, H., Ryugo, D.K., Oleskevich,S.

- **The role of taurine, a free radical scavenger, that mitigates noise-induced hearing loss in mice**
  The Otorhinolaryngologist. 2014.
  Sahota,R.S., Allen, J.A., Borecki,A.A., Hoehn, K.L., Pau, H., Ryugo, D.K., Oleskevich,S.

Publications (1):

- **Effect of epithelial stem cell transplantation on NIHL in adult mice**
  Neurobiology of Disease. 41 (2011) p552-559*.*
  Sullivan,J.M, Cohen,M.A., Pandit,S.R., Sahota,R.S., Borecki,A.A., Oleskevich,S.

Prizes (1):

- Winner of Registrars Gold Medal : ENT Masterclass at the Royal College of Surgeons (England)

Awards (1):

- Australian Neuroscience Society: Student Travel Award for National Conference 2012

Funding (2):

- Kennedy Foundation
- Fairfax Foundation

List of Content

Chapter 1: Noise-induced Hearing Loss Overview

1.1- Introduction and Epidemiology:

Deafness impairs communication, contributes to social isolation and may impact upon mental health, causing a global health problem with a profound socioeconomic impact. Over many years the organ of hearing (cochlea) undergoes damage from the loss of hair cells, the sensory cells of the auditory system. The leading causes of acquired hearing loss are as a consequence of modern day damage by man; from noise-induced hearing loss and ototoxic insults via medication. However, there are also other causes of congenital or acquired hearing loss, including (but not limited to) infectious disease (meningitis, measles, mumps), neonatal jaundice, trauma to the head and ear, as well as age related hearing loss (presbycusis).

Hearing loss is the third most prevalent chronic disability within America and is estimated to affect 29 million Americans of working age[1], equating to 16% of the American population. It is one of the most widespread, expensive and poorly understood disabilities. In 2013 the World Health Organisation (WHO) estimated that 360 million people suffer from moderate to profound hearing loss in both ears[2]. They also report that 80% of these cases live in low and middle-income countries. This number is expected to increase to 900 million by the year 2050[2]. In the United Kingdom there are more than 34,000 children who are deaf and approximately nine million deaf or hard of hearing people, of which 3.5 million are of working age (16-65 years)[3]. The estimated costs of communication disorders to the US economy equate to 154-186 billion dollars annually[2].

From a national health burden point of view, in relation to hearing aids, there are approximately two million used in the UK, but there are a further four million people who could benefit from

using hearing aids[3]. The cost of a hearing aid varies from around £800 to a few thousand pounds per unit. This equates to several billion pounds worth of equipment and a multi-billion pound industry in the UK alone. Current production of hearing aids meets less than 10% of global need[2]. In developing countries, fewer than one in 40 people who need a hearing aid have one[2].

The prevalence and health importance of NIHL is controversial due to multiple factors, including but not limited to:

- Difficultly to define NIHL- due to audiometric "shifts"
- Variable audiological definitions, these lead to different inclusion and exclusions from an epidemiological point of view hence the prevalence varies as a function of the used definition. Some audiological definitions include:
    - Occupation Safety & Health Administration (OSHA) Standard threshold shifts- 10dB from baseline in averages at 2,3,4 kHz when age corrected.
    - OSHA "recordable" hearing loss- 10dB shift from baseline as in averages at 2,3,4 kHz with an average of absolute hearing threshold at 2,3,4 kHz greater than or equal to 25dB
    - American Medical Association (AMA)- hearing threshold averages at 0.5,1,2,3 kHz greater than 25dB hearing loss with 1.5 % monoaural impairment for each decibel over 25dB.
- Near impracticable nature of removing confounding factors of other additive causes of SNHL, most notably the effects of presbycusis (age related hearing loss), these types of issues are well documented in multiple legal cases.

Current state-of-the-art technological treatment for hearing loss consists of devices such as hearing aids (including bone anchored hearing aids) for mild to severe hearing loss or cochlear implants for profound hearing loss. Hearing aids amplify sounds but require intact inner hair

cells to transfer sound to the cochlea nerve. Damage to inner hair cells can be bypassed by a cochlea implant, a neural prosthesis designed to electrically stimulate the spiral ganglion neurons comprising the cochlear nerve. In this procedure a curled linear array of up to two-dozen electrodes are placed into the scala tympani of the cochlea. Despite their successes, hearing aids and cochlear implants are far from perfect. In particular, the efficacy of cochlear implants differs greatly among patients and throughout the world a multitude of laboratories specialise in clinical research of this field. Cochlear implants are very good pieces of technology that allow people to hear but have many limitations. An obvious limitation is due to the small number of electrodes that cannot simply stimulate the tens of thousands of hairs cells that are normally found within a normally functioning cochlea. Cochlear implants are poor for allowing a significant appreciation of complicated music[4]. A vast quantity of research is being carried out in the subjects of gene therapy, nerve cell regeneration and stem cells to act as new, powerful and novel treatment modalities for hearing loss. There is a massive economic and social demand to develop therapies/treatments for hearing loss to ameliorate the associated disability.

Within the cochlear system, the sensory receptors of hearing or hair cells are the weakest link in the chain. Human hair cells are deficient in their ability to regenerate, but the mammalian cochlea evolved from common ancestors with the capacity for hair cell self-repair. Demonstrations of this can be seen in non-mammalian vertebrates that can regenerate auditory hair cells that restore sensory function[5-8]. Hair cells are divided into outer and inner hairs cells (OHC and IHC respectively), the former being more sensitive to acoustic damage and age-related loss, but the latter can also become damaged. Moreover the death of hair cells leads to a subsequent progressive degenerative atrophy of the primary afferent neurons; the spiral ganglion neurons[9,10].

Aging, drugs or other disease processes can cause hearing loss, however a large proportion is caused from exposure to acoustic trauma. NIHL is the second most common cause of sensorineural hearing loss, after age related hearing loss, and is the single largest industrial injury in the entire world. The National Institute of Deafness and other Communication Disorders estimates that 600 million people in the world are affected by NIHL. Though the aetiology of NIHL is multifactorial – involving a complex interplay between environmental and genetic factors – it is a disease predominately caused from acoustic trauma and a disease of modernised mankind, from an evolutionary point of view "natural" noises seldom broke past the 100dB range until the existence of modern tools and machinery. NIHL has been documented since the first occupational health publication, almost 300 years ago[11], in copper metal workers due to the acoustic trauma induced by hammering. Humans are at risk from significant acoustic trauma from both industrial and recreational injury[12].

The only treatments currently available for NIHL are prevention and symptomatic control (e.g. hearing aids or cochlear implants), but these fail to halt ongoing neurodegeneration and disease progression. The long-term goal of this research project is to develop a treatment or combination of treatments to ameliorate NIHL through prevention of disease onset and disease progression. These are the first few steps on the journey of otoprotection.

1.2- Occupational Health Issues:

The occupational significance of NIHL has been published for more than three centuries, first published in 1713 by Bernardino Ramazzini in his seminal occupational health publication De Morbis Artifactum (disease of workers)[11]. This identified 52 occupational types that were associated with afflictions caused by their vocation. Bernardino Ramazzini made vivid illustrations of the illnesses in these cases including, but not limited, to tailors slumped posture due to leaning forwards to use sewing machines and bakers becoming bow-legged from

kneading dough. He also identified coppersmiths in Venice who lived in the industrial quarter who would beat sheets of copper with wooden and then iron hammers to help it achieve the shape that was required. Due to the sound of the banging he stated they inevitably became hard of hearing or if they lived long enough completely deaf, the only comparative natural cause of such problems that he encountered was the people who dwelled near the Nile in Egypt whom were deaf from the excessive uproar of the falling water. His work highlights the important facts that NIHL is a disease of modern mankind, there are seldom causes in the natural world and the vast majority of causation has been associated with industrial growth using tools or machinery in different forms.

Industrial groups at particular risk include (i) military workers from a consequence of firearms, transport vehicles or machinery; (ii) transport workers especially involved in aviation or rail work; (iii) manufacturing, construction, mining, plumbing, forestry, farming, the list is potentially endless. Ten million people in the USA and 25-30 million people in Europe work daily in conditions that pose a potential threat to hearing[13]. The military has a particular interest in firearm related injury, for example the United States military spent almost one billion dollars on its annual disability cost related to NIHL in 2006 alone[14].

NIHL raises a very specific challenge for the military; the soldiers, sailors, aviation personnel, marines and civilians who serve beside them are exposed to noise levels that are much higher than the vast majority of the general population, placing them all as a significantly more high-risk group than the general population. The perverse issue is that military (and associated personnel) rely on their hearing in particular for communication in training and combat environments because they will need to determine multiple conflict related noise factors (including, but not limited to; direction of enemies, direction of enemy fire, distance of enemy, enemy versus friendly fire, ground vehicles, aircraft, radio signals, warning from fellow

combatants in field and the civilians who are in the field of battle). Obviously many military personnel are not directly involved in fighting, but are still at risk from the same noisy environments associated with training. For example, the sound output of an M16 rifle, used in basic military training and standard issue for American soldiers, has been measured to discharge at 156 dB impulse burst. It is physically difficult to protect against the acoustic trauma at such high levels, and code 29 of Federal Regulations (CFR 1910.95, 2009) states that exposure to impulse or impact noise should not exceed 140 dB peak SPL. Certainly sound exposure in the military can be at a much larger magnitude than a 140 dB, for example firing a 155 mm Howitzer, previously used by the US military, has an gigantic peak impulse output of 181 dB. This obviously makes this a very unique predicament due to the immense variability in military-related noise during training and combat environments, going from ambient noise levels to greater than 140 dB in a fraction of an instant.

Pure tone audiometry (PTA) is the foundation of clinical audiometric assessment. It is a psychoacoustic test that aims to establish a subject's hearing threshold, that is the minimum sound level at which a specific response can be obtained. The decibel scale is a logarithmic scale to the base of a factor 10, this is due to the huge variance in sounds that can be heard and the practical aspects of documenting hearing. The decibel scale is not dissimilar to the pH or Richter scale, both being logarithmic scales. There are a variety of decibel (dB) scales used in differing applications of sound intensity measurement. The most commonly used dB scales are:

- dB SPL (sound pressure level)- this scale ranges from 20- $200x10^6$ microPascals. The human auditory system is less efficient at detecting both low and high frequencies than middle frequencies. The detection of sounds in DB SPL produces an audiogram for a normal hearing subject that would not be flat, hence this type of test would make it more difficult to identify abnormalities in hearing due to non linear normal diagrams.

- dB HL (hearing level)- this scale was primarily designed so that 0 dB hearing level (HL) would be the expected threshold, it is important to note that the amount of energy at 0 dB HL at each frequency is not the same. As specified above there is variance in sensitivity within the human auditory system for the detection of sound at different frequencies. It is measured in relative terms where a reference zero has been set as internationally agreed standard. The standard represents the thresholds at each test frequency for otolgoically normal young adults. Due to the above reasons, a normal hearing individual would expect to have a flat audiogram on a dB HL scale (centred at 0 dB HL). This is used as a clinical PTA, allowing a subjects hearing to be tested and compared to a known accepted norm.
- dB A (A-weighted scale sometimes referred to as assessment weighted scale)- is used for measures of sound field assessments. The ear is most sensitive to 'speech frequencies' (500-4000 Hertz) and less so to frequencies outside of this range, interestingly the ear is damaged less easily at frequencies to which it is less sensitive. To take the above into account an A weighting is used that reduces the contribution of very low and very high frequencies to the overall noise level measurement. This scale is often used for open field testing of hearing which include industrial and noise exposure settings.

As many as 10-15% of US armed forces personnel show a significant change in their long–term hearing levels[15]. This is not an isolated risk to only American military personnel, however the majority of data is based on US citizens, 11% of Israeli soldiers have significant temporary changes in hearing 56 days after firearms exercises[16]. Noise-induced hearing loss alone was estimated to cost approximately 2% of the gross domestic product in America[2]. NIHL is not the only pathology that industrial workers suffer from as a consequence of repeated acoustic trauma, tinnitus is an important co-morbidity that has been estimated to be involved in almost half (41.7%-56.6%) of all occupational NIHL claims[17,18]. However, it is not simply a problem of impulse weapon associated noise, but also the industrial noise from equipment such as aircraft.

It is known that naval aircraft carrier flight deck noise levels can reach up to 152 dBA[19], this level of noise is double the level that can be protected by a combination of hearing protection devices (HPD) using both ear plugs and over ear noise muffs together.

The use of HPD is not a recent development and their use has been claimed since ancient Greece. In the major ancient Greek poem Odyssey by Homer, Odysseus's crew used wax earplugs to avoid to be ensnared by the song of Sirens that would cause sailors to hit rocks and sink. However modern earplugs have been available since early in the 1900s. The earliest mention of a widely commercial device to act as a HPD was from 1907. Maximilian Negwer, a pharmacist, founded the "Max Negwer factory for pharmaceutical and cosmetic specialities" in Berlin-Schöneberg the Germany in 1907. His first product was Ohropax, from German and Latin meaning, "ear peace". This company still exists today to produce HPDs. Their initial commercial HPD were incredibly basic and more modern earplugs have been around since 1967 and this is due to a change in the materials that were used to produce the HPDs. In 1967 Ross Gardener and his team from the USA were developing a resin that sealed joints and produced a resin with energy absorption properties. They produced what was termed E-A-R material, which was later developed into commercial memory foam earplugs. HPD are still changing, which demonstrates there is still emerging advancement in this field.

The problems associated with the military and acoustic trauma with consequential sequela is a predicament that does affect the UK even though we have a relatively small military compared to other countries. There is public information available under the freedom of information act from the Ministry of Defence (MOD) relating to hearing claims and poor hearing (MOD Ref: 19-12-2013-081103-002). As of 1st November 2013 there were 156,220 full time service personnel in the UK Armed Forces. Of these personnel 3,530 had impaired hearing of which 470 had a coding of NIHL recorded on their medical records, a further 630 personnel had poor hearing of

which 90 also were coded for NIHL in their medical records. This leads to a significant health burden as a direct consequence of acoustic over exposure.

There are significant industries that are at high risk from the consequences of excessive noise exposure and the associated financial compensation that is paid as a result of acoustic over exposure. The Department of Energy and Climate Change (DECC) in the UK is the body that is responsible for dealing with claims for compensation from former mineworkers, as the inheritor of liabilities of the former nationalised coal industry. Under the Freedom of Information Act 2000 they have released information regarding NIHL claims. Between April 2011 and up to July 2014 they received 11,230 claims, of which they have paid out for 3,147 claims for a total of £7,026,878, this equates to approximately £2,232 per claimant. See (Table 1) for further information. Claims are not limited to coal related work, there are widespread claims associated with NIHL and tinnnitus. As of November 2013 the UK police force has paid more that 135 million pounds in compensation to 8,641 former police officers, averaging £2,546 per claimant while many other claims are coming through. There are multiple claims within the armed forces and are supported by the Armed Forces Compensation Scheme (AFCS). AFCS came into force on 6th April 2005 for people injured, made ill or killed as a result of service in the armed force from that day onwards. This scheme operates on a "no fault basis", meaning that you do not need to show negligence by the forces to make a claim. Interestingly claiming for deafness does not have to caused by active service or combat to qualify, if for example a member of the armed forces contracted an ear infection as result of service that lead to deafness they can still claim. All claims must be carried out within 7 years from the day of the injury, first sought medical advice or retired from service (whichever is the earliest date applies). The condition is very costly for the U.S. military too, they receive more than 22,000 claims a year for NIHL and it is still the most common reason that U.S. soldiers cannot be redeployed. The estimated cost to the Department of Veterans Affairs (equivalent of AFCS) is more than $1billion annually.

Financial Year	Number of Claims Received	Number of Claims Paid	Compensation Paid
2014/15 (as at 31/7/14)	902	435	£961,005
2013/14	3589	1393	£3,033,947
2012/13	4081	953	£2,204,861
2011/12	2658	366	£827,065

Table 1- Department of Energy and Climate Change (DECC) claims for compensation from former mineworkers between April 2011 and July 2014.

Recreational groups at particular risk include (i) persons attending events with loud music exposure e.g. concerts, musicians, regular patrons of discos and nightclubs; (ii) users of recreational machinery including firearms, power tools or gardening equipment. NIHL has increased dramatically in adolescents, with a 30% increase in prevalence observed in the past two decades. Shargorodsky et al carried out a cross-sectional analysis of the prevalence American adolescents (aged 12-19 years old) comparing from 1988-1994 with a group from 2005-2006 and showed there was an increased prevalence of 14.9% to 19.5% in each group respectively, coining the term "MP3" generation[14]. NIHL is the most common cause of acquired hearing loss in the under 40-age group. The true extent of NIHL disease burden within the general populous remains poorly understood and investigated. However, it undoubtedly plays a significant role in the global burden of hearing loss.

Action on hearing loss, formally called Royal National Institute for Deaf people (RNID), launched "Don't lose the music" a national campaign to highlight the danger of listening to excessively loud music. The campaign focussed on exposure relating primarily to young people in nightclubs, concerts, gigs and using personal audio equipment. When this campaign was launched under RNID the focus was to promote the campaign at music festivals, gigs where earplugs were distributed and information on the dangers of excessive noise. This has subsequently been superseded by the "Loud music!" campaign, which has a broader remit and can also be supported by fundraising. They often use the pneumonic M.U.S.I.C. (MP3 player-

turn it down, Use chillout zones in clubs, Stand back from speakers, Invest in noise cancelling headphones, Carry and use earplugs) to help increase awareness of this important health problem. Action on hearing loss is a charity for hearing related issues, but there are people who have been affected from the effects of acoustic trauma and set up dedicated services. An example of a dedicated service would be H.E.A.R. (Hearing Education and Awareness for Rockers), this is a non-profit organisation that has been around since 1988 that was set up by Kathy Peck of The Contractions and Flash Gordon who is a General Practitioner (not to be confused with the comic book character). H.E.A.R. is a non-profit volunteer organisation that dedicates its work to preventing the deleterious effects of acoustic trauma causing hearing loss, primarily from loud rock music but not limited to rock music alone. The organisation came into existence due to the damage encountered to Kathy Peck that led her music career to be cut short and she wanted to educate others about the potential risks of NIHL and tinnitus.

Occupational health strategies (OHS) for hearing protection have generally only been in place since the early 1970s and recommendations for exposure have been implemented since then (Table 2). Incidentally, the US military have been aware of the problem since the 1800's and audiology grew throughout the world as a consequence of World War I veterans' needs as a direct consequence of NIHL. OHS in the work place involve environmental or physical protection against acoustic trauma in hazardous environments. Environmental protection decreases the absolute amount of noise by either decreasing the sound intensity or the exposure duration (Figure 1). Physical protection involves the use of hearing protection devices (HPD) as the mainstay for physical defence against acoustic trauma (Figure 1). Many military users of HPD when questioned often admit to non-compliance of HPD, often quoting poor fit, worsened head movement, discomfort, poor communication ability and worsened enemy detection[20]. A large body of current work is aiming to predict groups that will suffer from NIHL and tinnitus

from time in service or military occupation speciality. The Institute of medicine committees' recommendations have given guidelines circa 2005 for noise-induced hearing loss associated with military service from World War II to the present. These recommendations mean that an individual going into military service receives audiological assessments prior to entering service and at the end of service.

(a)

Exposure duration (hours)	Sound Level (dBA)
8	90
4	93
2	96
1	99
0.5	102
0.25	105
0.125	108
0.0625	111
0.03125	114

(b)

Area or equipment	**Typical noise level (dBA)**
Library	38-48
Typical office	50-60
Typical laboratory	55-65
Photocopier	59-71
Vacum cleaner	68-74
Typical factory	76-82
Noisy lawn mower	87-94
Belt sander	90-97
Hand drill	95-101
High pressure spray painter	98-103
Angle grinder	95-107
Chain saw	106-115

Table 2- (a) OHS permissible noise exposure levels over a working day, measured in dBA

(b) Typical examples of occupational sound exposure, measured in dBA

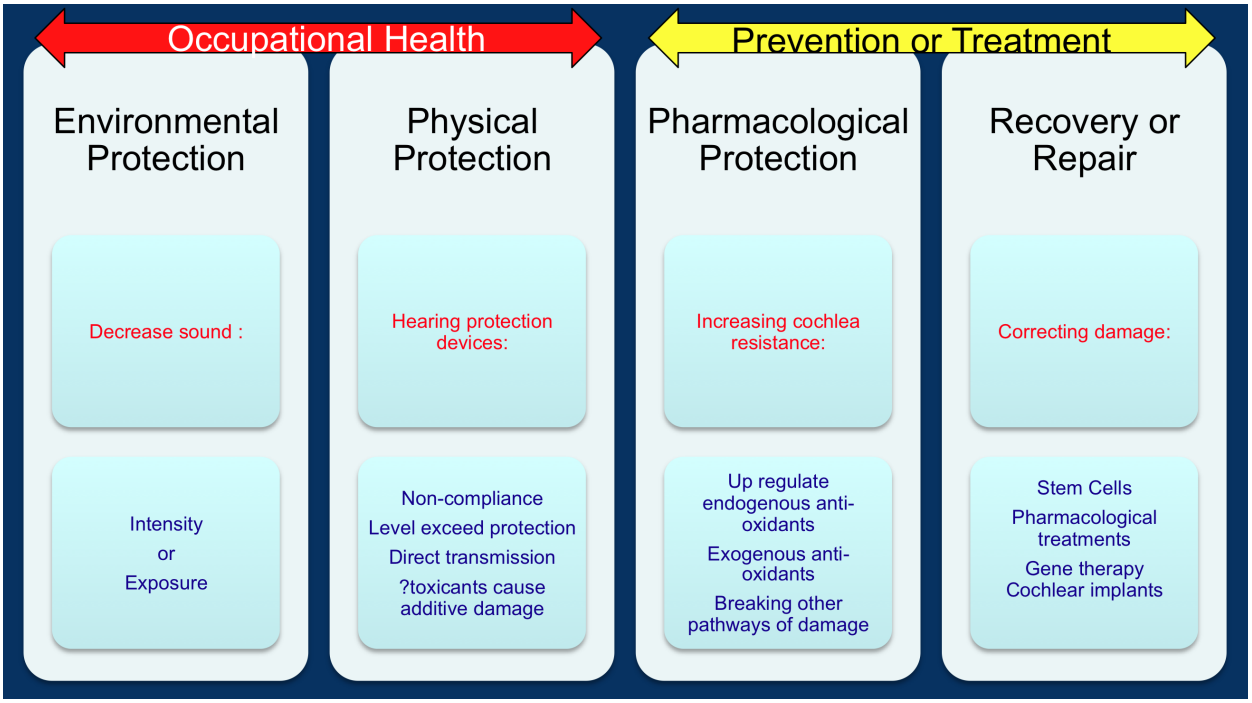

Figure 1- NIHL protection, prevention and repair strategies.

Figure made by author, Raguwinder Sahota, in 2012.

NIHL classically has been defined clinically through the use of so called "notch definitions". These determine if there is presence or absence of high frequency notches. Notches are characteristically (but not limited to) at 3-4 kHz with improvement at 8 kHz on an audiogram. An example of a typical audiogram of bilateral NIHL is demonstrated below. (Figure 2). This type of notch is suggestive of NIHL and is often used clinically to differentiate between NIHL and presbycusis. However, it can be difficult to attribute the cause of hearing loss in groups that have been exposed to high-noise and then have superimposed presbycusis. This is due to notches being less apparent when both conditions are present[21] and blurring of the audiological findings between these two conditions leaving no definite way of attributing the cause of the hearing loss.

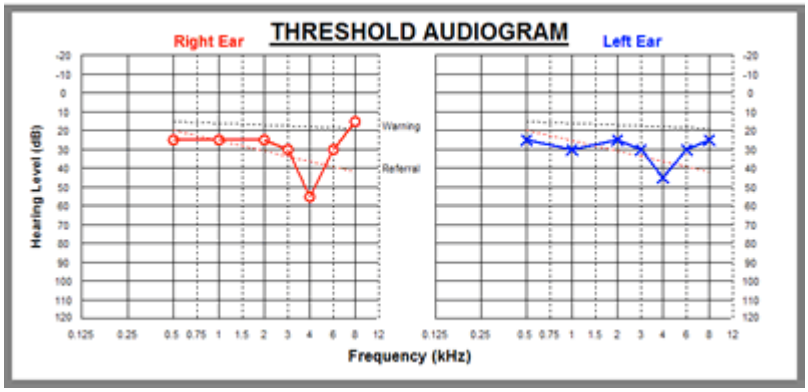

Figure 2- Audiogram demonstrating typical notching associated with NIHL

(image from www.audiometrics.co.uk/hearing_loss.html)

1.3- Pathological Process:

NIHL is the most frequent cause of acquired hearing loss in the under 40-age group[2]. Previously it was thought that NIHL was only caused by the mechanical insult of the acoustic trauma, however the pathophysiology is multi-factorial. Acoustic trauma causes cochlear damage by two main methods, firstly the direct mechanical forces and by secondary metabolic disruption.

Acoustic trauma leads to sound waves striking the tympanic membrane, the ossicles then move as a consequence, leading to a transmission of pressure into the oval window. This resulting pressure leads to displacement of the fluid within the cochlea and consequently displacement of the basilar membrane. If the force exerted on the basilar membrane is too high this leads to a shearing force upon the outer hair cells (OHCs). Disruption of the cellular structures within the cochlea caused by the mechanical force of acoustic trauma was previously ascribed as the principal and only pathological cause of NIHL[22-30]. There is well documented evidence of prolonged noise exposure causing tearing of the tectoral membrane, detachment of the basilar membrane[22,23,33-33], hair cell loss and loss of synaptic afferents[34,35]. Noise exposure causes mechanical forces to drive the basilar membrane to oscillate. The oscillation leads to excessive motion causing a cascade of structural changes in the cochlear sensory cells and their supporting

cells, which compromises cochlear function. It was not until the mid-1990s that two groups independently demonstrated the production of free radicals[36-40] that are part of the secondary metabolic disruption. Cochlear damage caused by free radicals is sustained once free radicals are in such high concentrations that they overwhelm the natural endogenous antioxidant defence mechanisms of the cochlea and of the supporting cells. In the past 10 years, there has been a rapid growth in interest regarding the role of free radical related damage within the cochlear and whether free radical inhibition could be manipulated as a potential therapy for NIHL. Scientists have highlighted a metabolic sequence of events that cause cochlear damage, at which free radicals sit centrally in the cascade. Other parts of the cascade include calcium excitotoxicity, glutamate toxicity, metabolic exhaustion/energy depletion and ischemia[41-43] (Figure 3). Evidence of cochlear vasoconstriction has been documented as being another pivotal secondary metabolic effect of acoustic trauma and potential therapeutic area of interest[41-44]. At high levels >125 db mechanical disruption predominates, but at low levels <115 dB NIHL pathology tends to be metabolically driven[45].

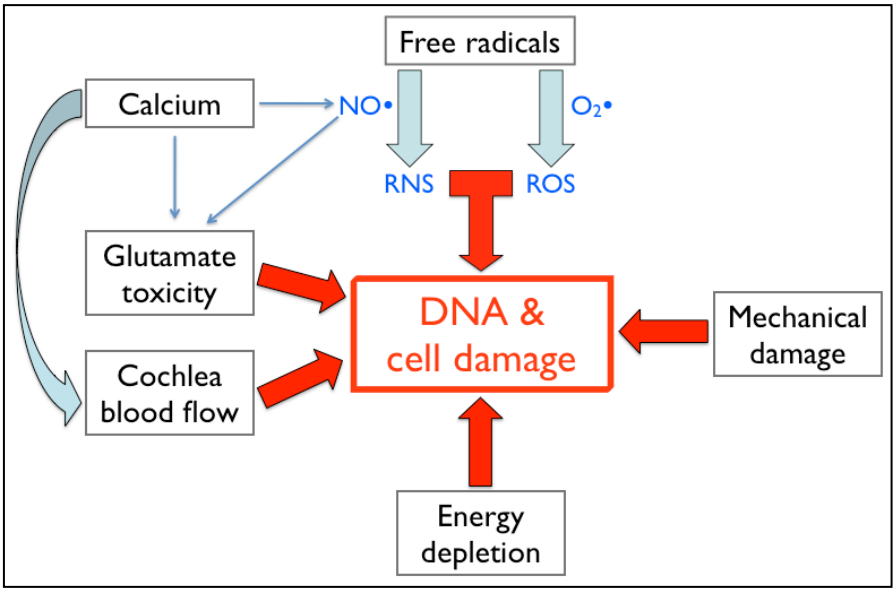

Figure 3- Simplified pathways involved in NIHL pathogenesis.

Figure made by author, Raguwinder Sahota, in 2012.

Mechanical damage is sustained only during the duration of noise exposure, where as metabolic disruption is initiated at the time of the acoustic trauma but continues to develop for days and even weeks after the initial insult. Structural changes within the cochlea can be classified as reversible or permanent. Mild structural deficits result in what is described as temporary threshold shifts on audiometric analysis, while severe structural deficits lead to permanent threshold shifts; leading to temporary or permanent hearing loss respectively. Temporary threshold shifts are thus larger than permanent threshold shifts.

Cochlear damage from acoustic trauma leads to multiple pathological issues, including (but not limited to):

I. **Hair cell lesions in the organ of Corti-** this is the most evident change in cochlear morphology after acoustic trauma. Hair cells within the cochlea are arranged in rows as single rows of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) within the organ of Corti. Acoustic damage happens in clusters forming lesions of hair cells, dependent on the sound intensity, time exposure and frequency of noise trauma[46] . In the centre of a lesion there is hair cell degradation and hair cell loss is apparent. There is a peripheral area of transition, where there is still normally active degeneration of hair cells occurring. The process of acoustic trauma leads to a compromise in the plasma membrane of the cells causing increased membrane permeability. This subsequently causes the release of cellular components to extracellular spaces and loss of structural integrity. This cascade often leads to hair cell death, and any irreversible features are the pathological basis of permanent hearing loss[47]. Lesions start during noise exposure but continue after termination of exposure to the acoustic trauma[35].

   The level of acoustic trauma is associated with the extent of damage that occurs, but is not parallel and therefore the correlation is linked[48]. At high levels >125db mechanical disruption predominates (damage to Reissner's membrane, basilar membrane cell

junctions, damage/loss of stereocillia bundles, leading to leaking of rich endolymh), but at low levels <115dB NIHL pathology tends to be metabolically driven (free radicals, calcium excitotoxicity, glutamate toxicity, metabolic exhaustion/energy depletion and ischemia)[49]. At lower levels the damage tends to lead to necrosis, but >120dB the damage increases dramatically and tends to be apoptotic in nature[48].

The distribution of hair cell loss is closely associated with the frequency of acoustic trauma. High frequency sounds traumatise the basal turn, while low frequency sounds preferentially damage the apical turn of hair cells within the cochleae respectively[50]. Therefore experimental lesions can be focused to damage different regions of hair cells[50]. High frequency noise damage is more severe than the equivalent low frequency damage in animal models[51].

II. **Stereocillia**- Hair cells are capped by extremely well organised bundles of stereocillia. Seterocillia are tiny finger like projections arranged in either a W or V pattern in rows. These have an actin core and are cross-linked together, which makes them act as a unit. When stereocillia are stretched from acoustic stimulation this opens transduction channels that activate the cell[48]. Acoustic trauma can cause a wide variety of damage to seterocillia, including but not limited to; disarray, detaching, separation, fusion, collapsing, shrinking, breaking and simply loss[52,53].

There is also a variation between the level of damage to stereocillia and the corresponding hair cells. OHC are more vulnerable than IHC to acoustic trauma, this is not true for the corresponding stereocillia and often demonstrate an opposite pattern[54]. It is not simply the stereocillia that can become damaged, the cross-links between stereocillia are susceptible to the effects of acoustic trauma. There is a capacity for stereocillium to recover after acoustic trauma, however it is not currently understood fully and is an active area of research.

III. **Reticular lamina and circular plates**- the reticular lamina is the upmost layer of the organ of Corti and contains the apical structures of IHC and OHC, called the circular plates. These structures are essential in the homeostasis of hair cells. Structural defects in the reticular lamina are often at the circular plates and are associated with hair cell degeneration. Defects can also take place at cell-cell junctions, these are predominately caused by acoustic trauma[29,55-57].

IV. **Plasma membrane**- is essential to maintain intracellular homeostatis, but can be damaged by acoustic trauma by direct mechanical stress or subsequent problems caused from metabolic disturbance. The direct injury is due to excessive motion of the basilar membrane causing a stretching injury but the metabolic disturbance is due to a result of metabolic oxidisation and energy exhaustion[57]. The effects of metabolic oxidisation leads to lipid peroxidation and the generation of free radicals that can cause direct and indirect damage (Figure 3).

V. **Variability of cochlea damage & susceptibility to acoustic overexposure**- there is a significant difference between individuals to the susceptibility of cochlear noise trauma. There is less variability between both ears of the same animal for NIHL[58]. Factors of the acoustic trauma that affect the level of damage include frequency, intensity and duration of acoustic trauma. There are a multitude of factors involving an individual that can cause variability to noise trauma, these can be extrinsic (shape of ear canal or pinna) or intrinsic (age, sex, level of intrinsic antioxidants or genetic factors for susceptibility for NIHL)

VI. **Cell death modes and pathways**- There are numerous intricate cell death pathways that can be activated as a consequence of NIHL. There are three global modes of cell death; apoptosis, necrosis or atypical cell death. Apoptosis, often termed programmed cell death, needs an energy supply to happen. There is characteristic shrinkage of cells due to condensation of cellular structures and subsequent fragmentation. However necrosis is

premature cell death caused by autolysis and is independent of energy. In this situation there is swelling of the cell body and nuclear structures leading to eventual rupture. A third pathway has been identified which is independent of apoptosis or necrosis[46]. In the atypical pathway there is maintaince of cell shape but cellular debris is evident. Bohne et al demonstrated the presence of cellular debris arranged in the shape of intact OHC with a nucleus deficient in nucleoplasm[46]. The reason for this atypical pathway is unclear currently but tends to happen at only low or moderate levels of sound exposure.

Free radicals are defined as any molecule or ion with a lone electron and they are products of normal cellular metabolism[59]. Free radicals can be subdivided into reactive nitrogen species (RNS) and reactive oxygen species (ROS) each containing a central nitrogen or oxygen atom, respectively. The most common RNS is the nitric oxide radical (NO•) and the most frequently generated ROS is the super oxide radical (O_2•). ROS and RNS cellular damage is termed oxidative and nitrosative stress respectively[59-61]. Free radicals were originally discovered in 1900 by Gomberg[62] , but the damaging effects were not realised till over half a century later[63]. The presence of unpaired electrons renders such radicals highly reactive, so they normally occur only as transient intermediates in reactions[64] and have the ability to cause profound cellular damage. Free radicals can both generate as well as propagate additional free radicals and can change enzymes or prevalency of metals leading to increased free radical production. At low to moderate levels, free radicals are beneficial, and actually even essential, to the existence of cells. For example they are used in cell signalling processes (in a dynamic form of homeostasis termed 'redox signalling'), immune responses and mitogenic responses, but they are deleterious in excess causing damage to tissues and hence a balance is needed[59,65]. Research has shown that exposure to acoustic trauma causes an increase in metabolic activity in the inner ear, with a peak in free radical production during the acoustic trauma (within hours) followed by a secondary peak at seven to ten days after the trauma[66,67].

Understanding how free radicals are produced within the inner ear needs an understanding of the principles of normal cell biology. Nutrients, such as glucose, and oxygen enter the cell. They are converted within mitochondria to a usable form of energy as ATP. This mitochondrial transport chain system that produces ATP to be available within the cell, consumes approximately 85% of all oxygen cells and in normal physiological conditions 1-3% are converted in the superoxide radical (O_2•), subsequently leading to the production of hydrogen peroxide (H_2O_2) if they react with water, which itself can then dissociate in to two hydroxy radicals (OH•) and any of these free radical by products can react causing more free radicals or can cause irreversible cellular damage[59,65,68]. There is a physiological "leakage" of electrons during normal metabolic processes and these are implicit in the formation of free radicals. Animals have naturally occurring antioxidants that act as natural defence systems to protect against the adverse effects of increased numbers of free radicals, however these systems can become saturated and fail when bombarded with scores of free radicals. Glutathione (GSH) is an antioxidant that compromises the largest antioxidant system within the cochlea[69-73] and GSH immunoreactivity distribution is observed within the cochlea in the stria vascularis, spiral ligament and vestibular endorgans[74]. Depletion of GSH has been demonstrated to enhance hearing loss as a consequence of the generation of free radicals[75,76]. There has been an increasing body of evidence on the use of exogenous antioxidants to help mitigate the effects of excessive acoustic trauma[15,44,67,77-96].

Agent	Type	Route	Subjects	Type of noise	References
Salicylate	[a]Antioxidant	IP	Mice	BBN, 113 dB, 3.5 h	Adelman et al. (2008)
D-Methionine	Antioxidant	IP	Mice	4 kHz OBN, 110 dB, 4 h	Samson et al. (2008)
Salicylate + trolox	Antioxidant, anti-nitrosative	IP, SC	Guinea pigs	4 kHz OBN, 120 dB, 5 h	Yamashita et al. (2005)
Tempol + creatine	Antioxidants	Oral	Guinea pigs	4 kHz OBN, 120 dB, 5 h	Minami et al. (2007)
Tempol or 3-aminobenzamide	Antioxidant, PARS inhibition	IP	Mice	4 kHz, 128 dB, 4 h	Murashita et al. (2006)
Vitamin E + idebenone	Antioxidants	IP, IM	Guinea pigs	6 kHz, 120 dB, 40 min	Fetoni et al. (2008)
N-acetyl-cysteine	Antioxidant	IP, oral	Chinchillas	Various high-kurtosis	Bielefeld et al. (2007)
Idebenone	Antioxidant	IP	Guinea pigs	6 kHz, 120 dB, 40 min	Sergi et al. (2006)
Vitamin C	Antioxidant	Diet	Guinea pigs	4 kHz OBN, 114 dB, 6 h	McFadden et al. (2005)
Edaravone	Antioxidant	Perilymph	Guinea pigs	4 kHz ?BN, 130 dB, 4 h	Tanaka et al. (2005)
Vitamin A + C + E + Mg^{++}	[b]Antioxidants + Ca^{++} inhibition	IP	Guinea pigs	4 kHz OBN, 120 dB, 5 h	Le Prell et al. (2007a)
Hydroxy-phenyl-*N*-tert-butylnitrone + *N*-acetyl-cysteine + acetyl-carnitine	[b]Antioxidants + energy enhancer	IP	Chinchillas	4 kHz OBN, 105 dB, 6 h	Choi et al. (2008)
N-acetyl-cysteine Acetyl-carnitine	Antioxidant Energy enhancer	IP	Chinchillas	4 kHz OBN, 105 dB, 6 h	Coleman et al. (2007a)
T-817MA	Antioxidant and neuroprotectant	Oral	Guinea pigs	4 kHz OBN, 120 dB, 5 h	Yamashita et al. (2008)
BN 82270	Antioxidant, calpain inhibitor	Perilymph	Guinea pigs	6 kHz, 120 dB, 30 min	Wang et al. (2007a)
Cyclosporin A FK506	Calcineurin Inhibitors	IP	Guinea pigs Mice	2 kHz, 120 dB, 10 min 4 kHz, 128 dB, 4 h	Uemaetomari et al. (2005)
Trimethadione ethosuximide	T-type Ca^{++} channel blockers	IP	Mice	BBN, 110 dB, 30 min	Shen et al. (2007)
Caroverine	Glutamate antagonist	SC	Rats	Impulse Noise 160 dB peak 50 pulses	Duan et al. (2006)
Geranylgeranyl acetone	Induces heat shock proteins	Oral	Guinea pigs	4 kHz OBN, 130 dB, 3 h	Mikuriya et al. (2005)
Human insulin-like growth factor 1	Growth factor	Round window	Rats	BBN, 120 dB, 2 h	Iwai et al. (2006)
Amitriptyline	Induces neurotrophic factor	IP	Guinea pigs	4 kHz OBN, 117 dB, 24 h	Shibata et al. (2007)
Retinoic acid	Anti-apoptotic	Oral	Mice	BBN, 122 dB, 3 h/d 3 days	Ahn et al. (2005)
AM-111	Anti-apoptotic	IP, round window	Chinchillas	Impulse noise 155 dB peak 150 pulses	Coleman et al. (2007b)
D-JNKI-1 peptide	Anti-apoptotic	Round window	Guinea pigs	6 kHz, 130 dB, 15 min	Wang et al. (2007b)

BBN, broadband noise; OBN, octave-band noise.
[a] Also intended to de-sensitize OHCs.
[b] Added benefit from combination.

Table 3- Therapies shown to be successful against NIHL 2005-2008 from Ohlemiller (2008)[97].

A recent review of the findings in treating NIHL in animal models summarised studies that investigated the protective ability of a wide variety of compounds against permanent NIHL[97] (Table 3). This review summarised the different types of pharmacological therapies that were protective against NIHL. The treatments were predominantly aimed at decreasing the amount of oxidative damage caused by reactive oxygen species (ROS) that are produced as a consequence of NIHL, or to pre-condition the hair cells against further damage. Therapies included antioxidants[44,67,92,93,95,98-105], growth factors, anti-apoptotics[106,107], calcium channel blockers[108], energy enhancers[106] and less than half a dozen published papers have used a combination of these. The ultimate aim being that a combination of treatments may be the perfect defensive mechanism to protect against metabolically driven acoustic trauma causing noise-induced hearing loss in humans, assuming they can ultimately be delivered orally. There has been a growing amount of interest into researching therapies to protect and/or treat NIHL in animal models with the aim of successful translational application to humans.

Further to this review, I carried out a more recent review of the literature using PubMed. A search was carried out using pubmed with keywords "noise-induced hearing loss" and "antioxidant". There were 195 search results in December 2015. I reviewed all 195 results and identified 77 suitable publications that identified antioxidants that had been used in pre-clinical (animal) models of NIHL. The findings are summarised in Appendix 1. The first identified antioxidant that was used to mitigate the effects of NIHL was glutathione in a guinea pig model in 1998. Glutathione was an obvious agent to start with because it is the major endogenous antioxidant within the inner ear. There were only a handful of studies prior to 2003 and after that point in time there was a growth in otoprotective antioxidant treatments. Reviewing these studies there is no set standard for subject strain, noise exposure, method of exposure, duration of noise exposure, method of administration of antioxidants, duration of administration of antioxidants or type of hearing test carried out. It clearly demonstrates the vast diversity in this field with a lack of comparable standards between studies.

From the review by Ohlemiller et al and my review of the literature, I have come to the following conclusions about pharmacological treatments for NIHL:

- Very few papers have studied the inhibition of nitrosative stress alone, often using generalised free radical inhibitors.
- The research papers that have studied nitrosative stress inhibition have mixed and conflicting evidence regarding their efficacy (see Chapter 3 for more information).
- Very few studies have tried combination pharmacological therapies against NIHL – there are a total of five papers that have used combination therapies.

1.4- Summary:

Hearing loss is one of the most common and significant conditions in humans, however, the morbidity and disability associated is often both under recognised and undertreated even by health professionals. NIHL is often overlooked as a major cause of hearing loss, but is the single largest industrial injury in the world. Hence, the health burden associated with NIHL is considerable but often underestimated. This coupled with a general increasing of an aging population and the subsequent confounding of presbycusis, that will also arise as a direct consequence of increased life expectancy, will lead to an increase in the overall burden of hearing loss. The military impact of NIHL is well documented and is a very complex problem to address. As a consequence there is a need for comprehensive hearing research programs in these types of fields. Research goals should mainly include prevention of hearing loss, but that does not mean treatment goals should be overlooked. Thus the field of NIHL research should aspire to expand in the fields of prevention, otoprotection and repair/regeneration.

Chapter 2: Methods

2.1- Introduction:

This chapter documents the generic methods that were used for chapters 3, 4 and 5. Specifics for each chapter are included in the relevant sections.

2.2- Experimental design:

All experiments were approved and carried out under the supervision of the Animal Ethics Committee (AEC) at the Garvan Institute of Medical Research, Sydney, Australia.

2.2.1- Animals:

Multiple different studies have used animal models to highlight the potential role of antioxidants and membrane stabilisers as a therapeutic agents that can mitigate the deleterious effects of noise-induced hearing loss[44,67,92-95,97-107,118]. We chose mice because they are a well-studied analogous model for NIHL research when compared to human hearing. Both the structure of the murine and human cochlea are similar macroscopically/histologicallly and show a similar tonotopic arrangement[140]. Many genes in mice have been identified in humans in association with hereditary hearing loss[141-144]. The frequency range of mouse hearing varies from 1 kHz- 71 kHz (most sensitive from 4-24 kHz), while human hearing ranges from 20 Hz-20k Hz (most sensitive 1-4kHz)

There are documented gender variations to the susceptibility of animals to oxidative stress[145]. Julicher et al demonstrated that male rats have increased rates of ROS damage, decreased ROS detoxification and greater vulnerability to NIHL damage probably secondary to a difference in

hepatic enzymes when compared to their female counterparts[145]. We decided to select only male mice because of these known gender variations relating to rates of ROS damage, rate of ROS detoxification and vulnerability to NIHL[145-147]. We used mice that were aged between 8-16 weeks as cochlear electrical potentials first appear on the eighth day after birth and gradually increase till the fourteenth day when the responses reach adult values[148]. We decided to use the CBA strain for this research; CBA mice have previously been well documented to have good hearing throughout their natural life and do not suffer from presbycusis until late in life around the age of 60 weeks[149]. Zheng et al. tested hearing in eighty different strains of mice and CBA mice were used as the gold standard that all other mice were compared against[150], this work was carried out the Jackson laboratory (JAX) which is an independent non-profit biomedical research institution that acts as a worlds source for more than 7,000 strains of mice. The response of the CBA strain mice to acoustic trauma over time is well documented and the types of thresholds that are achieved[154]. We used the CBA/CaH substrain (all animals were supplied from Australian BioResources Ltd, Moss Vale, NSW, Australia), the CaH sub-strain is one that has been imported to Australia by the Garvan Insitute from JAX and further information about this sub-strain is available at https://www.jax.org/strain/000655. Only male-pigmented mice were used in our experiments because of the known vulnerability of albino mice with increased susceptibility to acoustic trauma[151].

2.2.2- Experimental groups:

The mice were randomised into their groups, please see specific chapter for information pertaining to each group. All groups received once daily treatments via intra-peritoneal (IP) injection. The treatment started one week before acoustic trauma and continued until one week after acoustic trauma, totalling fourteen days of treatment per mouse. Treatment was given for

seven days after acoustic trauma because previous research has shown that exposure to acoustic trauma causes an increase in metabolic activity in the inner ear, with an initial peak in free radical production during the acoustic trauma (within hours) followed by a secondary peak at 7 to 10 days after the trauma[36] and this treatment duration would coincide with this second peak. Treatment was initiated seven days prior to acoustic trauma to allow suitable time for treatment concentrations to reach a sufficient level.

2.2.3- Acoustic trauma method:

All subjects were anesthetised (using a combination of ketamine and xylazine, 75mg/kg and 15mg/kg respectively) and exposed to broadband noise between 4-32kHz (delivered by ES1 free field speakers, Tucker-Davis technologies, Alachua, FL, USA) for 2 hours in a ventilated sound chamber. Please see the relevant chapter for information regarding the sound output of the acoustic trauma; this was either 90 or 120dB dependant on each individual experiment. (Figure 4).

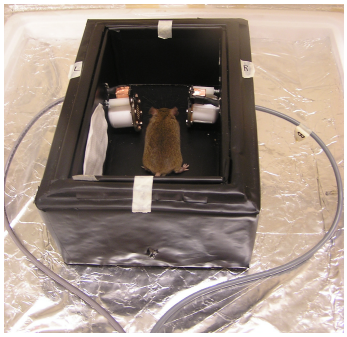

Figure 4- Ventilated chamber for acoustic traumatisation with ES1 speakers.

2.2.4- Auditory Brainstem Responses:

NIHL was confirmed with auditory brainstem responses (ABR). ABR thresholds were determined using tone pips from the right ears of all subjects at three different time points (Figure 5). The initial ABR was recorded before commencing treatment and provided a baseline ABR (t=-7 days). The second ABR was recorded one week after the deafening procedure (t=7 days) to confirm a temporary threshold shift (TTS). The third ABR was carried out one month after the initial ABR (t=28) to confirm a permanent threshold shift (PTS).

For ABR testing, all animals were anesthetised with a combination of IP ketamine (75 mg/Kg) and xylazine (15 mg/Kg). Mice had core temperature maintained at 37^{o}C +/- 0.5^{o}C using a thermal heat pad. The external auditory canal was inspected to ensure the tympanic membrane was intact, and to ensure a lack of effusion, congenital deformity and wax. Electrodes were inserted on the vertex, and below the test ear with a grounding electrode placed below the contralateral ear (Figure 6). Frequencies tested were at 8, 16, 24 kHz (5 ms duration, 0.5 ms rise-falls, 30/second for an average of 512 samples). All signals were generated with equipment from Tucker-Davis Technology (TDT, Alachua, FL, USA); TDT system hardware (RP2.1, PA5, MA3, SA1) and software (SigGen 3.2). Signals were presented with an open field speaker (delivered by ES1 speaker, TDT) and the counter lateral external auditory canal was blocked with an ear plug. Sounds were presented at 90 dB baseline, and then incrementally decreased at 5 dB intervals till 10 dB was reached. All hearing tests were performed in the hearing-testing Laboratory at the Garvan Institute.

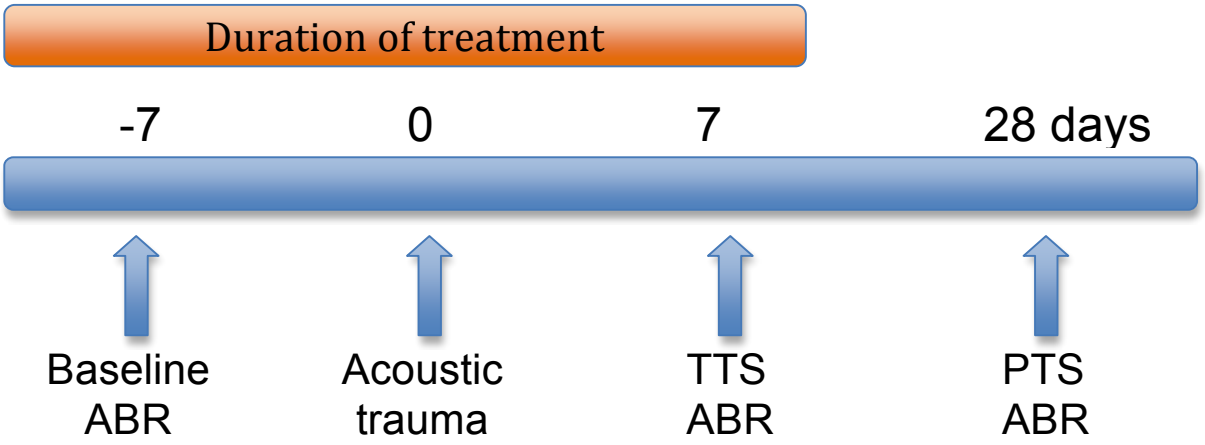

Figure 5- Frequency of ABR testing and acoustic trauma.

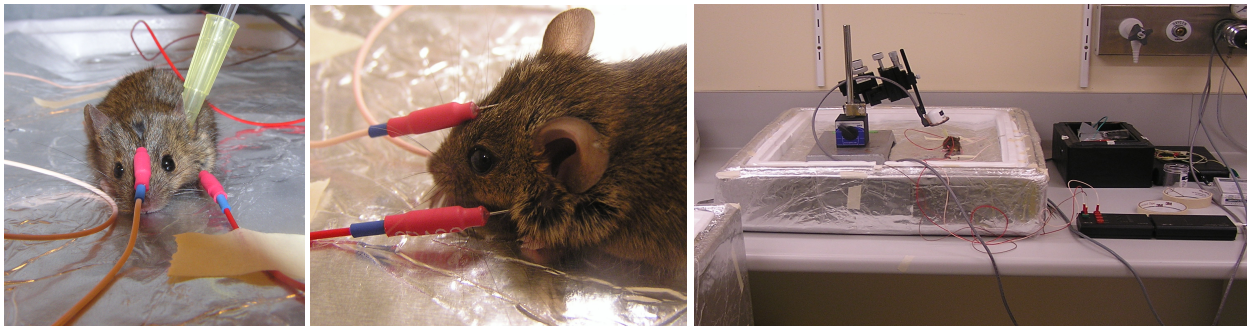

Figure 6- Positioning of mice and electrodes for ABR testing within acoustic apparatus.

2.2.5- ABR statistical analysis:

ABR analysis determines the sound intensity at which a neural response first appears (hearing threshold). Traditionally, threshold has been assessed by visual estimation of a series of ABRs evoked by different sound intensities. All ABR thresholds were analysed by visual inspection and by automated threshold detection as described by our laboratory[152] using Axograph software (Figure 7). Automated detection was carried out to remove any investigator bias when analysis of ABR thresholds was carried out.

We have previously developed, tested, validated and published this software in 2009[152]. It is available, as open source software to be used inside the Axograph program and subsequently has

been used in laboratories all over the globe, including at Oxford and in Montreal. The automated method is a robust computational procedure that detects the sound level at which the peak amplitude of the evoked ABR signal first exceeds four times the standard deviation of the baseline noise. The automated detection method avoids the subjectivity of visual analysis and offers a rapid, easily accessible approach to measure hearing threshold levels in ABR testing.

All data values in the text and figures are expressed as means±SEM; all statistical comparisons were performed using Graphpad Prism® software. Statistical reliability of group differences in ABR thresholds and threshold shifts were tested using ANOVA (Dunnets post-hoc testing).

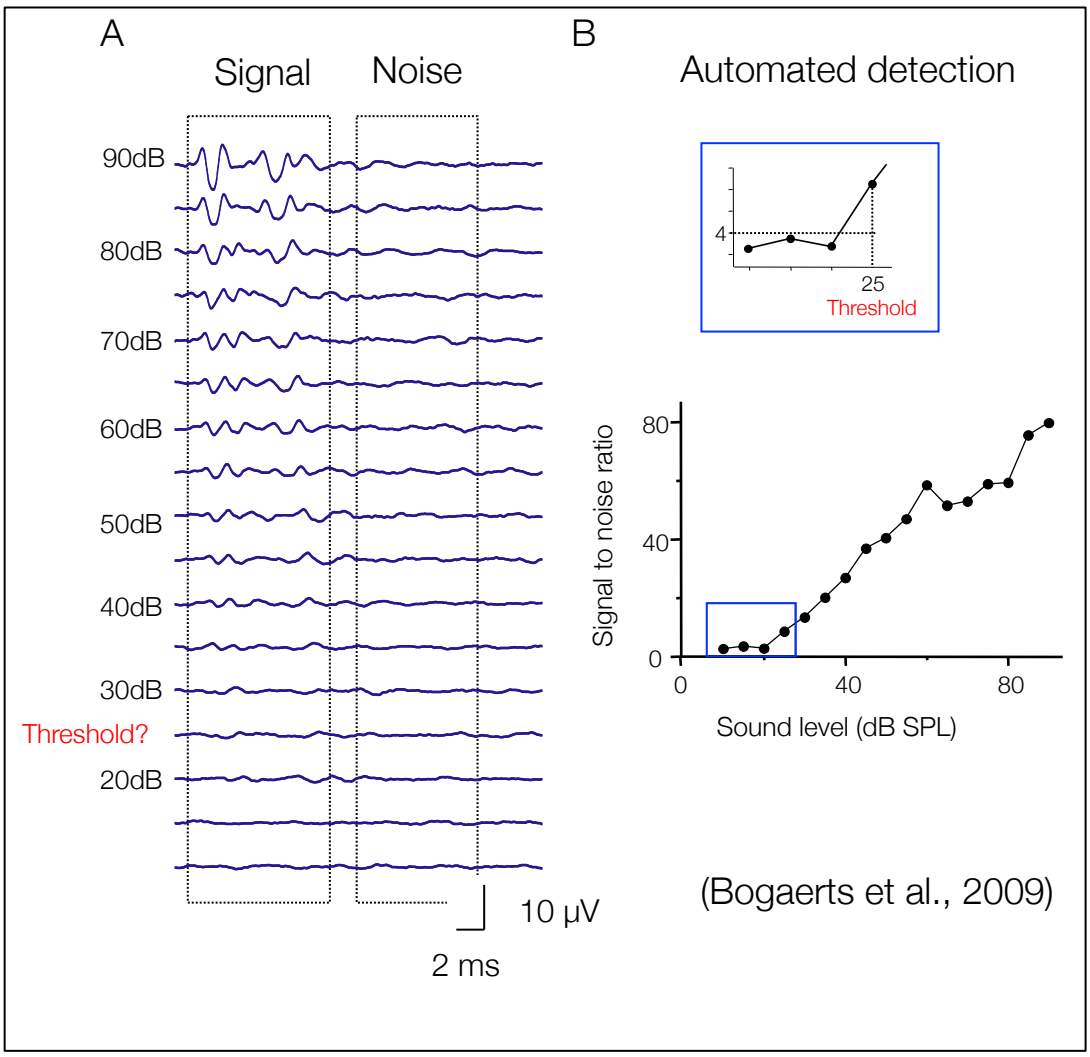

Figure 7- ABR threshold analysis. Modified from Bogaerts et al., 2009[152] with permission.

2.3- Summary:

This section relates to generic methodology in our experiments, however specifics of each experiment are in the relevant chapters.

Chapter 3: Treatment for Noise-induced Hearing Loss with Magnesium, Taurine and Manganese TBAP

3.1- Introduction:

Deafness is a widespread sensory disability throughout the world, often neglected due to the relative invisibility of its nature. Deafness impairs communication, contributes to social isolation and may impact upon mental health. Noise-induced hearing loss (NIHL) is the second largest cause of hearing loss. The principal pathological cause of NIHL was previously thought to be disruption of the cellular structures within the cochlea caused by the mechanical force of acoustic trauma[22-30]. There is well documented evidence of prolonged noise exposure causing tearing of the tectoral membrane, detachment of the basilar membrane[22,23,31-33], hair cell loss and loss of synaptic afferents[34,35] (see chapter 1 for further detail). Evidence of cochlear vasoconstriction has been documented as also being caused by acoustic trauma[41,43,109-111]. However, it was not until the mid-nineties that two groups independently demonstrated that the production of free radicals[36-40] to be deleterious to hearing. Free radicals overwhelm the natural antioxidant defence mechanisms of the cochlea and supporting cells. Over the past 10 years, there has been a rapid growth in interest a propos the role of free radical related damage within the cochlea and if inhibition of free radicals could be a potential therapy for NIHL[15,67,77-96,112-115].

A free radical is defined as any molecule or ion with a lone electron. The presence of unpaired electrons renders such radicals highly reactive, so they normally occur only as transient intermediates in reactions[64] and have the ability to cause profound cellular damage. Free radicals are products of normal cellular metabolism[59] and can be subdivided into reactive nitrogen species (RNS) and reactive oxygen species (ROS), each containing a central nitrogen or oxygen atom, respectively. The most common RNS is the nitric oxide radical (NO•) and the most

common ROS is the super oxide radical (O_2•) in humans. RNS and ROS cellular damage is termed nitrosative and oxidative stress respectively[61,116,117].

At low to moderate levels, free radicals are beneficial, even essential, to the existence and functioning of cells. For example, they are used in cell signalling processes (termed redox signalling), immune responses and mitogenic responses. However, they can be deleterious in excess, causing damage to tissues and hence a balance is needed[59,65]. Animals have naturally occurring antioxidants that act as endogenous defence systems to protect against the adverse effects of increased numbers of free radicals. These defence systems can become saturated and fail when bombarded with excessive free radicals.

A recent review of the findings in otoproection related to NIHL within animal models demonstrated the protective ability of a wide variety of pharmacological treatments against permanent NIHL[97] (Table 3, see chapter 1). The treatments were predominantly aimed at decreasing the amount of oxidative damage caused by reactive oxygen species (ROS) that are produced as a consequence of NIHL, or at pre-conditioning the hair cells against further damage. Therapies included antioxidants[44,67,92-95,98-102,105,106,118], growth factors[119-121], anti-apoptotics[106,107], calcium channel blockers[108] , energy enhancers[106] and a combination of these therapies. There is hope that some combination of treatments may be the perfect defensive mechanism against metabolically-driven NIHL in humans, assuming such treatments can ultimately be delivered orally.

From this review and the literature I have reviewed, I have come to the following conclusions about pharmacological treatments for NIHL:

- There is minimal research on the inhibition of nitrosative stress alone as treatments often used generalised free radical inhibitors.

- The research papers on nitrosative stress inhibition have mixed and conflicting evidence regarding their efficacy.
- Very few studies have tried a combination of pharmacological therapies against NIHL. Currently only 5 studies in the literature have used a combination of therapies to prevent or treat against the effects of NIHL.

This chapter demonstrates our preliminary work in which we identified multiple otoprotective agents that act on the different pathological processes arising from acoustic trauma. This work has not been previously published, but has been presented at international meetings.

3.1.1 Magnesium:

Magnesium (Mg^{2+}) is the 11th most abundant element in the human body. Mg^{2+}ions are essential to all living cells, as they manipulate important biological compounds and aid in the function of hundreds of enzymes. Mg^{2+}has previously been shown to be protective to hearing including gunshot noise trauma in guinea pigs[122,123] and prolonged acoustic trauma in guinea pigs[44].

Magnesium compounds are used medicinally as laxatives, antacids (e.g., milk of magnesia), for nerve stabilization of abnormal nerve excitation (e.g. treating eclampsia) and as a life saving measure in acute severe asthma. Low levels of magnesium in the body have been associated with the development of a number of human illnesses such as asthma, diabetes, and osteoporosis. The adult human daily nutritional requirement, which is affected by various factors including gender, weight and size, is in the region of 300-400 mg/day. We decided to administer an intra-peritoneal Mg^{2+}in form of magnesium sulphate at a dose of 343

mg/Kg/day (Volume=0.2 ml) for a two-week period as this dose was used in a previous study to ameliorate the effects of acoustic trauma[44].

3.1.2 Taurine:

Taurine, (2-aminoethanesulfonic acid), is one of the few known naturally occurring sulfonic acids (Figure 8). It is present in food, especially abundant in seafood and meat, but can be synthesised from cystine within the pancreas[122]. Taurine is a major constituent of bile and can be found in the lower intestine and, in small amounts, in the tissues of many animals, including humans[122]. Taurine is a metabolic product of sulphur containing amino acids and it is biosynthesised from cysteine in the liver[123]. It has a multitude of functions including: constituent of bile salts, neurotransmission[124], membrane stabilisation[125], immune response[126], calcium homeostasis[127], and protection from glutamate excitotoxicity[128]. Recently, cosmetic compositions containing taurine have been introduced for aesthetic treatments. Taurine is an antioxidant that acts as a potent NO• scavenger[129-135] and to protect against oxidative and nitrosative stress[129-135]. Liu et al 2008 showed that taurine administered to healthy guinea pigs prevented hearing impairment as a consequence of aminoglycoside induced hearing damage[134]. Since taurine has not been investigated in a murine model of NIHL, we investigated whether taurine supplementation can mitigate the effects of acoustic trauma in a mouse model of NIHL. Studies have reported no significant side effects with taurine supplementation. A 2003 study by the European Food Safety Authority found no adverse effects for up to 1,000 mg of taurine per kilogram of body weight per day[123]. We decided to administer an intra-peritoneal dose of taurine of 400 mg/Kg/day (administered in a volume=0.2ml) for a two-week period[134].

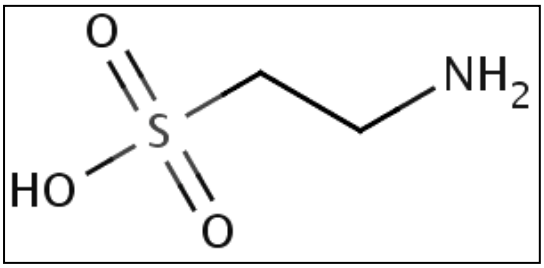

Figure 8- Structure of Taurine.

3.1.3 Manganese T BAP:

Manganese T BAP (MnTBAP), chemically known as manganese 5,10,15,20 tetrakis (4-benzoic acid) porphyrin (Figure 9), is a mitochondrial penetrating super oxide scavenger that works as a superoxise dismutase mimetic and a peroxynitrile scavenger, **but** does not scavenge the nitric oxide radical (NO•). Superoxide dismutases (SOD) are metalloenzymes that catalyse the conversion of superoxide radical (O_2•) to oxygen (O_2) and hydrogen peroxide (H_2O_2). Therefore SOD play a crucial role in protecting naturally occurring biological systems against damage generated from deleterious free radicals. They are essential for defending against O_2• that is a byproduct of the mitochondrial electron transport chain.

Mutations in genes associated with SOD have been associated with cardiomyopathy, motor neuron disease and cancer. Mice that lack SOD die shortly after birth, this is in keeping with the great importance that SOD plays within mammalian life[136]. Previous work at the Garvan Institute of medical research has shown that MnTBAP can protect against oxidative stress and the formation of insulin resistance in diabetes[137]. Since oxidative stress has been implicated as an important mechanism underlying noise induced hearing damage[97], we decided to investigate if MnTBAP could protect against NIHL. The effect of MnTBAP on NIHL has not been trialed previously and MnTBAP could act as a novel protective compound. There is no evidence of known MnTBAP toxicity in 279 research papers worldwide.

We administered an intra-peritoneal dose of MnTBAP of 15mg/Kg/day (Volume=0.2ml) for a two-week period as used by Hirschberg et al in 2010[138] and Adeagbo et al in 2005[139] in their studies, neither of these studies were related to hearing.

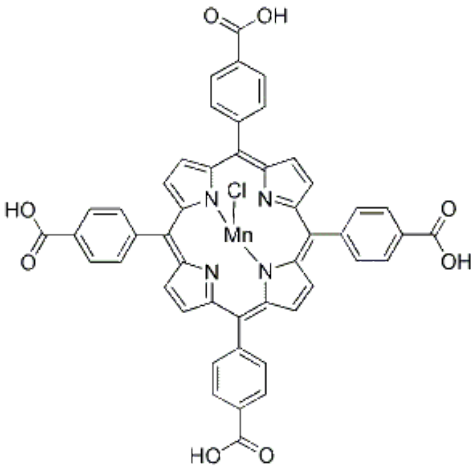

Figure 9- Structure of Manganese T BAP.

3.2- Aim:

Our aim was to identify if there was a role for our agents to mitigate the effects of acoustic trauma in a mouse model of NIHL. Briefly, magnesium is a membrane stabiliser and antagonises calciums excitotoxicty, taurine is a specific inhibitor of NO• and prevents further propagation of down stream metabolites which can cause significant DNA and cellular damage and MnTBAP is a mitochondrial penetrating super oxide scavenger that works as a superoxise dismutase mimetic and a peroxynitrile scavenger (Figure 10).

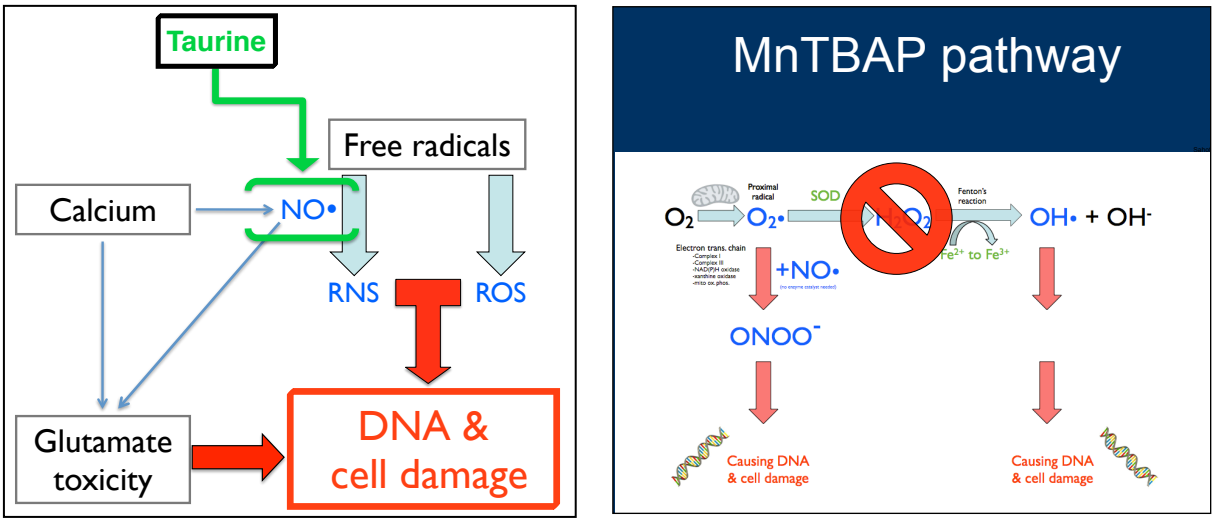

Figure 10- Action of taurine as a NO• scavenger, and action of MnTBAP (SOD)

3.3- Experimental design:

All experiments were approved and carried out under the supervision of the Animal Ethics Committee (AEC) at the Garvan Institute of Medical Research, Sydney, Australia. See chapter 2 for generic experimental details.

3.3.1- Experimental groups:

The mice were randomised into four different groups, we used the website randomizer.org to generate each mouse into groups. All groups received once daily treatments via intra-peritoneal (IP) injection. IP injections were administered by the author into the peritnem and rotated from left to right on a daily basis in each subject. The treatment started one week before acoustic trauma and continued until one week after acoustic trauma, totalling fourteen days of treatment per mouse. Treatment was given for seven days after acoustic trauma because previous research has shown that exposure to acoustic trauma causes an increase in metabolic activity in the inner ear, with an initial peak in free radical production during the acoustic trauma (within hours) followed by a secondary peak at 7 to 10 days after the trauma[36] and this treatment duration

would coincide with this second peak. Treatment was initiated seven days prior to acoustic trauma to allow suitable time for treatment concentrations to reach a sufficient level.

Group 1 compromised of the control animals (n=5) received 0.9% saline (0.2ml, IP daily). Group 2 received magnesium (n=3) in the form of magnesium sulphate of a dose of 343mg/kg/day in 0.2ml sterile water. Group 3 were administered taurine (n=5) at a dose of 400 mg/Kg in 0.2ml sterile water. Group 4 were treated with Manganese T-Bap (n=5) with a dose of 15mg/kg in 0.2ml sterile water (Figure 11). All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich, PO BOX 970, Castle Hill, NSW, 1765, Australia).

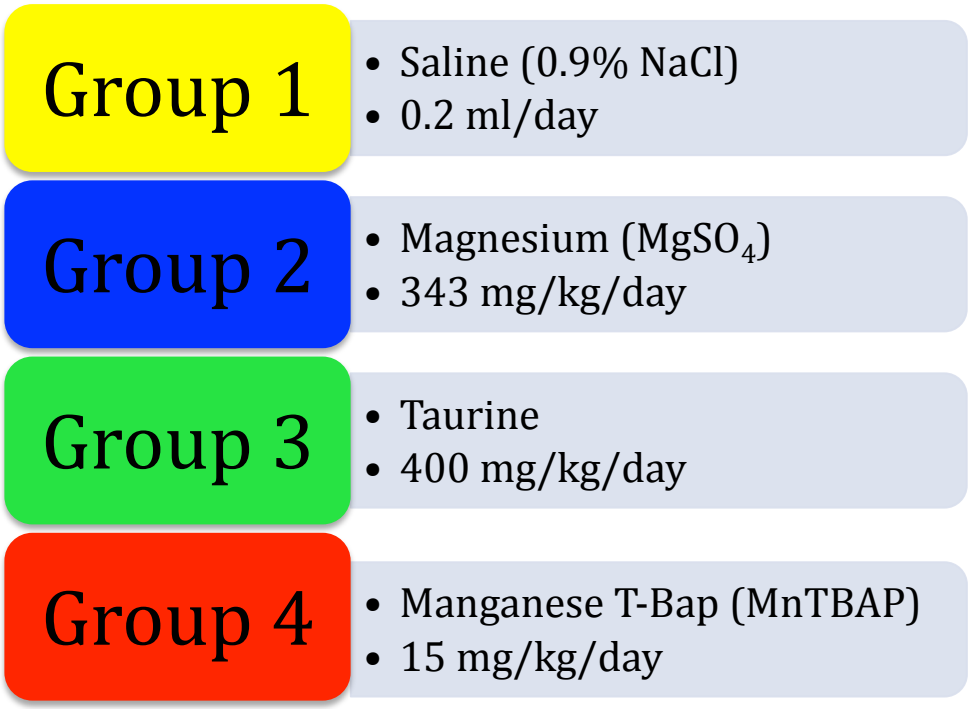

Figure 11- Schematic of experimental groups.

3.3.2- Acoustic trauma method:

Broad-band noise was delivered between 4-32kHz (delivered by ES1 free field speakers, Tucker-Davis technologies, Alachua, FL, USA) at **90 dB** for 2 hours in a ventilated sound chamber, see chapter 2 for further information.

3.4-Results:

3.4.1- Auditory brainstem responses:

Neither TTS nor PTS ABR responses were statistically improved in any treatment group when compared to saline controls (Figure 12). The lack of statistical difference may be due to the small numbers of mice in this preliminary study or due to problems associated with the small threshold shifts that arise as a consequence of 90dB trauma for only 2 hours. We decided not carry out labour intensive immunohistological analysis of the mice cochleae due to the lack of significant differences in the ABR responses. Our future experiments were modified by increasing the number of mice in each limb of treatment and by increasing the amount of acoustic trauma from 90 dB to 120 dB for 2 hours to establish larger threshold shifts (please see chapter 3 for further information).

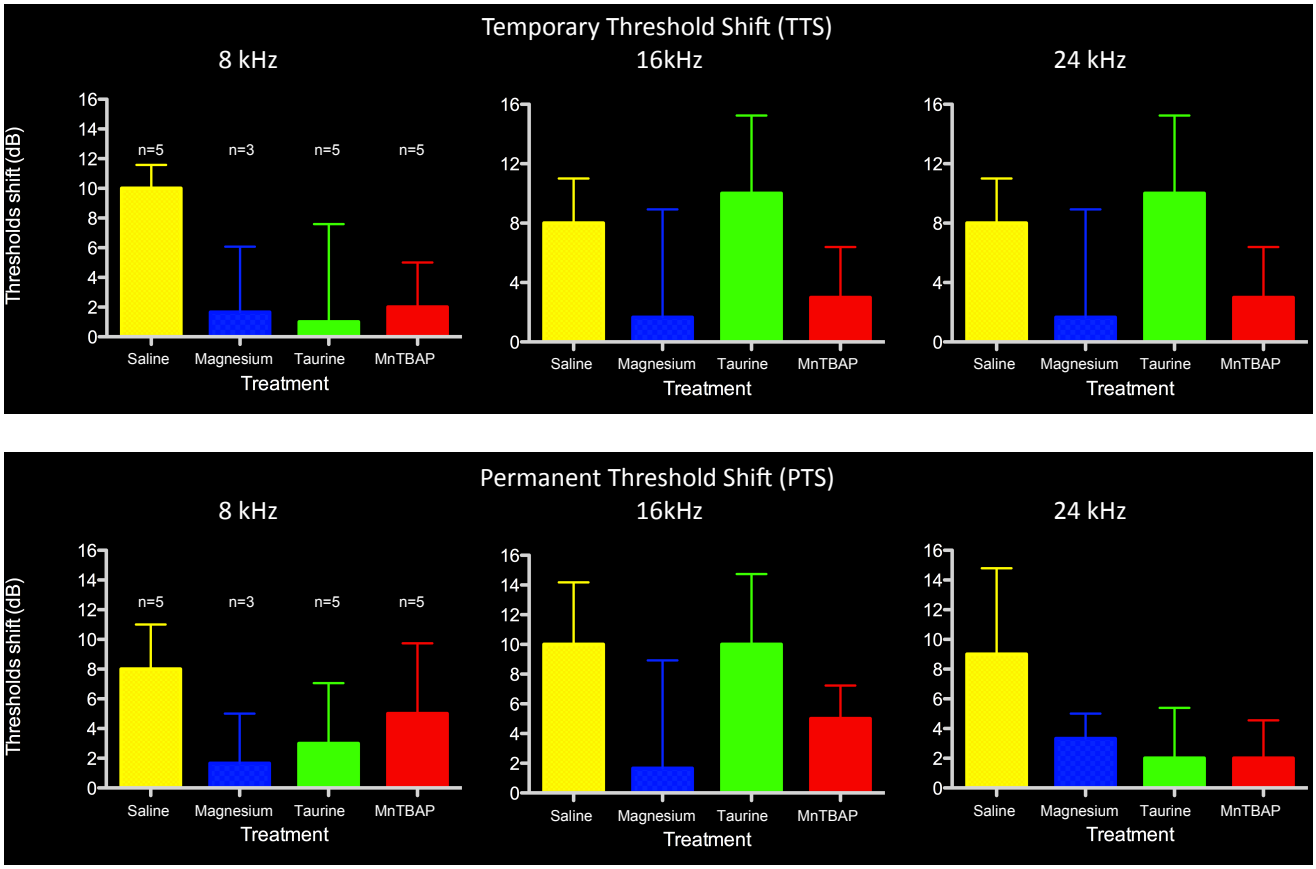

Figure 12- There was no significant statistical difference between any group and saline controls in either TTS or PTS when traumatised at 90dB for 2 hours**.** Temporary Threshold Shifts (TTS) represent the difference between ABRs measured 7 days after acoustic trauma compared to baseline ABRs measured prior to acoustic trauma. Permanent Threshold Shifts (PTS) represent the difference between ABRs measured 28 days after acoustic trauma compared to baseline ABRs measured prior to acoustic trauma. Statistical analysis was carried out via ANOVA (Dunnets post-hoc testing.

3.4.2- Results summary:

- There was no statistically significant difference between groups treated with magnesium, taurine or MnTBAP when compared to saline control animals when acoustic trauma was delivered at 90 dB for 2 hours. However, these data helped to identify areas of improvement in the experimental paradigm for further experiments, please see chapter 4 for more information.

3.5- Discussion:

These data have allowed our research group to advance our future experiments in this field. Modifications in our experimental paradigm were a direct consequence of these data. Modifications regarding acoustic trauma arose due to the low level of acoustic trauma, a level lower than what others have achieved with similar paradigms[44,93]. There is a significant variation when reviewing sound trauma in published work (see chapter 1). The variations include sound intensity, duration, method of delivery, and delivery format (impulse or continuous noise). There is no set gold standard or standard technique for acoustic trauma in animal models. A reason for this could be due to the simple fact there is no one single method that is representative of all types of noise trauma. There is a significant variation of the type of trauma that you would protect against with otoprotective agents. An example for this wide variation would be industrial

injury versus firearms, these two methods are both causative in NIHL, but the type of sound trauma that causes the acoustic injury is extremely different. In industry the type of sound tends to be continuous at certain intensity and therefore the damage is a cumulative effect of the intensity of the sound and the time exposed (an example being 100 dB for 8 hours in a factory). Comparatively to firearms, these tend to be rapid impulses of sound for a fraction of a second, but the sound intensity tends to be significantly higher (156 dB as an impulse burst for an M16 rifle). In our experiments, sound was delivered with anesthetised mice at 90 dB for 2 hours. We presented these data at the Australian Neuroscience Conference and it was suggested to increase the intensity of the sound exposure, meaning that we would have larger threshold shifts and there would be a larger amount of damage to the inner ear. It was suggested we should carry out histological analysis of the cochleae using cytoccochleogram analysis. These pilot data were fundamental for improving our experimental paradigm and for refining our future hypotheses, leading to the work that comprises Chapter 4.

Chapter 4: Taurine treatment for Noise-induced Hearing Loss

4.1- Introduction

From the work presented in Chapter 3 we identified agents that could act as otoprotective agents in a murine model. These experiments helped highlight how to modify and improve the experimental paradigm. There were three significant modifications to the paradigm. Firstly we used one agent, taurine, allowing us to look more closely at the damage caused by a RNS. Taurine showed significant potential and has not been used previously in the literature against the deleterious effects of NIHL. Secondly we used differing doses of taurine in an attempt to identify if there was a dose dependant relationship to the effects of taurine. Finally, we increased the intensity of the acoustic trauma from 90 dB up to 120 dB. We found that the threshold shifts described in Chapter 3 were small and we wanted to deliver a level of acoustic trauma that is more realistic to the dangerous levels experienced by humans. We kept the duration of noise exposure at 2 hours. This chapter describes our findings relating to the use of taurine to mitigate the effects acoustic trauma in a murine model.

4.1.1 Taurine

As described in Chapter 3, Taurine, (2-aminoethanesulfonic acid), is one of the few known naturally occurring sulfonic acids. Taurine is well documented to protect against oxidative and nitrosative stress[129-133]. Liu et al 2008 showed that taurine administered to healthy guinea pigs prevented hearing impairment as a consequence of aminoglycoside induced hearing damage[134]. Since taurine has not been investigated in a murine model of NIHL, we investigated whether taurine supplementation can mitigate the effects of acoustic trauma in a mouse model of NIHL.

4.2- Aim:

Our aims were two fold: (1) to identify if there was a role of taurine to mitigate the effects of acoustic trauma in a mouse model of NIHL, and (2) to establish if there was a dose dependant response to any effect observed.

Taurine is a specific inhibitor of the nitric oxide radical and prevents further propagation of down stream metabolites that cause DNA and cellular damage leading to the deleterious effects of NIHL (Figure 13). We hypothesised that taurine would mitigate the deleterious effects of NIHL.

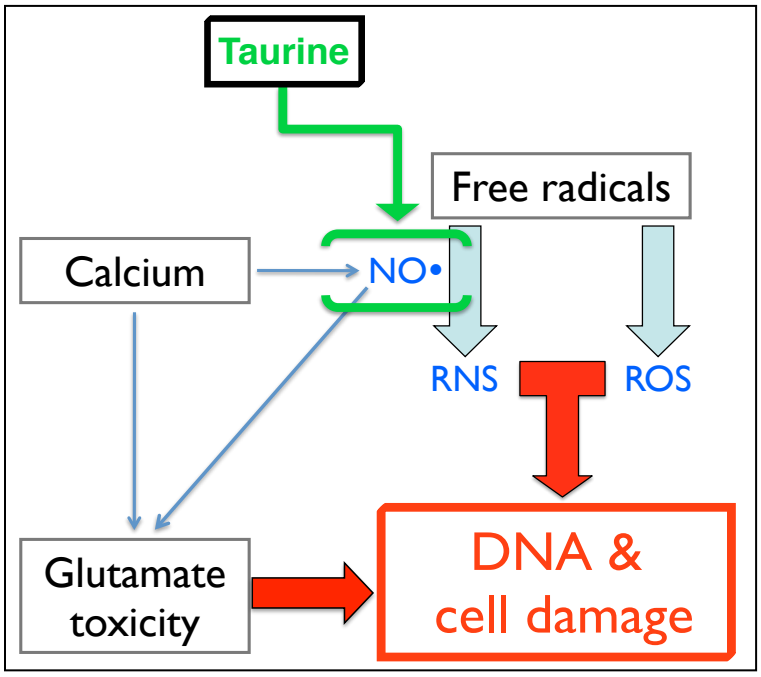

Figure 13- Action of taurine as a NO• scavenger.

4.3- Experimental design:

See chapter 2 for generic methodology, specific details to this experimental paradigm are included here.

4.3.1- Animals:

Recent studies highlight that antioxidants are beneficial against the occurrence of noise induced hearing loss in mice[44,67,92-95,97-107,118]. Mice are an excellent analogous model of hearing for NIHL research compared to human hearing because they have similar macroscopic/histological cochlea structure and genes in mice have been identified in humans[141-144,153]. Also the frequency range of mouse hearing is from 1kHz- 71kHz with greatest sensitivity from 4-24kHz, compared to the human hearing range of 20Hz-20kHz (greatest sensitivity from 1-4kHz)

There are known gender variations in the damage caused by oxidative stress in rats[145]. Julicher et al demonstrated that male rats have increased rates of ROS damage, decreased ROS detoxification and greater vulnerability to NIHL damage probably secondary to a difference in hepatic enzymes when compared to their female counterparts[145], see chapter 2 for more information. Therefore only male pigmented mice (aged 4-10 weeks initially, weighing average 24.5gm (range 21.9-26.9gm) were used in these experiments.

4.3.2- Experimental groups:

The mice were randomised into five different groups. All groups received once daily treatments via intra-peritoneal (IP) injection. The treatment started one week before acoustic trauma and continued until one week after acoustic trauma, totalling fourteen days of treatment per mouse. Control animals (n=10) received 0.9% saline (0.2ml, IP daily). Groups 2-5 (n=10/group) received taurine (2-aminoethanesulfonic acid), with different doses given to each group (50, 100, 200, 400 mg/Kg in 0.2ml sterile water, IP daily) (Figure 14). Taurine was purchased from Sigma-Aldrich (Sigma-Aldrich, PO BOX 970, Castle Hill, NSW, 1765, Australia). Doses were selected due to the normal dietary intake in humans and sequentially doubled. IP route was

chosen to remove issues relating to first pass metabolism. Treatment was given for seven days after acoustic trauma, see chapter 2 for more information.

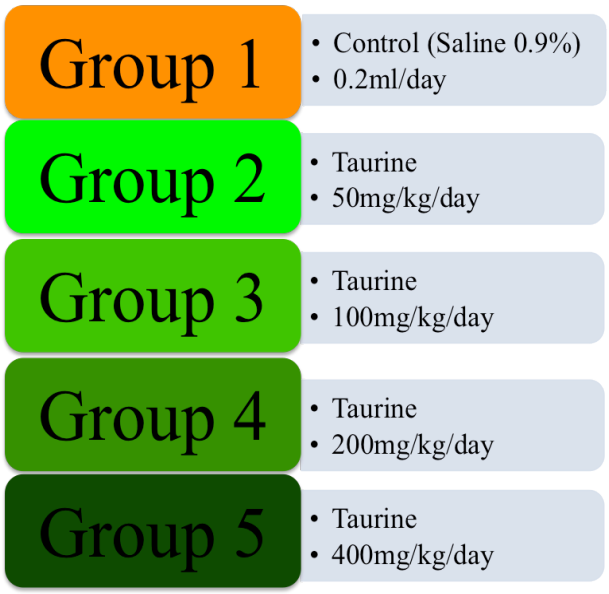

Figure 14- Schematic of experimental groups.

4.3.3- Acoustic trauma method:

Noise exposure was broad-band noise between 1-32kHz (delivered by MF1 free field speakers, Tucker-Davis technologies, Alachua, FL, USA) at 120 dB for 2 hours in a ventilated sound chamber. (Figure 4, see chapter 2).

4.3.4- ABR statistical analysis:

All ABR readings were analysed with the automated threshold detection as described by our laboratory[152] using Axograph software and thresholds were confirmed with a visual inspection method (Figure 7, see chapter 2). This was carried out to remove any investigator bias when analysis of ABR thresholds was carried out. All data values in the text and figures are means±SEM; all statistical comparisons were performed using Graphpad Prism® software.

Statistical reliability of group differences in threshold and threshold shift were via ANOVA (Dunnets post-hoc test). More information pertaining to this method is in chapter 2.

4.3.5- Histological analysis:

After the final ABR recording, the mice were sacrificed with decapitation under deep anaesthesia for cytocochleogram analysis. Noise exposed cochleae were dissected into cold PBS, the round and oval windows were opened and a small hole fashioned in the apex. The cochleae were perfused through the oval and round window with succinate dehydrogenase (SDH) and Nitro Blue tetrazolium (NBT) solution prepared immediately before dissection (0.2 M PBS; 0.2 M SDH, 0.2 % NBT) and incubated in the same solution at 37^{o}C for 1 hour to develop the reaction product. The cochleae were washed thoroughly in PBS before being fixed overnight in 4 % PFA in PBS at 4^{o}C. Cochleae were decalcified in 10 % EDTA in PBS for 2 days at 4^{o}C. Whole-mount dissections were prepared for cytocochleograms. The sections were viewed using a Zeiss Axioplan epifluorescence microscope equipped with Plan-Neofluar 10x0.30 NA and Plan-Neofluar 20x0.50 NA dry objective lens and an AxioCam MRm digital camera (zeiss, Germany). Hair cells (HC; inner & outer) were counted every 250 mm from the apex to the base using light microscopy. Alexander Borecki (research assistant) manually counted the HCs, he was blinded to the treatment that each mouse received. Results were plotted as a percentage of total hair cell loss as a function of percentage distance from the cochlear apex. A cochlear frequency place-map ($d(\%) = 156.5 - 82.5 \times \log(f)$ where d is the percentage distance from apex and f is frequency in Hz) was used to evaluate hair cell loss at specific frequencies[155]. Inner and outer hair cell losses were compared via ANOVA.

4.4-Results:

4.4.1- Auditory brainstem responses

The original experimental design from our initial project (see chapter 3) lead to several modifications including modification to the acoustic trauma to allow for larger threshold shifts to be achieved. All ABR readings were smaller for PTS than TTS, as expected, demonstrating that some cochlear damage was reversible TTS ABR results were improved and statistically significant ($p<0.05$) for every treatment dose group when compared to saline controls, with the exception of the 100mg dose at 8kHz tone testing (Figure 15). PTS thresholds were smaller in all taurine treated groups at 16 kHz when compared to saline controls. Taurine groups treated with 50, 200 and 400 mg/kg demonstrated improved TTS ABR thresholds when compared to saline controls in all groups. Prevention was essentially equivalent at all doses of taurine tested for TTS. Interestingly, only a taurine dose of 200mg for 8kHz testing during PTS was statistically significant, all other doses were lower than the control group but had $p>0.05$ (ANOVA, Dunnett's post-hoc test). All doses of taurine at 16 kHz for PTS decreased threshold shifts for PTS. At 24 kHz, all groups apart from the 100 mg dose were effective in attenuating ABR thresholds. One of the reasons for this difference in statistical results maybe due to the fact that two mice passed away for non-experimental reasons and therefore there are only results for 8 mice in this group. Figure 15 demonstrates Temporary Threshold Shifts (TTS) with (A) representing the difference between ABRs measured seven days after acoustic trauma compared to baseline ABRs measured prior to acoustic trauma. Permanent Threshold Shifts (PTS) and (B) representing the difference between ABRs measured 28 days after acoustic trauma compared to baseline ABRs measured prior to acoustic trauma. Asterisks indicate statistically significant results between treatment group and control group ($P<0.05$).

Our results show that taurine provides some protection against NIHL as it decreases hearing loss after acoustic trauma. Specifically, taurine significantly attenuated the effects of acoustic trauma as shown by TTS and PTS when compared to saline controls ($P<0.05$ at 8, 16 kHz for TTS and

8, 16, 24 kHz for PTS). Threshold shifts were on average 13.2 dB better in all taurine treated mice compared to the saline control group (Figure 16). A taurine dose of 200 mg/kg yielded the greatest effect in mitigating against NIHL compared to saline controls (P<0.05 at 8, 16, 24 kHz for TTS and PTS). Figure 17 demonstrates examples of ABR traces taken from a saline control and taurine-200mg treated animal for their baseline ABRs and temporary threshold ABRs. There does not appear to be a clear dose-response relationship, suggesting there may be a saturation effect of the taurine treatment. The full raw data is available for review in Appendix 1, as is the statically testing with p values.

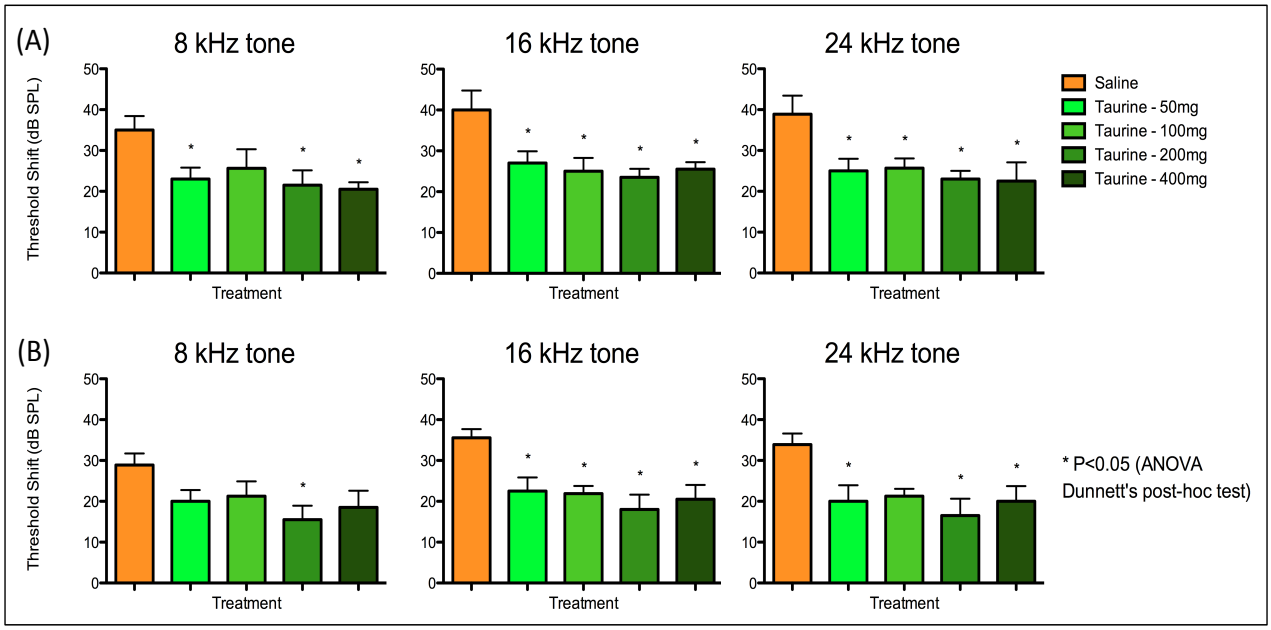

Figure 15- **Taurine attenuates both Temporary Threshold Shifts (TTS) and Permanent Threshold Shifts (PTS).** (A) Temporary Threshold Shifts (TTS) represent the difference between ABRs measured 7 days after acoustic trauma compared to baseline ABRs measured prior to acoustic trauma. (B) Permanent Threshold Shifts (PTS) represent the difference between ABRs measured 28 days after acoustic trauma compared to baseline ABRs measured prior to acoustic trauma. Asterisks indicate statistically significant results between treatment group and control group (P<0.05).

	Mean TTS (dB)			Mean PTS (dB)		
	8 kHz	16 kHz	24 kHz	8 kHz	16 kHz	24 kHz
Saline	35.00	40.00	38.89	28.89	35.56	33.89
Taurine-50mg	23.00	27.00	25.00	20.00	22.50	20.00
Taurine-100mg	25.63	25.00	25.63	21.25	21.88	21.25
Taurine-200mg	21.50	23.50	23.00	15.50	18.00	16.50
Taurine-400mg	20.50	25.50	22.50	18.50	20.50	20.00

Figure 16- **Raw Threshold Shifts for all groups.** These data are the averages for TTS and PTS in each group at 8, 16, 24 kHz. Number of mice that completed the experiment for each group are: Saline n=9, Taurine-50mg n=10, Taurine-100mg n=8, Taurine-200mg n=10, Taurine-400mg n=10 respectively.

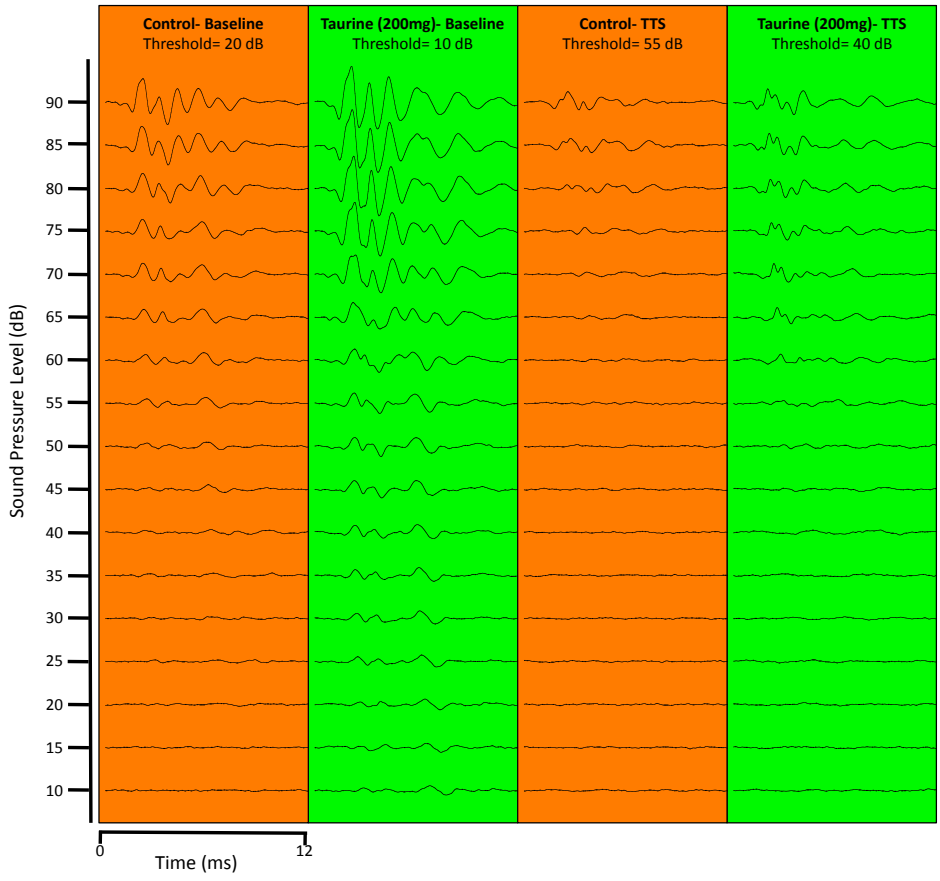

Figure 17- **ABR data.** This figure shows representative data, each group shows a time frame of 12ms against sound pressure level (dB). The first two traces show baseline ABR traces for a saline control mouse and a taurine mouse with 20 and 10 dB thresholds respectively. The third and forth traces are temporary threshold ABRs which are used

to calculate the temporary threshold shift (TTS) for the same saline control mouse and taurine mouse with 55 and 40 dB thresholds respectively.

4.4.2- Histological analysis:

Manual counting of both IHCs and OHCs along the whole cochlea was carried out for each treatment group. Figure 18 graphically represents all cytocochleogram data; average inner (A) and outer (B) hair cell loss after acoustic trauma as a function of distance from apex. The total length of the basement membrane (BM) was measured and normalized as a percentage distance from the apex. Average BM length was 5.255mm (SD 0.1399, SEM 0.02693, n=27). Frequency was mapped using the equation % distance from the apex = 156.5 -82.5 x log (kHz)[155]. The shaded region shows the frequency spectrum of the acoustic trauma (1-32 kHz SPL, 120 dB, 2 hours). Acoustic trauma caused more OHC loss than IHC loss in every treatment group. Taurine (50, 100, 200, 400mg/kg) decreased the amount of hair cell loss in all groups, but had a greater effect on OHCs than IHCs, suggesting that OHCs are more prone to NO• related damage.

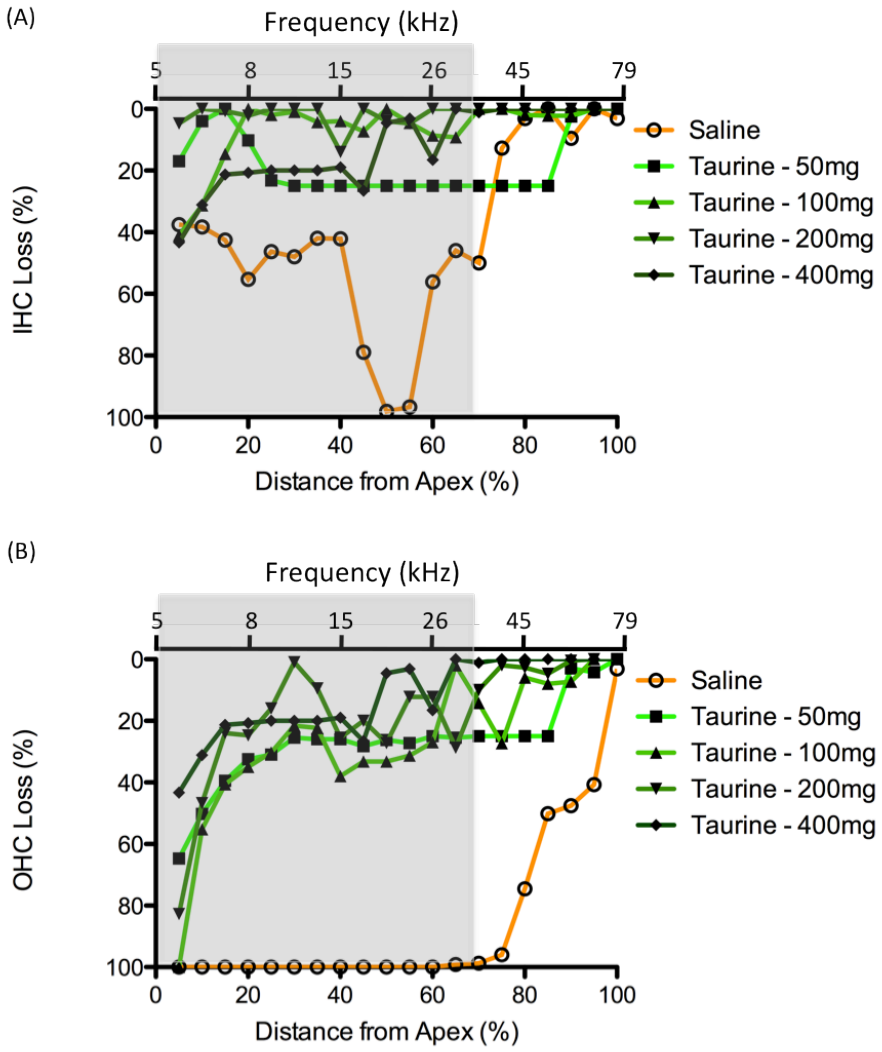

Figure 18- Averaged cochleograms (% distance from apex) showing total (%) noise-induced inner (A) and outer (B) hair cell loss. The total length of the BM was measured and normalised as percentage distance from the apex. Average BM length was 5.255 mm (SD 0.1399, S.E.M 0.02693, n=27). Frequency was mapped using the equation % distance from apex = 156.5 – 82.5 x log (kHz) (Müller et al., 2005). The shaded region shows the frequency spectrum of the acoustic trauma (1-32 kHz SPL, 120 dB, 2 hours). Taurine (50, 100, 200, 400 mg / kg; n=4, 3, 4, 5 respectively) protected inner and outer hair cells when compared to the saline treated control (n=5). These data are further quantified in Figure 19.

The data from Figure 18 shows cumulative data across the entire length of the cochlea. However, Figure 19 deconstructs this information into different sections across the entire cochlea. Each section was labelled to allow location identification, from the apical middle or basal turn of the cochlea. Figure 19 (A) demonstrates that there is a statistically significant difference between

taurine treated groups in both OHC and IHC across the entire length of the cochlea. These data were separated further depending upon which turn of the cochlea the specimen was from; apical, middle or basal turn. Figure 19 (B) shows data for IHC where the least effect is seen in the basal turn. Figure 19 (C) demonstrates OHC cell survival across each turn of the cochlea and that taurine treated animals in every group had increased HC survival in every turn and for every dose of taurine administered.

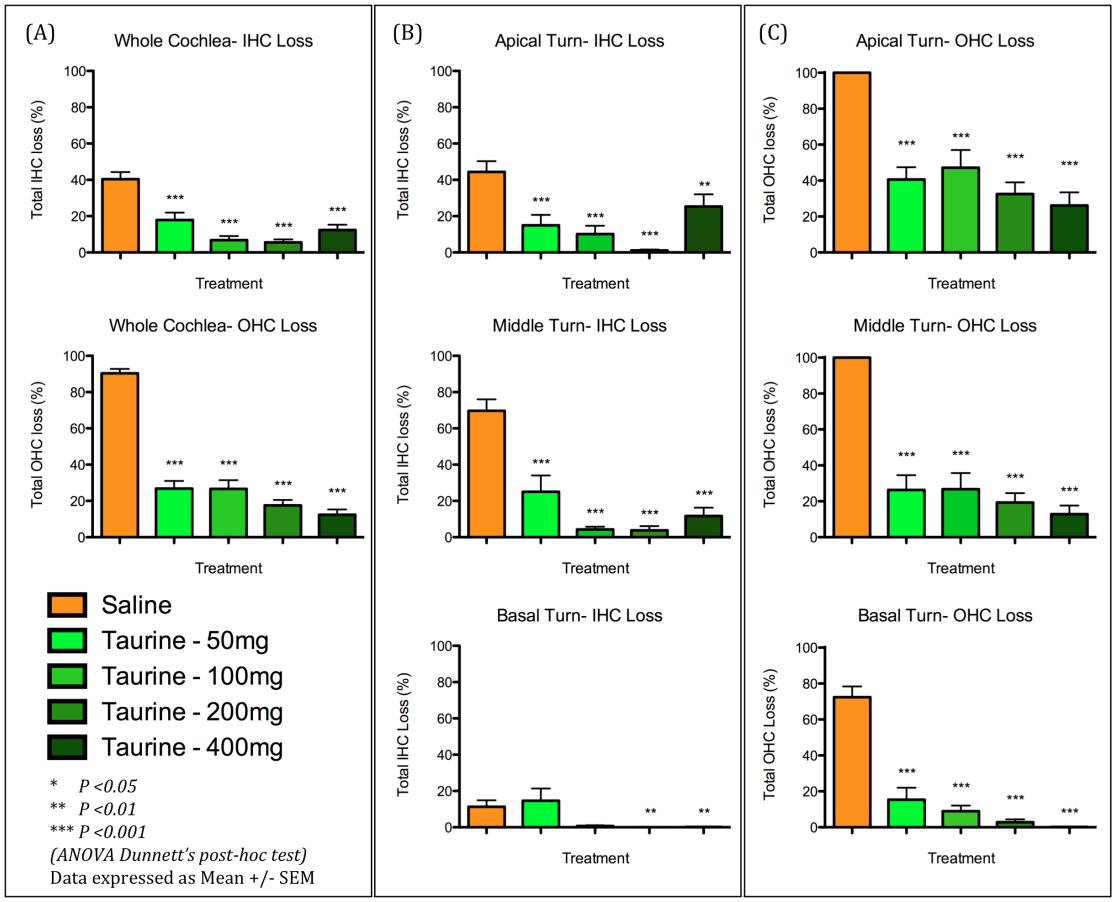

Figure 19- Mean cumulative data of hair cell counts for the entire cochlea for IHC and OHC (A). (B) and (C) are visual representations of IHC and OHC counts, depending on the location and categorised as apical, middle or basal. All treatment groups were compared to saline for statistical significance (n= 5 cochlea for saline group. n= 4, 3, 4, 5 cochlea for taurine 50, 100, 200, 400 mg/kg dose groups).

Figure 20 is a typical photomicrograph of cochlear tissue corresponding to the 7-14 kHz range of the tonotopic map. (A) demonstrates a saline-treated animals OHC, and to a lesser extent IHC, show extensive damage 28 days after acoustic trauma. (B) demonstrates a taurine-treated animals, OHC and IHC rows are predominately intact. Arrows point to areas of OHC loss.

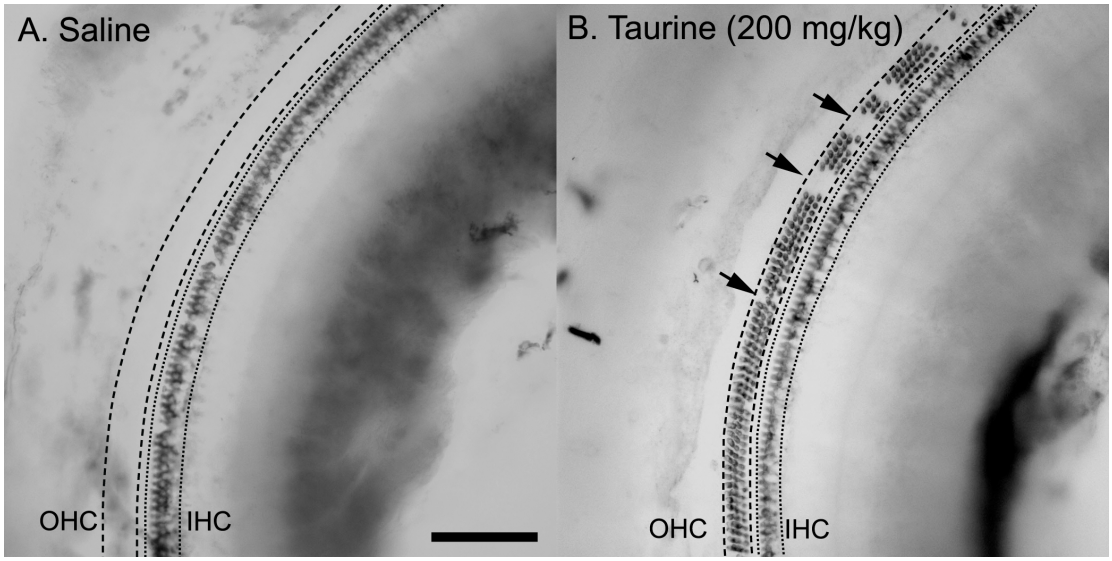

Figure 20- **Taurine reduces outer hair cell loss caused by acoustic trauma.**

A. Photomicrograph shows extensive damage to outer hair cells (OHC) and to a lesser extent to inner hair cells (IHC) 28 days after acoustic trauma in saline treated animals. **B.** In taurine treated animals, IHC and OHC layers showed minimal loss, the arrows demonstrate areas where there is OHC loss. The photomicrographs represent a section of the cochlea corresponding to the 7-14 kHz range. Scale bar = 100 μm.

4.4.3- Results summary:

- Nitric oxide scavengers can be used to mitigate the effect of NIHL in an animal model of NIHL.
- Taurine attenuates hearing loss (ABR) at both 7 and 28 days after acoustic trauma.

- All doses of taurine aid the survival of both inner and outer hair cells throughout the entire cochlea following acoustic trauma. A greater protective effect of taurine is observed for OHC.
- Taurine macroscopically reduces OHC loss after acoustic trauma.
- Taurine is most effective at a dose of 200 mg/kg both acoustically and histologically. However, there is no obvious dose-dependant response.
- This may have potential for translation to humans as a therapeutic agent in the prevention and/or treatment of NIHL.

4.5- Discussion:

Taurine is the most abundant intracellular amino acid in the human body. A 70Kg human contains about 560mmol (70g) of taurine[231]. It has a number of physiological functions that include, but not limited to, cell volume regulation and inhibitory neuro-modulation. Taurine is found in virtually all cells throughout the animal kingdom. In particular it is enriched in electricably excitable tissues such as brain, retina, heart and skeletal muscles. In the central nervous system taurine has been implicated in two major phenomena; cell volume regulation[232-234] and inhibitory neurotransmission[236-238]. In other tissues taurine has been shown to act as anitoxidant in cell protection and to have beneficial effects on cardiovascular function.

Taurine has proved to be neuroprotective in a number of situations. For example, it is effective in a rat hypoxic model, due to its membrane stabilising actions. Exogenously applied taurine alleviates neuronal damage evoked by a variety of pathological impacts, including ischemia and hypercalcaemia. Taurine can attenuate the excessive neuronal accumulation of Ca^{2+} ions which predisposes cells to damage[239], and protects neurons from glutamate-induced excitotoxicity[239], this by preventing or reducing the glutamate-induced elevation of intracellular calcium ions[239].

Tissues that are excitable and prone to generate free radicals, such as the retina, white blood cells, platelets, brain, CNS, heart, skeletal muscle and liver, all have high concentrations. Although it has antioxidant activity, it is not a classical free radical scavenger, therefore, its mechanism remains unclear. Taurine prevents the formation of superoxide by the mitochondria, a mechanism related to the formation of a post-transcriptional modification of tRNA in the mitochondria. This has been shown from taurine-depleted hearts being oxidatively stressed, as exemplified by decreased glutathione redox ratio[238]. Among other damage, taurine deficiency triggers cell death, as shown by an increase in the percentage of apoptotic cells. In the hamster, prophylactic dietary taurine can prevent acute NO• -induced bronchioles injury and may avert other oxidant-induced lung injuries[231]. Taurine is neither a classical scavenger nor a regulator of the antioxidative defenses, leaving uncertain the mechanism underlying the antioxidant activity of taurine.

NO• is catalysed by the nitric oxide synthase (NOS) enzyme. NOS is found in three distinct isoforms; inducible, endothelial and neuronal (iNOS, eNOS, nNOS respectively)[156,157]. iNos can be up regulated by immune cells and other tissue when it is required for the cellular immune response. Macrophages have calcium/calmodulin independent iNOS and it is induced by gamma-interferon or lipopolysaccharide (LPS)[158-161]. eNOS can regulate vascular tone; at normal calcium levels it is inactive but causes vasoconstriction as calcium levels change. nNOS is thought to be involved in signalling and responds to calcium changes. eNOS and nNOS are both calmodulin dependant and therefore are regulated by calcium[158].

There is a small body of evidence suggesting that NO scavengers fail to mitigate the effects of NIHL[114,162]. However, there is a much larger body of evidence supporting NO scavengers are

effective in protecting against the deleterious effects of free radicals[163-173]. Our work is in agreement with the larger body of evidence.

Work by Murishita et al has tested the effects of a plethora of direct therapeutic agents: an O_2• scavenger (tempol), a Poly ADP-ribose inhibitor (3-aminobenzamide), a non-selective iNOS inhibitor (N-Nitro-L-arginine), a selective nNOS inhibitor (7-nitroindazole) and a selective iNOS inhibitor (aminoguanidine)[162]. Each agent was tested in a mouse model of NIHL and only the O_2• scavenger and the Poly ADP-ribose inhibitor were shown to mitigate the effect of acoustic trauma, while none of the NOS inhibitors showed statistically significant results. Ohinata et al demonstrated that L-Name (an NOS inhibitor) attenuated threshold shifts at 2 kHz but increased them at 20 kHz in a guinea pig model of NIHL, and attenuated the production of 8-isoprostane (a by-product of lipid peroxidation) in both stria and vascular core. However, L-Name had no overall protective effect for hair cell survival[115].

Tabuch et al. have shown that NOS inhibitors protect animal cochleae from the ischemia-reperfusion injury associated with NIHL[163,164]. Nuttall's group demonstrated that nNOS knockout mice have increased defence against acoustic trauma when compared to controls[166]. Shi et al showed an up-regulation of iNOS in LPS induced mice in NIHL when compared to controls. This was especially evident in HC and stria vascularis marginal cells. There is a known association with increasing calcium concentrations with eNOS expression and both increase after NIHL[168].

From this current body of work multiple questions have been raised and our future aims include (but are not limited to):

1. The presence of NO• and related enzymes will be tracked with immunocytochemical probes (eg, anti-nitric oxide synthase, anti-nitrotyrosine etc.) and analysed with light microscopy at selected frequency locations in the cochlea. We will test whether taurine mitigates NIHL by attenuating the production of nitrogenous free radicals in cochlea tissues.
2. Our further studies will test the hypothesis that taurine helps to prevent the loss of afferent nerve terminals of primary neurons by labelling C-terminal binding protein 2 (CtBP2) with fluorescent immunohistochemistry (see below). Quantitative analysis will be performed with confocal microscopy at selected frequency locations in the cochlea between cohorts of normal, traumatised and traumatised taurine treated mice.

To investigate these future aims, cochleae will be histologically prepared following noise trauma using methods that are routine in our laboratory, including succinate dehydrogenase histochemistry (Figure 20). Recent work has shown that noise trauma can leave cochlear sensory cells intact despite acute loss of afferent nerve terminals and recovery of threshold sensitivity measured by ABRs[2,35]. Our study proposes to quantify the ability of taurine to rescue sensory cell synapses following noise trauma by immunohistochemically labelling C-terminal binding protein 2 (CtBP2) in both fixed and decalcified whole mount preparations. CtBP2 is a structural component of hair cell afferent synapses (i.e. 'presynaptic ribbon') and will be analysed with confocal microscopy at selected frequency locations in the cochlea that span the site of exposure (i.e., 4 – 64 KHz).

Our future work also proposes to track the production of nitrogen free radicals (i.e., NO•) and nitric oxide synthetase (NOS; and related isoforms) following noise trauma using

immunohistological methods including anti-nitrotyrosine, anti-iNOS, and anti-eNOS in fixed, decalcified and cut cochlea sections. Confocal and/or fluorescence microscopy will be used to analyse and compare sections at different frequencies (i.e., 4, 8, 16, 24, 48 kHz) to describe the effect of taurine in the pathophysiology of NIHL, specifically in the organ of Corti, lateral wall, and spiral ganglion. These experiments can be applied to describe the effect of other free radical scavengers on the pathophysiology of NIHL in the future. There does not appear to be a clear dose-dependent effect of taurine to mitigate the effects of acoustic trauma. This might be explained by a saturation effect, whereby at low doses the taurine has little effect, and at higher doses it has a better effect up to a point whereby any greater doses do not evoke further improvement in the mitigatation of acoustic trauma. It would also be prudent to review the metabolism of taurine and if there is a 1st pass effect to establish the pharmokinetics of taurine within mamals.

Figure 21 demonstrates the proposed multifactorial actions and effects of taurine to mitigate against NIHL including:

- As an antioxidant acting as a potent free radical scavenger- having direct effect upon NO• and indirectly by decreasing the production of 8-isoprostane-F2α. 8-isoprostane-$F_{2\alpha}$ is a vaso-active by-product of free radical formation[3,176] and therefore antioxidants that reduce free radical formation may stop this mechanism of noise-induced vasoconstriction. Reduced cochlear blood flow has significant implications for metabolic homeostasis within the cochlea.
- As a membrane stabiliser due to its capacity to prevent suppression of membrane bound NaK-ATPase[2,174,175].
- Being involved with calcium homeostasis[3,176]. It is well documented that taurine compromises over 50% of the total free amino acid pool of the heart. Its actions have a positive ionotrophic effect on cardiac tissue and in part most of the abilities of taurine are

due to its ability to protect the heart from the adverse effects of excessive or inadequate calcium levels. Taurine regulates calcium both directly be regulating intracellular calcium ion levels by modulating the activity of voltage dependant calcium channels and, indirectly, by regulating sodium channels[2,127,177]. It has been demonstrated in a situation where there is an adequate amount of taurine then calcium induced myocardial damage is significantly reduced[2,178].

- Protection from glutamate excitotoxicity is known but poorly understood. Theories include the prevention of membrane depolarization, neuronal excitotoxicity and/or mitochondrial energy failure[4,179]. In addition, events that occur downstream of glutamate stimulation, including altered enzymatic activities, apoptotic pathways and necrosis triggered by the increased serum calcium levels, can be inhibited by taurine[5-8,175,179]. Large concentrations of glutamate are released as a consequence of acoustic trauma and the toxic levels of this excitatory amino acid lead to large and rapid influxes of both sodium and potassium, leading to an osmotic imbalance. The sequela of this leads to swelling and subsequent rupture of cell membranes[9,10,180].

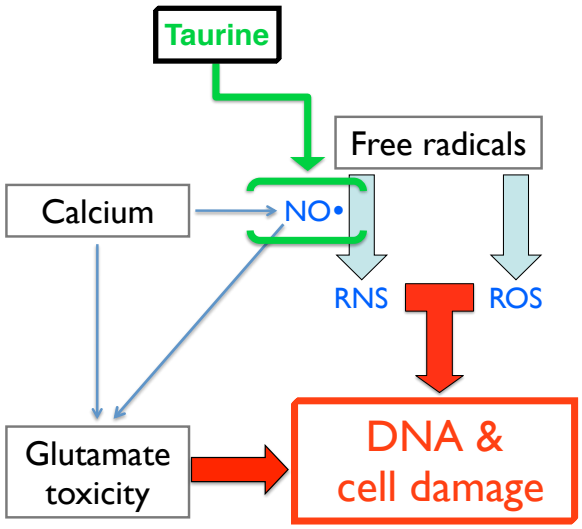

Figure 21- **Proposed action of Taurine to attenuate Noise-Induced Hearing Loss.**

There are multiple different pathological pathways that are closely interwoven to cause necrosis or apoptosis as a

result of NIHL. Taurine is known to have physiological effects as a membrane stabiliser, in calcium homeostasis, protection from glutamate excitotoxicity and an antioxidant that scavengers NO•.

Noise trauma leads to permanent loss of sensory receptor hair cells within the inner ear. Current prevention and treatment strategies are inadequate to deal within the extent of this disease. We predict from current work that both ROS and RNS inhibition will both be beneficial, but not 100% effective in preventing against all damage caused from acoustic trauma. It is unlikely that a single agent will be the panacea to protect completely against NIHL, but multiple agents will be used that act on different parts of the pathological process underlying NIHL. In the long term, antioxidants will act as adjuncts in the prevention or, acutely, in the treatment of NIHL and have a significant effect upon the long-term health costs associated with this prevalent disease. Ultimately, these data may lead to new therapies for this disease of the modern age.

Chapter 5: Stem cells to repair against Noise-induced Hearing Loss

5.1- Introduction:

Hearing loss due to noise overexposure is one of the most common sensory disabilities in humans, particularly in industrialised countries. This debilitating disease significantly reduces quality of life by negatively impacting upon communication in social and professional settings. The ætiology of NIHL is multifactorial involving a complex interplay between environmental and genetic factors[97,240]. Acute noise trauma results in mechanical damage, enhanced mitochondrial free radical formation, and reduced cochlear blood flow[44,47]. The sequelae of these changes are extensive and include necrosis, apoptosis and sublethal pathologies in tissues throughout the cochlea[92,97] (see Chapter 1). Stem cell transplantation is rapidly gaining interest as a potential therapy to prevent or reverse this cell loss and thereby provide a treatment for NIHL[3, 241-243].

The effects of noise trauma include both transient and persistent increases in hearing threshold levels (temporary and permanent threshold shifts, respectively)[244,245]. Susceptibility to the permanent effects of noise exposure differs markedly between individuals in humans[21,246] and animal[247-249] models of NIHL with respect to both the extent of hearing loss and the cochlear tissues affected. The CBA/Ca inbred mouse strain family has proven to be an invaluable model for the study of the pathology and treatment of NIHL as their hearing levels remain stable with age[250-252], thus eliminating conflicting contributions of presbycusis.

Several studies have identified the cellular targets of noise trauma in CBA/Ca mice. Depending upon the degree of trauma, these can include the cochlear lateral wall (fibrocytes of the spiral ligament, and marginal, intermediate and basal cells of the stria vascularis), the organ of Corti (hair cells and supporting cells), and the spiral limbus[249,253,254]. As several of these cochlear cell

types are epithelial in origin (e.g., hair cells, supporting cells, marginal cell layer of the stria vascularis), our group postulated that epithelial stem/progenitor cell transplantation could possess the potential to ameliorate NIHL. Transplanted stem cells can repair tissues by replacing damaged cells or by secreting factors that enhance the survival and/or proliferation of endogenous cells[255-257].

Our group and others have demonstrated that the epithelium of the tongue represents an accessible and abundant source of adult stem and progenitor cells[258-261]. Adult stem/progenitor cells have a number of advantages for cochlear transplantation in that they can be used for autologous transplantation (to resist host rejection) and are less tumourigenic than embryonic stem cells[262]. Tissue homeostasis in adult epithelia is maintained by stem cells residing in the basal-most cell layer that give rise to progenitor cells which proliferate for a finite number of times generating several epithelial and taste bud cell types[263-265].

In this study, we isolated adult stem/progenitor cells from CBA/CaH mouse tongue epithelium and characterised their proliferative capacity and phenotypes in vitro. Subsequently, we examined the efficacy of cochlear transplantation of these cells in reducing noise ototoxicity. Epithelial stem/progenitor cells were transplanted into the cochleae of CBA/CaH mice shortly after noise trauma (48 h) and hearing levels were then measured after 4 weeks. Survival and incorporation of the transplanted cells were also investigated by cell fate analyses. Together, the results of these studies provide evidence that epithelial stem/progenitor cell transplantation can engender a functional rescue of hearing in an animal model of NIHL.

5.2- Materials and methods:

All procedures were approved by the Garvan Institute of Medical Research/St Vincent's Hospital Animal Ethics Committee and con- ducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004) of the National Health and Medical Research Council of Australia. Every effort was taken to minimise discomfort to the animals.

5.3- Epithelial stem/progenitor cell isolation and culture:

CBA/CaH mice (6 weeks; n = 5) were anaesthetised with CO_2 and decapitated. The tongue was dissected free and injected with a solution of 2 mg/ml collagenase D (Roche Pharmaceuticals). Following 90 min, the dorsal epithelium at the rear of the tongue (surrounding and including the circumvallate papilla) was peeled off the underlying muscle with fine forceps. This region was selected as the circumvallate papilla represents a readily identifiable landmark, thus enabling the same tissue to be isolated across animals. Tissues were minced with fine scissors and incubated in TrypLE Express (Invitrogen Life Technologies) containing 1 mg/ml collagenase D and 1 mg/ml hyaluronidase (Sigma Aldrich) at 37 °C for 1 h. Dissociated cells were cultured in Advanced DMEM/F12 medium containing 20 mM glutamine, 10% fetal bovine serum, B-27 supplement minus vitamin A, 20 ng/ml EGF, 20 ng/ml bFGF, 100 U/ml penicillin G and 100 μg/ml streptomycin on plastic tissue culture dishes coated with rat-tail collagen (5 $\mu g/cm^2$; Roche Pharmaceuticals) at 37 °C with 5% CO_2. Cells at passage 6 were used for transplantation experiments.

5.4- Immunocytochemistry :

Cells were grown on glass coverslips coated with rat-tail collagen (5 $\mu g/cm^2$; Roche Pharmaceuticals) and fixed at confluency for 10 min in methanol at −20°C (for cytokeratin 8 and cytokeratin 14 immunolabelling) or 4% paraformaldehyde in 0.1 M phosphate-buffered saline

pH 7.4 (PBS; for p63 immunolabelling) at 4 °C. Cells were then blocked for 1 hour in 10% normal goat serum in PBS containing 0.3% Triton X-100 (NS-PBSTx). Primary antibodies were diluted in NS-PBSTx and applied for 2 hours at room temperature. The following primary antibodies were used: monoclonal mouse anti-p63 (1:50; Santa Cruz Biotechnology; sc-8431), monoclonal rat anti-cytokeratin 8 (1:20; Developmental Studies Hybridoma Bank; TROMA1), monoclonal mouse anti-cytokeratin 14 (1:50; Chemicon; CBL197), monoclonal rat anti-5′-bromo-2′-deoxyuridine (BrdU; 1:250; AbD Serotec; MCA2060) and monoclonal mouse anti-BrdU (1:20; Developmental Studies Hybridoma Bank; G3G4). Cells were then rinsed for 4 hours in several changes of PBS and incubated for 1 hour at room temperature in the appropriate secondary antibodies diluted in PBS. Fluorescent secondary antibodies used were as follows: Alexa 488- conjugated goat anti-mouse IgG (1:100; Invitrogen; A-11029) and DyLight 649-conjugated goat anti-rat IgG (1:100; Jackson ImmunoR- esearch; 112-495-167). Sections were counterstained by incubation overnight at 4°C in rhodamine-conjugated Phaseolus vulgaris leucoag- glutinin (PHAL; 1:100; Vector Laboratories; RL-1112), fluorescein- conjugated Jacalin (1:100; Vector Laboratories; FL-1151), or the nuclear stain DAPI (0.3 μM; Invitrogen Life Technologies). In experiments examining mitotic activity, BrdU (Sigma Aldrich) was added to the culture medium at a final concentration of 10 μM 2 h prior to fixation. The fixed cells were then incubated in 2 N HCl at room temperature for 20 min. Following rinsing in 0.1% Triton X-100 in PBS for 20 min, cells were processed for BrdU immunolabelling as described above.

5.5- Noise trauma and hearing threshold detection:

CBA/CaH mice (male and female; 4–6 weeks; n = 11) were deafened in both ears by noise overexposure (120 dB SPL, 1–80 kHz broadband noise, 2–2.5 hours under general anaesthesia) in a foam-padded, shielded acoustic chamber. This strain of mice was selected to match the stem/

progenitor cell donors and minimise immunorejection. Animals of this age were selected as vulnerability to noise exposure declines after 8 weeks of age in CBA/CaJ mice[34].

Auditory function was assessed by measuring auditory brainstem response (ABR) thresholds to click and pure tone stimuli, as described previously[152]. Briefly, acoustic stimuli were delivered to anaesthetised mice via an electrostatic insert speaker (Tucker Davis Technologies) fitted into the external ear canal (see Chapter 2 for more information). Clicks and pure tone bursts (20kHz) were delivered and ABRs were recorded while sound intensity was reduced in 5dB SPL steps beginning at 90dB SPL. ABR thresholds were determined by identifying the lowest sound intensity level at which the peak amplitude of the evoked ABR signal exceeded four times the standard deviation of the baseline noise[152].

To assess the extent of NIHL, permanent ABR threshold shifts were determined by comparing the pre-trauma threshold levels in the operated (left) ear to threshold levels in the non-operated (right) ear 30 days post-trauma[266]. Animals that did not display a permanent threshold shift (i.e., shift b 10 dB SPL) were excluded from study.

5.6- Stem/progenitor cell transplantation:

Prior to transplantation, isolated adult epithelial stem/progenitor cells were grown in flasks (Corning) to 70–80% confluency and then harvested using TrypLE Express. The collected cells were rinsed in DMEM/F12 (Invitrogen), centrifuged for 5 min at 300 × g, resuspended in PBS at 2000–4000 cells/μl, and stored on ice until transplanted.

To investigate the functional effects of stem/progenitor cell transplantation, mice with equivalent hearing levels at 2 days post- trauma were divided into two cohorts: Transplant and Sham. The

Transplant cohort received a unilateral cochlear injection of epithelial stem/progenitor cells (n = 7) and the Sham cohort received a unilateral injection of the vehicle solution alone (n = 4). Cochleostomies were performed in the lateral wall of the left cochlea at the basal turn, posterior to the stapedial artery and in line with the round window as described previously[152]. This cochleostomy site corresponds to the 51.4 ± 2.8 kHz (n = 3) region of the mouse cochlea according to the place-frequency map of Müller et al.[155]. It is important to note, however, that the place-frequency map of the mouse cochlea can shift by up to one octave following noise damage[155]. This cochleostomy site has been shown to deliver transplanted cells primarily to the two perilymphatic compartments, scala vestibuli and scala tympani[267]. For stem/progenitor cell transplantations, 1µl of cells suspended in PBS was injected over 1 min to transplant 2000– 4000 cells. The cochleostomy was then sealed with bone wax, with all surgeries completed in 30–40 min.

5.7- Cell fate analyses of transplanted adult epithelial stem/progenitor cells:

To investigate the fate of transplanted cells in the mouse cochlea, stem/progenitor cells (prepared as above) were labelled with the lipophilic dye Vybrant CM-DiI (5 µl/ml; Invitrogen) and injected into the cochleae of CBA/CaH mice 4–5 weeks old (10,000 cells/µl; n = 6) using the microsurgical approach described above. Mice were sacrificed 1–4 weeks after surgery by perfusion through the left ventricle with 4% paraformaldehyde. Transplanted cochleae were removed and postfixed in the same fixative for 24 h at 4 o C. For decalcification, cochleae were exposed to 10% EDTA (Sigma) for 48 h at 4 °C. Tissues were rinsed in PBS for 1 h, cryoprotected in graded sucrose/PBS solutions to 30% sucrose (w/v), frozen in OCT (Sakura Finetek), sectioned along the modiolar axis at 7 µm on a cryostat, counterstained with NeuroTrace 500/525 green fluorescent Nissl stain (1:50; Invitrogen), and mounted in Gelmount. Sections containing transplanted cells were then immunolabelled for Na^+/K^+-ATPase expression

using a rabbit monoclonal anti-Na^+/K^+-ATPase α anti- body (1:100; Epitomics; 2047-1) and standard immunohistochemical methods[261].

To examine the distribution of transplanted cells along the cochlear axis, Vybrant CM-DiI-labelled cells were injected into the cochleae of CBA/CaH mice (4 weeks of age; 4000 cells; n=5) 2 days post-noise trauma. Mice were sacrificed 5 days after surgery by perfusion through the left ventricle with 4% paraformaldehyde. Transplanted cochleae were postfixed in the same fixative for 24 h at 4 o C and decalcified by exposure to 10% EDTA for 48 h at 4 °C. Surface preparations of the cochlear spiral were prepared from the apex to the base and the frequency locations of transplanted cells were determined using the place-frequency map of Müller et al.[155].

5.8- Microscopy and image processing:

Specimens were viewed using a Zeiss Axioplan epifluorescence microscope equipped with Plan-Neofluar 10 × 0.30 NA and Plan- Neofluar 20 × 0.50 NA dry objective lenses and an AxioCam MRm digital camera (Zeiss). Images were processed to adjust brightness and contrast using Adobe Photoshop 8.0 (Adobe Systems).

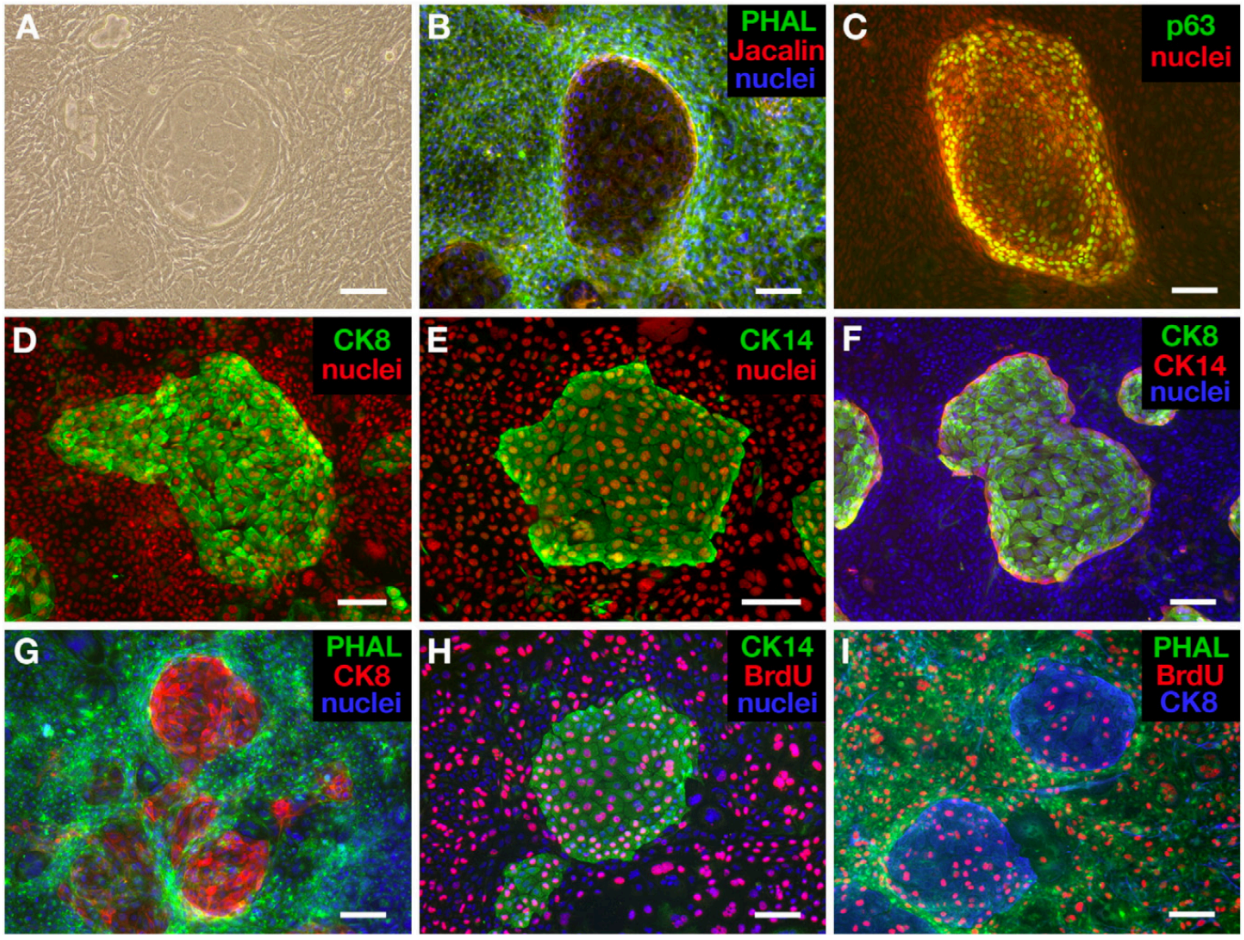

Figure 21- Characterisation of tongue epithelial stem/progenitor cells in vitro. A. Light micrograph showing detail of a colony generated by cells isolated from the dorsal tongue epithelium. These colonies are comprised of small, densely packed cells surrounding islands of squamous cells. A representative island can be seen in the centre of the image. B. The small, densely packed cells within the colonies bind the lectins PHAL and Jacalin. C–G. Cells comprising the squamous islands express the epithelial stem cell marker p63 (C), and the epithelial markers CK8 (D, F, and G) and CK14 (E and F). H and I. Immunolabelling for BrdU incorporation showing that mitotic activity is distributed throughout the colonies, including both the populations of lectin-binding cells and the islands of squamous cells. Abbreviations: CK8, cytokeratin 8; CK14, cytokeratin 14; PHAL, Phaseolus vulgaris leucoagglutinin. Scale bars = 100 μm.

5.9- Statistical analysis:

Statistics are quoted as mean ± standard error of the mean (SEM). Significant differences in mean threshold values were determined using the non-parametric Mann–Whitney one-tailed test

for comparison of ABR thresholds before and after noise trauma (Fig. 22), and for comparison of ABR thresholds in Transplant versus Sham cohorts (Figs. 23 and 24). All statistical analyses were performed using Prism 5.0a (GraphPad).

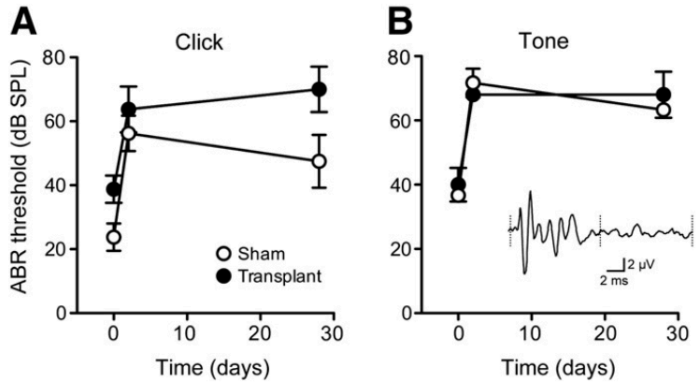

Figure 22- Transplant and Sham cohorts exhibit similar levels of NIHL. A and B. Noise trauma caused significant increases in mean ABR threshold levels (Pb0.05) in the Transplant and Sham cohorts for click (A) and pure tone (B; 20 kHz) stimuli 2 days and 30 days (permanent threshold shift) post-trauma. Mean ABR threshold shifts did not differ between the Transplant and Sham cohorts, indicating comparable levels of deafening in the two groups. Inset: Representative averaged ABR signal prior to noise trauma in response to click stimuli. Mean ± SEM.

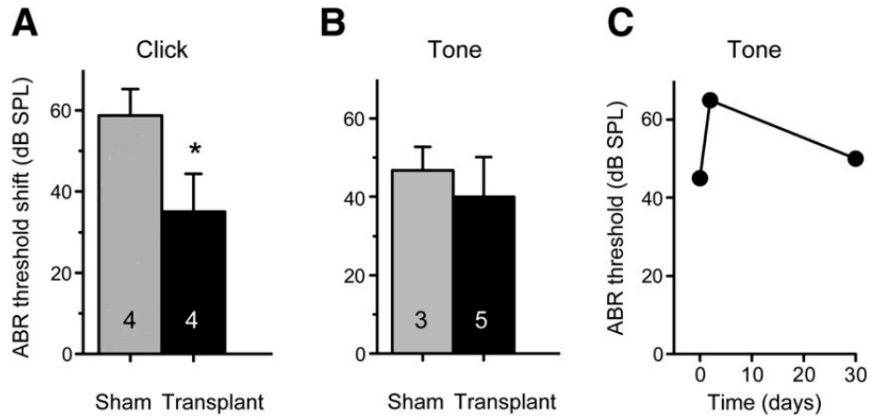

Figure 23- Transplanted epithelial stem/progenitor cells attenuate NIHL (Analysis 1). A and B. The mean difference between pre-trauma and 28 day post-surgery ABR threshold levels (ABR threshold shift) to click stimuli (A) in the operated (left) ear was significantly less for mice transplanted with stem/progenitor cells (Transplant) than for sham-injected mice (Sham). No difference was observed between the two cohorts in the threshold shifts to pure tone (20 kHz) stimuli (B). Number of animals indicated in each bar. Mean ± SEM; * Pb0.05. C. Return to approximate pre-trauma levels after stem cell transplantation in an animal's response to pure tone stimuli.

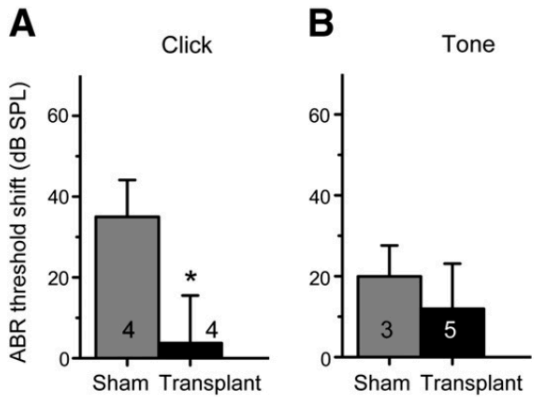

Figure 24- Transplanted epithelial stem/progenitor cells attenuate NIHL (Analysis 2). A and B. The mean difference between the post-surgery ABR threshold levels of the operated (left) and non-operated (right) ears in the Transplant cohort was significantly less than in the Sham cohort for click stimuli (A). Differences were not observed in the threshold shifts to pure tone (20 kHz) stimuli (B). Number of animals indicated in each bar. Mean ± SEM; * Pb0.05.

5.10- Results - Tongue epithelium as a source of adult stem/progenitor cells:

Isolated cells from the posterior tongue epithelium gave rise to a rapidly growing colony after 7 days in vitro (Fig. 21), capable of propagating into additional colonies upon serial passage (n = 12 passages). These colonies were comprised of small, densely packed cells whose plasma membranes bound the lectins Jacalin and PHAL (Fig. 21B), which label rodent tongue epithelial cells of the basal and granular layers in vivo[268]. Squamous cells expressing the epithelial stem cell marker p63[269] (Fig. 21C), and the epithelial markers cytokeratin 8 and 14[270] were observed in islands within the colonies (Fig. 21D–G). Mitotic activity was extensive throughout the colonies, including both the lectin- binding cells and the islands of squamous epithelial cells, as shown by cell proliferation assays using the thymidine analogue BrdU (Fig. 21H and I). The extensive proliferative potential of these colonies is characteristic of holoclones, stem cell derived keratinocyte colonies[271-272]. Similarly, previous studies have reported the isolation of holoclone-forming cells from the anterior portion of the adult mouse tongue epithelium[259].

5.11- Results - Transplant and sham cohorts exhibit similar levels of NIHL:

Animals were exposed to noise trauma and separated into two cohorts: Transplant and Sham. To assess the extent of NIHL, ABR threshold levels were tested 30 days post-trauma and compared to pre-trauma levels. Previous studies indicate that noise-induced threshold shifts reach permanent levels 2–4 weeks after exposure[266]. For both Transplant and Sham cohorts, ABR threshold levels for click and pure tone stimuli were significantly increased at 2 days post-trauma and at 30 days post-trauma in the non-operated ear (permanent threshold shift) compared to pre-trauma levels (Fig. 22). Mean ABR threshold shifts did not differ between the Transplant and Sham cohorts at either time point, indicating comparable levels of NIHL were present in animals assigned to the two cohorts (P N 0.05; Fig. 22). Permanent threshold shifts of 31 ± 8 and 28 ± 6 dB SPL were observed in the Transplant cohort to click and pure tone (20 kHz) stimuli, respectively, while threshold shifts of 24 ± 5 and 27 ± 3 dB SPL were present in the Sham cohort. Consistent with previous studies examining noise-induced threshold shifts of less than 40 dB SPL[34, 273], significant hair cell loss was not observed at 30 days post-trauma.

5.12- Results - Transplantation of epithelial stem cells attenuates NIHL:

To examine the effects of epithelial stem/progenitor cell trans- plantation on NIHL, mice within the Transplant cohort received a unilateral cochlear injection of epithelial stem/progenitor cells, while those of the Sham cohort received a unilateral injection of the vehicle solution. Surgeries were performed 2 days post-trauma, the time point providing maximal integration of transplanted stem/progenitor cells following noise trauma[242]. Transplanted cells occurred primarily within the region of the cochlea spanning 8.5 ± 1.8 to 14.0 ± 1.2 kHz (n = 5), according to the place-frequency map of Müller et al.[155], though small numbers of cells were distributed sparsely along the remainder of the cochlea (data not shown).

Two distinct analyses were performed to assess the effects of epithelial stem/progenitor cell transplantation following noise trauma (Figs. 23 and 24). In Analysis 1, hearing threshold shifts were determined for the operated ear by comparing pre-trauma levels in this ear with the levels observed at 28 days post-surgery (30 days post-trauma; Fig. 23A). Threshold shifts in the Transplant and Sham cohorts were then compared. This analysis revealed that the ABR threshold shift between pre-trauma and post-surgery levels in the operated (left) ear of mice in the Transplant cohort was significantly less than in mice of the Sham cohort in response to click stimuli (P b 0.05). For pure tone stimuli, the ABR threshold shift was similar in the two cohorts (P N 0.05; Fig. 23B). Interestingly, one animal showed a return to approximate pre-trauma levels for pure tone stimuli following cell transplantation (Fig. 23C), an outcome not observed in the Sham cohort.

In Analysis 2, the non-operated (right) ear of each animal was used as an internal control and compared against the operated (left) ear. Differences between the hearing threshold levels of the two ears at 28 days post-surgery were compared between the Transplant and Sham cohorts (Fig. 4). This second analysis was consistent with Analysis 1 in that mice in the Transplant cohort again showed a significantly smaller ABR threshold shift for click stimuli (Pb0.05) than those of the Sham cohort (Fig. 24A). For pure tone stimuli, the ABR threshold shift did not differ significantly between the two cohorts (Fig. 24B). No correlation was observed in the Transplant cohort between improvements in hearing levels and either sex or the number of transplanted stem/progenitor cells (data not shown).

5.13- Results - Stem/progenitor cells survive and integrate into the cochlea:

To examine the fate of adult epithelial stem/progenitor cells upon cochlear transplantation, cells were injected into the cochleae of mice and tracked using Vybrant CM-DiI labelling (Fig. 25). Incorporation of stem/progenitor cells was observed primarily into the suprastrial regions of the spiral ligament (Fig. 25A and B), Reissner's membrane (Fig. 25A), and the simple squamous epithelial lining of scala tympani 1–4 weeks after transplantation (Fig. 25A–C). These locations are consistent with the epithelial origins of the transplanted cells.

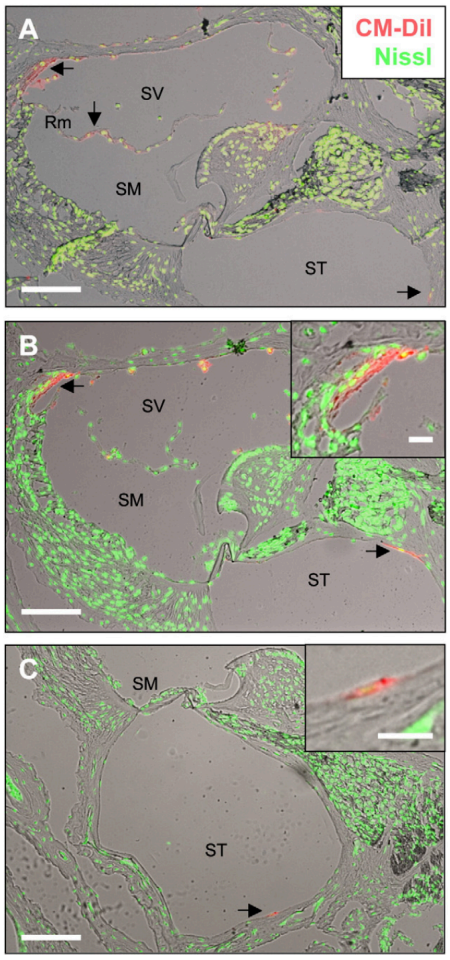

Figure 25- Stem/progenitor cells survive and integrate into the cochlea. A and B. Overlaid fluorescence and light micrographs showing the locations of transplanted stem/ progenitor cells (arrows) labelled with Vybrant CM-DiI (red) in the spiral ligament of scala vestibuli (SV), Reissner's membrane (Rm), and the squamous epithelial lining of scala tympani (ST) 1 week after transplantation. C. Transplanted cells are present 4 weeks post-surgery and incorporated into the epithelial lining of scala tympani. Preparations were counterstained with the nuclear dye NeuroTrace 500/525 (green). Insets: High magnification views showing incorporation of stem/progenitor cells into the spiral ligament (B) and into the simple squamous epithelial lining of the scala tympani (C). Abbreviation: SM, scala media. Scale bars = 10 μm (insets) and 50 μm.

Transplanted cells integrating into suprastrial regions expressed the enzyme Na^+ /K^+ -ATPase (Fig. 26), a protein abundantly expressed by superficial fibrocytes of the suprastrial region[274-277]. Immunolabelling for Na^+ /K^+ -ATPase was not observed in transplanted cells present in other regions of the cochlea (Fig. 26). Examination of the epithelial stem/progenitor cells in vitro indicated that cells of the squamous islands express Na^+/K^+-ATPase (data not shown). As only those transplanted cells integrating into suprastrial regions showed expression of this enzyme in

vivo, the observed immunolabelling suggests either that cells of the squamous islands specifically integrate into the cochlear lateral wall or that expression was induced in lectin-binding cells following their integration.

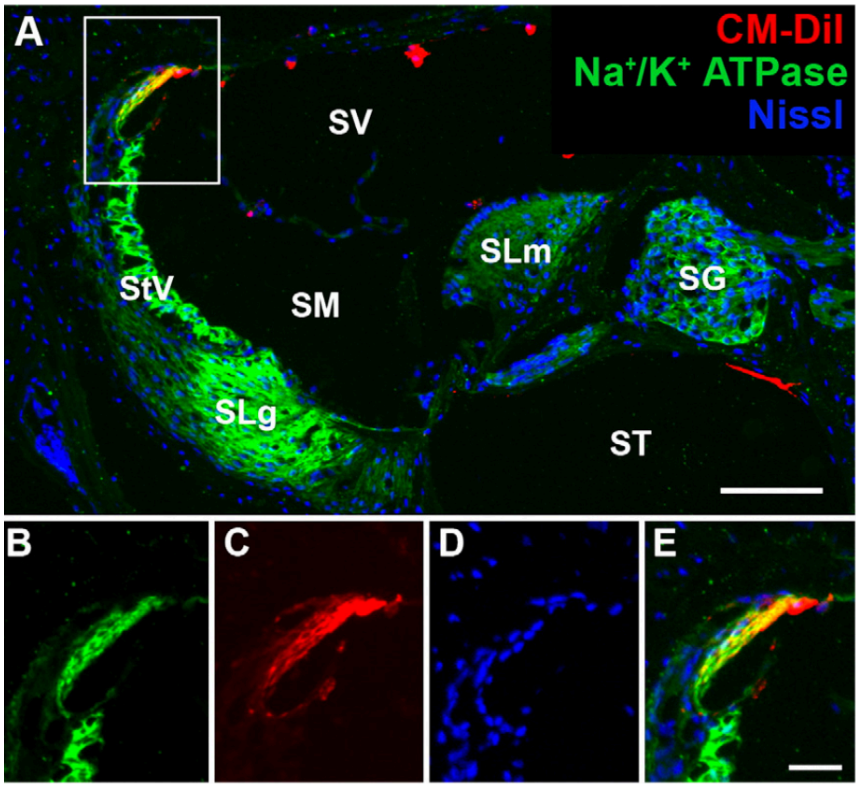

Figure 26- Transplanted stem/progenitor cells express markers of endogenous cochlear cells. A. Transplanted stem/progenitor cells labelled with Vybrant CM-DiI (red) integrate into the lateral wall of the cochlea (square) and express the ion transport-mediating enzyme Na^+/K^+-ATPase (green), strongly expressed in cells of the spiral ligament (SLg) and stria vascularis (StV). Note that immunolabelling for Na^+/K^+-ATPase is not observed in transplanted cells present in other regions of the cochlea. Cell nuclei are labelled with a fluorescent Nissl stain (blue). B–E Higher magnification views of the region highlighted by the square in (A) showing labelling for Na^+/K^+-ATPase (B), Vybrant CM-DiI (C), cell nuclei (D), and a merged image of the three labels (E). Abbreviations: SG, spiral ganglion; SLg, spiral ligament; SLm, spiral limbus; SM, scala media; ST, scala tympani; StV, stria vascularis; SV, scala vestibuli. Scale bars = 100 µm in A; 25 µm in B–E.

Together, these results indicate that the cochleostomy site used in this study delivers epithelial stem/progenitor cells primarily to scala vestibuli and scala tympani of the mouse cochlea, and that these stem/ progenitor cells survive within the cochlea for at least 4 weeks (the time period of the study) following transplantation.

5.14- Discussion:

Previous studies have tested for a functional rescue of hearing via stem cell transplantation in animal models of cochlear ischaemia and aminoglycoside exposure[278-280]. This study is the first to examine the functional effect of stem cell transplantation on NIHL. As several of the cellular targets damaged by NIHL are of epithelial origin, we focussed our attention on the effects of epithelial stem cell transplantation. Our findings demonstrate, via two distinct analyses, that allotransplantation of epithelial stem/progenitor cells into adult mice following noise trauma resulted in a significantly reduced ABR threshold shift to click stimuli. These findings provide evidence that epithelial stem/progenitor cell transplantation can lessen permanent threshold shifts resulting from noise trauma.

The exposure of animals to noise levels capable of producing permanent cochlear damage leads to large thresholds shifts that recover exponentially to smaller stable shifts at 2–4 weeks after exposure[266]. The permanent threshold shifts arise from mechanical, metabolic, and vascular changes that result in apoptotic and sublethal pathologies in the organ of Corti and lateral wall[92,97]. The nature and extent of these pathologies differ across frequencies in the cochlear tonotopic map[104, 281, 282]. Our finding of an improvement in the ABR threshold shift for click but not pure tone stimuli (20 kHz) suggests that the observed effects of epithelial stem/progenitor cell transplantation occurred primarily outside the 20 kHz cochlear location (the mid-point of the cochlear axis in normal hearing mice[283]. As the transplanted cells were located primarily within the portion of the cochlea corresponding to 8.5–14.0 kHz, effects in this region could mediate the observed improvement to click stimuli. Determination of the specific region/s involved, however, will be complicated by the shifts that occur in the place-frequency map of the mouse cochlea following noise damage[155].

Transplanted stem cells can repair tissues by replacing damaged cells or by secreting bioactive factors that enhance the survival and/or proliferation of endogenous cells[255-257]. Cell fate analyses in the present study show incorporation of transplanted cells into the spiral ligament, one of the principal sites of cochlear damage in NIHL. Constitutive fibrocyte turnover in the spiral ligament within the cochlear lateral wall is thought to be essential in maintaining normal cochlear function by regulating potassium recycling, and enhancement of this turnover may act as an endogenous cochlear repair mechanism[284-285]. Consistent with the importance of potassium recycling for cochlear function, several NIHL susceptibility genes in humans are linked to potassium homeostasis[246]. As upregulation of local proliferation via paracrine signalling is commonly observed following stem cell transplantation, enhancement of fibrocyte turnover may represent one avenue by which the transplanted epithelial stem/progenitor cells can influence hearing levels. Additional mechanisms of intervention could include enhancement of cochlear blood flow or normalisation of mitochondrial free radical levels, which peak at 7–10 days following noise[67,181], as transplanted adult stem cells can upregulate angiogenesis[290,291] and efficiently scavenge reactive oxygen and nitrogen species[292]. As the transplanted cells integrating into suprastrial regions of the spiral ligament expressed the ion transport- mediating enzyme Na^+ /K^+ -ATPase, these cells could potentially also contribute directly to the maintenance of cochlear fluid homeostasis[293].

Our cell fate studies indicated that transplanted epithelial stem/ progenitor cells survive for at least 4 weeks within the cochlea and incorporate within tissues lining the perilymphatic compartments. Interestingly, integration of neural stem cells injected into noise- deafened mice was not observed in these tissues, but instead within the spiral ganglion, spiral limbus, and organ of Corti[242]. Together these results suggest that cochlear integration sites may be stem cell type specific. Stem cell therapies combining diverse stem cell types may therefore enable intervention at multiple sites within the cochlea and provide additive beneficial effects on NIHL.

Chapter 6: Overview Discussion

6.1- Pathology of NIHL:

NIHL is a significant health problem that has multifaceted implications clinically, socially and financially. This body of work has aided towards the development of otoprotective agents. Novel therapeutic agents are an essential part of otoprotection and will make great adjuncts to other health strategies that are employed to decrease the deleterious effects of excess noise exposure. The role of free radicals in the causation of NIHL is well documented, but a better understanding of the specific oxidative and nitrostative processes that underpin the acoustic injury is needed. These processes can lead to cell death in the form of apoptosis or necrosis, leading to the associated irreversible nature of acoustic trauma. However, there are multiple types of acoustic injury that occur and lead to sublethal pathology (see chapter 1 for further details)[34,35]. Currently most of the research in the field of otoprotection is laboratory based in pre-clinical animal studies. Realistically the natural progression in this field is to lead towards clinical acceptance through the demonstration of the effects of otoprotective agents that reduce PTS in prospective randomised, placebo-controlled human clinical trials. Such studies would need to be carried out in at-risk populations, who are already exposed to hazardous noise levels and develop permanent levels of hearing-loss despite the use of conventional Hearing Protection Devices (HPDs). These types of studies have a multitude of pitfalls and problems. The studies are costly, time intensive because NIHL normally happens over a period of years, labour intensive due to long term follow-up that would be needed with regular assessment of hearing and the ethical clearance that is needed to conduct these types of studies.

When considering injury caused by excessive noise, the physical damage effects of direct loud noise can be reduced with appropriate use of HPDs. The problem in this scenario simply comes down to the issue that insult is added to injury as a consequence of the metabolic changes that

arise from excessive noise exposure. Clearly impulse noise does cause a substantial amount of direct trauma, but in a large proportion of acoustic trauma it is the secondary metabolic effects of free radicals, changes in blood flow, glutamte excitotoxicity and energy depletion that lead to damage. Free radical formation in the inner ear is well documented, with an initial peak arising with 1-2 hours[12,67,181] and a secondary peak arising as a consequence of free radicals 7-10 days post initial exposure[38,67]. All animals have naturally occurring antioxidants that act as natural defence systems to protect against the adverse effects of increased numbers of free radicals due to normal electron "leakage" as part of energy production. The principle of using free radical scavengers to mitigate the effects of acoustic trauma can either be carried out by increasing the naturally occurring endogenous antioxidants within the cochlea or by introducing exogenous antioxidants. Endogenous antioxidants production within the cochlea can be increased by effecting glutathione, super oxide dismutase (SOD) enzyme, catalyse enzyme or other smaller antioxidant pathways.

6.2- Free radical scavengers used to mitigate the effects of NIHL:

Free radical scavengers used in the prevention of NIHL include (but are not limited to):

- N-Acetylcystine (NAC) is an agent that has he largest amount of information for its use in the prevention of NIHL as an otoprotective agent. More than 20 studies have identified it to work in this field. NAC is a derivative of cysteine with an adjoined acetyl group. It is an acceptable medication that is commonly used in humans for the treatment of paracetamol poisoning, as a mucolytic agent, nephroprtective agent, along with other uses. NAC is a pro-drug of L-cystenine, which itself of a precursor of glutathione and hence administering NAC replenishes glutathione. As previously mentioned glutathione is the major antioxidant within the inner ear and is crucial for redox homeostasis. Studies

have shown a reduction of up to 25dB in differing animal models when delivered either singly prior to noise trauma[13,115], multiple dose prior to sound exposure[14,96], or with multiple treatments after noise exposure[106]. However, there is also some contrary evidence from Hamernick et al[35,182] where NAC did not provide protection against prolonged acoustic trauma of 8 hours over a 5-day period in chinchillas. Given the numbers of animal studies that demonstrate positive results to defend against the deleterious effects of acoustic trauma, it has lead to 2 clinical trials in humans using NAC as an otoprotective agent (see later in this chapter for more information).

- D-Methionine is a micronutrient in the form of an amino acid that has been demonstrated to reduce noise-induced lipid peroxidation and increases both catalyse and SOD to lead to a decreased amount of free radicals due to effect on GSH[16,99]. There has been a significant amount of interest for the use of D-Methionine to mitigate the effects of acoustic trauma. A group in Southern Illinois headed by Prof Campbell have carried out several studies in animal models, which have yielded positive results in animal due to acoustic trauma and also due to drug-induced hearing loss[2,183,184]. This group have now moved forward and been approved for a phase 3 clinical trial by the U.S. Food and Drug Administration (FDA). Studies have shown a reduction of up 15-20dB in differing animal models, smaller than treatment with NAC. At this current time there are many unanswered questions relating to the differences between D-Methionine and NAC, this will need to be investigated further.
- Ebselin is a compound that contains selenium and acts as a catalyst similar to glutathione peroxidase, which acts to catalyse the reduction of free radicals by GSH. Fascinatingly, ebselin is more efficient than glutathione peroxidase. Ebselin has been shown to reduce ischemia-reperfusion injury in a model of acute lung injury[17,18]. Lynch and Kil, amongst others, have shown definite effects at reducing PTS thresholds[17,18,185]. Currently there are

no clinical trials that I am aware of using ebselin and Lynch & Kil have registered a patent relating to this therapy, this is expanded in more detail shortly.

- Vitamin A (retanoic acid) & ß-carotene (Vitamin A precursor that is converted within the human body into Vitamin A) have been shown to prevent against the deleterious effects of NIHL. Biesalski et al identified those deficiencies in Vitamin A led to an increase in the damaging effects of NIHL[186]. Both Vitamin A and ß-carotene act as an antioxidant and have demonstrated promising effects to protect against acoustic trauma[186-188].
- Vitamin C (often termed ascorbic acid or ascorbate) is synthesised by all mammals, with the exceptions of guinea pigs, monkeys and humans[21,189,190]. Vitamin C increases endogenous amount of antioxidants, induces the effects of enzymes involved in production of antioxidants (including SOD and catalase) and it also acts dirreclty as a free radical scavenger[2,91]. So far studies have shown a reduction of up to 20dB in animal models for NIHL[22,24-30,92]. It is often used in combination with magnesium and vitamins A & E by Coleen Le Prell's group, see below for more information[22,31-33,44,191].
- Vitamin E is the generic term for members the tocopherol family; alpha-tocopherol is the most biologically active of this group. Vitamin E is involved in prevention of lipid peroxidation by scavenging lipid peroxyl radicals that are extremely deleterious to constituents of the cell and in particular DNA. Studies have shown a mitigation of the deleterious effects of acoustic trauma when using Vitamin E[34,35,192,193] in a dose dependant fashion[36-40,82,193,194]. Some studies have shown protection up to 45dB compared to controls[41-43,194,195].
- Magnesium was previously discussed in detail in Chapter 2, it has been shown to be protective to hearing including in gunshot noise trauma in guinea pigs[41-44,109,191,195] and prolonged acoustic trauma[16,44,79,191,195-206]. There has been a dose dependant relationship documented[195,207-209]. The effects of magnesium to mitigate the effects of excessive noise are attributed to the prevention of reduction in coclear blood flow[47,202] due to ischemia

and the subsequent reperfussion injury. The ischemia causes hypoxia, with loss of aerobic metabolism and accumulation of local metabolites, all of which lead to damage directly and or indirectly. Calcium also has effects on calcium permeability, influx of calcium into the coclear hair cells and glutamate, which is known to cause excitotoxic damage[35,210]. There is also work now suggesting that magnesium is increasingly considered to mediate oxidative stress and DNA damage[48,211-213].

- There has been a significant amount of interest of using combination therapies to compliment each other synergistically and act on different pathways involved in pathological process that underpins NIHL. A combination of magnesium with Vitamins A/C/E, often termed MACE, is the combination therapy that has had the most interest in pre-clinical studies and currently has a clinical trial registered. Le Prell's group lead on this combination with multiple publications[44,45,191,204,205]. In their initial work the vitamins or the magnesium alone did not give rise to statically significant protection when compared to controls in an guinea pig model, however, when given together this gave rise to a synergistic effect that lead to up to 35dB reductions in NIHL[44,48].

6.3- Military implications of noise exposure:

Basic laboratory research in this field does not allow for direct systematic comparisons due to variation in species, noise insult, administration routes, and treatment time schedules. There is a considerable amount of basic auditory science work being carried out in the field of otoprotection, but it is fundamental to allow for translation from the laboratory bench to the bedsides of patients. The logical advancement in this research area is to move towards research in humans. Moving to human subjects adds a very important dimension of the ethical challenges faced when designing and conducting studies in humans without placing research subjects at risk of the deleterious effects of acoustic trauma. Such studies would need to identify populations

who are all ready at risk from acoustic trauma even though they actively use appropriate HPDs. There would need to be very high levels of noise exposure on a regular basis and the obvious groups would be high risk of industrial or military acoustic exposure, who are already well established to gain problems with PTS with associated hearing impairment. It is possible that military personnel, already exposed to excessive noise due to weapons and machinery, are an obvious choice of group. As alluded to in chapter 1, military personnel are in a fairly unique predicament due to the profound variability in military-related noise during training and combat environments. Noise can range from ambient low noise levels to exceptionally loud sounds (greater than 140dB) in a mere fraction of a second. Military weapons are producers of excess sound in milliseconds, and standard issue weapons for the American military such as a M16 rifle has a discharge of 156dB as an impulse burst. These are also the same weapons that are used in weapons training and military personnel are given HPDs, but it is physically impossible to protect against the acoustic trauma at such high levels. Weapons training makes up a crucial and fundamental part of military training. This leads to both a fortuitous and distinctive environment, where groups of young, healthy individuals, who have been screened for illnesses, are exposed to sounds in excess of 140dB, which is technically against code 29 of Federal Regulations (CFR 1910.95, 2009). These cohorts of military personnel would be ideal to enter studies for otoprotective agents to mitigate the effect of acoustic trauma. The design for said studies would involve using HPDs alone (as is currently being practiced) versus HPDs with added protection with novel otoprotective agents. These types of studies would need to be carried out over sufficient longevity to allow NIHL to develop and would therefore need long-term hearing testing with follow up. There is controversy over the type of test metrics that should be used in these types of studies: conventional pure-tone audiometry (PTA), extended high frequency (highPTA) or distortion product otoacoustic emissions (DPOAE).

6.4- Metrics used to test the effects of NIHL:

Conventional PTA is the normal metric that is used for assessment of hearing. There are multiple reasons for this, including that they are easy to carry out, cheap, generally reliable, reproducible and there is a massive amount of historical data available in the medical field. Most audiometric diagnoses are confirmed via PTA, see Chapter 1 for more information. Legally PTAs are used to define if a patient has NIHL and for the grounds for a claim due to excessive noise exposure. Human hearing ranges from 20Hz-20kHz and PTA are limited because the upper limit is normally at 8kHz. Using highPTA in conjunction with conventional PTA would be a very useful metric tool. The range for highPTA ranges from 9-20kHz, extending into the high-frequency range. Since 1969 the use of highPTA have been shown to detect ototoxic changes before a change arises in the conventional PTA range[50,214-218]. Even though this information has been recognised for over 4 decades, it is still not standard practice for testing high-risk groups. However, highPTA has been proven to be variable, which reduces usefulness in clinical settings. This brings us onto DPOAE as a significant metric because of the unique fact that this test specifically is an objective and sensitive test of outer hair cell function (OHC)[50,219-221]. As discussed in chapter 4, OHC are particularly prone to the insults of acoustic trauma and are more sensitive to damage when compared to inner hair cells (IHC)[51,222]. Over many years there have been a number of suggestions that DPOAE are predictive for subsequent elevated threshold changes in PTA[48,223-226] and as a consequence, have been suggested by many that they should be used for surveillance and early diagnosis of the consequences of acoustic trauma in high risk industries[52,53,226-230]. DPOAEs are not standard practice in high-risk groups and conventional PTAs dominate the hearing surveillance market. I am not sure if this is due to problems with equipment, funding, learning new techniques, apprehension towards change or a failure to review the current evidence as part of evidence-based medicine from a surveillance point of view. There is also the problem that there is a lack of national and international standards for DPAOE settings, calibration and testing, coupled with the fact that normative data

for large populations is missing. The usefulness of DPAOE testing in early diagnosis of NIHL is an expanding field and the Department of Veteran Affairs (USA) is currently inviting people to be recruited into a clinical trial [clinicaltrials.gov identifier NCT01022710, https://clinicaltrials.gov/ct2/show/NCT01022710]. This trial has been registered since 2009, started recruiting in January 2010, is still recruiting in mid 2015 and aims to be completed by December 2015, however no data has been published as of yet. The goal of this study is to identify features of DPOAEs that will improve clinical methods for the early detection of NIHL. There is testing for subtle post-noise changes in DPOAE and this will give rise to a new diagnostic metric. I hope this will help to develop an effective early diagnostic and monitoring test for NIHL.

6.5- Current clinical trials to treat and/or prevent NIHL:

There are limited clinical studies using otoprotective agents in NIHL. A review of clinicaltrials.gov, which is a service of the US National Institutes of Health and the National Library of Medicine, confirms the sparse number of these type of trials. This service is a registry of clinical trails and acts as a results database of clinical studies of human participants conducted around the world. I carried out a review of clinicaltrials.gov, the website was searched for "Noise-induced hearing loss", identifying 19 studies either in progress or completed. When these were further scrutinised 12 were not relevant to the specific topic relating to otoprotective agents. 7 trials were identified that were registered with clinicaltrials.gov that related to both NIHL and the use of otoprotective agents in a clinical trail. The findings are summarised in Table 4. Of the seven trials, two involved NAC, one involved steroids, the other four used different antioxidants. Three of the seven studies used a combination of treatments. Currently only two trials are completed. All study participants are adults, often-young adults specifically,

with no gender bias, who are already exposed to excessive amounts of noise trauma. Patients enrolled must have excessive noise exposure while using conventional methods of hearing protection, occupations in studies included (but not limited to) drill sergeant instructors or steel workers. Dependent on the precise trial, patients are/were either treated with a single medication or combination of treatments or a placebo. An interesting point that arose when reviewing the study designs is that only one of the studies, Guo et al, crosses over treatment arm between treatment and placebo. Currently these data appears to be limited, however promising, and will pave the way forward in the field of otoprotection entering the clinical realm.

Name of study	Senior Author(s)	Location	Testing	Registered date	Completion date	Weblink
Micronutrients to Prevent NIHL	Miller, Le Prell	Florida and Sweden	Mg, Vit A,C,E (Soundsbites and Auraquell)	Dec-08	Completed	https://clinicaltrials.gov/ct2/show/NCT00808470
Protective Effects of EPI-743 on NIHL	Le Prell	Florida	EPI-743	Aug-14	Feb 15 (delayed)	https://clinicaltrials.gov/ct2/show/NCT02257983
Phase 3 Clinical Trial: D-Methionine to reduce NIHL and tinnitis	Campbell, Bimson, Anderson	Southern Illinois University/Fort Jackson, USA	D-Methionine	Apr-11	Mar-17	https://clinicaltrials.gov/ct2/show/NCT01345474
Prevention of NIHL using zonisamide or methylprednisolone	Lieu	Washington School of Medicine, USA	zonisamide or methylprednisolone	Jan-14	Jan-19	https://clinicaltrials.gov/ct2/show/NCT02049073
Prevention of NIHL using Antioxidants-NAC & magnesium combo	Giles	University Hospital Antwerp, Belgium	NAC and magnesium combo (Antiocidantia)	Sep-12	Unknown	https://www.clinicaltrials.gov/ct2/show/NCT01727492
Antioxidation Medication in NIHL-NAC vs placebo (cross over)	Guo, Shih	National Taiwan University Hospital	NAC vs placebo with cross over	Nov-07	Completed	https://clinicaltrials.gov/ct2/show/NCT00552786
Study to determine	Tyson,	North Carolina,	HPN-07 vs HPN-	Oct-14	Jan-15	https://clinicaltri

safety, tolerability, & pharmacokinetic profile of HPN-07 or HPN-07 & NAC	Kopke	USA	07 with NAC vs placebo			als.gov/ct2/show/NCT02259595

Table 4- Current clinical trials registered with clinicaltrials.gov in the field of otoprotective Noise-Induced Hearing Loss, currently only 7 registered.

The military has an interest in otoprotection and are funding projects in this field. For example Prof Campbell's group from Illinois have been awarded a $1.2 million by the U.S. Department of Defence for their clinical trail of D-methionine. Intriguingly there are more patents registered for the use of otoprotective agents in NIHL than there are clinical trials registered. Reviewing the United States Patent and Trademark office there are quite a few patents awarded and registered. These include (but are not limited to):

- Formulation involving NAC in the form of "The Hearing Pill", which is already sold by American BioHealth Group. The patent for which as been registered to Richard Kopke, Donald Henderson and Micheal Hoffer since 2003.
- Magnesium with Vitamins A/C/E in the form of Auraquell® developed by OtoMedicine Incorporated. The funding for which was provided by General Motors and the United Auto Workers and registered in 2009. Subsequently a similar formation in the form of chewable mints has been released termed "Soundsbites", both of which are the brain childs of Joseph Miller and Coleen Le Prell.
- MRX-1,024 has been patented by Kathleen Campbell with the Molecular Therapeutics company. This is based upon D-Methionine.
- Ebselin which is being developed by Sound Pharmaceuticals and marked as SPI-1,005 and has been register to Kil and Lynch since 2010.
- The hearing loss pill by Audiens which contains Vitamins D3, Methyl B12, magnesium, L-glutathione, alpha lipoic acid, vinpocetine, Quercetin, Acetyl-L-Carnitine.

There are many more patents registered than these 5 that I have mentioned. The reason for patenting is often due to intellectual property but it may also be due to the potential financial incentives that could arise as a consequence of patenting such otoprotecive agents. Such financial incentives may account for the difference to the number of trails registered when compared to the larger number of patents registered in this field.

6.6- Closing summary:

Antioxidants that are able to halt the deleterious effects of free radicals are novel and have significant appeal clinically. Free radical production has been implicated in many diseases including, but not limited to, Parkinson's disease, Alzheimer's disease, diabetes[137] , stroke, and age related diseases involving degeneration[54]. There is widespread evidence to support the role of free radical scavengers in reducing neurodegenerative processes and NIHL. When agents are trialled against the deleterious effects of acoustic trauma, they should ideally be compared to the same type of acoustic trauma and animal model, use similar metrics to assess hearing, and use immunohistochemical analysis. Only then will this allow for comparison of different therapeutic agents. As discussed in chapter 1, there is a widespread variety in models including (but not limited to) animal selection (type, strain), acoustic trauma (impulse or continuous, broad or narrow band), hearing assessment (ABR, DPOAE, ECOG, CAP). However, it is important to establish a standard paradigm to allow for direct comparison of results, but in real life there is a variation in the type of sound and acoustic trauma that leads to hearing loss, therefore as a consequence of this fact it gives rise to the need for multiple scientific paradigms for different models to test otoprotective agents against the deleterious effects of NIHL. There is not a "single glove" that fits all experiments in this situation. Initially, agents need to demonstrate an efficacy

against the deleterious effects of acoustic trauma, ideally in a paradigm where they can be compared directly. Once a primary effect has been demonstrated, these agents should be tested at differing doses to identify the lowest dose to have an effect and a maximal dose to have an effect without any significant deleterious effects, leading to a dose-response curve for the agent. Dose-response curves will be pivotal for otoprotective agents to lead towards clinical trails, even though drug metabolism differs vastly from species to species. This will lead to the inevitable translation of bench work to the bedside and improve the care that patients with NIHL receive.

7.1- Appendix 1:

Raw data and threshold shifts for chapter 3 experiments. Any mice thresholds that are marked – detonate a mouse that did not complete the experiment.

i.) Saline Control Absolute Thresholds:

SALINE (Controls)	Baseline 8kHz	Baseline 16kHz	Baseline 24kHz	Temporary threshold 8kHz	Temporary threshold 16kHz	Temporary threshold 24kHz	Permanent threshold 8kHz	Permanent threshold 16kHz	Permanent threshold 24kHz
Mouse ID 2435	40	30	35	60	50	60	70	65	60
Mouse ID 2436	30	20	25	80	50	55	70	55	55
Mouse ID 2437	40	25	35	90	85	85	70	60	70
Mouse ID 2438	35	30	25	60	50	45	55	60	55
Mouse ID 2439	30	20	25	60	55	50	45	55	45
Mouse ID 2454	40	25	35	-	-	-	-	-	-
Mouse ID 2455	35	25	30	70	75	80	70	75	75
Mouse ID 2556	35	30	25	65	75	75	70	60	65
Mouse ID 2461	35	30	25	75	80	70	70	60	65
Mouse ID 2462	35	20	30	70	70	85	55	60	70
Average threshold	**35.50**	**25.50**	**29.00**	**70.00**	**65.56**	**67.22**	**63.89**	**61.11**	**62.22**

ii.) Saline Control Threshold Shifts:

SALINE (Controls)	TTS 8kHz	TTS 16kHz	TTS 24kHz	PTS 8kHz	PTS 16kHz	PTS 24kHz
Mouse ID 2435	**20**	**20**	**25**	**30**	**35**	**25**
Mouse ID 2436	**50**	**30**	**30**	**40**	**35**	**30**
Mouse ID 2437	**50**	**60**	**50**	**30**	**35**	**35**
Mouse ID 2438	**25**	**20**	**20**	**20**	**30**	**30**
Mouse ID 2439	**30**	**35**	**25**	**15**	**35**	**20**
Mouse ID 2454	-	-	-	-	-	-
Mouse ID 2455	**35**	**50**	**50**	**35**	**50**	**45**
Mouse ID 2556	**30**	**45**	**50**	**35**	**30**	**40**
Mouse ID 2461	**40**	**50**	**45**	**35**	**30**	**40**
Mouse ID 2462	**35**	**50**	**55**	**20**	**40**	**40**
Average Shift	**35.00**	**40.00**	**38.89**	**28.89**	**35.56**	**33.89**

iii.) Taurine 50mg Absolute Thresholds:

Taurine (50mg)	Baseline 8kHz	Baseline 16kHz	Baseline 24kHz	Temporary threshold 8kHz	Temporary threshold 16kHz	Temporary threshold 24kHz	Permanent threshold 8kHz	Permanent threshold 16kHz	Permanent threshold 24kHz
Mouse ID 1833	35	15	15	50	25	35	35	35	40
Mouse ID 1834	30	10	10	50	30	30	55	35	45
Mouse ID 1838	20	10	15	60	45	55	50	45	55
Mouse ID 1839	30	10	25	55	50	55	50	45	45
Mouse ID 1840	30	25	30	40	60	35	40	50	30
Mouse ID 1787	25	20	15	40	50	45	45	40	40
Mouse ID 1788	35	10	10	60	30	40	55	15	20
Mouse ID 1792	25	10	10	55	40	30	50	35	35
Mouse ID 1761	20	15	30	50	35	55	45	20	40
Mouse ID 1762	15	10	25	35	40	55	40	40	35

Average threshold	**26.50**	**13.50**	**18.50**	**49.50**	**40.50**	**43.50**	**46.50**	**36.00**	**38.50**

iV.) Taurine 50mg Threshold Shifts:

Taurine (50mg)	TTS 8kHz	TTS 16kHz	TTS 24kHz	PTS 8kHz	PTS 16kHz	PTS 24kHz
Mouse ID 1833	**15**	**10**	**20**	**0**	**20**	**25**
Mouse ID 1834	**20**	**20**	**20**	**25**	**25**	**35**
Mouse ID 1838	**40**	**35**	**40**	**30**	**35**	**40**
Mouse ID 1839	**25**	**40**	**30**	**20**	**35**	**20**
Mouse ID 1840	**10**	**35**	**5**	**10**	**25**	**0**
Mouse ID 1787	**15**	**30**	**30**	**20**	**20**	**25**
Mouse ID 1788	**25**	**20**	**30**	**20**	**5**	**10**
Mouse ID 1792	**30**	**30**	**20**	**25**	**25**	**25**
Mouse ID 1761	**30**	**20**	**25**	**25**	**5**	**10**
Mouse ID 1762	**20**	**30**	**30**	**25**	**30**	**10**
Average Shift	**23.00**	**27.00**	**25.00**	**20.00**	**22.50**	**20.00**

V.) Taurine 100mg Absolute Thresholds:

Taurine (100mg)	Baseline 8kHz	Baseline 16kHz	Baseline 24kHz	Temporary threshold 8kHz	Temporary threshold 16kHz	Temporary threshold 24kHz	Permanent threshold 8kHz	Permanent threshold 16kHz	Permanent threshold 24kHz
Mouse ID 1810	35	10	10	50	45	45	50	40	35
Mouse ID 1811	-	-	-	-	-	-	-	-	-
Mouse ID 1812	20	10	10	35	40	40	25	30	30
Mouse ID 1818	25	10	10	40	30	30	45	30	30
Mouse ID 1819	20	15	20	65	35	35	45	35	35
Mouse ID 1820	-	-	-	-	-	-	-	-	-
Mouse ID 1823	20	15	20	65	30	45	55	35	40
Mouse ID 1824	30	10	15	55	35	45	50	30	40
Mouse ID 1829	25	10	10	40	50	40	40	40	40
Mouse ID 1830	10	20	25	40	35	45	45	35	40
Average threshold	**23.13**	**12.50**	**15.00**	**48.75**	**37.50**	**40.63**	**44.38**	**34.38**	**36.25**

Vi.) Taurine 100mg Threshold Shifts:

Taurine (100mg)	TTS 8kHz	TTS 16kHz	TTS 24kHz	PTS 8kHz	PTS 16kHz	PTS 24kHz
Mouse ID 1810	**15**	**35**	**35**	**15**	**30**	**25**
Mouse ID 1811	-	-	-	-	-	-
Mouse ID 1812	**15**	**30**	**30**	**5**	**20**	**20**
Mouse ID 1818	**15**	**20**	**20**	**20**	**20**	**20**
Mouse ID 1819	**45**	**20**	**15**	**25**	**20**	**15**
Mouse ID 1820	-	-	-	-	-	-
Mouse ID 1823	**45**	**15**	**25**	**35**	**20**	**20**
Mouse ID 1824	**25**	**25**	**30**	**20**	**20**	**25**
Mouse ID 1829	**15**	**40**	**30**	**15**	**30**	**30**
Mouse ID 1830	**30**	**15**	**20**	**35**	**15**	**15**
Average Shift	**25.625**	**25**	**25.625**	**21.25**	**21.875**	**21.25**

Vii.) Taurine 200mg Absolute Thresholds:

Taurine (200mg)	Baseline 8kHz	Baseline 16kHz	Baseline 24kHz	Temporary threshold 8kHz	Temporary threshold 16kHz	Temporary threshold 24kHz	Permanent threshold 8kHz	Permanent threshold 16kHz	Permanent threshold 24kHz
Mouse ID 1795	20	10	10	50	25	30	45	25	30
Mouse ID 1796	20	10	15	40	40	40	30	40	50

Mouse ID 1797	30	10	10	50	30	30	40	15	25
Mouse ID 1798	40	30	30	65	45	50	50	45	45
Mouse ID 1799	25	10	10	40	35	40	40	35	10
Mouse ID 1802	30	10	15	45	35	40	45	20	20
Mouse ID 1744	20	10	20	60	30	40	60	35	55
Mouse ID 1800	15	10	15	30	30	45	25	20	20
Mouse ID 1803	40	10	25	40	45	35	40	15	30
Mouse ID 1804	20	10	25	55	40	55	40	50	55
Average threshold	**26.00**	**12.00**	**17.50**	**47.50**	**35.50**	**40.50**	**41.50**	**30.00**	**34.00**

Viii.) Taurine 200mg Threshold Shifts:

Taurine (200mg)	TTS 8kHz	TTS 16kHz	TTS 24kHz	PTS 8kHz	PTS 16kHz	PTS 24kHz
Mouse ID 1795	**30**	**15**	**20**	**25**	**15**	**20**
Mouse ID 1796	**20**	**30**	**25**	**10**	**30**	**35**
Mouse ID 1797	**20**	**20**	**20**	**10**	**5**	**15**
Mouse ID 1798	**25**	**15**	**20**	**10**	**15**	**15**
Mouse ID 1799	**15**	**25**	**30**	**15**	**25**	**0**
Mouse ID 1802	**15**	**25**	**25**	**15**	**10**	**5**
Mouse ID 1744	**40**	**20**	**20**	**40**	**25**	**35**
Mouse ID 1800	**15**	**20**	**30**	**10**	**10**	**5**
Mouse ID 1803	**0**	**35**	**10**	**0**	**5**	**5**
Mouse ID 1804	**35**	**30**	**30**	**20**	**40**	**30**
Average Shift	**21.50**	**23.50**	**23.00**	**15.50**	**18.00**	**16.50**

iX.) Taurine 400mg Absolute Thresholds:

Taurine (400mg)	Baseline 8kHz	Baseline 16kHz	Baseline 24kHz	Temporary threshold 8kHz	Temporary threshold 16kHz	Temporary threshold 24kHz	Permanent threshold 8kHz	Permanent threshold 16kHz	Permanent threshold 24kHz
Mouse ID 1751	30	10	20	45	40	35	30	30	35
Mouse ID 1752	25	10	10	40	40	40	55	30	35
Mouse ID 1753	20	10	10	35	35	15	35	15	10
Mouse ID 1754	30	25	20	50	45	30	55	60	45
Mouse ID 1755	40	25	15	55	40	55	45	30	40
Mouse ID 1756	20	20	10	50	45	50	50	40	45
Mouse ID 1757	30	10	15	55	40	45	45	25	40
Mouse ID 1758	10	10	20	35	30	40	50	35	40
Mouse ID 1759	35	15	35	55	45	35	40	35	35
Mouse ID 1760	25	20	20	50	50	55	45	60	50
Average threshold	**26.50**	**15.50**	**17.50**	**47.00**	**41.00**	**40.00**	**45.00**	**36.00**	**37.50**

X.) Taurine 400mg Threshold Shifts:

Taurine (400mg)	TTS 8kHz	TTS 16kHz	TTS 24kHz	PTS 8kHz	PTS 16kHz	PTS 24kHz
Mouse ID 1751	**15**	**30**	**15**	**0**	**20**	**15**
Mouse ID 1752	**15**	**30**	**30**	**30**	**20**	**25**
Mouse ID 1753	**15**	**25**	**5**	**15**	**5**	**0**
Mouse ID 1754	**20**	**20**	**10**	**25**	**35**	**25**
Mouse ID 1755	**15**	**15**	**40**	**5**	**5**	**25**
Mouse ID 1756	**30**	**25**	**40**	**30**	**20**	**35**
Mouse ID 1757	**25**	**30**	**30**	**15**	**15**	**25**
Mouse ID 1758	**25**	**20**	**20**	**40**	**25**	**20**
Mouse ID 1759	**20**	**30**	**0**	**5**	**20**	**0**
Mouse ID 1760	**25**	**30**	**35**	**20**	**40**	**30**
Average Shift	**20.50**	**25.50**	**22.50**	**18.50**	**20.50**	**20.00**

The statistical testing associate with data for chapter 3 experiments. Results from multiple 1-way ANOVA.

Xi.) 1-way ANOVA of 8kHz TTS data:

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Saline vs 50mg	12.00	2.648	Yes	*	0.2394 to 23.76
Saline vs 100mg	9.375	1.957	No	Non-significant	-3.062 to 21.81
Saline vs 200mg	13.50	2.980	Yes	*	1.739 to 25.26
Saline vs 300mg	11.50	2.538	No	Non-significant	-0.2606 to 23.26
Saline vs 400mg	14.50	3.200	Yes	*	2.739 to 26.26

Xii.) 1-way ANOVA of 16kHz TTS data:

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Saline vs 50mg	13.00	3.000	Yes	*	1.754 to 24.25
Saline vs 100mg	15.00	3.274	Yes	**	3.107 to 26.89
Saline vs 200mg	16.50	3.808	Yes	**	5.254 to 27.75
Saline vs 300mg	15.00	3.462	Yes	**	3.754 to 26.25
Saline vs 400mg	14.50	3.347	Yes	**	3.254 to 25.75

Xiii.) 1-way ANOVA of 24kHz TTS data:

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Saline vs 50mg	13.89	2.899	Yes	*	1.452 to 26.33
Saline vs 100mg	13.26	2.618	Yes	*	0.1117 to 26.42
Saline vs 200mg	15.89	3.316	Yes	**	3.452 to 28.33
Saline vs 300mg	11.39	2.377	No	Non-significant	-1.048 to 23.83
Saline vs 400mg	16.39	3.421	Yes	**	3.952 to 28.83

XiV.) 1-way ANOVA of 8kHz PTS data:

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Saline vs 50mg	8.889	1.749	No	Non-significant	-4.304 to 22.08
Saline vs 100mg	7.639	1.421	No	Non-significant	-6.314 to 21.59
Saline vs 200mg	13.39	2.634	Yes	*	0.1955 to 26.58
Saline vs 300mg	7.889	1.552	No	Non-significant	-5.304 to 21.08
Saline vs 400mg	10.39	2.044	No	Non-significant	-2.804 to 23.58

XV.) 1-way ANOVA of 16kHz PTS data:

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Saline vs 50mg	13.06	2.991	Yes	*	1.724 to 24.39
Saline vs 100mg	13.68	2.963	Yes	*	1.697 to 25.66
Saline vs 200mg	17.56	4.021	Yes	***	6.224 to 28.89
Saline vs 300mg	14.56	3.334	Yes	**	3.224 to 25.89
Saline vs 400mg	15.06	3.449	Yes	**	3.724 to 26.39

XVi.) 1-way ANOVA of 24kHz PTS data:

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Saline vs 50mg	13.89	2.988	Yes	*	1.825 to 25.95
Saline vs 100mg	12.64	2.571	No	Non-significant	-0.1198 to 25.40
Saline vs 200mg	17.39	3.741	Yes	**	5.325 to 29.45
Saline vs 300mg	9.889	2.128	No	Non-significant	-2.175 to 21.95
Saline vs 400mg	13.89	2.988	Yes	*	1.825 to 25.95

7.2- Appendix 2:

There were 195 search results in December 2015. All 195 results were reviewed and identified 77 suitable publications that identified antioxidants that had been used in pre-clinical (animal) models of NIHL. This appendix summarises the findings of these 77 studies.

Author	Year	Agent(s) Name	Agent action	Subject Strain	Noise Trauma	Hearing Testing
Yamosaba (1)	1998	Glutathione	Antioxidant (ROS primarily)	Guinea Pig (M, pigmented)	BBN, 102dB, 3hrs for 5 consectuive days	ABR
Yamosaba (2)	1999	(1) Deferoxamine mesylate, (2) Deferoxamine mesylate with manitol	(1) Iron chelator/Antioxidant (works on Fenton reaction) (2) Iron chelator with hydroxyl scavenger	Guinea Pig (F, pigmented)	4kHz, 115dB, 5hrs,	ABR
Attanasio	1999	Alopurinol	Antioxidant (Xanthine oxidase inhibitor)	Guniea Pig	2-3kHz, 125dB, 1.8hrs	CAP
Ohinata	2000	Glutathione	Antioxidant (ROS primarily)	Guinea Pig (M, pigmented)	4kHz (octave), 115dB, 5hrs	ABR
Rao	2000	PBN (Phenyl-N-tert-Butylnitrone)	Antioxidant	Rat (M, Long Evans)	13.6kHz (octave), 100dB, 2hrs, *also exposed to carbon monoxide	CAP
Kopke	2000	NAC (N-acetyl-cysteine) and salicylate	Antioxidant (Glutiathione pre-cursor/ROS primarily) and COX inhibitor	Chincillias (F, Lanigers)	4kHz (octave), 105dB, 6hrs	ABR
Karlidag	2002	Melatonin	Anitoxidant	Guinea Pig (M, albino)	0.25-10kHz, 100+/-2dB, 60hrs	ECOG
Hight	2003	Glutathione monoethylester and R-PIA	Antioxidant (Glutiathione pre-cursor/ROS primarily)	Chincillias	Impulse (100 presentations), 145dB SPL or Continous noise 4kHZ (ocatve), 105dB, 4hrs	EP
Canlon	2003	PBN	Antioxidant	Rat (F, Sprague-Dowley)	6-12kHz, at 105dB SPL for 2hrs or 110dB SPL for 4 hrs	ABR
Hou	2003	Alpha-tocopherol	Antioxidant (Xanthine oxidase inhibitor)	Guinea Pig (M, pigmented)	4kHz (octave), 100dB, 8hr for 3 consecutive days	ABR
Franzé	2003	Alopurinol	Antioxidant (Xanthine oxidase inhibitor)	Guniea Pig	White noise, 120dB SPL, 2hrs or Impulse noise, 114dB SPL, 5hrs	ABR
Cassandro	2003	Alopurinol and Cu/Zn SOD	Antioxidant (Xanthine oxidase inhibitor) and Antioxidant (ROS	Guniea Pig	Impulse, 2-3kHz, (presentation	ECOG and CAP

			primarily)		4/second), 1.8hrs	
Diao	2003	Alpha-Lipolic acid	Antioxidant	Guniea Pig	4kHz (ocatve), 115db SPL, 5hrs	ABR
Ohinita	2003	(1) N-acetyl-cysteine (2) +MK801	(1) Antioxidant (Glutiathione pre-cursor/ROS primarily) (2) NMDA Blocker	Guinea Pig (M, pigmented)	4kHz (octave), 115dB, 5hrs	ABR
Lynch	2004	Ebselen	Antioxidant (Glutiathione peroxide mimic)	Rat (F, F-344)	4-16kHz, 110/113/115dB SPL, 4hrs	ABR
Zhuraushii	2004	Carnosine	Antioxidant	Rat (M, Wistar)	5kHz, 103-107dB, 4hrs	Preyers reflex alone
Takemoto	2004	Endaverone	Anitoxidant	Guniea Pig (Hartley)	4kHz, 130dB, 3hrs	ABR
Scholik	2004	Vitamin E	Anitoxidant	Minnows (M, Fathead)	0.3-4kHz, 142dB, 2 or 20hrs	ABR
Duan	2004	NAC (N-acetyl-cysteine)	Anitoxidant (Glutiathione pre-cursor/ROS primarily)	Rat (Sprague-Dowley)	Impulse (50 presentations), 0.5-7kHz, 160dB SPL	ABR
Derekög	2004	Vitamin C (Ascorbic acid)	Anitoxidant	Rabbit (New Zealand strain)	1kHz, 100dB, 1hr	OAE
Yamashita	2005	Salicylates and trolox	Anitoxidant (ROS primarily) and Antioxidant (xanthine oxidase inhibitor, pre-cursor of alpha-tocopherol)	Guinea Pig (M, pigmented)	4kHz (octave), 120dB, 5hrs	ABR
Yamosaba	2005	Ebselen	Antioxidant (Glutiathione peroxide mimic)	Guinea Pig (M, albino)	4kHz, 115dB, 3hrs	ABR
Bielefeld	2005	NAC (N-acetyl-cysteine) and Src inhibitor	Antioxidant (Glutiathione pre-cursor/ROS primarily) and Glutiathione pro-drug	Chinchilla	4kHz (octave), 100dB, 6hrs for 4 consecutive days	EP
Kopke	2005	NAC (N-acetyl-cysteine) and Acetyl-L-Carnitine	Antioxidant (Glutiathione pre-cursor/ROS primarily) and Energy enhancer	Chinchilla	Impulse (150 presentations), 155dB	ABR
McFadden	2005	Vitamin C (Ascorbic acid)	Anitoxidant	Guinea Pig (Hartley albino)	4kHz (octave), 114dB, 6hrs	ABR
Tanaka	2005	Endaverone	Anitoxidant	Guinea Pig (M, Hartley)	4Khz, 130dB SPL, 3hrs	ABR
Hou	2005	Vitamin E	Anitoxidant	Guniea Pig	4kHz (octave), 100dB, 8hrs for 3 consecuive days	ABR
Murishita	2006	(1) Tempol (2) 3-aminobenzamine	(1) Antioxidant (ROS primarily) (2) Antioxidant (Poly ADP Synthase inhibitor)	Mice (F, ddY)	4kHz (pure tone), 110-128dB, 4hrs	ABR
Sergi	2006	Idebenone	Anitoxidant	Guinea Pig (Hartley albino)	6kHz (pure tone), 120db SPL, 0.66hrs	ABR
Lorito	2006	LNAC (L-N-acetyl-cysteine)	Antioxidant (Glutiathione pre-cursor/ROS primarily)	Rat (Sprague-Dowley)	8kHz (octace), 105dB, 4hrs	OAE
Duan	2006	Caroveine	Anitoxidant and Glutamate blocker	Rat (Sprague-Dowley)	Impulse (50 presentations), 160dB SPL	ABR
Kil	2007	Ebselen	Antioxidant (Glutiathione peroxide mimic)	Rat	4-16kHz, 113dB, 4hrs	ABR
Campbell	2007	D-Methionine	Anitoxidant	Chinchilla (F, Langier)	4kHz (narrow band), 105dB	ABR

					SPL, 6hrs	
Coleman	2007	NAC (N-acetyl-cysteine) and Acetyl-L-Carnitine	Antioxidant (Glutiathione pre-cursor/ROS primarily) and Energy enhancer	Chincillias (F, Lanigers)	4kHz (ocatve), 105db SPL, 6hrs	ABR
Le Prell	2007	Magnesium and Vitamins A/C/E	Blood flow regulator and antioxidants	Guinea Pig (M, pigmented)	4kHz (ocatve), 120db SPL, 5hrs	ABR
Minami	2007	Tempol and Creatine	Anitoxidant and Energy enhancer	Guinea Pig (M, pigmented)	4kHz (ocatve), 120db SPL, 5hrs	ABR
Bielefeld	2007	NAC (N-acetyl-cysteine)	Antioxidant (Glutiathione pre-cursor/ROS primarily)	Chinchilla	4kHz (ocatve), 105db SPL, 6hrs	ABR
Diao	2007	L-NAME (Nitro-L-Arginine Methyl Ester)	Antioxidant (RNS primarily)	Guinea Pig (M, pigmented Long-Evans)	4kHz (ocatve), 115db SPL, 5hrs	ABR
Adelman	2008	Salicylic acid	Anitoxidant	Mice (M, Albino)	0.25-8kHz, 113dB SPL, 3.5hrs	ABR
Cheng	2008	D-Methionine	Anitoxidant	Guinea Pig (M, albino)	0.125-15kHz, 105dB, 0.167hrs	ABR
Fetoni	2008	Idebenone and Vitamin E	Anitoxidants	Guinea Pig (albino)	6kHz (pure tone), 120db SPL, 0.66hrs	ABR
Samson	2008	D-Methionine	Anitoxidant	Mice (C57BL/6)	4kHz (octave), 110dB SPL, 4hrs	ABR
Heinrich	2008	Vitamin C (Ascorbic acid)	Anitoxidant	Guinea Pig (M, pigmented)	90dB, 1hr	ABR
Choi	2008	4-OHPBN (4-hydroxy phenyl N-tert-butylnitrone)	Anitoxidant	Chinchilla (F, Langier)	4kHz (ocatve), 105db SPL, 6hrs	ABR
Lorito	2008	LNAC (L-N-acetyl-cysteine)	Anitoxidant (Glutiathione pre-cursor/ROS primarily)	Rat (M, Sprague Dawley Albino)	8kHz, 105db SPL, 4hrs	ABR and OAE
Hirose	2008	Co-enzyme Q10	Anitoxidant	Guniea Pig (M, Hartley)	4kHz (centred), 130dB SPL, 3hrs	ABR
Gao	2009	Endaverone	Anitoxidant (Glutiathione pre-cursor/ROS primarily)	Guniea Pig	125dB SPL, 2hrs	ABR
Fetoni	2009	NAC (N-acetyl-cysteine)	Anitoxidant	Guinea Pig (F)	16kHz (pure tone), 120dB SPL, 0.5hrs	CAP
Fischer	2009	Vitamin C (Ascorbic acid)	Anitoxidant	Guniea Pig	90dB, 1hr	ABR
Coleman	2010	Salicylic acid and NAC (N-acetyl-cysteine)	Anitoxidant	Chinchilla (F, Langier)	4kHz (octave), 105dB, 6hrs	ABR
Tamir	2010	(1) NAC (2) Frusemide (3) NAC & Frusemide (4) Magnesium & Vitamins A/C/E (5) Magnesium & Vitamins A/C/E & Frusemide	(1) Anitoxidant (Glutiathione pre-cursor/ROS primarily) (2) Diuertic (4) Blood flow regulator and antioxidants	Mice (M, Sabra Albino)	2kHz (centred), 113dB SPL, 3.5hrs	ABR
Wu	2010	NAC (N-acetyl-cysteine)	Anitoxidant (Glutiathione pre-cursor/ROS primarily)	Rat (M, Wistar)	1-20kHz, 110dB, 8hrs for 10 consecutive days	ABR
Fetoni	2010	Ferulic acid	Anitoxidant	Guniea Pig (Hartley)	6kHz (pure tone), 120db SPL, 1hr	ABR
Nagashima	2010	L-NAME (Nitro-L-Arginine Methyl Ester) and Tempol	Antioxidants (RNS and ROS scavenger repectively)	Mice (M, Std-ddY)	8kHz (octace), 110dB, 1hr	ABR
Lin	2010	Hydrogen	Anitoxidant	Guniea Pig (M, Hartley)	4kHz (octave), 115dB, 3hrs	ABR and OAE

Bielefeld	2011	NAC (N-acetyl-cysteine) and Src inhibitor	Anitoxidant (Glutiathione pre-cursor/ROS primarily) and Glutathione pro-drug	Chinchilla	4kHz (ocatve), 107db SPL, 2hrs	ABR
Le Prell	2011	Magnesium and Vitamins A/C/E	Blood flow regulator and antioxidants	Guniea Pig	4kHz (ocatve), 110db SPL, 4hrs	CAP
Xiong	2011	Astragaloside	Anitoxidant (inhibits RNS)	Guinea Pig (pigmented)	Impulse (assult rifle), 10 shots (0.35ms each), 176dB SPL	ABR
Choi	2011	4-OHPBN (4-hydroxy phenyl N-tert-butylnitrone), NAC (N-acetyl-cysteine) and acetyl-L-carnitine	Antioxidant and energy enhancer	Chinchilla (F, Langier)	4kHz (ocatve), 105db SPL, 6hrs	ABR
Clifford	2011	D-Methionine and NAC (N-acetyl-cysteine)	Anitoxidant	Chinchilla (F, Langier)	4kHz (ocatve), 105db SPL, 6hrs	ABR
Cascella	2012	ACUVEL 400 (Coenzyme Q10, Vitamin B1/B2/B6/B12/E, melatonin)	Anitoxidant	Rat (Sprague-Dowley)	6kHz, 115dB SPL, 2hrs	ABR
Du	2012	4-OHPBN (4-hydroxy phenyl N-tert-butylnitrone), NAC (N-acetyl-cysteine) and acetyl-L-carnitine	Antioxidant and energy enhancer	Chinchilla	4kHz (octave) 105dB, 6hrs	ABR
Xiong (1)	2012	Radix Astragali, alpha-lipoic acid, vitamin E	Anitoxidant	Guniea Pig	1.05-20.3kHz, Impulse (Assualt riffle), 10 shots, 176dB SPL	ABR
Xiong (2)	2012	Astragalode	Antioxidant and involved with calcium homeostasis	Guinea Pig (pigmented)	1.05-20.3kHz, Impulse (Assualt riffle), 10 shots, 176dB SPL	ABR and OAE
Ewert	2012	NAC (N-acetyl-cysteine) and HPN-07	Anitoxidant (Glutiathione pre-cursor/ROS primarily) and Antioxidant	Rat (Black)	Blast stimulator, 3 consecutive 14psi blasts	ABR and OAE
Fetoni	2012	Co-enzyme Q10	Anitoxidant	Rat (Wistar)	10kHz, 120dB, 4hrs	ABR
Zhou	2012	Hydrogen	Anitoxidant	Guniea Pig	4kHz, 115dB, 4hrs	ABR and OAE
Fetoni	2013	Co-enzyme Q10	Anitoxidant	Rat (Wistar)	10kHz centred, 100dB SPL, an hour for 10 consecutive days	ABR
Rewerska	2013	D-Methionine	Anitoxidant	Mice (C57BL/6)	4kHz (ocatve), 110db SPL, 3hrs	ABR
Seidman	2013	Resveratol	Anitoxidant	Rat (Fischer)	105dB, 24hrs	N/A
Pourbakht	2013	Celecoxib	Antioxidant/NSAID (COX 2 Blocker)	Guinea Pig (M, albino)	4kHz (ocatve), 102db SPL, 3hrs	ABR
Yenigun	2013	Ozone (O3)	Anitoxidant	Rat (Wistar)	White band noise, 105dB, 4hrs	ABR
Mohamadkhani	2013	Silgmarin	Anitoxidant (flavanoid)	Guniea Pig	4kHz (ocatve), 102db SPL, 6hrs	ABR
Chen	2014	Hydrogen-saturadted saline	Anitoxidant	Guinea Pig (albino)	2.5-3.5kHz, 130dB SPL, 1hr	ABR and OAE
Park	2014	Renexin	Anitoxidant	Mice (C57BL/6)	110dB SPL, 1hr	ABR
Choi	2014	4-OHPBN (4-hydroxy phenyl N-tert-	Anitoxidant (Glutiathione pre-	Chinchilla	4kHz (octave) 105dB, 6hrs	OAE

		butylnitrone) and NAC (N-acetyl-cysteine) and acetyl-L-carnitine	cursor/ROS primarily) and Antioxidant			
Lu	2014	NAC (N-acetyl-cysteine) and HPN-07	Anitoxidant	Rat	10-20kHz, 115dB, 1hr	ABR and OAE

7.2- Bibliography:

1. Agrawal, Y., Platz, E. A., Niparko, J. K. (2008). Prevalence of hearing loss and differences by demographic characteristics among US adults: data from the National Health and Nutrition Examination Survey, 1999-2004. Archives of Internal Medicine, 168(14), 1522–1530.
2. World Health Organization. (2013). World Health Organization: Deafness and hearing loss – Fact Sheet No. 300.
3. Brigande, J. V., Heller, S. (2009). Quo vadis, hair cell regeneration? Nature Neuroscience, 12(6), 679–685.
4. Looi, V., Gfeller, K., Driscoll, V. (2012). Music appreciation and training for cochlear implant recipients: a review. Seminars in Hearing. 04, 307-334.
5. Corwin, J. T., Cotanche, D. A. (1988). Regeneration of sensory hair cells after acoustic trauma. Science (New York, NY), 240(4860), 1772–1774.
6. Ryals, B. M., Rubel, E. W. (1988). Hair cell regeneration after acoustic trauma in adult Coturnix quail. Science (New York, NY), 240(4860), 1774-1776.
7. Warchol, M. E., Lambert, P. R., Goldstein, B. J., Forge, A. (1993). Regenerative proliferation in inner ear sensory epithelia from adult guinea pigs and humans. *Science (New York, NY)*, 259, 1619-1622.
8. Forge, A., Li, L., Corwin, J. T., Nevill, G. (1993). Ultrastructural evidence for hair cell regeneration in the mammalian inner ear. *Science (New York, NY)*, 259(5101), 1616-1619.
9. Leake, P. A., Hradek, G. T. (1988). Cochlear pathology of long term neomycin induced deafness in cats. *Hearing Research*. 33(1), 11-33.

10. Waltzman, S. B. (2006). Cochlear implants: current status. *Expert Review of Medical Devices*, 3(5), 647-655.

11. Ramazzini, B. (2001). De morbis artificum diatriba [diseases of workers]. 1713. *American journal of public health*, 91, 1380–1382.

12. Lynch, E. D., Kil, J. (2005). Compounds for the prevention and treatment of noise-induced hearing loss. *Drug Discovery Today*, *10*(19), 1291–1298.

13. Quaranta, A. (2001). Noise Induced Hearing Loss: Basic Mechanisms. Prevention and Control. London: NRN Publications.

14. Shargorodsky, J., Curhan, S. G., Curhan, G. C., Eavey, R. (2010). Change in prevalence of hearing loss in US adolescents. *JAMA : the Journal of the American Medical Association*, *304*(7), 772–778.

15. Kopke, R., Bielefeld, E., Liu, J., Zheng, J., Jackson, R., Henderson, D., Coleman, J. K. M. (2005). Prevention of impulse noise-induced hearing loss with antioxidants. *Acta Oto-Laryngologica*, *125*(3), 235–243.

16. Attias, J., Sapir, S., Bresloff, I., Reshef-Haran, I., Ising, H. (2004). Reduction in noise-induced temporary threshold shift in humans following oral magnesium intake. *Clinical Otolaryngology and Allied Sciences*, *29*(6), 635–641.

17. Phoon, W. H., Lee, H. S., Chia, S. E. (1993). Tinnitus in noise-exposed workers. *Occupational Medicine (Oxford, England)*, *43*(1), 35–38.

18. Griest, S. E., Bishop, P. M. (1998). Tinnitus as an early indicator of permanent hearing loss. A 15 year longitudinal study of noise exposed workers. *AAOHN*, *46*(7), 325–329.

19. Wilt, J., Bjorn, V. (2006). Noise and advanced hearing protection. 45th Navy Occupational Health Preventive Medicine Conference.

20. Ylikoski, M. E., Pekkarinen, J. O., Starck, J. P. (1995). Physical characteristics of gunfire impulse noise and its attenuation by hearing protectors. *Scandinavian Audiology, 24(1), 3-11.*

21. Taylor, W., Pearson, J., Mair, A., Burns, W. (1965). Study of noise and hearing in jute weaving. *J Acoust Soc Am, 38, 113-120.*

22. Jones, R. C., Stevens, S. S., Lurie, M. H. (1940). Three mechanisms of hearing by electrical stimulation. *J Acoust Soc Am, 12(2).*

23. Davis, H. (1953). Energy into nerve impulses; hearing. *Medical Bulletin. St. Louis University*, *5*(3), 43–48.

24. Spoendlin, H. (1971). Primary structural changes in the organ of Corti after acoustic overstimulation. *Acta Oto-Laryngologica*, 71(2), 166-176

25. Spoendlin, H. (1972). Noise and cochlea. JFORL, 21(2), 107-113.

26. Spoendlin, H. (1972). Innervation densities of the cochlea. *Acta Oto-Laryngologica*, 73(2), 235-248.

27. Spoendlin, H., Brun, J. P. (1973). Relation of structural damage to exposure time and intensity in acoustic trauma. *Acta Oto-Laryngologica*, 75(2), 220-226.

28. Salvi, R. J., Hamernik, R. P., Henderson, D. (1979). Auditory nerve activity and cochlear morphology after noise exposure. *Archives of Oto-Rhino-Laryngology*, *224*(1-2), 111–116.

29. Roberto, M., Hamernik, R. P., Salvi, R. J., Henderson, D., Milone, R. (1985). Impact noise and the equal energy hypothesis. *J Acoust Soc Am*, *77*(4), 1514–1520.

30. Henderson, D., Hamernik, R. P. (1986). Impulse noise: critical review. *J Acoust Soc Am, 80(2).*

31. Beagley, H. A. (1965). Acoustic Trauma in the Guinea Pig: I. Electrophysiology and Histology. *Acta Oto-Laryngologica*, *60*(1-6), 437–451.

32. Beagley, H. A. (1965). Acoustic Trauma in the Guinea Pig: II. Electron microscopy including the morphology of cell junctions in the organ of Corti. *Acta Oto-Laryngologica*, *60*(1-6), 479–495.

33. Lim, D. J., Melnick, W. (1971). Acoustic damage of the cochlea. A scanning and transmission electron microscopic observation. *Archives of Otolaryngology (Chicago, Ill. : 1960)*, *94*(4), 294–305.

34. Kujawa, S. G., Liberman, M. C. (2006). Acceleration of age-related hearing loss by early noise exposure: evidence of a misspent youth. *The Journal of Neuroscience*, *26*(7), 2115–2123.

35. Kujawa, S. G., Liberman, M. C. (2009). Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss. *The Journal of Neuroscience*, 29(45), 14077-14085.

36. Yamane, H., Nakai, Y., Takayama, M., Konishi, K., Iguchi, H., Nakagawa, T., et al. (1995). The emergence of free radicals after acoustic trauma and strial blood flow. *Acta Oto-Laryngologica Supplementum*, *519*, 87–92.

37. Yamane, H., Nakai, Y., Takayama, M., Iguchi, H., Nakagawa, T., Kojima, A. (1995). Appearance of free radicals in the guinea pig inner ear after noise-induced acoustic trauma. *European Archives of Oto-Rhino-Laryngology*, *252*(8), 504–508.

38. Clerici, W. J., Hensley, K., DiMartino, D. L., Butterfield, D. A. (1996). Direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants. *Hearing Research*, *98*(1-2), 116–124.

39. Clerici, W. J., Yang, L. (1996). Direct effects of intraperilymphatic reactive oxygen species generation on cochlear function. *Hearing Research*, *101*(1-2), 14–22.

40. Clerici, W. J. (1996). Effects of superoxide dismutase and U74389G on acute trimethyltin-induced cochlear dysfunction. *Toxicology and Applied Pharmacology*, *136*(2), 236–242.

41. Hawkins, J. E. (1971). The role of vasoconstriction in noise-induced hearing loss. *The Annals of Otology, Rhinology, and Laryngology*, *80*(6), 903–913.

42. Nara, T., Goto, N., Nakae, Y., Okada, A. (1993). Morphometric development of the human auditory system: ventral cochlear nucleus. *Early Human Development*, 32(2-3), 93-102.

43. Quirk, W. S., Seidman, M. D. (1995). Cochlear vascular changes in response to loud noise. *The American Journal of Otology*, *16*(3), 322–325.

44. Le Prell, C. G., Hughes, L. F., Miller, J. M. (2007). Free radical scavengers vitamins A, C, and E plus magnesium reduce noise trauma. *Free Radical Biology Medicine*, *42*(9), 1454–1463.

45. Kopke, R. D., Hoffer, M. E., Wester, D., O'Leary, M. J., Jackson, R. L. (2001). Targeted topical steroid therapy in sudden sensorineural hearing loss. *Otology Neurotology*, *22*(4), 475–479.

46. Bohne, B. A., Clark, W. W. (1982). Growth of hearing loss and cochlear lesion with increasing duration of noise exposure. New perspectives on noise-induced hearing loss, 283-302.

47. Hu, B. H., Henderson, D., Nicotera, T. M. (2006). Extremely rapid induction of outer hair cell apoptosis in the chinchilla cochlea following exposure to impulse noise. *Hearing Research*, *211*(1-2), 16–25.

48. Subramaniam, M., Campo, P., Henderson, D. (1991). Development of resistance to hearing loss from high frequency noise. *Hearing Research*, *56*(1-2), 65–68.

49. Kopke, R. D., Hoffer, M. E., Wester, D., O'Leary, M. J., Jackson, R. L. (2001). Targeted topical steroid therapy in sudden sensorineural hearing loss. *Otology Neurotology*, *22*(4), 475–479.

50. Harding, G. W., Bohne, B. A. (2009). Relation of focal hair-cell lesions to noise-exposure parameters from a 4- or a 0.5-kHz octave band of noise. *Hearing Research*, *254*(1-2), 54–63.

51. Nilsson, P., Erlandsson, B., Håkanson, H., Ivarsson, A., Wersäll, J. (1980). Morphological damage in the guinea pig cochlea after impulse noise and pure tone exposures. *Scandinavian Audiology Supplementum*, (Sp12), 155–162.

52. Robertson, D., Johnstone, B. M. (1980). Acoustic trauma in the guinea pig cochlea: early changes in ultrastructure and neural threshold. *Hearing Research*, *3*(2), 167–179.

53. Gao, W. Y., Ding, D. L., Zheng, X. Y., Ruan, F. M., Liu, Y. J. (1992). A comparison of changes in the stereocilia between temporary and permanent hearing losses in acoustic trauma. *Hearing Research*, *62*(1), 27–41.

54. Tsuprun, V., Schachern, P. A., Cureoglu, S., Paparella, M. (2003). Structure of the stereocilia side links and morphology of auditory hair bundle in relation to noise exposure in the chinchilla. *Journal of Neurocytology*, *32*(9), 1117–1128.

55. Henderson, D., Hamernik, R. P. (1986). Impulse noise: critical review. *J Acoust Soc Am, 80(2).*

56. Hamernik, R. P., Turrentine, G., Roberto, M., Salvi, R., Henderson, D. (1984). Anatomical correlates of impulse noise-induced mechanical damage in the cochlea. *Hearing Research*, *13*(3), 229–247.

57. Hamernik, R. P., Turrentine, G., Wright, C. G. (1984). Surface morphology of the inner sulcus and related epithelial cells of the cochlea following acoustic trauma. *Hearing Research*, *16*(2), 143–160.

58. Lipscomb, D. M., Axelsson, A., Vertes, D., Roettger, R., Carrol, J. (1977). The effect of high level sound on hearing sensitivity, cochlear sensorineuroepithelium and vasculature of the chinchilla. *Acta Oto-Laryngologica*, *84*(1-2), 44–56.

59. Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry Cell Biology*, *39*(1), 44–84.

60. Kovacic, P., Jacintho, J. D. (2001). Mechanisms of carcinogenesis: focus on oxidative stress and electron transfer. *Current Medicinal Chemistry*, *8*(7), 773–796.

61. Mancardi, D., Ridnour, L. A., Thomas, D. D., Katori, T., Tocchetti, C. G., Espey, M. G., et al. (2004). The chemical dynamics of NO and reactive nitrogen oxides: a practical guide. *Current Molecular Medicine*, *4*(7), 723–740.

62. Lafont, O. (2007). Life and death of free radicals. *Revue D'histoire De La Pharmacie*, 54(352), 475-478.

63. Gerschman, R., Nadig, P. W., Snell, A. C., Nye, S. W. (1954). Effect of high oxygen concentrations on eyes of newborn mice. *The American Journal of Physiology*, *179*(1), 115–118.

64. Tomioka, H., Iwamoto, E., Itakura, H., Hirai, K. (2001). Generation and characterization of a fairly stable triplet carbene. *Nature*, *412*(6847), 626–628.

65. Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., Mazúr, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions*, *160*(1), 1–40.

66. Desaki, Y., Miya, A., Venkatesh, B., Tsuyumu, S., Yamane, H., Kaku, H., et al. (2006). Bacterial lipopolysaccharides induce defense responses associated with programmed cell death in rice cells. *Plant Cell Physiology*, *47*(11), 1530–1540.

67. Yamashita, D., Jiang, H.-Y., Schacht, J., Miller, J. M. (2004). Delayed production of free radicals following noise exposure. *Brain Research*, *1019*(1-2), 201–209.

68. Jang, A. S., Choi, I. S. (1999). Nitric oxide metabolites in patients with asthma: induced sputum versus blood. *Respiratory Medicine*, *93*(12), 912–918.

69. Hoffman, M., Weinberg, J. B. (1987). Tumor necrosis factor-alpha induces increased hydrogen peroxide production and Fc receptor expression, but not increased Ia antigen expression by peritoneal macrophages. *Journal of Leukocyte Biology*, *42*(6), 704–707.

70. Hoffman, D. W., Whitworth, C. A., Jones-King, K. L., Rybak, L. P. (1988). Potentiation of ototoxicity by glutathione depletion. *The Annals of Otology, Rhinology, and Laryngology*, *97*(1), 36–41.

71. Lautermann, J., McLaren, J., Schacht, J. (1995). Glutathione protection against gentamicin ototoxicity depends on nutritional status. *Hearing Research*, *86*(1-2), 15–24.

72. Lautermann, J., Crann, S. A., McLaren, J., Schacht, J. (1997). Glutathione-dependent antioxidant systems in the mammalian inner ear: effects of aging, ototoxic drugs and noise. *Hearing Research*, *114*(1-2), 75–82.

73. Ravi, R., Somani, S. M., Rybak, L. P. (1995). Mechanism of cisplatin ototoxicity: antioxidant system. *Pharmacology Toxicology*, *76*(6), 386–394.

74. Usami, S., Hjelle, O. P., Ottersen, O. P. (1996). Differential cellular distribution of glutathione–an endogenous antioxidant–in the guinea pig inner ear. *Brain Research*, 743(1-2), 337-340.

75. Lautermann, J., Crann, S. A., McLaren, J., Schacht, J. (1997). Glutathione-dependent antioxidant systems in the mammalian inner ear: effects of aging, ototoxic drugs and noise. *Hearing Research*, *114*(1-2), 75–82.

76. Ravi, R., Somani, S. M., Rybak, L. P. (1995). Mechanism of cisplatin ototoxicity: antioxidant system. *Pharmacology Toxicology*, *76*(6), 386–394.

77. Attanasio, G., Barbara, M., Buongiorno, G., Cordier, A., Mafera, B., Piccoli, F., et al. (1999). Protective effect of the cochlear efferent system during noise exposure. *Annals of the New York Academy of Sciences*, *884*, 361–367.

78. Rao, D., Fechter, L. D. (2000). Protective effects of phenyl-N-tert-butylnitrone on the potentiation of noise-induced hearing loss by carbon monoxide. *Toxicology and Applied Pharmacology*, *167*(2), 125–131.

79. Kopke, R. D., Weisskopf, P. A., Boone, J. L., Jackson, R. L., Wester, D. C., Hoffer, M. E., et al. (2000). Reduction of noise-induced hearing loss using L-NAC and salicylate in the chinchilla. *Hearing Research*, *149*(1-2), 138–146.

80. Karlidağ, T., Yalçin, S., Oztürk, A., Ustündağ, B., Gök, U., Kaygusuz, I., Susaman, N. (2002). The role of free oxygen radicals in noise induced hearing loss: effects of melatonin and methylprednisolone. *Auris, Nasus, Larynx*, *29*(2), 147–152.

81. Canlon, B., Erichsen, S., Nemlander, E., Chen, M., Hossain, A., Celsi, G., Ceccatelli, S. (2003). Alterations in the intrauterine environment by glucocorticoids modifies the developmental programme of the auditory system. *The European Journal of Neuroscience*, *17*(10), 2035–2041.

82. Hou, F., Wang, S., Zhai, S., Hu, Y., Yang, W., He, L. (2003). Effects of alpha-tocopherol on noise-induced hearing loss in guinea pigs. *Hearing Research*, *179*(1-2), 1–8.

83. Hou, F., Wang, S., Hu, Y. (2003). Effects of noise on antioxidant enzymes of cochlea in guinea pigs. *Chinese Journal of Industrial Hygiene and Occupational Diseases*, *21*(2), 121–123.

84. Franzé, A., Sequino, L., Saulino, C., Attanasio, G., Marciano, E. (2003). Effect over time of allopurinol on noise-induced hearing loss in guinea pigs. *International Journal of Audiology*, *42*(4), 227–234.

85. Cassandro, E., Sequino, L., Mondola, P., Attanasio, G., Barbara, M., Filipo, R. (2003). Effect of superoxide dismutase and allopurinol on impulse noise-exposed guinea pigs--electrophysiological and biochemical study. *Acta Oto-Laryngologica*, *123*(7), 802–807.

86. Diao, M.F., Liu, H.Y., Zhang, Y. M., Gao, W.Y. (2003). Changes in antioxidant capacity of the guinea pig exposed to noise and the protective effect of alpha-lipoic acid against acoustic trauma. *Acta Physiologica Sinica*, *55*(6), 672–676.

87. Zhuravskii, S. G., Aleksandrova, L. A., Ivanov, S. A., Sirot, V. S., Lopotko, A. I., Zhloba, A. A. (2004). Protective effect of carnosine on excitable structures of the

auditory apparatus in albino rats with acute acoustic trauma. *Bulletin of Experimental Biology and Medicine*, *137*(1), 98–102.

88. Takemoto, T., Sugahara, K., Okuda, T., Shimogori, H., Yamashita, H. (2004). The clinical free radical scavenger, edaravone, protects cochlear hair cells from acoustic trauma. *European Journal of Pharmacology*, *487*(1-3), 113–116.

89. Duan, M., Qiu, J., Laurell, G., Olofsson, A., Counter, S. A., Borg, E. (2004). Dose and time-dependent protection of the antioxidant N-L-acetylcysteine against impulse noise trauma. *Hearing Research*, *192*(1-2), 1–9.

90. Duan, M., Chen, Z., Qiu, J., Ulfendahl, M., Laurell, G., Borg, E., Ruan, R. (2006). Low-dose, long-term caroverine administration attenuates impulse noise-induced hearing loss in the rat. *Acta Oto-Laryngologica*, *126*(11), 1140–1147.

91. Dereköy, F. S., Köken, T., Yilmaz, D., Kahraman, A., Altuntaş, A. (2004). Effects of ascorbic acid on oxidative system and transient evoked otoacoustic emissions in rabbits exposed to noise. *The Laryngoscope*, *114*(10), 1775–1779.

92. McFadden, S. L., Woo, J. M., Michalak, N., Ding, D. (2005). Dietary vitamin C supplementation reduces noise-induced hearing loss in guinea pigs. *Hearing Research*, *202*(1-2), 200–208.

93. Tanaka, K., Takemoto, T., Sugahara, K., Okuda, T., Mikuriya, T., Takeno, K., et al. (2005). Post-exposure administration of edaravone attenuates noise-induced hearing loss. *European Journal of Pharmacology*, *522*(1-3), 116–121.

94. Bielefeld, E. C., Hynes, S., Pryznosch, D., Liu, J., Coleman, J. K., Henderson, D. (2005). A comparison of the protective effects of systemic administration of a pro-glutathione drug and a Src-PTK inhibitor against noise-induced hearing loss. *Noise Health*, *7*(29), 24–30.

95. Sergi, B., Fetoni, A. R., Paludetti, G., Ferraresi, A., Navarra, P., Mordente, A., Troiani, D. (2006). Protective properties of idebenone in noise-induced hearing loss in the guinea pig. *Neuroreport*, *17*(9), 857–861.

96. Lorito, G., Giordano, P., Prosser, S., Martini, A., Hatzopoulos, S. (2006). Noise-induced hearing loss: a study on the pharmacological protection in the Sprague Dawley rat with N-acetyl-cysteine. *Acta Otorhinolaryngologica Italica*, *26*(3), 133–139.

97. Ohlemiller, K. K. (2008). Recent findings and emerging questions in cochlear noise injury. *Hearing Research*, *245*(1-2), 5–17.

98. Adelman, C., Freeman, S., Paz, Z., Sohmer, H. (2008). Salicylic acid injection before noise exposure reduces permanent threshold shift. *Audiology Neuro-Otology*, *13*(4), 266–272.

99. Samson, J., Wiktorek-Smagur, A., Politanski, P., Rajkowska, E., Pawlaczyk-Luszczynska, M., Dudarewicz, A., et al. (2008). Noise-induced time-dependent changes in oxidative stress in the mouse cochlea and attenuation by D-methionine. *Neuroscience*, *152*(1), 146–150.

100. Minami, S. B., Yamashita, D., Ogawa, K., Schacht, J., Miller, J. M. (2007). Creatine and tempol attenuate noise-induced hearing loss. *Brain Research*, *1148*, 83–89.

101. Murashita, H., Tabuchi, K., Hoshino, T., Tsuji, S., Hara, A. (2006). The effects of tempol, 3-aminobenzamide and nitric oxide synthase inhibitors on acoustic injury of the mouse cochlea. *Hearing Research*, *214*(1-2), 1–6.

102. Fetoni, A. R., Ralli, M., Sergi, B., Parrilla, C., Troiani, D., Paludetti, G. (2009). Protective effects of N-acetylcysteine on noise-induced hearing loss in guinea pigs. *Acta Otorhinolaryngologica Italica* , *29*(2), 70–75.

103. Bielefeld, E. C., Kopke, R. D., Jackson, R. L., Coleman, J. K. M., Liu, J., Henderson, D. (2007). Noise protection with N-acetyl-l-cysteine (NAC) using a variety of noise

exposures, NAC doses, and routes of administration. *Acta Oto-Laryngologica*, *127*(9), 914–919.

104. Lim, H.-W., Choi, S. H., Kang, H. H., Ahn, J. H., Chung, J. W. (2008). Apoptotic pattern of cochlear outer hair cells and frequency-specific hearing threshold shift in noise-exposed BALB/c mice. *Clinical and Experimental Otorhinolaryngology*, *1*(2), 80–85.
105. Coleman, J. K. M., Littlesunday, C., Jackson, R., Meyer, T. (2007). AM-111 protects against permanent hearing loss from impulse noise trauma. *Hearing Research*, *226*(1-2), 70–78.
106. Coleman, J. K. M., Kopke, R. D., Liu, J., Ge, X., Harper, E. A., Jones, G. E., et al. (2007). Pharmacological rescue of noise induced hearing loss using N-acetylcysteine and acetyl-L-carnitine. *Hearing Research*, *226*(1-2), 104–113.
107. Ahn, J. H., Kang, H. H., Kim, Y.-J., Chung, J. W. (2005). Anti-apoptotic role of retinoic acid in the inner ear of noise-exposed mice. *Biochemical and Biophysical Research Communications*, *335*(2), 485–490.
108. Shen, H., Zhang, B., Shin, J.-H., Lei, D., Du, Y., Gao, X., et al. (2007). Prophylactic and therapeutic functions of T-type calcium blockers against noise-induced hearing loss. *Hearing Research*, *226*(1-2), 52–60.
109. Hawkins, J. E., Johnsson, L. G., Preston, R. E. (1972). Symposium on basic ear research. I. Cochlear microvasculature in normal and damaged ears. *The Laryngoscope*, 82(7), 1091-104.
110. Okada, H., Yamane, H., Nakai, Y. (1991). Morphological changes of the spiral vessel after rock music exposure. *Acta Oto-Laryngologica Supplementum*, *486*, 61–65.
111. Quirk, W. S., Seidman, M. D. (1995). Cochlear vascular changes in response to loud noise. *The American Journal of Otology*, *16*(3), 322–325.

112. Yamasoba, T., Schacht, J., Shoji, F., Miller, J. M. (1999). Attenuation of cochlear damage from noise trauma by an iron chelator, a free radical scavenger and glial cell line-derived neurotrophic factor in vivo. *Brain Research*, *815*(2), 317–325.

113. Ohinata, Y., Yamasoba, T., Schacht, J., Miller, J. M. (2000). Glutathione limits noise-induced hearing loss. *Hearing Research*, *146*(1-2), 28–34.

114. Ohinata, Y., Miller, J. M., Altschuler, R. A., Schacht, J. (2000). Intense noise induces formation of vasoactive lipid peroxidation products in the cochlea. *Brain Research*, *878*(1-2), 163–173.

115. Ohinata, Y., Miller, J. M., Schacht, J. (2003). Protection from noise-induced lipid peroxidation and hair cell loss in the cochlea. *Brain Research*, *966*(2), 265–273.

116. Kovacic, P., Jacintho, J. D. (2001). Mechanisms of carcinogenesis: focus on oxidative stress and electron transfer. *Current Medicinal Chemistry*, *8*(7), 773–796.

117. Valko, M., Morris, H., Mazúr, M., Rapta, P., Bilton, R. F. (2001). Oxygen free radical generating mechanisms in the colon: do the semiquinones of vitamin K play a role in the aetiology of colon cancer? *Biochimica Et Biophysica Acta*, *1527*(3), 161–166.

118. Choi, C.-H., Chen, K., Vasquez-Weldon, A., Jackson, R. L., Floyd, R. A., Kopke, R. D. (2008). Effectiveness of 4-hydroxy phenyl N-tert-butylnitrone (4-OHPBN) alone and in combination with other antioxidant drugs in the treatment of acute acoustic trauma in chinchilla. *Free Radical Biology Medicine*, *44*(9), 1772–1784.

119. Arpornchayanon, W., Canis, M., Ihler, F., Settevendemie, C., Strieth, S. (2013). TNF-α inhibition using etanercept prevents noise-induced hearing loss by improvement of cochlear blood flow in vivo. *International Journal of Audiology*, *52*(8), 545–552.

120. Inaoka, T., Nakagawa, T., Kikkawa, Y. S., Tabata, Y., Ono, K., Yoshida, M., et al. (2009). Local application of hepatocyte growth factor using gelatin hydrogels attenuates noise-induced hearing loss in guinea pigs. *Acta Oto-Laryngologica*, *129*(4), 453–457.

121. Iwai, K., Nakagawa, T., Endo, T., Matsuoka, Y., Kita, T., Kim, T.-S., et al. (2006). Cochlear protection by local insulin-like growth factor-1 application using biodegradable hydrogel. *The Laryngoscope*, *116*(4), 529–533.

122. Bouckenooghe, T., Remacle, C. (2006). Is taurine a functional nutrient?. Curr Opin Nutr Metab Care, 9(6), 728-733.

123. Schaffer, S. W., Shimada, K., Jong, C. J., Ito, T., Azuma, J., Takahashi, K. (2014). Effect of taurine and potential interactions with caffeine on cardiovascular function. *Amino Acids*, *46*(5), 1147–1157.

124. Olive, M. F. (2002). Interactions between taurine and ethanol in the central nervous system. *Amino Acids*, *23*(4), 345–357.

125. Birdsall, T. C. (1998). Therapeutic applications of taurine. *Alternative Medicine Review 3(2),128-136.*

126. Tappaz, M., Almarghini, K., Legay, F., Remy, A. (1992). Taurine biosynthesis enzyme cysteine sulfinate decarboxylase (CSD) from brain: the long and tricky trail to identification. *Neurochemical Research*, *17*(9), 849–859.

127. Foos, T. M., Wu, J.-Y. (2002). The role of taurine in the central nervous system and the modulation of intracellular calcium homeostasis. *Neurochemical Research*, *27*(1-2), 21–26.

128. Leon, R., Wu, H., Jin, Y., Wei, J., Buddhala, C. (2009). Protective function of taurine in glutamate-induced apoptosis in cultured neurons. *J Neurosci Res, 87(5), 1185-1194.*

129. Gurujeyalakshmi, G., Wang, Y., Giri, S. N. (2000). Suppression of bleomycin-induced nitric oxide production in mice by taurine and niacin. *Nitric Oxide : Biology and Chemistry / Official Journal of the Nitric Oxide Society*, *4*(4), 399–411.

130. Manna, P., Sinha, M., Sil, P. C. (2009). Taurine plays a beneficial role against cadmium-induced oxidative renal dysfunction. *Amino Acids*, *36*(3), 417–428.

131. Balkan, J., Doğru-Abbasoğlu, S., Kanbağli, O., Cevikbaş, U., Aykaç-Toker, G., Uysal, M. (2001). Taurine has a protective effect against thioacetamide-induced liver cirrhosis by decreasing oxidative stress. *Human Experimental Toxicology*, *20*(5), 251–254.

132. Doğru-Abbasoğlu, S., Kanbağli, O., Balkan, J., Cevikbaş, U., Aykaç-Toker, G., Uysal, M. (2001). The protective effect of taurine against thioacetamide hepatotoxicity of rats. *Human Experimental Toxicology*, *20*(1), 23–27.

133. Hwang, D. F., Wang, L. C., Cheng, H. M. (1998). Effect of taurine on toxicity of copper in rats. *Food and Chemical Toxicology*, 36(3), 239-244.

134. Liu, H.Y., Chi, F.L., Gao, W.Y. (2008). Taurine attenuates aminoglycoside ototoxicity by inhibiting inducible nitric oxide synthase expression in the cochlea. *Neuroreport*, *19*(1), 117–120.

135. Liu, H.Y., Chi, F.L., Gao, W.Y. (2008). Taurine modulates calcium influx under normal and ototoxic conditions in isolated cochlear spiral ganglion neurons. *Pharmacological Reports : PR*, *60*(4), 508–513.

136. Li, P. F. (1995). Oxidative modification of cupro-zinc superoxide dismutase by reactive oxygen species. *Progress in Physiology*, *26*(1), 50–52.

137. Hoehn, K. L., Salmon, A. B., Hohnen-Behrens, C., Turner, N., Hoy, A. J., Maghzal, G. J., et al. (2009). Insulin resistance is a cellular antioxidant defense mechanism. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(42), 17787–17792.

138. Hirschberg, K., Radovits, T., Korkmaz, S., Loganathan, S., Zöllner, S., Seidel, B., et al. (2010). Combined superoxide dismutase mimetic and peroxynitrite scavenger protects against neointima formation after endarterectomy in association with decreased proliferation and nitro-oxidative stress. *European Journal of Vascular and Endovascular Surgery*, *40*(2), 168–175.

139. Adeagbo, A. S. O., Zhang, X., Patel, D., Joshua, I. G., Wang, Y., Sun, X., et al. (2005). Cyclo-oxygenase-2, endothelium and aortic reactivity during deoxycorticosterone acetate salt-induced hypertension. *Journal of Hypertension*, *23*(5), 1025–1036.

140. Nayagam, B. A., Muniak, M. A., Ryugo, D. K. (2011). The spiral ganglion: connecting the peripheral and central auditory systems. *Hearing Research*, *278*(1-2), 2–20.

141. Holley, M., Rhodes, C., Kneebone, A., Herde, M. K., Fleming, M., Steel, K. P. (2010). Emx2 and early hair cell development in the mouse inner ear. *Developmental Biology*, *340*(2), 547–556.

142. Hilton, J. M., Lewis, M. A., Grati, M., Ingham, N., Pearson, S., Laskowski, R. A., et al. (2011). Exome sequencing identifies a missense mutation in Isl1 associated with low penetrance otitis media in dearisch mice. *Genome Biology*, *12*(9), R90.

143. Lewis, M. A., Quint, E., Glazier, A. M., Fuchs, H., de Angelis, M. H., Langford, C., et al. (2009). An ENU-induced mutation of miR-96 associated with progressive hearing loss in mice. *Nature Genetics*, *41*(5), 614–618.

144. Mencía, A., Modamio-Høybjør, S., Redshaw, N., Morín, M., Mayo-Merino, F., Olavarrieta, L., et al. (2009). Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss. *Nature Genetics*, *41*(5), 609–613.

145. Julicher, R. H., Sterrenberg, L., Haenen, G. R., Bast, A., Noordhoek, J. (1984). Sex differences in the cellular defence system against free radicals from oxygen or drug metabolites in rat. *Archives of Toxicology*, *56*(2), 83–86.

146. Barbary, el, A., Altschuler, R. A., Schacht, J. (1993). Glutathione S-transferases in the organ of Corti of the rat: enzymatic activity, subunit composition and immunohistochemical localization. *Hearing Research*, *71*(1-2), 80–90.

147. McFadden, S. L., Henselman, L. W., Zheng, X. Y. (1999). Sex differences in auditory sensitivity of chinchillas before and after exposure to impulse noise. *Ear and Hearing*, *20*(2), 164–174.

148. Mikaelian, D., Alford, B. R., Ruben, R. J. (1965). Cochlear potentials and 8 nerve action potentials in normal and genetically deaf mice. *The Annals of Otology, Rhinology, and Laryngology*, *74*, 146–157.

149. Yoshida, N., Liberman, M. C. (2000). Sound conditioning reduces noise-induced permanent threshold shift in mice. *Hearing Research*, *148*(1-2), 213–219.

150. Zheng, Q. Y., Johnson, K. R., Erway, L. C. (1999). Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. *Hearing Research*, *130*(1-2), 94–107.

151. Bartels, S., Ito, S., Trune, D. R., Nuttall, A. L. (2001). Noise-induced hearing loss: the effect of melanin in the stria vascularis. *Hearing Research*, *154*(1-2), 116–123.

152. Bogaerts, S., Clements, J. D., Sullivan, J. M., Oleskevich, S. (2009). Automated threshold detection for auditory brainstem responses: comparison with visual estimation in a stem cell transplantation study. *BMC Neuroscience*, *10*, 104.

153. Pau, H., Fuchs, H., de Angelis, M. H., Steel, K. P. (2005). Hush puppy: a new mouse mutant with pinna, ossicle, and inner ear defects. *The Laryngoscope*, *115*(1), 116–124.

154. Wang, Y., Liberman, M. C. (2002). Restraint stress and protection from acoustic injury in mice. *Hearing Research*, *165*(1-2), 96–102.

155. Müller, M., Hünerbein, von, K., Hoidis, S., Smolders, J. (2005). A physiological place–frequency map of the cochlea in the CBA/J mouse. *Hearing Research*.

156. Shi, H. M., He, L. H., Zhang, Y., Ye, K. P., Chang, D. (2007). Changes of nitric oxide and nitric-oxide synthase in the development of cold-induced hypertension. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi, 25(4), 197-199.*

157. Loihl, A. K., Asensio, V., Campbell, I. L., Murphy, S. (1999). Expression of nitric oxide synthase (NOS)-2 following permanent focal ischemia and the role of nitric oxide in infarct generation in male, female and NOS-2 gene-deficient mice. *Brain Research*, 29:830(1), 155-164.

158. Nowicki, M. J., Shi, D., Cai, Z., Bishop, P. R., May, W. L. (2003). Developmental expression of endothelial nitric oxide synthase (eNOS) in the rat liver. *Pediatric Research*, 54(5), 732-738.

159. Shi, X., Nuttall, A. L. (2003). Upregulated iNOS and oxidative damage to the cochlear stria vascularis due to noise stress. *Brain Research*, 28;967(1-2),1-10.

160. Takumida, M., Anniko, M., Popa, R., Zhang, D. M. (2000). Lipopolysaccharide-induced expression of inducible nitric oxide synthase in the guinea pig organ of Corti. *Hearing Research*, *140*(1-2), 91–98.

161. Watanabe, S., Ono, H., Ishimitsu, T., Matsuoka, H., Ono, Y., Fujimori, T. (2000). Calcium antagonist inhibits glomerular cell apoptosis and injuries of L-NAME exacerbated nephrosclerosis in SHR. *Hypertension Research : Official Journal of the Japanese Society of Hypertension*, *23*(6), 683–691.

162. Murashita, H., Tabuchi, K., Hoshino, T., Tsuji, S., Hara, A. (2006). The effects of tempol, 3-aminobenzamide and nitric oxide synthase inhibitors on acoustic injury of the mouse cochlea. *Hearing Research*, *214*(1-2), 1–6.

163. Tabuchi, K., Kusakari, J., Ito, Z., Takahashi, K., Wada, T., Hara, A. (1999). Effect of nitric oxide synthase inhibitor on cochlear dysfunction induced by transient local anoxia. *Acta Oto-Laryngologica*, *119*(2), 179–184.

164. Tabuchi, K., Takahashi, K., Ito, Z., Hara, A. (2000). Effect of 7-nitroindazole upon cochlear dysfunction induced by transient local anoxia. *Annals of Otology*, 109(1), 715-719.

165. Shi, X., Ren, T., Nuttall, A. L. (2001). Nitric oxide distribution and production in the guinea pig cochlea. *Hearing Research*, 153(1-2), 23-31.

166. Shi, X., Ren, T., Nuttall, A. L. (2002). The electrochemical and fluorescence detection of nitric oxide in the cochlea and its increase following loud sound. *Hearing Research*, *164*(1-2), 49–58.

167. Shi, X., Nuttall, A. L. (2003). Upregulated iNOS and oxidative damage to the cochlear stria vascularis due to noise stress. *Brain Research*, 967(1-2), 1-10.

168. Heinrich, U. R., Selivanova, O., Feltens, R., Brieger, J. (2005). Endothelial nitric oxide synthase upregulation in the guinea pig organ of Corti after acute noise trauma. *Brain Research*, 1047(1), 85-96.

169. Chen, Y. K., Huse, S. S., Lin, L. M. (2005). Inhibitory effect of inducible nitric oxide synthase inhibitors on DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis. *Nitric Oxide, 13(4), 232-239.*

170. Watanabe, T., Suzuki, J., Yamawaki, H., Sharma, V. K. (2005). Losartan Metabolite EXP3179 Activates Akt and Endothelial Nitric Oxide Synthase via Vascular Endothelial Growth Factor Receptor-2 in Endothelial Cells Angiotensin …. *Circulation*, 112(12),1798-1805.

171. Diao, M., Gao, W., Sun, J. (2007). Nitric oxide synthase inhibitor reduces noise-induced cochlear damage in guinea pigs. *Acta Oto-Laryngologica*, *127*(11), 1162–1167.

172. Nagashima, R., Yamaguchi, T., Tanaka, H., Ogita, K. (2010). Mechanism underlying the protective effect of tempol and Nω-nitro-L-arginine methyl ester on acoustic injury: possible involvement of c-Jun N-terminal kinase pathway and connexin26 in the cochlear spiral ligament. *Journal of Pharmacological Sciences*, *114*(1), 50–62.

173. Wang, H.-Q., Xiong, Y., Guo, W.-J. (2011). Expression of iNOS protein and gliacyte apoptosis in neonatal rats with white matter damage. *Chinese Journal of Contemporary Pediatrics*, *13*(4), 309–312.

174. Qi, B., Yamagami, T., Naruse, Y. (1995). Effects of taurine on depletion of erythrocyte membrane Na-K ATPase activity due to ozone exposure or cholesterol enrichment. *J Nutr Sci Vitaminol, 41(6), 627-34.*

175. Idrissi, El, A., Shen, C. H., L'Amoreaux, W. J. (2013). Neuroprotective role of taurine during aging. *Amino Acids*, 45(4), 735-750.

176. Miller, J. M., Brown, J. N., Schacht, J. (2003). 8-iso-prostaglandin F2α, a product of noise exposure, reduces inner ear blood flow. *Audiology and Neurotology*, 8(4),207-221.

177. Satoh, H. (1998). Cardiac actions of taurine as a modulator of the ion channels. *Adv Exp Med Biol, 442, 121-128.*

178. Kramer, J. H., Chovan, J. P. (1981). Effect of taurine on calcium paradox and ischemic heart failure. *Am J Physiol, 240(2), 238-246.*

179. Ye, H. B., Shi, H. B., Yin, S. K. (2013). Mechanisms underlying taurine protection against glutamate-induced neurotoxicity. *Can J Neurol Sci, 40(5), 628-634.*

180. Le Prell, C. G., Niemiec, A. J., Moody, D. B. (2001). Macaque thresholds for detecting increases in intensity: Effects of formant structure. *Hearing Research*, 161(1-2), 29-42.

181. Ohlemiller, K. K., Dugan, L. L. (1999). Elevation of reactive oxygen species following ischemia-reperfusion in mouse cochlea observed in vivo. *Audiology Neuro-Otology*, *4*(5), 219–228.

182. Hamernik, R. P., Qiu, W., Davis, B. (2008). The effectiveness of N-acetyl-L-cysteine (L-NAC) in the prevention of severe noise-induced hearing loss. *Hearing Research*, *239*(1-2), 99–106.

183. Campbell, K. C. M., Meech, R. P., Klemens, J. J., Gerberi, M. T., Dyrstad, S. S. W., Larsen, D. L., et al. (2007). Prevention of noise- and drug-induced hearing loss with D-methionine. *Hearing Research*, *226*(1-2), 92–103.

184. Campbell, K., Claussen, A., Meech, R., Verhulst, S., Fox, D., Hughes, L. (2011). D-methionine (D-met) significantly rescues noise-induced hearing loss: timing studies. *Hearing Research*, *282*(1-2), 138–144.

185. Hamacher, J., Stammberger, U., Weber, E., Lucas, R., Wendel, A. (2009). Ebselen improves ischemia-reperfusion injury after rat lung transplantation. *Lung*, *187*(2), 98–103.

186. Biesalski, H. K., Wellner, U., Weiser, H. (1990). Vitamin A deficiency increases noise susceptibility in guinea pigs. *The Journal of Nutrition*, *120*(7), 726–737.

187. Ylikoski, M. E., Pekkarinen, J. O., Starck, J. P. (1995). Physical characteristics of gunfire impulse noise and its attenuation by hearing protectors. *Scand Audiol, 24(1), 3-11*.

188. Shim, H. J., Kang, H. H., Ahn, J. H., Chung, J. W. (2009). Retinoic acid applied after noise exposure can recover the noise-induced hearing loss in mice. *Acta Oto-Laryngologica*, *129*(3), 233–238.

189. Chatterjee, I. B. (1973). Evolution and the biosynthesis of ascorbic acid. *Science (New York, NY)*, *182*(4118), 1271–1272.

190. Nandi, A., Mukhopadhyay, C. K., Ghosh, M. K., Chattopadhyay, D. J., Chatterjee, I. B. (1997). Evolutionary significance of vitamin C biosynthesis in terrestrial vertebrates. *Free Radical Biology Medicine*, *22*(6), 1047–1054.

191. Le Prell, C. G., Yamashita, D., Minami, S. B., Yamasoba, T., Miller, J. M. (2007). Mechanisms of noise-induced hearing loss indicate multiple methods of prevention. *Hearing Research*, *226*(1-2), 22–43.

192. Rabinowitz, P. M., Pierce Wise, J., Hur Mobo, B., Antonucci, P. G., Powell, C., Slade, M. (2002). Antioxidant status and hearing function in noise-exposed workers. *Hearing Research*, *173*(1-2), 164–171.

193. Yamashita, D., Jiang, H.-Y., Le Prell, C. G., Schacht, J., Miller, J. M. (2005). Post-exposure treatment attenuates noise-induced hearing loss. *Neuroscience*, *134*(2), 633–642.

194. Fetoni, A. R., Ferraresi, A., La Greca, C., Rizzo, D., Sergi, B. (2008). Antioxidant protection against acoustic trauma by coadministration of idebenone and vitamin E. *Neuroreport, 19(3), 277-281*.

195. Abaamrane, L., Raffin, F., Gal, M., Avan, P., Sendowski, I. (2009). Long-term administration of magnesium after acoustic trauma caused by gunshot noise in guinea pigs. *Hearing Research*, *247*(2), 137–145.

196. Tamir, S., Adelman, C., Weinberger, J. M. (2010). Uniform comparison of several drugs which provide protection from noise induced hearing loss. *J Occup Med Toxicol, 1(5), 26*

197. Ising, H., Handrock, M., Günther, T., Fischer, R., Dombrowski, M. (1982). Increased noise trauma in guinea pigs through magnesium deficiency. *Archives of Oto-Rhino-Laryngology*, *236*(2), 139–146.

198. Joachims, Z., Babisch, W., Ising, H., Günther, T. (1983). Dependence of noise-induced hearing loss upon perilymph magnesium concentration. *J Acoust Soc Am, 74(1), 104-108.*

199. Joachims, Z., Netzer, A., Ising, H., Rebentisch, E., Attias, J., Weisz, G., Günther, T. (1993). Oral magnesium supplementation as prophylaxis for noise-induced hearing loss: results of a double blind field study. *Schriftenreihe Des Vereins Für Wasser-, Boden-Und Lufthygiene*, *88*, 503–516.

200. Scheibe, F., Haupt, H., Vlastos, G. A. (2000). Preventive magnesium supplement reduces ischemia-induced hearing loss and blood viscosity in the guinea pig. *European Archives of Oto-Rhino-Laryngology*, *257*(7), 355–361.

201. Scheibe, F., Haupt, H., Ising, H. (2000). Preventive effect of magnesium supplement on noise-induced hearing loss in the guinea pig. *European Archives of Oto-Rhino-Laryngology* , *257*(1), 10–16.

202. Haupt, H., Scheibe, F. (2002). Preventive magnesium supplement protects the inner ear against noise-induced impairment of blood flow and oxygenation in the guinea pig. *Magnesium Research,15(1-2),17-25.*

203. Attias, J., Sapir, S., Bresloff, I., Reshef-Haran, I., Ising, H. (2004). Reduction in noise-induced temporary threshold shift in humans following oral magnesium intake. *Clinical Otolaryngology and Allied Sciences*, *29*(6), 635–641.

204. Le Prell, C. G., Dolan, D. F., Bennett, D. C., Boxer, P. A. (2011). Nutrient plasma levels achieved during treatment that reduces noise-induced hearing loss. *Translational Research : the Journal of Laboratory and Clinical Medicine*, *158*(1), 54–70.

205. Le Prell, C. G., Gagnon, P. M., Bennett, D. C., Ohlemiller, K. K. (2011). Nutrient-enhanced diet reduces noise-induced damage to the inner ear and hearing loss. *Translational Research : the Journal of Laboratory and Clinical Medicine*, *158*(1), 38–53.

206. Le Prell, C. G., Johnson, A. C., Lindblad, A. C., Skjönsberg, A., Ulfendahl, M., Guire, K., et al. (2011). Increased vitamin plasma levels in Swedish military personnel treated with nutrients prior to automatic weapon training. *Noise Health*, *13*(55), 432–443.

207. Bohne, B. A., Clark, W. W. (1982). Growth of hearing loss and cochlear lesion with increasing duration of noise exposure, in Hamernik, R.P. et al, New perspectives on noise-induced hearing loss. New York Raven Publishing, 283-302.

208. Scheibe, F., Haupt, H., Ising, H., Cherny, L. (2002). Therapeutic effect of parenteral magnesium on noise-induced hearing loss in the guinea pig. *Magnesium*, *15*(1-2), 27–36.

209. Sendowski, I., Abaamrane, L., Raffin, F., Cros, A., Clarençon, D. (2006). Therapeutic efficacy of intra-cochlear administration of methylprednisolone after acoustic trauma caused by gunshot noise in guinea pigs. *Hearing Research*, *221*(1-2), 119–127.

210. Vass, Z., Brechtelsbauer, P. B., Nuttall, A. L., Miller, J. M. (1996). Nitric oxide mediates capsaicin-induced increase in cochlear blood flow. *Hearing Research*.

211. Wolf, F. I., Trapani, V. (2008). Cell (patho)physiology of magnesium. *Clinical Science (London, England : 1979)*, *114*(1), 27–35.

212. Wolf, F. I., Trapani, V., Cittadini, A. (2008). Magnesium and the control of cell proliferation: looking for a needle in a haystack. *Magnesium Research*, *21*(2), 83–91.

213. Wolf, F. I., Trapani, V., Simonacci, M., Boninsegna, A., Mazur, A., Maier, J. A. M. (2009). Magnesium deficiency affects mammary epithelial cell proliferation: involvement of oxidative stress. *Nutrition and Cancer*, *61*(1), 131–136.

214. Jacobson, E. J., Downs, M. P., Fletcher, J. L. (1969). Clinical findings in high-frequency thresholds during known ototoxic drug usage. *Journal of Auditory Research, 9(4), 379-385.*

215. Fausti, S. A., Schechter, M. A., Rappaport, B. Z., Frey, R. H. (1984). Early detection of cisplatin ototoxicity selected case reports. *Cancer*. 53(2), 224-234.

216. Fausti, S. A., Rappaport, B. Z., Schechter, M. A., Frey, R. H., Ward, T. T., Brummett, R. E. (1984). Detection of aminoglycoside ototoxicity by high-frequency auditory evaluation: selected case studies. *American Journal of Otolaryngology*, *5*(3), 177–182.

217. Rappaport, B. Z., Fausti, S. A. (1985). Detection of ototoxicity by high-frequency auditory evaluation. Am J Otolaryngol, 5(3), 177-182.

218. Kopelman, J., Budnick, A. S., Sessions, R. B., Kramer, M. B., Wong, G. Y. (1988). Ototoxicity of high-dose cisplatin by bolus administration in patients with advanced cancers and normal hearing. *The Laryngoscope*, *98*(8 Pt 1), 858–864.

219. Kujawa, S. G., Glattke, T. J., Fallon, M., Bobbin, R. P. (1994). A nicotinic-like receptor mediates suppression of distortion product otoacoustic emissions by contralateral sound. *Hearing Research*, 74(1-2), 122-134.

220. Kemp, D. T. (1997). Otoacoustic emissions in perspective.

221. Hall, J. W. (2000). Handbook of otoacoustic emissions.

222. Hamernik, R. P., Qiu, W. (2000). Correlations among evoked potential thresholds, distortion product otoacoustic emissions and hair cell loss following various noise exposures in the chinchilla. *Hearing Research*, 150(1-2), 245-257.

223. Mensh, B. D., Patterson, M. C., Whitehead, M. L., Lonsbury-Martin, B. L., Martin, G. K. (1993). Distortion-product emissions in rabbit: I. Altered susceptibility to repeated pure-tone exposures. *Hearing Research*, *70*(1), 50–64.

224. Mensh, B. D., Lonsbury-Martin, B. L., Martin, G. K. (1993). Distortion-product emissions in rabbit: II. Prediction of chronic-noise effects by brief pure-tone exposures. *Hearing Research*, *70*(1), 65–72.

225. LePage, E. L., Murray, N. M. (1998). Latent cochlear damage in personal stereo users: a study based on click-evoked otoacoustic emissions. *The Medical Journal of Australia*, *169*(11-12), 588–592.

226. Seixas, N. S., Goldman, B., Sheppard, L. (2005). Prospective noise induced changes to hearing among construction industry apprentices. *Occup Environ Med, 62(5), 309-317.*

227. Prasher, D., Sułkowski, W. (1999). The role of otoacoustic emissions in screening and evaluation of noise damage. *International Journal of Occupational Medicine and Environmental Health*, *12*(2), 183–192.

228. Sliwińska-Kowalska, M., Zamyslowska-Szmytke, E., Szymczak, W., Kotylo, P., Fiszer, M., Wesolowski, W., Pawlaczyk-Luszczynska, M. (2003). Ototoxic effects of occupational exposure to styrene and co-exposure to styrene and noise. *Journal of Occupational and Environmental Medicine*, *45*(1), 15–24.

229. Vinodh, R. S., Veeranna, N. (2010). Evaluation of acoustic shock induced early hearing loss with audiometer and distortion product otoacoustic emissions. *Indian Journal of Medical Sciences*, *64*(3), 132–139.

230. Atchariyasathian, V., Chayarpham, S., Saekhow, S. (2008). Evaluation of noise-induced hearing loss with audiometer and distortion product otoacoustic emissions. *Journal of the Medical Association of Thailand*, *91*(7), 1066–1071.

231. Taurine: a conditionally essential amino acid in humans? An overview in health and disease R. Lourenço*, ** and M. E. Camilo**

232. Oja, S. S. and Saransaari, P. (1992) *J. Neurosci. Res.* 32, 551-561.

233. Oja, S. S. and Saransaari, P. (1996) *Metab. Brain Dis.* 11, 153-164.

234. Pasantes-Morales, H. and Franco, R. (2002) *Cerebellum* 1, 103-109.

235. Oja, S. S. and Kontro, P. (1983). Taurine. Pages 501-533, In Lajtha, A., editor. *Handbook of Neurochemistry*, vol. 3, 2nd edn, Plenum Press, New York.

236. Frosini, M., Sesti, C., Dragoni, S., Valoti, M., Palmi, M., Dixon, H. B. F., Machetti, F., and Sgaragli, G. (2003) *Br. J. Pharmacol.* 138, 1163-1171.

237. Frosini, M., Sesti, C., Saponara, S., Ricci, L., Valoti, M., Palmi, M., Machetti, F., and Sgaragli, G. (2003) *Br. J. Pharmacol.* 139, 487-494.

238. El Idrissi, A. and Trenkner, E. (2004) *Neurochem. Res.* 29, 189-197.

239. Mechanism underlying the antioxidant activity of taurine -- Jong and Schaffer 27 (1 Supplement): 1086.

240. Konings, A., et al., 2009. Genetic studies on noise-induced hearing loss: a review. Ear Hear. 30, 151–159.

241. Martinez-Monedero, R., Edge, A.S., 2007. Stem cells for the replacement of inner ear neurons and hair cells. Int. J. Dev. Biol. 51, 655–661.

242. Parker, M.A., et al., 2007. Neural stem cells injected into the sound-damaged cochlea migrate throughout the cochlea and express markers of hair cells, supporting cells, and spiral ganglion cells. Hear. Res. 232, 29–43.

243. Revoltella, R.P., et al., 2008. Cochlear repair by transplantation of human cord blood $CD133^+$ cells to Nod-Scid mice made deaf with kanamycin and noise. Cell Transplant. 17, 665–678.

244. Nordmann, A.S., et al., 2000. Histopathological differences between temporary and permanent threshold shift. Hear. Res. 139, 13–30.

245. White, C.H., et al., 2009. Genome-wide screening for genetic loci associated with noise-induced hearing loss. Mamm. Genome 20, 207–213.

246. Pawelczyk, M., et al., 2009. Analysis of gene polymorphisms associated with K ion circulation in the inner ear of patients susceptible and resistant to noise-induced hearing loss. Ann. Hum. Genet. 73, 411–421.

247. Erway, L.C., et al., 1996. Genetics of age-related hearing loss in mice. III. Susceptibility of inbred and F1 hybrid strains to noise-induced hearing loss. Hear. Res. 93, 181–187.

248. Davis, R.R., et al., 2001. Genetic basis for susceptibility to noise-induced hearing loss in mice. Hear. Res. 155, 82–90.

249. Ohlemiller, K.K., Gagnon, P.M., 2007. Genetic dependence of cochlear cells and structures injured by noise. Hear. Res. 224, 34–50.

250. Hunter, K.P., Willott, J.F., 1987. Aging and the auditory brainstem response in mice with severe or minimal presbycusis. Hear. Res. 30, 207–218.

251. Schone, G., et al., 1991. The effect of noise exposure on the aging ear. Hear. Res. 56, 173–178.

252. Jimenez, A.M., et al., 1999. Age-related loss of distortion product otoacoustic emissions in four mouse strains. Hear. Res. 138, 91–105.

253. Wang, Y., et al., 2002. Dynamics of noise-induced cellular injury and repair in the mouse cochlea. J. Assoc. Res. Otolaryngol. 3, 248–268.

254. Hirose, K., Liberman, M.C., 2003. Lateral wall histopathology and endocochlear potential in the noise-damaged mouse cochlea. J. Assoc. Res. Otolaryngol. 4, 339–352.

255. Bernardo, M.E., et al., 2009. Mesenchymal stromal cells. Ann. NY Acad. Sci. 1176, 101–117.

256. da Silva Meirelles, L., et al., 2009. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. Cytokine Growth Factor Rev. 20, 419–427.

257. Lai, R.C., et al., 2010. Exosome secreted by MSC reduces myocardial ischemia/ reperfusion injury. Stem Cell Res. 4, 214–222.

258. Ookura, T., et al., 2002. Fibroblast and epidermal growth factors modulate proliferation and neural cell adhesion molecule expression in epithelial cells derived from the adult mouse tongue. In Vitro Cell. Dev. Biol. Anim. 38, 365–372.

259. Luo, X., et al., 2009. Culture of endodermal stem/progenitor cells of the mouse tongue. In Vitro Cell. Dev. Biol. 45, 44–54.

260. Okubo, T., et al., 2009. Cell lineage mapping of taste bud cells and keratinocytes in the mouse tongue and soft palate. Stem Cells 27, 442–450.

261. Sullivan, J.M., et al., 2010. Stem and progenitor cell compartments within adult mouse taste buds. Eur. J. Neurosci. 31, 1549–1560.

262. Bithell, A., Williams, B.P., 2005. Neural stem cells and cell replacement therapy: making the right cells. Clin. Sci. (Lond.) 108, 13–22.

263. Haegebarth, A., Clevers, H., 2009. Wnt signaling, lgr5, and stem cells in the intestine and skin. Am. J. Pathol. 174, 715–721.

264. Blanpain, C., Fuchs, E., 2009. Epidermal homeostasis: a balancing act of stem cells in the skin. Nat. Rev. Mol. Cell Biol. 10, 207–217.

265. Fuchs, E., Horsley, V., 2008. More than one way to skin. Genes Dev. 22, 976–985.

266. Miller, J.D., et al., 1963. Deafening effects of noise on the cat. Acta Oto Laryngologica Suppl. 476, 74–88.

267. Bogaerts, S., et al., 2008. Microsurgical access for cell injection into the mammalian cochlea. J. Neurosci. Meth. 168, 156–163.

268. Wakisaka, S., 2005. Lectin histochemistry of taste buds in the circumvallate papilla of the rat. Chem. Senses 30, i46–i47.

269. Senoo, M., et al., 2007. p63 is essential for the proliferative potential of stem cells in stratified epithelia. Cell 129, 523–536.

270. Hudson, D.L., 2002. Keratins as markers of epithelial cells. Meth. Mol. Biol. 188, 157–167.

271. Barrandon, Y., Green, H., 1987. Three clonal types of keratinocyte with different capacities for multiplication. Proc. Natl Acad. Sci. USA 84, 2302–2306.

272. Pellegrini, G., et al., 2001. p63 identifies keratinocyte stem cells. Proc. Natl Acad. Sci. USA 98, 3156–3161.

273. Liberman, M.C., Beil, D.G., 1979. Hair cell condition and auditory nerve response in normal and noise-damaged cochleas. Acta Otolaryngol. 88, 161–176.

274. Schulte, B.A., Adams, J.C., 1989. Distribution of immunoreactive Na+, K + -ATPase in the gerbil cochlea. J. Histochem. Cytochem. 37, 127–134.

275. Spicer, S.S., Schulte, B.A., 1991. Differentiation of inner ear fibrocytes according to their ion transport related activity. Hear. Res. 56, 53–64.

276. Furukawa, M., et al., 1996. Na+, K + -ATPase activity in the cochlear lateral wall of the gerbil. Neurosci. Lett. 213, 165–168.

277. Xia, A., et al., 1999. Expression of connexin 26 and Na, K-ATPase in the developing mouse cochlear lateral wall: functional implications. Brain Res. 846, 106–111.

278. Hakuba, N., et al., 2005. Neural stem cells suppress the hearing threshold shift caused by cochlear ischemia. NeuroReport 16, 1545–1549.

279. Hildebrand, M.S., et al., 2005. Survival of partially differentiated mouse embryonic stem cells in the scala media of the guinea pig cochlea. J. Assoc. Res. Otolaryngol. 6, 341–354.

280. Yoshida, T., et al., 2007. Hematopoietic stem cells prevent hair cell death after transient cochlear ischemia through paracrine effects. Neuroscience 145, 923–930.

281. Hamernik, R.P., et al., 1989. The quantitative relation between sensory cell loss and hearing thresholds. Hear. Res. 38, 199–211.

282. Ou, H.C., et al., 2000. Noise damage in the C57BL/CBA mouse cochlea. Hear. Res. 145, 111–122.

283. Viberg, A., Canlon, B., 2004. The guide to plotting a cochleogram. Hear. Res. 197, 1–10.

284. Lang, H., et al., 2003. Effects of chronic furosemide treatment and age on cell division in the adult gerbil inner ear. J. Assoc. Res. Otolaryngol. 4, 164–175.

285. Yamasoba, T., et al., 2003. Changes in cell proliferation in rat and guinea pig cochlea after aminoglycoside-induced damage. Neurosci. Lett. 347, 171–174.

286. Mahmood, A., et al., 2004. Marrow stromal cell transplantation after traumatic brain injury promotes cellular proliferation within the brain. Neurosurgery 55, 1185–1193.

287. Munoz, J.R., et al., 2005. Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice. Proc. Natl Acad. Sci. USA 102, 18171–18176.

288. Einstein, O., et al., 2009. Transplanted neural precursors enhance host brain-derived myelin regeneration. J. Neurosci. 29, 15694–15702.

289. Madhavan, L., et al., 2009. Transplantation of subventricular zone neural precursors induces an endogenous precursor cell response in a rat model of Parkinson's disease. J. Comp. Neurol. 515, 102–115.

290. Kim, H.M., et al., 2009. Ex vivo VEGF delivery by neural stem cells enhances proliferation of glial progenitors, angiogenesis, and tissue sparing after spinal cord injury. PLoS ONE 4, e4987.

291. Zou, Z., et al., 2010. More insight into mesenchymal stem cells and their effects inside the body. Expert Opin. Biol. Ther. 10, 215–230.

292. Valle-Prieto, A., Conget, P.A., 2010. Human mesenchymal stem cells efficiently manage oxidative stress. Stem Cells Dev. doi:10.1089/scd.2010.0093

293. Kikuchi, T., et al., 2000. Potassium ion recycling pathway via gap junction systems in the mammalian cochlea and its interruption in hereditary nonsyndromic deafness. Med. Electron Microsc. 33, 51–56.