Association of Caffeine and Hearing Recovery After Acoustic Overstimulation Events in a Guinea Pig Model

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Dedication

To my father Tarif, my mother Norah, my sister Sarah and my uncle Mahmoud.

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List of Abbreviations

ABR: Auditory brainstem response

AoP: Apnea of Prematurity

AOSE: acoustic overstimulation event

ATP: adenosine triphosphate

dB: Decibels

H&E: Hematoxylin and Eosin

IHC: Inner hair cells

IP: Intraperitoneal

LM: Light microscopy

LSO: Lateral superior olivary complex

MSO: medial superior olivary nucleus

NICU: neonatal Intensive Care Unit

NIHL: Noise induced hearing loss

NRHI: Noise-related hearing loss

OHC: Outer hair cells

SEM: Scanning Electron microscopy

SL: Sensation Level

SOC: Superior Olivary Complex

SPL: Sound Pressure Level

THFC: The Hearing Foundation of Canada

TSS: Temporary Threshold shift

WHO: World Health Organization

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Abstract

Importance:

Noise induced hearing loss is an increasingly worrisome problem. Although caffeine intake is common in people who are in noisy environments, the effect of caffeine on the recovery of hearing after a temporary threshold shift requires further understanding.

Objectives:

To determine whether caffeine impairs hearing recovery in guinea pigs exposed to acoustic overstimulation.

Design, Setting, Subjects:

Twenty four female guinea pigs were divided into three groups. This study was approved by McGill University Ethics review board and the University animal care committee. The experiment took place at the McGill University Auditory Sciences Laboratory.

Intervention:

Group 1: received caffeine; Group 2: exposed to acoustic overstimulation events (AOSEs); Group 3: exposed to AOSEs and caffeine. Daily caffeine dose for groups 1 and 3 was 25 mg/kg administered intraperitoneally. AOSEs were administered on days 1 and 8 and consisted of a one hour of 110 dB SPL pure-tone sound. Serial auditory brain stem response tests (ABRs) were measured to determine the audiological threshold shift and recovery. Scanning Electron Microscopy (SEM) and Light Microscopic (LM) analysis were performed.

Main Outcomes and Measures:

ABRs at mid first week, one week mark, mid second week and two weeks mark. In addition to SEM and LM analysis of the cochlea

Results:

The 1 hour post acoustic overstimulation resulted in a similar threshold shift between all animals in groups 2 and 3 at all frequencies tested (8, 16, 20 and 25khz). Maximum threshold shift was at 16khz with a mean of 66 and 54 dB respectively. By day 8, group 2 threshold shift recovered completely in all frequency except at 20 kHz. Group 3 hearing impairment persisted in 3 frequencies (8, 16 and 25khz) with a threshold of 22, 28 and 26 dB respectively (p value 0.001). Following a second acoustic overstimulation at day 8, similar threshold shift and outcome was recorded on day 15, as compared to day 8. SEM & light microscope showed more aggressive changes in the group that received AOSEs and caffeine (Group 3) compared to the group that was only exposed to AOSEs (Group 2). The difference in ABR threshold recovery was in concordance with SEM and light microscopic findings of each group.

Conclusion:

A daily dose of caffeine was found to impair the recovery of hearing after an acoustic overstimulation event.

Résumé

Importance:

La perte d'audition due au bruit est un problème de plus en plus inquiétant. Bien que la consommation de caféine est fréquente chez les personnes impliquées dans des environnements bruyants, l'effet de la caféine sur la reprise de l'audition après un déplacement temporaire du seuil d'audibilité nécessite une meilleure compréhension.

Objectifs:

Déterminer si la caféine affecte la reprise d'audition chez les cochons d'Inde après une surstimulation acoustique.

Sujets:

Vingt-quatre cochons d'Inde femelles ont été divisés en trois groupes. Cette étude a été approuvée par le department d'éthique en recherche et du comité de protection des animaux de l'Université de McGill. L'expérience a eu lieu au Laboratoire des Sciences Auditives de l'Université de McGill.

Interventions:

Le groupe 1 a reçu de la caféine. Le groupe 2 a été exposés à des événements de surstimulation acoustiques (ESAs). Le groupe 3 a été exposée à des ESAs et à de la caféine. La dose de caféine par jour pour les groupes 1 et 3 était de 25 mg / kg, administrée par voie intrapéritonéale. Les ESAs ont été administrées aux jours 1 et 8 et consistait d'une heure de son pur de 110 dB. Des tests des potentiels évoqués *auditifs du tronc cérébral* (ABR) ont été mesurés pour déterminer le décalage du seuil d'audibilité et de sa récupération. Une analyse avec la microscopie électronique à balayage (MEB) et la microscopie optique (MO) ont été effectuées.

Mesures principales:

Des tests des potentiels évoqués *auditifs du tronc cérébral* (ABR) ont été mesurés au milieu de la première semaine, à une semaine, au milieu de la deuxième semaine et à deux semaines. En plus, une analyse de la cochlée a été faite avec la MEB et la MO.

Résultats:

Une heure après une surstimulation acoustique, le déplacement du seuil d'audibilité etait similaire entre tous les animaux dans les groupes 2 et 3 dans les fréquences testées (8, 16, 20 et 25 kHz). Ce changement de seuil etait maximal à 16 kHz avec une moyenne de 66 et 54 dB, respectivement. Au jour 8, le changement s'est normalisé complètement dans toutes les fréquences sauf à 20 kHz. La déficience auditive a persisté dans le groupe 3 à trois fréquences (8, 16 et 25 kHz) avec un seuil de 22, 28 et 26 dB, respectivement (valeur p de 0,001). Après une deuxième surstimulation acoustique au jour 8, des résultats similaires ont été enregistrées au jour 15. La différence entre les deux groupes était en concordance avec les résultats de la MEB et de la MO.

Conclusion:

Une dose quotidienne de caféine a été trouvé à altérer la reprise de l'audition après un événement de surstimulation acoustique.

PART 1: Introduction

Chapter 1: Introduction

Noise-induced hearing loss (NIHL) is a common problem among young adults and adolescents. Loud noise exposure is present in various settings, which can be in a recreational or a work environment[1]. The mechanisms of this type of hearing loss are: auditory hair cell trauma, promotion of a hypoxic environment with strial atrophy, and spiral ganglion neuronal degeneration[2,3].

The most ingested psychoactive substance is caffeine[4]. It is found in common beverages such as soda, energy drinks, coffee, and tea. Caffeine has been found to increase the release of dopamine, serotonin, and norepinerpherine[5]. It also has behavioral effects by increasing arousal and decreasing fatigue. Nevertheless, caffeine has a long list of side effects including insomnia, tremors, seizures, and anxiety[6].

Teenagers and adolescents consume caffeine in alarming amounts. Pollak et al. (2003) reported the caffeine intake up to 800 mg/day in eighth and ninth grade students[7]. When comparing these numbers to adults, a recent review of the association between caffeine and urinary incontinence noted that some patients consume more than 2.4 L of caffeine containing drinks per day[8].

Due to the increased consumption, there has been a growing interest in studying the relationship of caffeine with hearing loss. These studies are clinically relevant and based on the environments and activities where regular caffeine consumptions are either noisy leisure settings (e.g., concerts and loud music clubs) or occupational noise exposure (e.g., machine workers and aviation industry).

A recent pilot study by our group has demonstrated that caffeine-treated animals suffer from a delay in hearing recovery after being exposed to loud noise. There are many possible mechanisms in which caffeine may induce such a phenomenon. None of which is well understood[9].

The objective of the current thesis study is to determine the effect of daily caffeine intake on the hearing recovery after acoustic overstimulation events in a guinea pig animal model. The working hypothesis is that daily intake of caffeine impairs the recovery of hearing after an acoustic overstimulation event.

PART 2: Background and Literature Review

Chapter 2: Basic Anatomy and Physiology of The Ear

2.1 The Cochlea

The cochlea is part of the inner ear and is enclosed in the temporal bone. It is the main hearing organ and comprises 3 compartments filled with fluid and coiled in two and half turns[10]. Within the scala media, the organ of Corti rests above the basilar membrane in a partition bound by the Reissner's membrane as well as the lateral cochlea wall. In its basal end, the scala vestibuli has the oval window, usually connected to the stapes footplate, which is the region where vibrations induced by sound are transmitted to the cochlea friends within the ear[10]. The auditory hair cells situated in the organ Corti serve as transducers by the use of their stereocilia and effectively converts the vibration induced by sound into electrical activity encouraging spiral ganglion neurons[10].

Such hair cells are categorized into inner hair cells Inner hair cells (IHC) as well as outer hair cells (OHC)[11]. Moreover, the tectorial membrane, which is quite thin, follows the movement of hair cells after the vibrations (sound-induced) arrive at the cochlea[10]. An arrangement such as this facilitates the right conduction of mechanical energy to the hair cells together with each sound-induced transmitted vibration into the cochlear fluids.

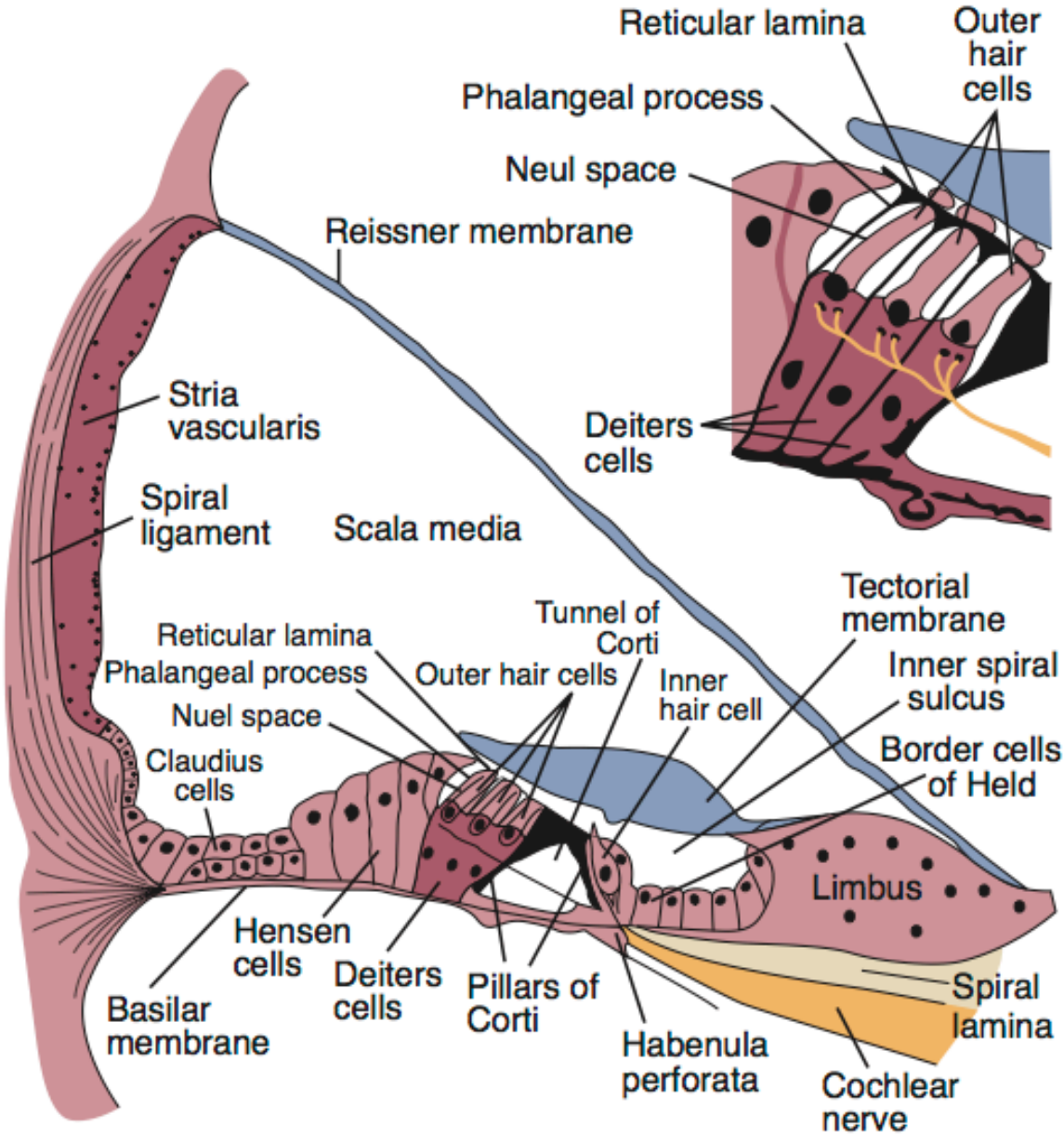

Figure 1: Cross Section of Organ of Corti

This cross section figure illustrates the major cellular structures of the organ of Corti. (Adapted from Cumming's Otolaryngology – Head and Neck Surgery sixth edition with permission)[12]

2.2 Perilymph, Endolymph and Auditory hair cells.

OHC's integrity alongside homeostatic ionic constitution in the cochlear fluids acts as a physiological basis of the Otoacoustic emissions. On the other hand, the perilymph ionic constitution looks like extracellular fluid having low potassium (3 millimol/L) and high sodium composition(150 millilol/L), whereas the endolymph is made up of ionic concentration similar to intracellular fluid[13]. Maintenance of the above concentrations is dependent on adenosine triphosphate (ATP) dependent pumps situated within the striavascularis. Such dissimilarity in concentration of ions sets the potential of the membrane for suitable depolarization of hair cells[10].

The hair cells' stereocilia are a formation of actin filaments groups that are attached with side links close to their base[14]. Links such as those facilitate all the stereocilia to travel in a single direction whenever the sound-induced vibrations arrive at the cochlea. With this disposition in mind, stereocilia's deflection leads to potassium channels opening in the hair cell leading to depolarization as well as conduction to the spiral ganglion neurons[15].

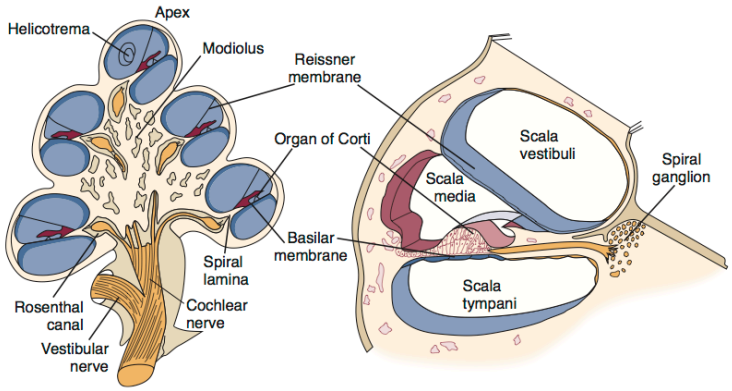

Figure 2: Cross section of the cochlea

Left: This is a cross section figure of the cochlea that shows the cochlear nerve passage through the modiolus to the organ of corti. Right: A higher magnification figure

demonstrating the osseous and membranous compartments of the cochlea. . (Adapted from Cumming's Otolaryngology – Head and Neck Surgery sixth edition with permission)[12]

2.3 Cochlear Mechanics and Nerve Transmission

According to Gillespie, the cochlea serves as a selective transducer depending on the frequency and intensity of the stimulus availed[14]. Because of mass (gradual increase) as well as variation stiffness (gradual decrease) from base to apex, the basilar membrane shakes at particular frequencies alongside the cochlea[14]. Furthermore, due to these features, sounds of high frequency creates a wave which peaks in the base, with low frequency sounds peaking in the apex. Thus, the basilar membrane acts similar to piano keyboard[11], with higher frequencies at one end, and lower frequencies at the other enc.

The auditory nerve is formed of the axons of the spiral ganglion neurons that make synapses with cochlear nucleus at brainstem's level, where the middle pathways convey in ascendant order[11].

2.4 The Central Auditory Pathway

The key relays of the pathway act as an anatomical root for the Auditory Brainstem Response (ABR), which will be utilized in the experimental testing of this thesis. Demarcation of the central auditory pathway is done by the cochlear nucleus as well as the auditory cortex located in the temporal lobe and is tasked with the integration of sound stimuli[16].

Evidence indicated that unilateral injury at the cochlear nuclei may lead to hearing disorders that are similar to auditory nerve dysfunction, signifying that cochlear nucleus bears only ipsilateral contribution[17]. The following conveyance is the contralateral superior olivary complex (SOC) comprising of 3 key groups of nuclei: the nucleus of the trapezoid body (MNTB), the medial superior olivary nucleus (MSO) and the lateral superior olivary complex (LSO)[16]. In addition, the lateral lemniscus joining the SOC together with the inferior colliculus consists of fibers of bilateral origins of the SOC and cochlear nucleus[18]. The SOC as well as the inferior colliculus have bilateral input implying that they serve a critical role in understanding of binaural sound stimuli.

The inferior colliculus, at the midbrain level, consists of neurons having sharply defined frequency sensitivity, which implies that is a conduit where the stimuli are noticeably differentiated so as to be adequately integrated before passing to advanced pathway levels[19]. Eventually, the medial geniculate body within the thalamus is the last convey before arriving at the auditory cortex[16].

As Musiek's work describes in detail[20], and akin to the cochlea arrangement, the neurons of the middle auditory passageways always uphold a tonotopic arrangement, upholding the perspective of an extremely organized system charged with sound integration within the central nervous system[21].

Moreover, the middle passageway is linked to the reticular system, which facilitates information integration gotten from sound stimuli within the cortex and elicits a reaction[16]. The reticular system comprises of 2 key systems: the motor activating system and the ascending reticular activating system[22]. With such an interconnection, whenever the sounds signify threat to the subject, the cortex is activated and capable of promoting awareness as well as activation of the motor system to jump into reaction.

Whereas the auditory nerve cells within the cochlea are the major signal transducers for sound stimuli, the central auditory passageway incorporates the information to bring out a response to sounds. Moreover, the auditory passageway has a high degree of organization so as to achieve these tasks.

Chapter 3: The Auditory Brainstem Response (ABR)

The ABR can be described as a window of the electrical movement of neurons of the auditory pathway. The test comprises of a series of waves in response to a sound stimulus. Such waves are frequently plotted in a graph showing time in milliseconds (msec) on the X axis and amplitude (microvolts) on Y axis at various intensity levels, decibels (dB)[23,24].

Akin to an electrocardiogram, the ABR waves are obtained using an electrode attached to the earlobe of the ear stimulated and another one attached at the vertex. A third electrode is positioned on the contralateral side as ground electrode[23,24]. With this arrangement, a differential electrical action is obtained following stimuli, leading to recordable waveforms[23,24].

Because every peak characterizes the reaction from neurons situated in the relays of the auditory pathway[23,24], a lot of ABR knowledge has been acquired from experimental research studying damage to particular brainstem points. Most studies are in agreement that wave I symbolizes the reaction of neurons in the lateral aspect of the auditory nerve whereas wave II originates from the medial section of the 8th nerve[25]. More than a single generator has been postulated to account for wave III; however the cochlear nucleus appears to be the main one[20]. For wave IV, the SOC has been proposed as the key source. On the hand, wave V, which is formed from the lateral lemniscus and fractional contribution of the lesser colliculus, is perhaps the most appropriate wave for the ABR[26,27].

Due to the regular shape of wave V as well as a strong association with behavioral audiometric thresholds[28], wave V is most frequently used to establish hearing thresholds[29]. While testing, as the stimulus intensity is reduced, the various waves' latencies increase and these turn out to be less clear[23,24]. For these reasons, the wave V is used in assessment of hearing thresholds in the procedures utilized in the McGill Auditory Sciences Laboratory.

There are two kinds of acoustic stimulus that can be employed during ABR testing: clicks and tone bursts. Clicks are pulses of comparatively long duration (200msec) and are useful to estimate hearing sensitivity[23,24]. However,, they cannot be utilized in assessment of particular frequencies. To do this, tone bursts of at defined frequencies having a shorter

duration than clicks are utilized[23,24,28]. Thus, the ABR is an instrument that can be used as a screening technique or to establish hearing thresholds.

One benefit of ABR testing is that test results are not strongly affected by sleep, attention changes, or sedation -- in contrast to behavioral audiometry[23,24]. Thus, the ABR is mostly utilized in such settings as hearing assessment of unconscious patients, newborns as well as in experimental animals, which is the basis for this thesis.

Chapter 4: Noise related Hearing impairment.

Most medical professionals, especially Otolaryngologists, are familiar with the term Noise related Hearing Loss (NRHI). Although, NIHL is usually not curable, preventive measures are well know - especially those based on occupational health guidelines are where much current research has focused on.

NRHL can be sub-classified into three categories; Temporary Threshold Shift (TTS), noise-induced hearing loss (NIHL), and acoustic trauma.

4.1 Temporary Threshold Shift

Temporary threshold shift (TTS) occurs after a period of loud sound exposure. This results in a temporary and mild hearing loss. This is a period when a person perceives that everything sounds quieter than they really are[30]. This phenomena tends to not be worrisome since most people fully recover[30]. However, repeated TTS can turn into permanent hearing loss. TTS causes an alteration of the fine and delicate cochlear micromechanics that includes the linkage between the stereocilia of the hair cells. The reversibility of TTS may not be complete. For that reason, people should avoid noise exposure that can lead to TTS[30].

4.2 Noise-Induced Hearing Loss

In NIHL, the patient is chronically exposed to a noise levels of >85 dB SPL for long periods per day. An example of this is occupational related noise exposure. When cochlear hair cells are deflected by an acoustic signal, they cause depolarization of the hair cells which in turn leads to excitation of the neural activity of their respective afferent neurons. Each group of hair cells is cross-linked into bundles[30,31]. For that reason, when sound displaces a whole bundle, these links open the ion channels' membrane located on the hair cell's surface. Potassium ions form the majority of flowing ions through the channels resulting in changes

in the hair cell receptor potential[30-32]. It is important to note that these changes: micromechanical arrangements, cross-linkage and ion channels, are very delicate and can get damaged in an acoustic overstimulation event[30-32].

Health Canada has set specific occupational exposure limits that are based on the maximum permitted duration of exposure for various noise levels.[33]. These guidelines were specified to help minimize NIHL[33].

Allowable Level of dB	Maximum Permitted daily duration
85	8 hours
88	4 hours
91	2 hours
94	1 hour
97	0.5 hour
100	0.25 hour

Table 1: Nose Exposure Limits

This table is based on the Canadian Centre for Occupational Health and Safety recommendations.[33]

4.3 Acoustic Trauma:

Patients can be exposed to a sudden high intensity sound pressure that causes abrupt mechanical damage to the middle or inner ear directly from the acoustic energy[34]. Such damage can be either related to TM rupture and fracture of the ossicles, or to cochlear membrane rupture resulting in mixing of the endolymph and perilymph, thereby injuring the hair cells[34]. This sudden acoustic trauma is usually occurs when sound intensity is in the range of 140-180 dB SPL or more. An example of this subcategory is the noise produced by a rifle or a gunshot[34].

Chapter 5: Noise Induced Hearing Loss in Children:

Usually, while evaluating hearing loss, healthcare professionals depend on the results of clinical tests that include speech threshold measures and the audiogram[30]. An audiogram reveals whether the hearing loss is mild, moderate, severe, or profound based on the audiometric definition being used. Otoacoustic emissions (OAEs) or auditory-evoked potentials are largely employed to find hearing loss in infants. As such, hearing threshold loss is measured in decibels (dB); these measures are certainly useful. However, unfortunately, it does not always inform about a hearing deficiency. Often, a child is found to have a normal hearing thresholds, but may also have difficulty understanding speech. This means that before testing a child with complex sounds, the dhild should be testing with pure tone audiometric sounds. This also means that a comprehensive hearing evaluation is necessary - including speech discrimination tests to find critical information on possible hearing losses of a child[30].

The World Health Organization (WHO) defines and assesses hearing issues in several ways such as 'hearing impairment', 'disability' and 'handicap'. A person is said to have 'impaired hearing' when his or her sensory functions do not respond adequately during the clinical tests as described above. 'Disability' describes the activity limitation that has resulted from their hearing impairment. For example, person's comprehension may not be not normal or adequate due to their hearing loss. The term 'Handicap" describes a reduced participation due to hearing problems. A person affected from hearing loss may not be able to make friends or participate in school activities and games, or they may not be able to choose career options freely. Learning is greatly affected because an affected person may fail to make appropriate interactions with his or her parents, sibling or other family members in a normal way. Interaction with teacher and fellow students is also greatly affected. Constraints in educational achievements also lead to reduced employment opportunities for the affected person impacting his or her quality of life. Overall, society is also affected due to hearing impairment issues of an individual when seen in the context of economic,

social, or educational achievements; an affected person fails to contribute to their full potential in nation-building activities[30].

Apart from profound or significant hearing loss, mild or moderate noise-induced hearing loss can also be observed in a child. Moderate hearing loss also prevents effective communication from taking place due to presence of multiple sounds of different thresholds. A typical example is in a classroom environment in a school. Unfortunately, moderate hearing losses are not easily recognizable for a child, for the same reasons that an elderly person might not be aware of their own age-related hearing loss. Physicians and parents need to be cautious because hearing screen tests or an audiogram will not reveal a hearing issue unless speech discrimination measures are employed to find it. If such deficiency persists in in a young child, then language development would be greatly hampered. Moreover, the child's educational accomplishments would also be affected because of the inability to understand information given at school. As child grows, social isolation may become another issue due to communication problems faced. Sometimes, such conditions may drive some people to commit suicide. Even if a doctor prescribes hearing aids, it is may be difficult for a young adolescent to use it regularly because of the social stigma attached; For example, the device acts as a spoiler to cosmetic appearance. Eventually, a child may prefer to go in isolation rather than using hearing aids. In either case, a child's growth and quality of life both may be significantly impacted[30,35].

While there have been publicity campaigns to educate people on hearing loss prevention in children, the Hearing Foundation of Canada (THFC) has recently employed a strategy that aims at communicating "save your hearing for the music" (meaning you, with hearing loss, would not be able to enjoy your music that you love so much now). Hearing loss may also lead to educational failures, or to reduced educational accomplishments[36]. Obviously, the condition could also lead to fewer job opportunities, and to a reduced earning potential for the person affected from hearing loss[30,35].

Chapter 6: Caffeine consumption and adverse effects

Caffeine, the most widely used stimulant in the world, is consumed by more than 85% of adults in the United States[37,38]. Caffeine is commonly found in coffee, and it is increasingly being used as an additive in soda and energy drinks at extremely high concentrations[38]. Half of the consumers of energy drinks are adolescents or young adults[38]. With a regular cup of coffee containing between 45 and 145 mg of caffeine, the reported consumption of caffeine above 500 mg per day is alarming, for a 70 kg man, this translates to > 7 mg / kg[7].

Caffeine is also used in medicine to treat several conditions. In the setting of neonatal intensive care units (NICU), caffeine is considered one of the go to drugs to treat apnea of prematurity (AoP)[39-41]. The typical loading dose to treat AoP is 10-20mg/kg followed by a maintenance dose of 5 mg / kg. Another condition that is often treated with Caffeine is drowsiness. The typical dose of a child of 12 years of age is 100~200 mg every 4 hours. That could translate to 4 mg / kg every 4 hours[39-41].

Although caffeine is often ingested to improve alertness and performance, it should be avoided in pregnant women, in children with comorbidities, and in patients with complex medical history including cardiovascular disease and anxiety disorders. Furthermore, recent studies demonstrated a link between increased risk of miscarriage and caffeine ingestion. Additionally, ingesting more than 300 mg per day of caffeine by pregnant women was found to be associated with smaller head circumference, lower birth weight, as well as with a doubling of the risk of miscarriage when compared to women whose caffeine intake was less than 151 mg per day[42].

In children, one study assessed the physiological effects of caffeine on young boys and girls, aged 7 to 9 years old. It demonstrated that, caffeine could produce a lower heart rate and higher blood pressure. Caffeine may also affect sleep patterns in teenagers[7,43].

Chapter 7: Rationale of this study

With the increasing awareness of the side effects of caffeine, further studies addressing the effect of caffeine on Otolaryngology related disease have surfaced.

Caffeine is consumed in various settings, social, occupational, and as a treatment especially in NICU settings. In many of these settings, a noisy environment can be present. Thus, it is imperative to understand the effect of caffeine on hearing in general and the changes it may cause in the presence of an environment that overstimulate the auditory system.

To understand the effect of caffeine on acoustic overstimulation event, an experiment was designed to assess these ideas in a guinea pig model. The methodology, results and discussion will be described in detail in the next part of this thesis.

PART 3: Experiment Study: Methods, Results, Discussion & Conclusion

Chapter 8: Methods and Materials

This study was approved by McGill University Ethics review board and the University animal care committee.

8.1. Animals

Twenty four female albino guinea pigs (6 months of age weighing 500-600 gms) were used. The animals were housed at 22 °C ±4 °C with a light/dark cycle of 12 h. They had access to food and water ad libitum.

The guinea pigs were divided randomly into three groups. The first group (control) (n=8) received a daily inraperitoneal injection (IP) of 25 mg/kg of caffeine for 15 days. The second group (n=8) received two acoustic overstimulation events (AOSEs). The third group (n=8) received a daily 25 mg/kg IP of caffeine for 15 days and two AOSEs.

The randomization process was done by a researcher who was not involved in the auditory brainstem response (ABR) testing.

8.2. Caffeine Administration

The choice of caffeine dose was based on the literature review and a previous study conducted on our lab. In the literature, a dosage of more than 25mg/kg/day was considered an excessive dose of caffeine that resulted in significant physiological changes including urinary incontinence. As a result, each dose was calculated based on the daily weight of the animal, and a daily dose of (25 mg/kg/day IP) was administered between 9-10:00 am.

The weight and behavior of the animals were recorded everyday throughout the experiment.

8.3. Acoustic Overstimulation Events (AOES)

The current study used the same setup employed in previously published studies[9,44,45]. In a sound proof room, the guinea pigs were anesthetized using ketamine (50 mg/kg) and xylazine (1 mg/kg). Each AOSE consisted of a continuous 6 kHz of pure-tone produced by a generator (Intelligent Hearing Systems, Miami, FL) and amplified by a D-75A amplifier (Crown Audio Inc., Elkhart, IN). The tone was then projected using two loudspeakers at a distance of 5 cm from the animal's head in a free field while in the sound proof room. A calibrated Bruel and Kjaer sound level meter was used to monitor the sound level (dB SPL). The sound was amplified to 110 dB SPL and was sustained for an hour[9,44,45].

Each animal in group 2 and 3 (AOSEs and AOSEs + Caffeine groups) received 2 AOSEs. The first was at Day 1 and Day 8 of the testing.

8.4. Auditory Brainstem response

ABR testing was done under general anesthesia using 5% inhaled isoflurane for induction and 2% for maintenance. Animals with abnormal anatomy of the ear were excluded.

Prior to measurement, the ABR machine was calibrated to industrial standard, as specified by the company, in a similar manner to the pilot study[9]. ABRs were measured at the following intervals: baseline (prior to the start of the experiment), Day 1 (1 h after AOSE), Day 4, Day 8 (before and 1 h after the second AOSE), Day 11 and Day 15.

The testing was performed only on the right ear of all animals.

The system used was the SmartEP program (Intelligent Hearing System, Miami FL). Electrodes were placed sub-dermally on the vertex (reference), Contralateral ear (ground) and tested ear; right ear (active). Frequencies tested were 8, 16, 20 and 25 kHz tone burst stimuli[9].

The stimuli, presented to the right ear, were initially presented at 100 dB SPL then decreased, using 5 DB decrements, to 5 dB SPL. The responses were filtered, amplified and averaged over 1600 sweeps. The hearing threshold was defined as the lowest intensity at which both wave III and wave V were clearly visible.

A threshold shift was calculated based on the difference between thresholds at the specific time point and the baseline at that specific frequency. Sensation levels (SL) were used to quantify the threshold shift.

8.5. Scanning Electron Microscopy and Light Microscopy

On day 15 of the experiment, and after the final ABR, four randomly selected animals from each group had their right cochlea removed and fixed with 2.5% glutaraldehyde for 2 h. Afterwards, it was soaked in 0.1 M PBS solution for 24 h at 4 °C. Post-fixation, the cochlea was treated with osmium tetroxide for 90 min; then it was dehydrated in 70% ethanol. Subsequently, the cochlea was drilled and the organ of Corti was dissected. Then the sample was dehydrated again in 100% ethanol and scanning electron microscopy was performed. To quantify the effect on the outer hair cells (OHC), this study adopted a modified version of the Saito et al (1995) 4-grade scale[46]. This scale is as follows: grade (N) = Normal; grade 1 = 10-50% OHC loss or damage; grade (2) = OHC count less than 50%; grade (3) = cuticular plate rupture and missing hair cells. Results are for each section, apical, middle and basal turns.

The other four animals in the each group had their right cochlea extracted immediately after they were euthanized. Fixation was performed with 10% formalin for 48 h. Following 3 weeks of decalcification, the cochleas were then dehydrated using ethanol 50-100% and then cut into 5 μm. Hematoxylin and eosin (H&E) staining was performed. At the end, they were mounted for analysis by light microscopy.

The turns of the cochlea were then viewed at 200x magnification, using a Zeiss AxioCam MR3 camera (Carl Zeiss, Germany).

8.6. Statistical analysis

Initially, analysis of the control group was performed between the baseline and day 14 to determine the effect of caffeine on hearing. After calculating the threshold shift for each frequency at each time-frame, the means of each group were analyzed using Kruskal Wallis Analysis of Variance to determine if there was a significance between all three groups. Next, to determine differences between groups 2 & 3 Mann-Whitney U test was utilized. The results were considered statistically significant at p-value ≤ 0.05. Analysis was performed using SPSS © v23.0.

Chapter 9: Results

9.1 ABR Thresholds

9.1.1 Baseline ABR testing

The baseline ABR threshold analysis showed no difference between any of the three groups (*p*-value >0.05).

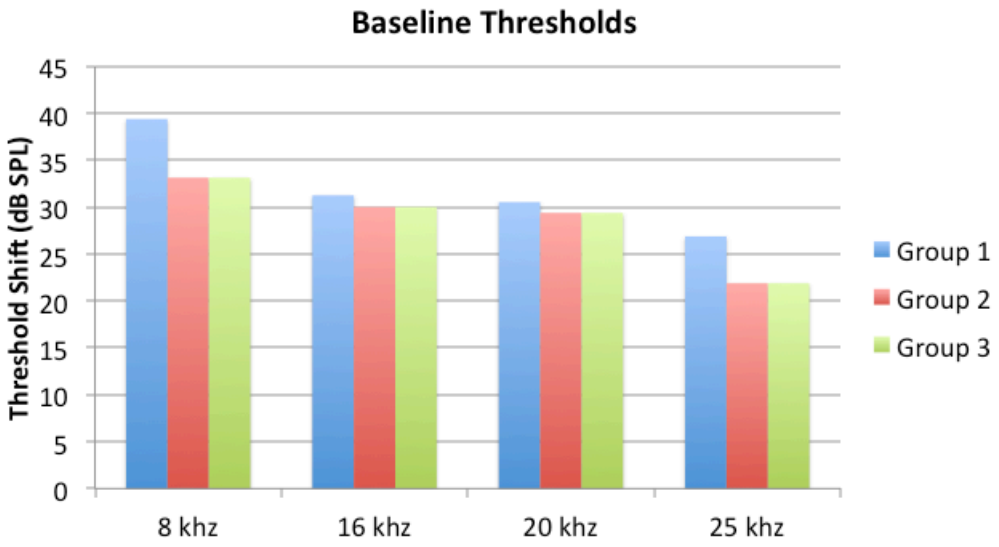

Figure 3: Baseline Thresholds

This chart shows ABR baseline thresholds for all 3 groups. Using Kruskal Wallis testing, there were no differences between these groups (p > 0.05)

9.1.2 Daily Caffeine and hearing

Group 1 was injected daily with caffeine. ABR analysis showed no threshold shift at this group that was not subjected to AOSEs. For that reason, daily IP caffeine was not found to cause ABR threshold shift up to 15 days of injections.

9.1.3 One-hour post AOSE threshold shift

On day one, 1 h after AOSE, a threshold shift was noted in groups 2 and 3. This shift was not significantly different between the groups (*p*-value >0.05, Kruskal Wallis). The maximum shift was observed at 16 kHz frequency (66.00 dB SL).

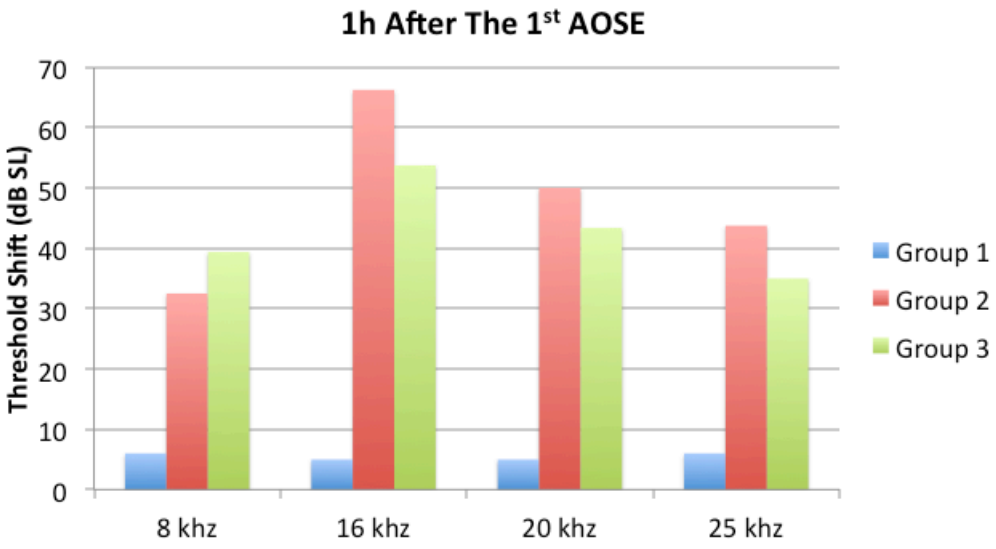

Figure 4: Threshold Shift 1h After 1st AOSE

There were no difference between group 2 and 3 after exposure to the 1st AOSE.

9.1.4: Threshold shift @ 8 khz

ABR testing at 8 kHz revealed full recovery at Day 4, and at day 8 for group 2. Furthermore, this recovery continued after the second AOSE, until day 15. When compared to group 1, group 2 did not show any statistically significant threshold shift at days 8 and 15 ($p > 0.05$).

In contrast, group 3, had a mean persistent threshold shift of 30.00 dB SPL at day 4, and 21.88 dB SPL at day 8. These results were statistically significant when compared to group 2 ($p < 0.0001$ and $p = 0.001$).

After the second AOSE, group 3 had a higher threshold shift (55.00 dB SL) in comparison to group 2 (31.88 dB SL). These results were statistically significant ($p = 0.015$).

At day 11 and 15, ABR testing showed impaired recovery with a threshold shift that persisted at 31.88 and 29.38 dB SL respectively. These results were statistically different from group 2 ($p < 0.0001$ and $p < 0.0001$).

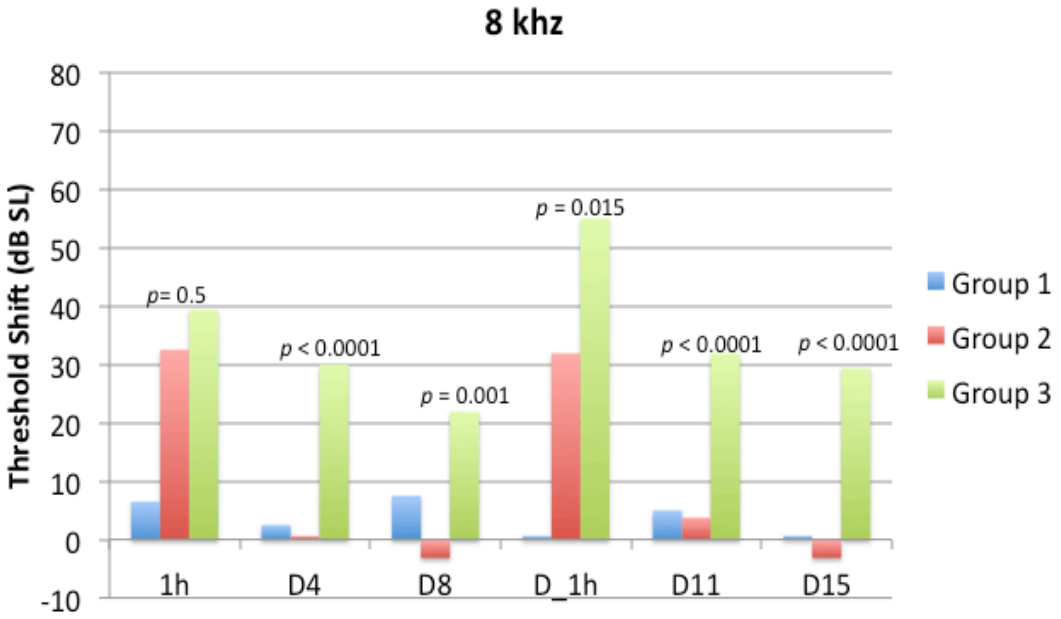

Figure 5: Threshold shift @ 8 khz

This chart shows ABR threshold shifts at 8 khz for all three groups at 6 intervals; 1 hour after AOSE (1h), Day 4 (D4), Day 8 (D8), Day 8 1 hour after AOSE (D8_1h), Day 11 (D11) and Day 15 (D15). Mann Whitney U test was used to compare Group 2 & 3 at each interval. There was a statistically significant imparement of recovery of group 3 (AOSEs + Caffeine) noted on D4, D8, D11 and D15.

9.1.5 Threshold shift @ 16 khz

ABR testing at 16 kHz, demonstrated full recovery at Day 4 and day 8 for group 2. Furthermore, this recovery was then replicated after the second AOSE until day 15. When compared to group 1, group 2 did not show any statistically different threshold shift at days 8 and 15 ($p > 0.05$).

In comparison, group 3, had a mean persistent threshold shift of 41.25 dB SL at day 4, and 28.13 dB SL at day 8. These results were found to be statistically significant when compared to group 2 ($p = 0.002$ and $p = 0.001$).

After the second AOSE, group 3 had a higher threshold shift (70.63 dB SL) in comparison to group 2 (63.75 dB SL). These results were statistically significant ($p = 0.015$).

At day 11 and 15, ABR testing revealed impaired recovery with threshold shift that persisted at 40.63 and 35.63 dB SL respectively. These results were statistically different from group 2 ($p < 0.0001$ and $p < 0.0001$).

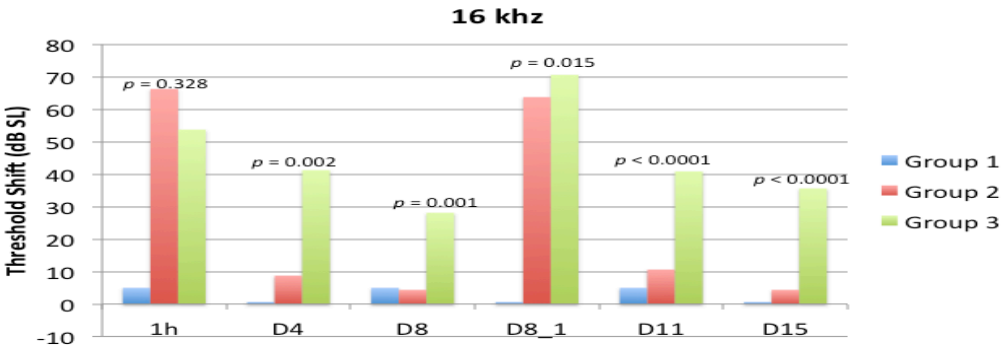

Figure 6: Threshold Shift @ 16 khz

This chart shows ABR threshold shifts at 16 kHz for all three groups at 6 intervals; 1 hour after AOSE (1h), Day 4 (D4), Day 8 (D8), Day 8 1 hour after AOSE (D8_1h), Day 11 (D11) and

Day 15 (D15). Mann Whitney U test was used to compare Group 2 & 3 at each interval. There is a statistically significant impaired recovery of group 3 (AOSEs + Caffeine).

9.1.6 Threshold shift @ 20 khz

ABR testing at 20 kHz showed impaired recovery of hearing at Day 4 and day 8 for group 2, with mean threshold shifts of 0.50 and 25.63 dB SL respectively. Furthermore, these were also noted on day 11 and 15 where threshold shift persisted at 33.75 and 28.38 dB SPL respectively.

Group 3 had a mean persistent threshold shift of 38.75 dB SL at day 4, and 36.25 dB SL at day 8. These results were not found to be statistically significant when compared to group 2 (p = 0.161 and p = 0.065).

After the second AOSE, group 3 had a similar threshold shift, 50.63 dB SPL, when compared to group 2, 49.38 dB SL (p = 0.798).

At day 11 and 15, ABR testing showed impaired recovery with threshold shift persisting at 41.25 and 40.63 dB SL respectively. These results were similar to group 2 (p = 0.234 and p = 0.083).

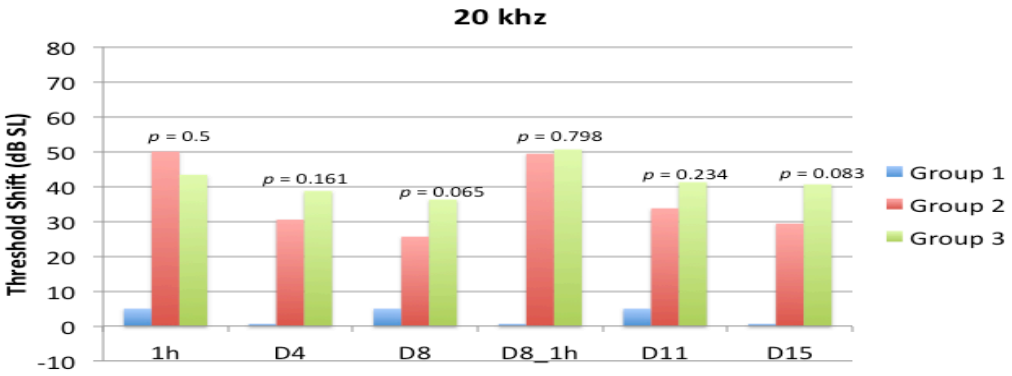

Figure 7: Threshold Shift @ 20 khz

This chart shows ABR threshold shifts at 20 khz for all three groups at 6 intervals; baseline 1 hour after AOSE (1h), Day 4 (D4), Day 8 (D8), Day 8 1 hour after AOSE (D8_1h), Day 11 (D11) and Day 15 (D15). Mann Whitney U test was used to compare Group 2 & 3 at each interval. This impairment was not statistically different.

9.1.7 Threshold shift @ 25 khz

For group 2, ABR testing at 25 kHz demonstrated full recovery at Day 4 and day 8. Furthermore, this recovery was then continued after the second AOSE until day 15. When compared to group 1, group 2 did not show any statistically different threshold shift at days 8 and 15 ($p > 0.05$).

In comparison, group 3, had a mean persistent threshold shift of 30.00 dB SPL at day 4, and 47.50 dB SPL at day 8. These results were found to be statistically significant when compared to group 2 ($p = 0.021$ and $p = 0.007$).

After the second AOSE, group 3 had a higher threshold shift (60.00 dB SL) in comparison to group 2 (31.88 dB SL). These results were statistically significant ($p = 0.038$).

At day 11 and 15, ABR testing revealed impaired recovery with threshold shift persisting at 41.25 and 38.75 dB SL respectively. These results were statistically different from group 2 ($p = 0.005$ and $p < 0.0001$).

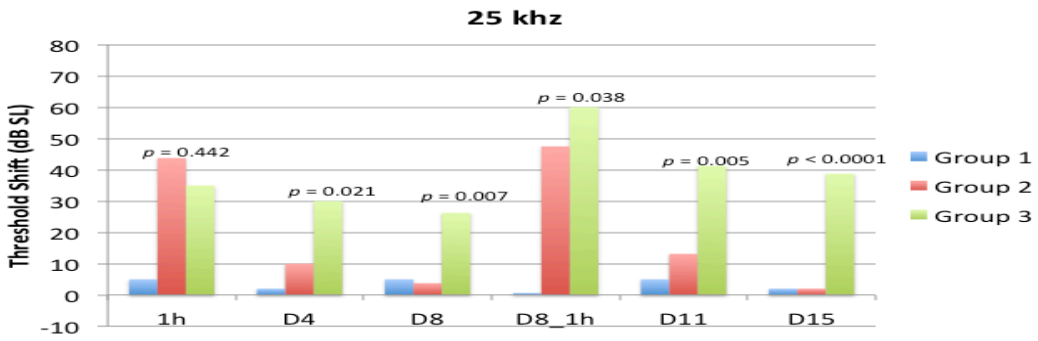

Figure 8: Threshold Shift @ 25 khz

This chart shows ABR threshold shifts at 25 khz for all three groups at 6 intervals; baseline 1 hour after AOSE (1h), Day 4 (D4), Day 8 (D8), Day 8 1 hour after AOSE (D8_1h), Day 11 (D11) and Day 15 (D15). Mann Whitney U test was used to compare Group 2 & 3 at each interval.

There is a statistically significant impaired recovery of group 3 (AOSEs + Caffeine) noted on D4, D8, D11 and Day 15.

9.1.8 Summary of Threshold Shift Outcomes

Group 2 (AOSEs only) ABRs showed complete recovery of the threshold shift by day 8 at all frequencies except 20 kHz, where a mean threshold shift of 20.625 dB SL was present. Similar results were obtained after the second AOSE. At day 15, the ABRs of group 2 guinea pigs showed hearing recovery in all frequencies except 20 kHz, where a mean threshold shift of 29.375 dB SL persisted.

In Group 3 (AOSEs + Caffeine), there was an impared threshold shift recovery at both day 4 and 8, in all frequencies. The mean threshold shifts on day 8 at 8, 16, 20 and 25 khz were 21.88, 28.13, 36.25, 26.25 dB SPL. Krusksal Wallis analysis of variance showed that these results were statistically significant compared to the bases line (p-value < 0.05). These results were then reproduced at the second week of the testing. At day 15, the mean threshold shifts at 8, 16, 20, and 25 kHz were 29.39, 35.63, 40.63, and 38.75 dB SL, respectively. These results were statistically different from baseline levels (p-value < 0.05, Kruskal Wallis).

When comparing Groups 2 and 3, during the hearing recovery period, there were threshold shift differences that were statistically significant at the following frequencies 8, 16, and 25 kHz between group 2 and 3 (p-values <0.001, 0.001, and 0.001, respectively, Mann Whitney U).

9.2. Scanning Electron Microscopy.

Based on the 4-grade scale previously mentioned, the control group, group 1, was normal in all animals in all sections tested. These results were statistically different when compared to either group 2 or 3. When comparing groups 2 and 3 using Mann Whitney-U test, there was no statistical difference between them except at the apex where group 3 was noted to have a more apparent loss of hair cell (p = 0.029, Mann Whitney U). This was based on a significant loss of hair cell count and disarrangement of the stereocilia. At the apex, group 3 had three animals with grade 2 and one animal with grade 4 whereas in group 2, all four animals did not exceed grade 1.

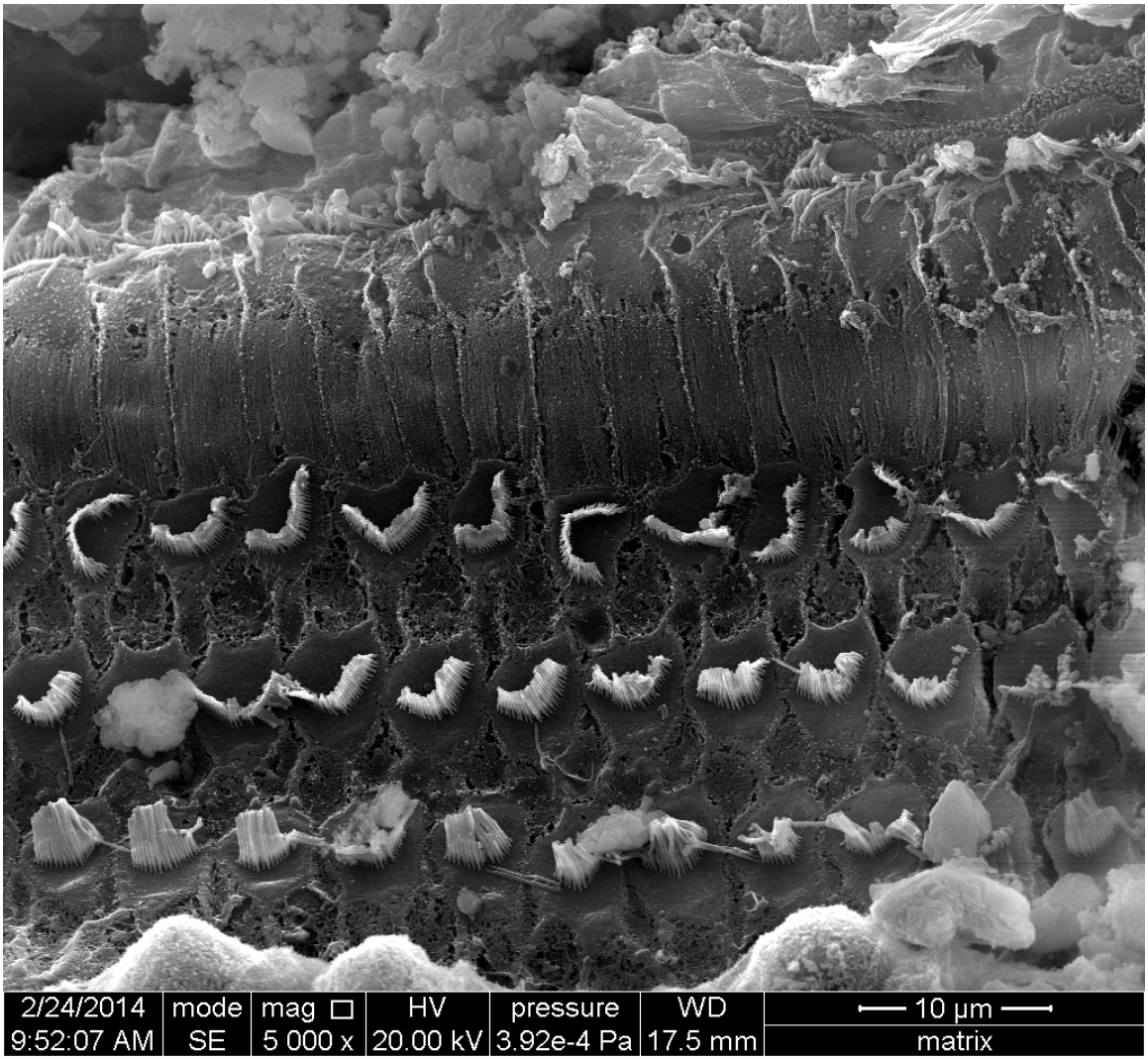

Figure 9: SEM of the Apex of the cochlea (Group 1)

This is a SEM of the apex of the cochlea of one of the group 1 animals demonstrating normal looking OHCs with normal arrangements.

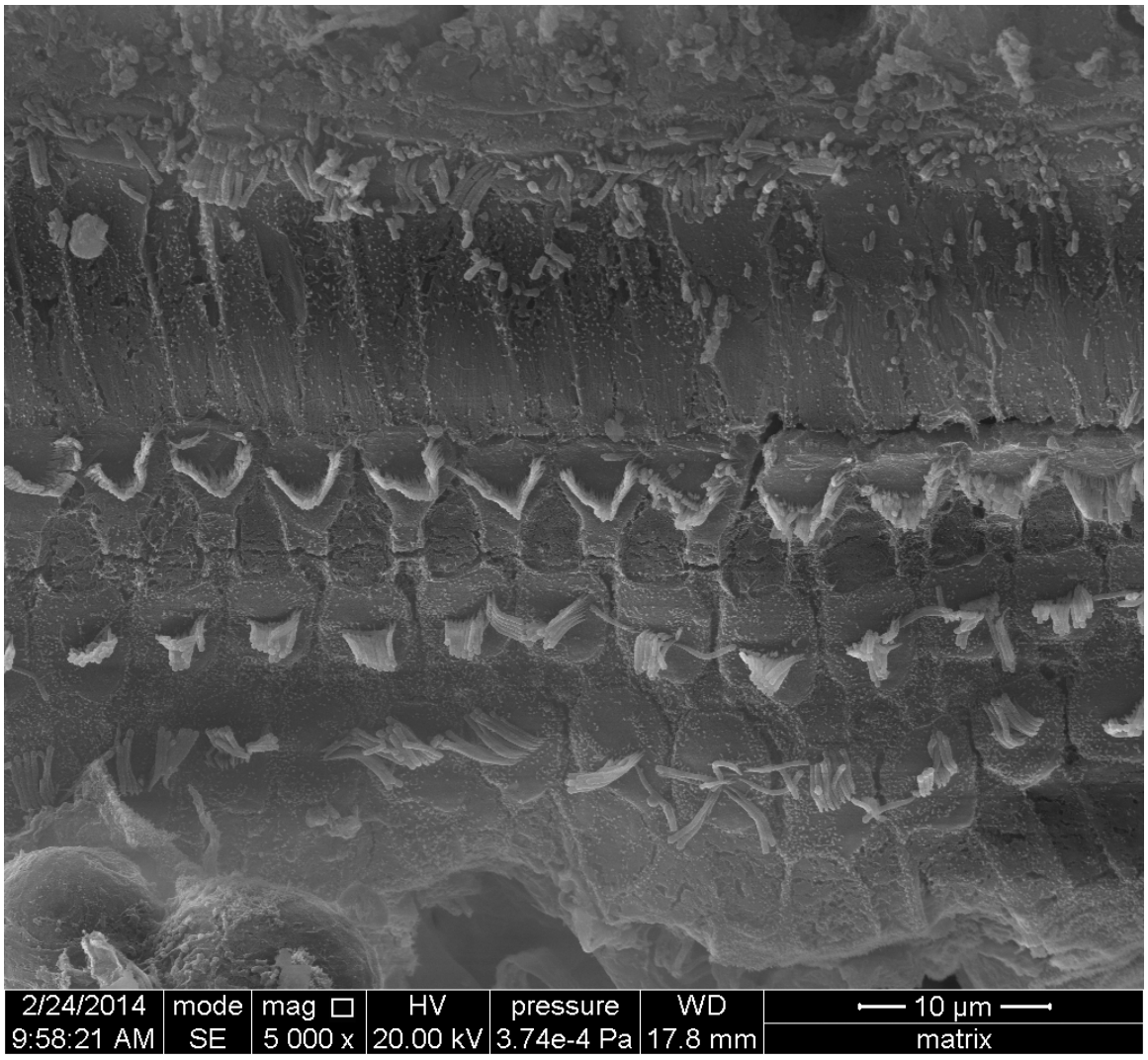

Figure 10: SEM of the Apex of Cochlea (Group 2)

This is an SEM This is a SEM of the apex of the cochlea of one of the animals of group 2 showing grade 1 OHC damage

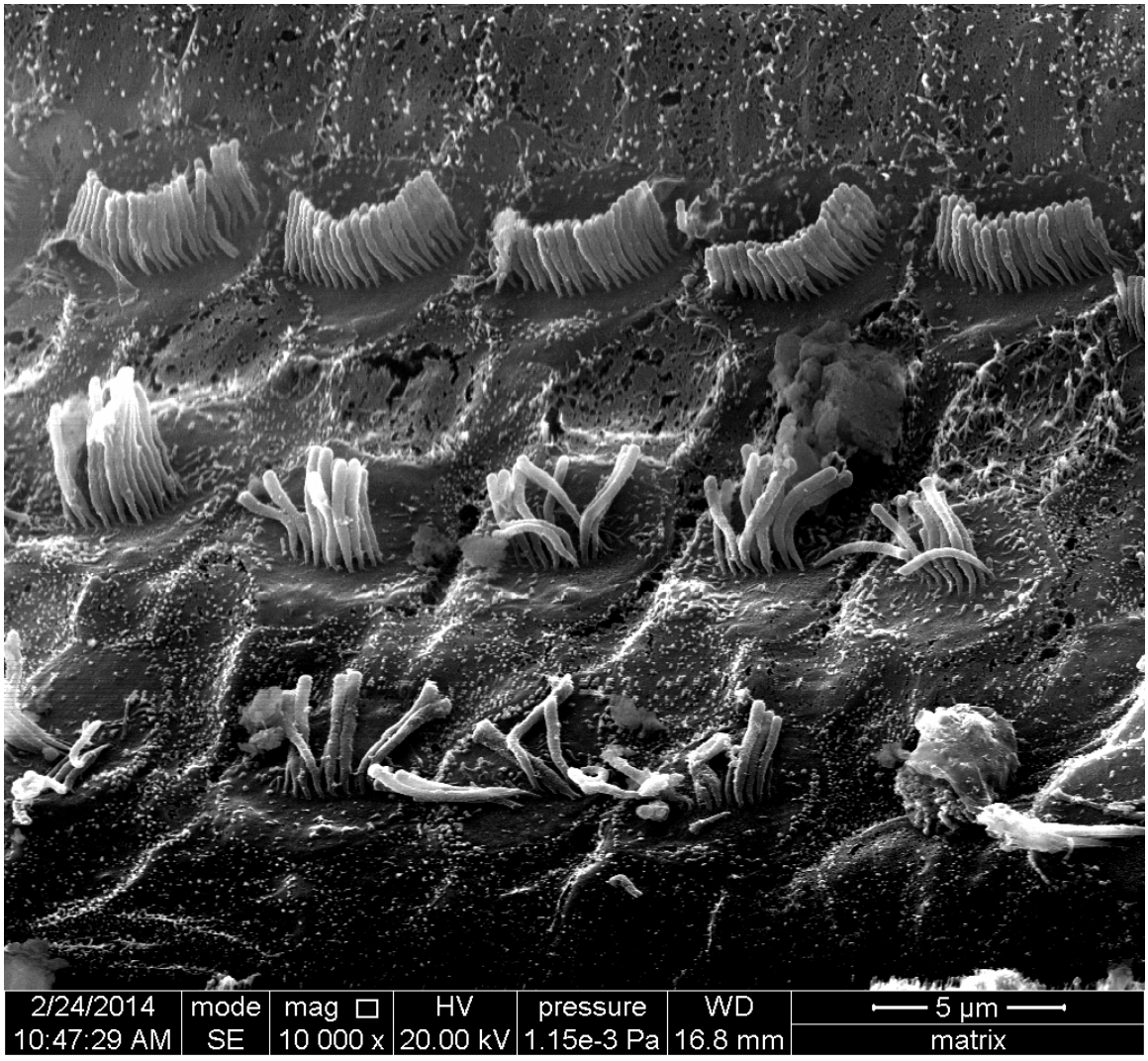

Figure 11: SEM of the Apex of the Cochlea (Group 3)

This is an SEM This is a SEM of the apex of the cochlea of one of the animals of group 2 showing grade 2 OHC damage

9.3 Light microscopy

Light microscopic analysis of the cochlea revealed that for the animals subjected to Caffeine only (Group1), there was preserved hair cell morphology at the organ of cotri and the stria vascularis seemed intact . In the group subjected to AOSEs only (Group 2) there were minimal changes in morphology and arrangement of the tunnel of Corti, while the stria vascularis was preserved. In the group subjected to the combined Caffeine and AOSEs (Group 3, there was abnormal morphology and arrangement of the tunnel of Corti and the stria vascularis showed dilation of vessels, with evidence of microscopic bleeding that might explain our results.

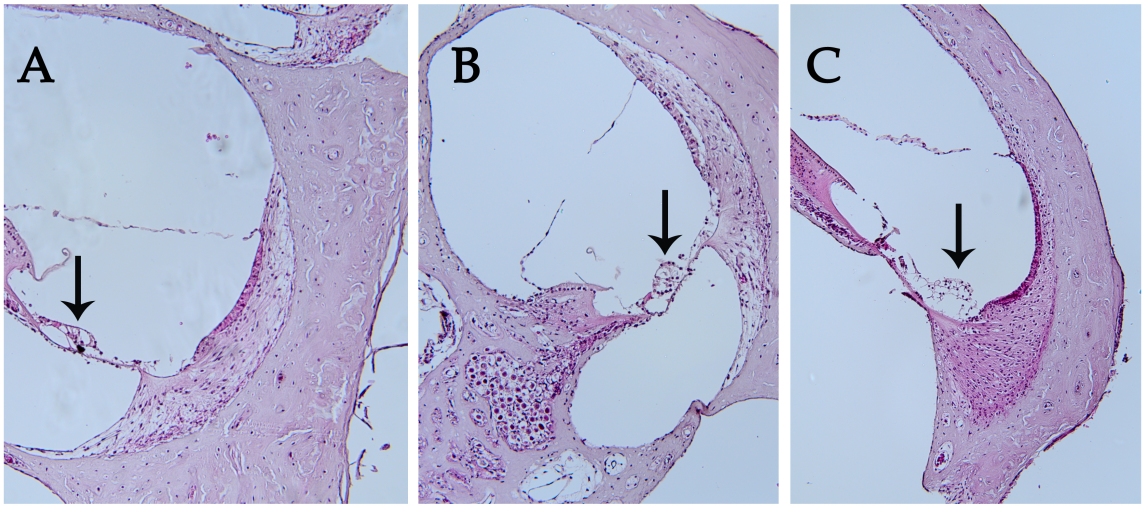

Figure 12: LM of all three groups

In the ears subjected to Caffeine only (A), there was preserved hair cell morphology as the organ of cotri (arrows) and stria vascularis seems intact . In the group subjected to AOSE only (B), there was minimal change in morphology and arrangement of the tunnel of Corti, while the stria vascularis was preserved. In the group subjected to combined Caffeine and AOSEs (C), there was abnormal morphology and arrangement of the tunnel of Corti, while the stria vascularis showed dilation of vessels, with evidence of microscopic bleeding that might explain our results.

9.4. Animals body weight and behavior

All the three groups (caffeine treated and non-caffeine treated) showed similar growth and behavior at daily doses of 25 mg/kg. The animals did not experience any seizures or gastroenteritis. No significant change in alertness or response to stimuli was noted. Kruskal Wallis analysis of variance did not show any difference in weight gain between the three groups (p-value >0.05, Kruskal Wallis).

Chapter 10: Discussion

In recent years, there has been an increased interest in studying the effect of caffeine both on normal physiological function and on known medical conditions. The current thesis examines the effect of caffeine on ABR threshold shift in guinea pigs exposed to acoustic overstimulation events.

The study initially addressed the effect of caffeine alone on hearing. The control group (group 1), which was exposed to caffeine for 15 days, did not show any changes in ABR threshold, SEM analysis or Light microscopy findings. This is in accordance with a previously published studies on caffeine. Dixit et al. (2006) reported that caffeine improved auditory pathway transmission by reducing the latency and raising the amplitudes of ABR waves[47].

In another pilot study, a chronic exposure to caffeine following noise reported a trend toward the delayed recovery of hearing. In that study, caffeine-exposed animals had a delayed recovery of hearing only at one frequency (8 kHz) that was evident on day 14 after the noise exposure[9]. The difference between that pilot study and this thesis is that the previous study used pure-tone acoustic stimuli that were higher in amplitude than those used in this study (120 dB SPL verses 110 dB SPL). This may have resulted in a permanent threshold shift at several frequencies, which could be one of the reasons why the difference between the groups was not more evident. Our studyfound that in 3 of 4 frequencies, there was a complete recovery of ABR threshold by day 8 after acoustic overstimulation of animals not receiving caffeine.

Furthermore, the current study also used a lower, more reasonable dose of caffeine i.e., 25 mg/kg. In a study comparing the effect of caffeine on autoimmune encephalomyelitis in guinea pigs, a dose of 30 mg/kg was found to exert neuroprotection against autoimmune encephalitis[48]. Additionally, in a human study, urinary incontinence was associated with higher doses of daily caffeine intake. In fact, the extremely high caffeine intake groups were ingesting as much as 2.4 L/day (~25-30 mg/kg)[8].

The results of this thesis showed that daily dose of caffeine had a negative effect on the hearing recovery after AOSEs at multiple frequencies. When comparing group 2 and 3, the latter had impaired ABR threshold shifts at frequencies 8, 16 and 25 kHz that were statistically significant (*p*-values <0.001, 0.001, and 0.001, respectively, Mann Whitney U). It was noted that at 20 kHz, there was no difference between both groups. This is likely due to a permanent impairment at that frequency, as both groups did not recover well. These results are in concordance with the previously published pilot study that showed a trend toward hearing impairment at 14 days post pure-tone exposure[9].

Another important result from this thesis is that the animals in both groups 2 and 3 recovered in a similar manner after both the first and second AOSE. Specifically in group 2, there was a complete ABR threshold recovery at 3 of 4 frequencies. The importance of this finding is that it can be considered as an animal model for other projects addressing acoustic overstimulation in guinea pigs. These results are also in agreement with that of Fetoni et al. (2010), where hearing recovery was noted between 7-21 days after pure-tone induced hearing threshold shift[44].

The scanning electron microscopy results showed increased OHC damage at the apex in the group that received caffeine and AOSEs (group 3) compared to the AOSEs only (group 2). Additionally, the light microscope slides showed tunnel of corti and stria vascularis changes in group 3 that were larger than the changes seen in group 2. These results are in keeping with the persistent ABR threshold shift that was noted with group 3. In the pilot study by Mujica-Mota et (2013), the SEM results showed more damage to the inner hair cell[9]. The difference in results between this study and the pilot could be explained by the difference in the caffeine dose and AOSE's sound amplitude.

There are many possible mechanisms on how caffeine impacts the auditory system in the recovery period after an AOSE. The organ of Corti, lateral wall, SGC and cochlear blood vessels contain high-affinity adenosine receptors[49]. During an AOSE, the cochlea becomes hypoperfused and may become ischemic[50]. Adenosine receptors help promote vascular blood flow to aid the reperfusion of the cochlea[51,52]. Caffeine has a non-selective adenosine receptors antagonist property, which may interfere with the activities of adenosine receptors, causing further ischemia resulting in an impaired recovery of hearing[53].

Another mechanism by which caffeine may interfere with hearing recovery is by increasing the release of corticosterone in response to AOSE[54]. This has been reported in a human study when caffeine intake prevented the morning drop of cortisol concentration[55].

A third mechanism may involve changes in intracellular calcium levels[56]. After acoustic trauma, there is a significant rise in the intracellular calcium levels[56]. This mechanism could lead to apoptosis of hair cells[57]. Caffeine functions by releasing further calcium that might cause shortening of the outer hair cells[58,59].

This thesis has shown that the additive effect of caffeine and AOSE exposure resulted in impaired recovery of hearing at multiple frequencies. These results are of clinical importance. The results of this study would predict that patients who continue consuming their caffeinated drinks while being exposed to noise, or after an AOSE, may be reducing their chances of full recovery of their hearing.

There are several limitations to this study. One limitation is that the AOSE used was a pure-tone sound, which in real life does not represent a typical noisy environment. Another limitation is that this study used a higher, but not unreasonable, dosage of Caffeine. Future studies should look into reducing the dose of caffeine to identify specifically at which dose caffeine might start to affect the recovery of hearing after an acoustic overstimulation event. A final limitation is that this study was not a long-term study. Due the relatively short follow up period (15 days), the long-term effects of caffeine remain uncertain.

The findings of this thesis could also lead the way to further research to better understand the mechanism behind the effect of caffeine on hearing after AOSEs with the goal of lowering the general incidence of noise induced hearing loss.

Chapter 11. Conclusion and Future Directions

11.1 Conclusion

1- An animal model using guinea pigs demonstrated threshold shift and recovery with pure-tone acoustic stimuli at 110 dB SPL.

2- Caffeine ingestion by itself did not show any effect on hearing up to 15 days, when delivered IP to guinea pigs.

3- Caffeine, in a daily IP dose, impaired the recovery of ABR threshold shift at multiple frequencies after acoustic overstimulation events in a guinea pig model. This was evident at 3 frequencies; 8, 16, and 25 khz, with the following threshold shifts at 15 days 29.39, 35.63, and 38.75 dB SL respectively. These results were statistically significant ($p < 0.05$).

4- Daily IP doses of 25mg/kg of caffeine had no effect on the animal growth or behavior.

11.2 Future Directions

The findings of this experiment were highly valuable and could lead to future discoveries in relation to caffeine use in noisy environments.

The future studies should focus on the following aspects:

1- Determine if caffeine would impair recovery of hearing if the AOSE was produced by sounds different than pure-tone, e.g. white noise.

2- Determine the caffeine's dose-specific effects on hearing recovery after AOSEs.

3- Corrosive casting of the cochlea could be used to try to determine the actual damage to the stria vascularis.

4- Determine if these results are reproducible on other animals and more importantly on human beings, by conducting clinical prospective cohort studies.

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