



Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN  
Neurobiologia e Neurofisiologia

**CICLO - XXI**

**Coordinatore Prof. Alessandro Martini**

**A Novel Oto-Protection Strategy in Cisplatin Induced Ototoxicity**

**Settore Scientifico Disciplinare MED/32**

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**Anni 2006/2009  
Ferrara 2009**

# Novel Oto-Protection Strategy in Cisplatin Induced Ototoxicity

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## **Abstract**

It has been almost forty years since cisplatin was introduced in clinical practice as a potent and promising anti-neoplastic drug. Since then, the usage of cisplatin for treating a variety of cancers in both children and young adults has increased. Ototoxicity is one of the dose limiting side effects of cisplatin. Currently, there is not a single good otoprotecting drug against cisplatin ototoxicity in clinical practice.

We planned to study the effect of noise stress against cisplatin ototoxicity alone and in combination with two other thiol based otoprotectors, namely L-NAC and D-MET, at very low dosages which had shown otoprotection against cisplatin ototoxicity. Although these two otoprotectors have shown promising results in animal studies at high dosages, there is concern that high dose of L-NAC and D-MET could have negative effects on chemotherapy, leading to reduced chemotherapeutic efficacy. In an attempt to avoid this negative chemotherapeutic effect, studies have been conducted administrating the otoprotecting drug at varying times and space.

The underlined hypothesis of this thesis is that a tolerable stress before an intolerable cisplatin insult could better prepare the hair cells for the intolerable cisplatin insult. Above all, an acute noise stress alone could potentially activate antioxidant enzymes, heat shock proteins, glucocorticoids, and stress activated protein kinases that could protect the hair cells from cisplatin toxicity.

We used noise stress to enhance the otoprotective effects of L-NAC and D-MET. Moreover, we were interested in studying the effect of noise stress alone.

Results of our studies have demonstrated that the noise stress technique maximizes the otoprotecting efficacy of 275 mg/kg L-NAC and 300 mg/kg D-MET, where 300 mg/kg D-MET + noise stress being the best among all treated groups. More interestingly, we found that noise stress alone showed better results against cisplatin ototoxicity when compared with the cisplatin only group. Both Hematoxylin and Eosin staining and TRITC-conjugated phalloidin staining were essentially consistent with the ABR findings.

Even minor otoprotection by an acute noise stress could change the current course of drug-only otoprotection approach against cisplatin ototoxicity. In clinic, this would let us use certain frequencies of sound by a headphone before or after the cisplatin treatment to protect at least the speech perception frequencies of patients.

*Key words:* Cochlea; hair cells; otoprotection; noise stress; D-MET; L-NAC; JNK; antioxidant; glucocorticoid; HSP

## **Una nuova strategia otoprotettiva per l'ototossicità indotta da cisplatino**

*Theneshkumar S.*

E' ormai da quasi quarant'anni che il cisplatino e' stato introdotto nella pratica clinica come un potente e promettente farmaco anti-neoplastico. Da allora l'uso del cisplatino e' aumentato nel trattamento di diversi tipi di cancro sia nei bambini che negli adulti.

L'ototossicità e' uno degli effetti collaterali dose limitante del cisplatino. Attualmente non vi e' un buon farmaco otoprotettivo nei confronti della tossicità del cisplatino. Abbiamo voluto studiare l'effetto dello stress da rumore contro l'ototossicità da solo e in combinazione con altri due otoprotettori tiolici, chiamati L-NAC e D-MET, a livelli di dosaggio molto bassi, che hanno mostrato otoprotezione contro l'ototossicità del cisplatino. Anche se questi due otoprotettori hanno dimostrato risultati promettenti negli studi sugli animali ad alto dosaggio, vi è una preoccupazione riguardo al fatto che alte dosi di L-NAC e D-MET potrebbero avere effetti negativi sulla chemioterapia, portando ad una riduzione dell'efficacia chemioterapeutica. Nel tentativo di evitare questo effetto negativo chemioterapeutico, sono stati condotti degli studi di somministrazione del farmaco otoprotettivo a diversi tempi e spazi.

L'ipotesi sostenuta in questa tesi e' che uno stress tollerabile, prima dell'insulto intollerabile del cisplatino, potrebbe meglio preparare le cellule cigliate all'insulto del cisplatino. Soprattutto, uno stress da rumore acuto, da solo, potrebbe potenzialmente attivare gli enzimi antiossidanti, proteine heat shock, glucocorticoidi e proteine chinasi attivate da stress che potrebbero proteggere le cellule cigliate dalla tossicità del cisplatino. Abbiamo usato stress da rumore per aumentare l'effetto protettivo da parte di L-NAC e D-MET. Inoltre, eravamo interessati a studiare l'effetto dello stress da rumore da solo.

I risultati dei nostri studi hanno dimostrato che la tecnica dello stress da rumore massimizza l'efficacia otoprotettiva di 275 mg / kg di L-NAC e 300 mg / kg di D-MET, dove 300 mg / kg di D-MET combinato con lo stress da rumore è il migliore tra tutti i gruppi trattati. Molto interessante, e' stato aver trovato che lo stress da rumore da solo,

mostri dare migliori risultati contro l'ototossicità del cisplatino, rispetto al gruppo trattato col solo cisplatino. Sia la colorazione Ematossilina-Eosina che la colorazione TRITC-coniugata alla falloidina, hanno dato sostanzialmente risultati in linea con quelli ottenuti con la registrazione ABR.

Anche una minima protezione ottenuta da stress da rumore acuto potrebbe cambiare l'approccio attualmente usato del solo farmaco otoprotettivo nei confronti della tossicità del cisplatino. Ciò clinicamente ci permetterebbe di utilizzare determinate frequenze del suono attraverso l'uso di cuffie prima o dopo il trattamento con il cisplatino per permettere ai pazienti di percepire almeno le frequenze della voce.

**Parole chiave:** Coclea; cellule cigliate; otoprotezione; stress da rumore; D-MET; L-NAC; JNK; antiossidante; glucocorticoidi; HSP

*Important families are like potatoes. The best parts are underground.*

*-- Francis Bacon*

***To My Family***

## **Acknowledgements**

I would like to express my sincerer gratitude to all who have helped me in different ways to make this work possible.

Especially, I would like to thank:

Professor Alessandro Martini, my coordinator and my supervisor, for your support, encouragement and guidance all the time.

Professor Stavros Hatzopoulos, Pietro Giordano, Vincenza Cascella and Guiscardo Lorito thanks a lot for your collaboration, understanding and support throughout this research.

My warm thanks are also extending to all my teachers and friends in St.Petersburg, Russia. Especially to Dr. Zuravski Sergei Grigoryevich, and Professor Thompson Vladimir Viktorovich for all what they have given and done for me.

To all my friends in residence, especially Mohammed Aldhoun, Teddy Rango, Bellal Abuhelaleh, Ahmed Rjoob , Chiranjibi Prasad Pokheral, and Sotiris Sboras for their encouragement and friendship.

Special thanks to my friend Roland Ferrucci for helping me in manuscript preparation and giving me valuable advice.

Sara Magosso and Laura Astolfi for helping me in histology and letting me use your lab.

I also owe a debt of gratitude to the Government and people of Italia, for proving this wonderful opportunity.

Above all I would like thank my parents, brother, sister, brother-in-law, and my wonderful wife for love, understanding, and encouragement.

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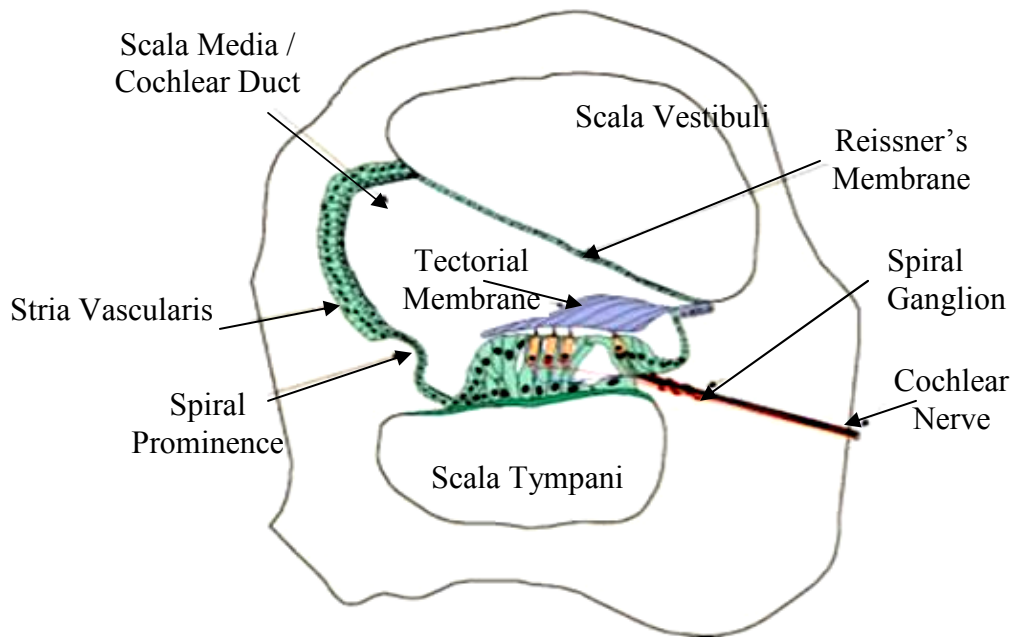
## Abbreviations.....

ABR	Auditory Brainstem Response
AIF	Apoptosis Inducing Factor
c-JNK	c-Jun N-terminal Kinase
dB	Decibel
HMG	High Mobility Group Protein
HSP	Heat Shock Proteins
IHC	Inner Hair Cell
iNOS	Inducible Nitric Oxide-Synthase
MAPK	Mitogen-Activated Protein kinases
MET	Methionine
NAC	L-N-Acetylcysteine
NOS	Nitric Oxide Synthetase
OHC	Outer Hair Cell
ROS	Reactive Oxygen Species

## **Anatomy of the Cochlear and Corti organ.....**

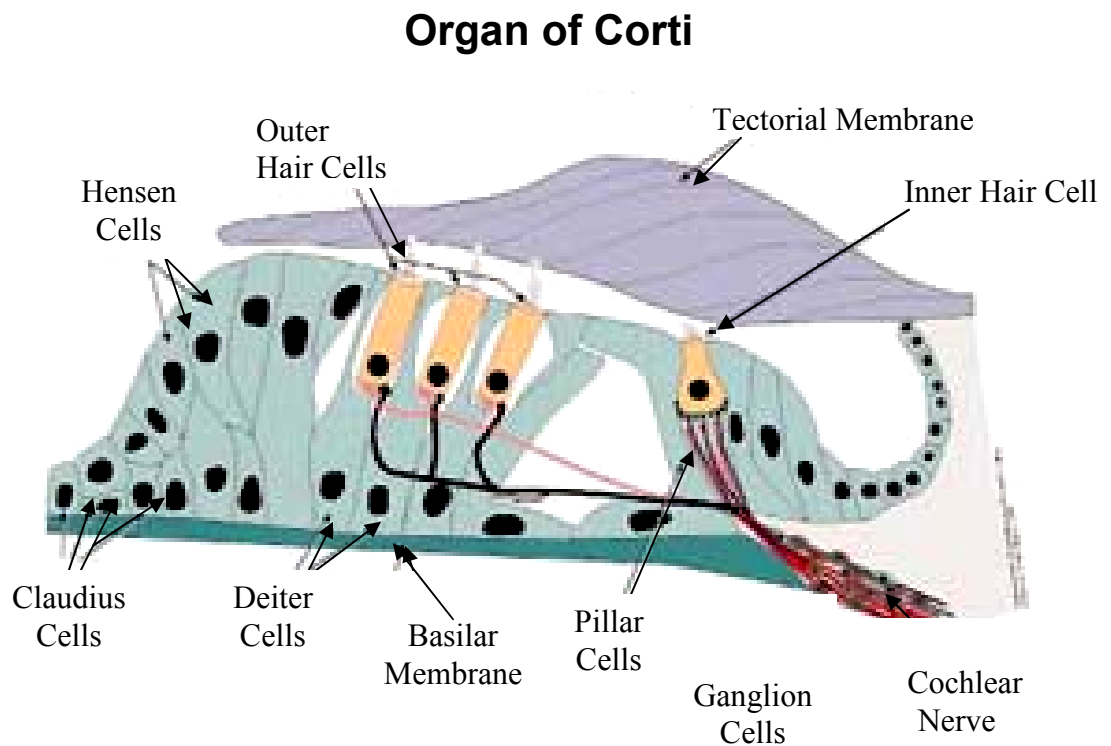
A part of the inner ear contains three spiral canals within the temporal bone that makes up the cochlea. The bony canal of the cochlea is divided into an upper chamber, the scala vestibule, and a lower chamber, the scala tympani, by a membranous labyrinth, the scala media, which houses the organ of Corti. While the bony labyrinth contains perilymph, the membranous labyrinth, also known as the cochlear duct, contains endolymph. Endolymph is similar in ionic content to the intracellular fluid (high K, low Na), whereas the perilymph resembles the extracellular fluid (low K, high Na). Perilymph acts as a cushioning agent for the delicate structures within the cochlear duct. The floor and roof of the cochlea duct are created by the basilar and Reissner's membrane, respectively.

### **Section of the Cochlea**



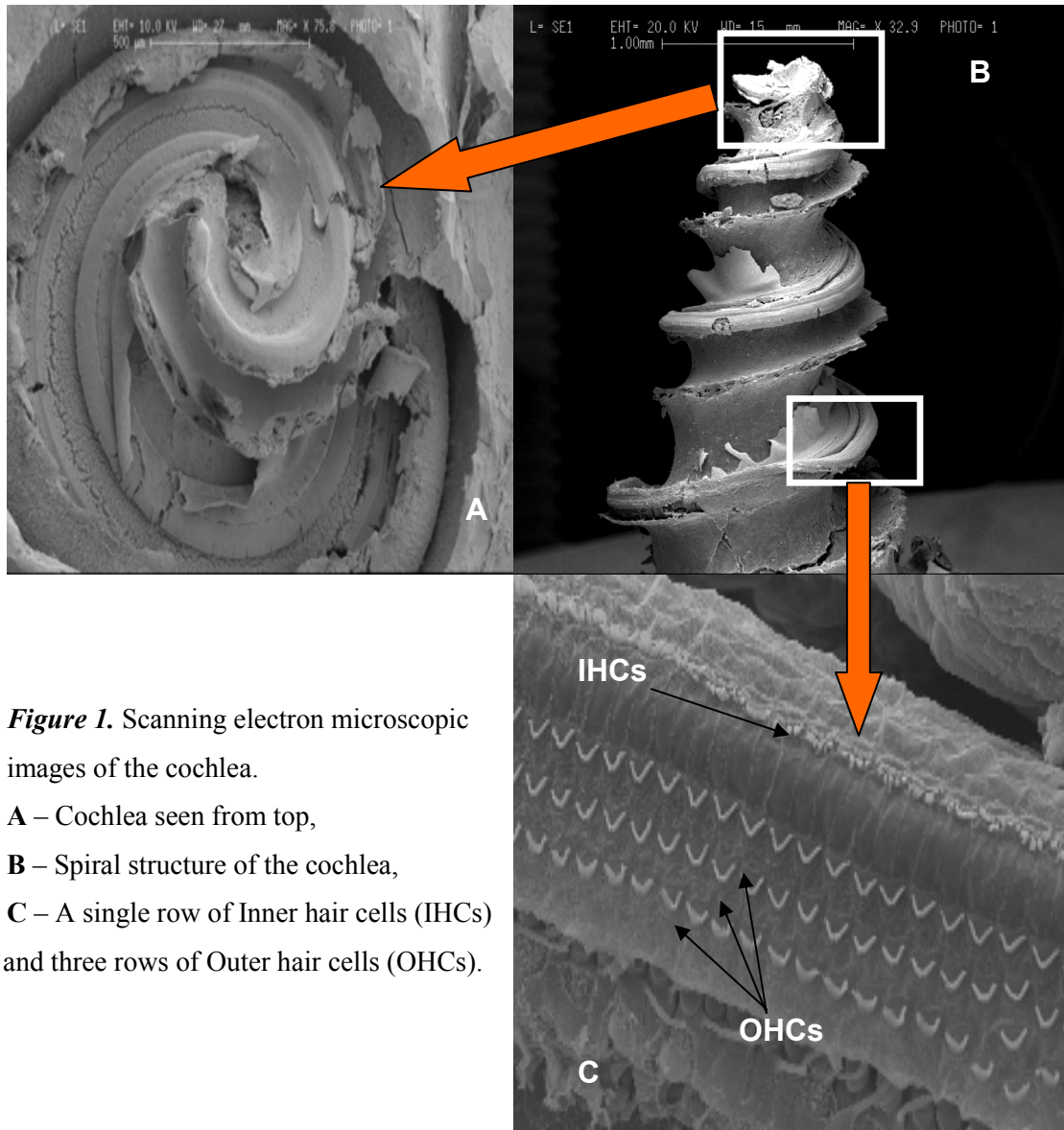
<http://depts.washington.edu/hearing/InnerEarHairCellRegeneration.php>

The organ along the basilar membrane is called the organ of **Corti**, which is composed of many specialized cells. Among them is a single row of **Inner Hair Cells** (IHCs) medially and three rows of **Outer Hair Cells** (OHCs) laterally. High and low frequency sounds are detected at the base and apex of the cochlea, respectively, by these specialized cells, which have specialized stereocilia on their apical surfaces. Attached to the medial aspect of the scala media is a fibrous structure known as the tectorial membrane, which roofs the inner and outer hair cells and attached to their stereocilia. The basal end of the hair cells synapses with the afferent and efferent nerve fibers of the cochlear branch of vestibulocochlear nerve.



<http://depts.washington.edu/hearing/InnerEarHairCellRegeneration.php>

The human cochlea contains approximately 20,000 auditory hair cells; 5000 of which are the IHCs and 15,000, the OHCs. Hearing deficits are mostly caused by death of these auditory hair cells, which do not have an innate ability to regenerate themselves since they are only produced during embryonic development [1, 2]. Permanent hearing loss can result when these hair cells are damaged and not repaired or regenerated. In drug-induced hearing loss these auditory hair cells mainly die by an **Apoptotic** pathway.



**Figure 1.** Scanning electron microscopic images of the cochlea.

**A** – Cochlea seen from top,

**B** – Spiral structure of the cochlea,

**C** – A single row of Inner hair cells (IHCs) and three rows of Outer hair cells (OHCs).

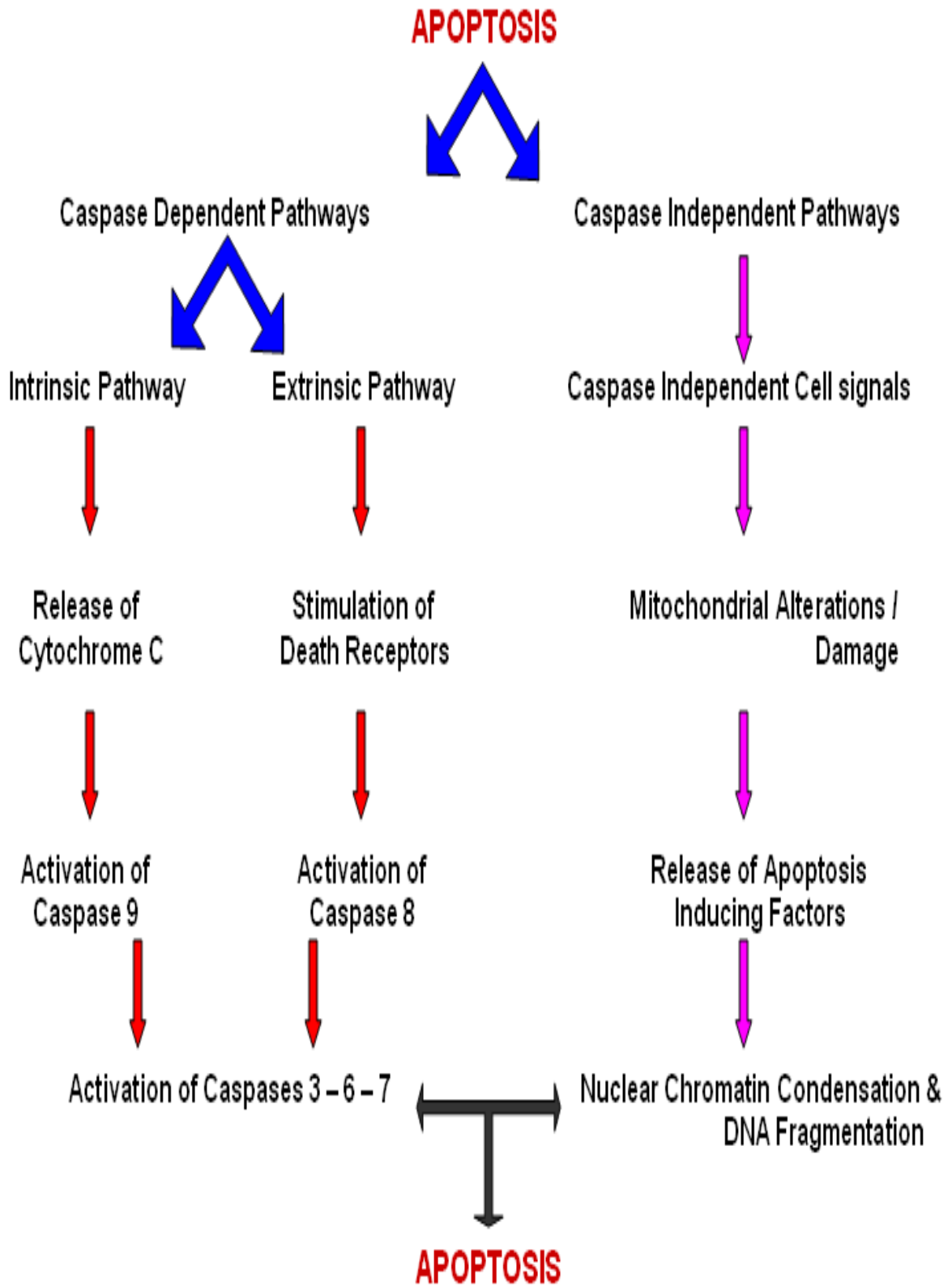
**Apoptosis.....**

A tightly regulated form of programmed cell death in multicellular organisms, apoptosis involves a series of biochemical events leading to a variety of morphological changes including blebbing, changes to the cell membrane, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Harmless, large protein bodies known as apoptosomes are formed at the end of apoptosis. In contrast, cells undergoing **Necrosis** release their intracellular contents, damaging surrounding cells and often causing inflammation.

The process of apoptosis is tightly controlled by a variety of cell signals, which may originate either extracellularly or intracellular. Extracellular apoptotic signals include toxins, hormones, growth factors, or cytokines, whereas an intracellular apoptotic signal is initiated by a cell in response to stress, and may ultimately result in cell suicide. During apoptosis, the cell is killed by a class of proteases called caspases. More than 10 caspases have been identified at present. While caspases 2, 8, 9, and 10 are involved in the initiation of apoptosis, caspases 3, 6, and 7 execute the death order by destroying essential proteins in the cell. Some caspases, such as 1, 4, and 5, are important for cytokine processing. The apoptotic process can be reviewed in three main stages: activation of initiating caspases by intracellular or extracellular specific signals; activation of executing caspases by the initiating caspases, which in turn cleave inactive caspases at specific sites to activate them; and degradation of essential cellular proteins by the executing caspases with their protease activity. Apart from these caspase-dependent apoptotic pathways, there is a caspase-independent pathway which is triggered by Apoptosis Inducing Factor (AIF), which causes DNA fragmentation, chromatin condensation, and NADH oxidization.

Cisplatin-induced ototoxicity is regulated by some important cell signaling events, including activation and redistribution of cytosolic Bax, release of cytochrome C from damaged mitochondria, and activation of caspases-9 and 3. It is likely that cisplatin ototoxicity is mediated by mitochondrial damage in the affected hair cells, with sequential activation of initiator and executing caspases, resulting in apoptosis, hair cell destruction, and hearing loss.

**Possible pathways of Apoptosis.....**



## **Cisplatin.....**

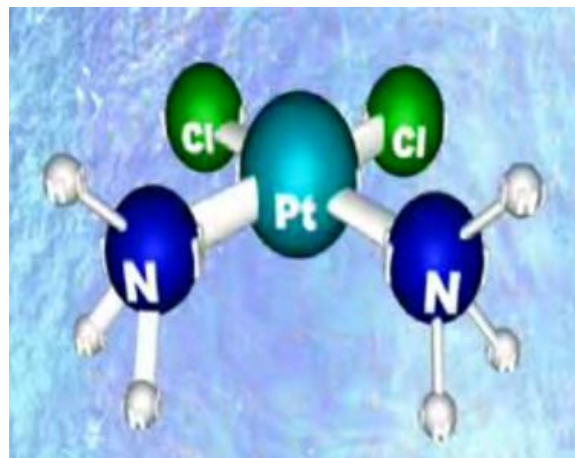
### **History**

Though cisplatin was first synthesized by M. Peyrone in 1845, its cytotoxic effect which inhibited *E-coli* cell division was discovered 120 years later by *Barnett Rosenberg et al.* [3]. In 1971, cisplatin went into clinical trials as a chemotherapeutic agent, with the results were published in 1972 [4]. Later, two more platinum-based chemotherapeutic drugs, namely carboplatin and oxaliplatin, were introduced into clinical practice. Until today, cisplatin continues to hold its place as a potent and highly studied platinum-based, anticancer drug [5].

### **Cisplatin in modern cancer therapy**

Cisplatin has been used for more than three decades and still holds an important role in modern cancer therapy. Cisplatin is highly effective in treating head and neck cancer, soft-tissue neoplasms, and lung, squamous cell, testicular, ovarian, cervical, and bladder cancers. Moreover, cisplatin is frequently used as part of a combination chemotherapy regimen with other drugs and radiation therapy. The chemo-radiation therapy where cisplatin has a good therapeutic effect is with advanced lung and uterine cervix cancer [6, 7, 8].

## Mechanisms of Cisplatin



**Figure 2.** Molecular structure of Cisplatin - cis-diamminedichloridoplatinum(II) (CDDP)

Although the exact intra-cellular mechanism of cisplatin is not completely understood, some evidence reveals that cisplatin is intra-cellularly converted into a cytotoxic monohydrated complex. This is the most important cytotoxic complex that mediates the DNA reaction which causes cell death [9]. Furthermore, it is believed that this monohydrated complex is partially responsible for cisplatin's most common nephrotoxic, neurotoxic, and ototoxic side effects [10, 11, 12, 13].

*Li G., et al. 2006* [14] described another possible cytotoxic mechanism of cisplatin, where the high-mobility group protein (HMG1) and inducible nitric oxide-synthase (iNOS) play major roles in cisplatin toxicity. They found that elevated levels in the expression of HMG1 and iNOS in response to cisplatin chemotherapy are responsible for cisplatin toxicity. Moreover, this HMG is believed to form a complex with DNA, which in turn impairs the DNA repairing mechanism, hence causing cell death.

But the core reason for cisplatin-induced ototoxicity is thought to be the generation of reactive oxygen species (ROS) and the depletion of endogenous antioxidant-glutathione levels within the hair cells during cisplatin treatment [15, 16].



### **Side effects of Cisplatin**

Cisplatin has a number of side-effects that can limit usage, including Nephrotoxicity, Neurotoxicity, and Ototoxicity.

#### **Nephrotoxicity**

During cisplatin treatment, nephrotoxicity may manifest as acute renal failure or as chronic disease with signs of electrolyte wasting [17]. Clinically, cisplatin induced nephrotoxicity can be easily revealed by elevated levels of creatinine in blood biochemical analysis, as in other cases of renal failure. The main region which is affected by cisplatin toxicity in kidney is the proximal tubule, as this is the most sensitive part of the kidney for drugs and toxins [18]. Nowadays, nephrotoxicity can be ameliorated by pre-hydration and diuresis; maintaining high chloride concentrations in kidney cells by chloride diuresis prevents aquation and activation of cisplatin [19, 20, 21].

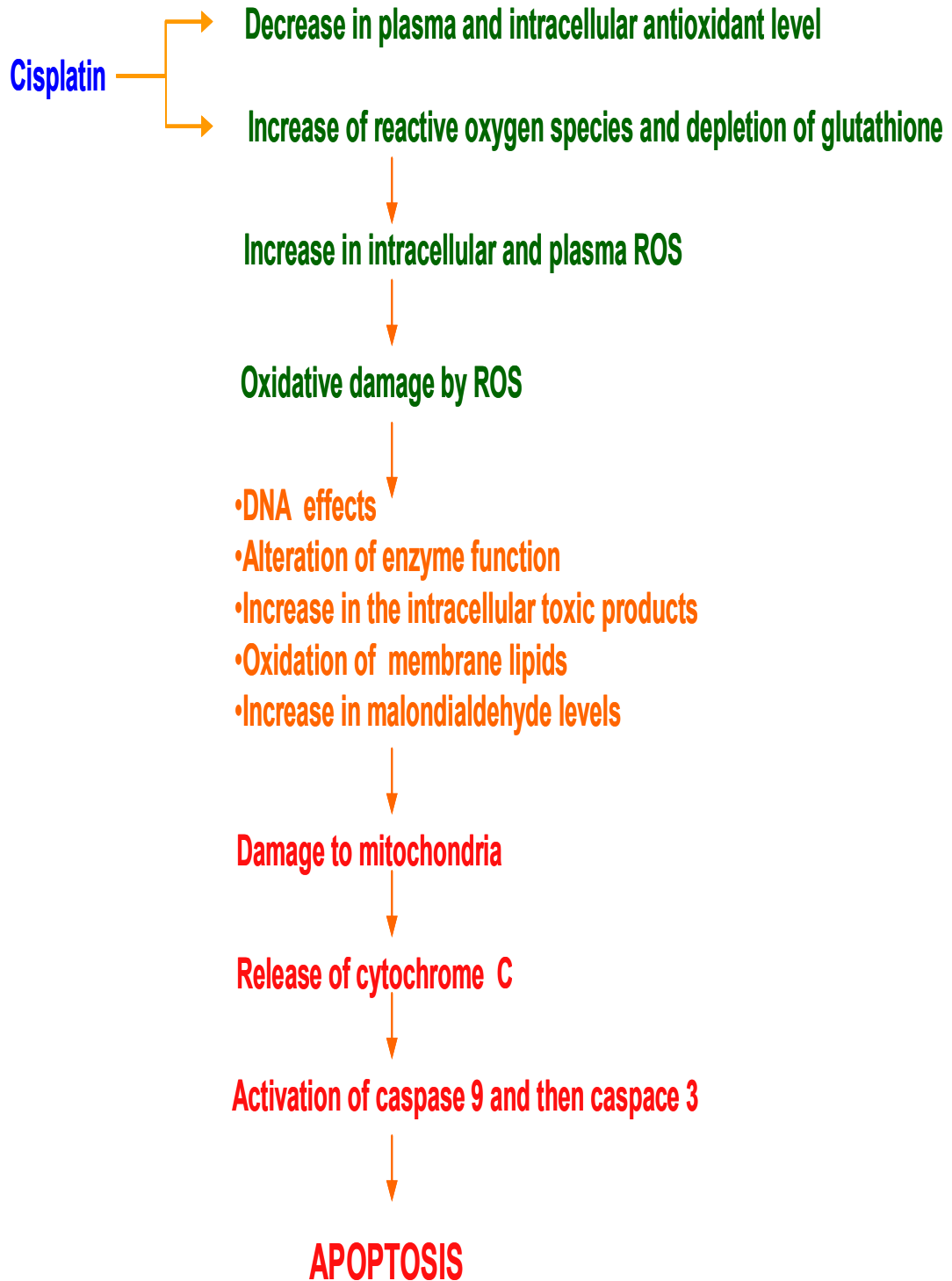
#### **Neurotoxicity**

Neurotoxicity was first reported soon after clinical usage of the drug began. This neurotoxicity is directly related to total cumulative drug dose. Significant peripheral neurotoxicity is evident in patients who receive a dosage of 400–500 mg/m<sup>2</sup> of cisplatin [22 - 27]. The neurotoxicity is initially manifested as paresthesiae in the distal extremities and may progress to severe sensory ataxia. Pathologically, cisplatin-induced neurotoxicity shows demyelination of nerve fibers and axonal degeneration [27, 28]. Electrophysiological studies in cancer patients treated with cisplatin show a predominant involvement of sensory axons [29, 30]. This predominant sensory neuron toxicity could be due to direct cisplatin contact with sensory neurons rather than selective neuronal damage. Normally, cisplatin does not cross the blood–brain barrier. Therefore, central nervous system neurons and motor neurons are not directly exposed to cisplatin.

### **Ototoxicity**

Unfortunately, there is no effective treatment to prevent this side effect at present. Audiometric analysis is used to assess the severity of ototoxicity in clinics. Clinically, cisplatin administration can cause tinnitus and high frequency sensorineural hearing loss, which may be permanent or progressive, involving also lower frequencies. There is evidence in the literature that, with an increase in total administration dose of cisplatin, almost every patient has the risk of developing at least some degree of hearing loss [31, 32]. The hearing impairment in cisplatin ototoxicity has been demonstrated by the effect of cisplatin on the OHCs located in the organ of Corti of the inner ear [33 - 36]. This phenomenon is more severe at the first row of OHCs in the basal turn of the cochlea and then progresses to the other two rows of OHCs [33]. This apoptotic damage has been partly explained by the presence of high-mobility group protein, during cisplatin treatment in the organ of Corti [14]. But the core reason for the cisplatin-induced ototoxicity is believed to be the generation of ROS and the decrease of endogenous antioxidant- glutathione levels in the hair cells during cisplatin treatment [15, 16]. Therefore, scientists have mainly used strong antioxidant such as L-NAC and D-MET to counter cisplatin-induced ototoxicity [14, 37 - 42].

**Possible pathway of cisplatin induced ototoxicity.....**

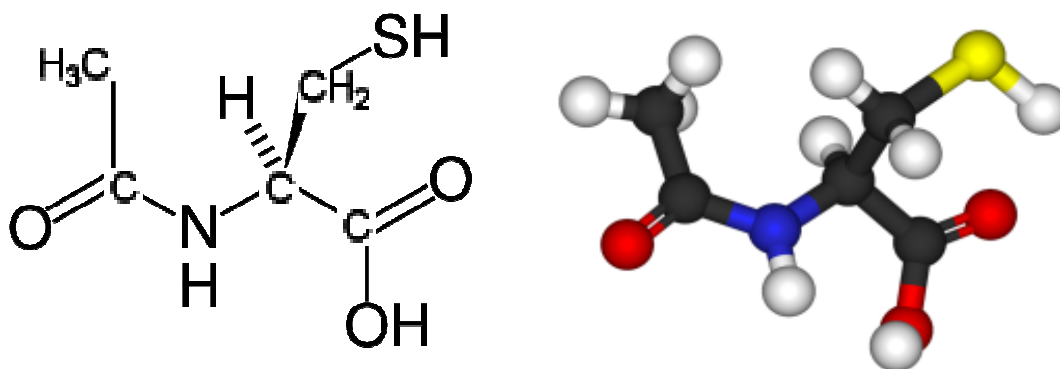


## Widely used drugs, to prevent cisplatin induced ototoxicity in animal models .....

Drugs that are used to protect against cisplatin-induced ototoxicity mainly take part in endogenous antioxidant production and recycling, or by chemically binding to the reactive oxygen species

### N- Acetylcysteine (NAC)

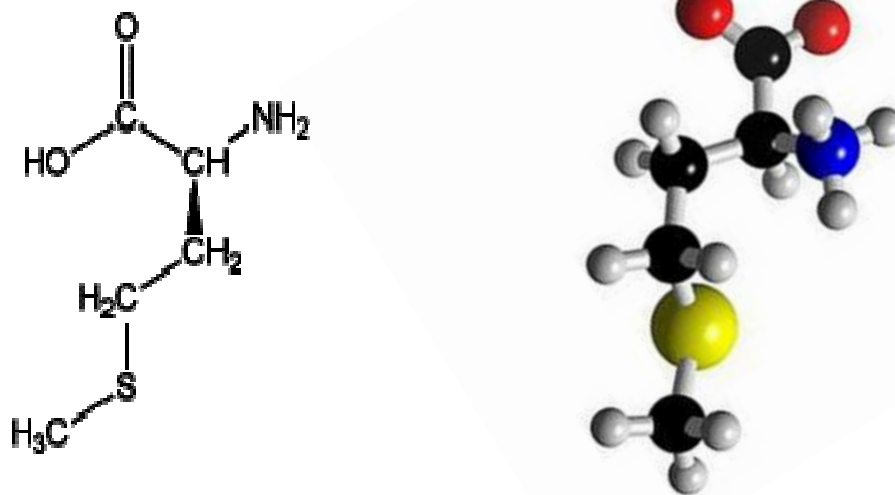
N-Acetylcysteine (NAC) is an amino acid form best utilized by the body and widely used in clinical practice as a mucolytic agent. NAC is a cysteine analog with strong antioxidant activity. It induces the synthesis of glutathione, which contributes to long-term protection against ROS, and its sulf-hydryl group is thought to play a main role in the observed otoprotection. As a glutathione precursor and an antioxidant, it has many important functions including hearing preservation [37, 38, 39]. *Dickey et al.* 2004, demonstrated that rats treated with 400 mg/kg of I.V. NAC 15 minutes before cisplatin therapy (6 mg/kg) exhibited very good auditory brainstem response (ABR), while the control group showed poor high frequency ABR, reflecting clear ototoxicity [43]. Data from studies, including ours, showed that best results were observed in animals receiving higher dosages of L-NAC.



**Figure 3.** Molecular structure of L-N-Acetylcysteine

### Methionine (MET)

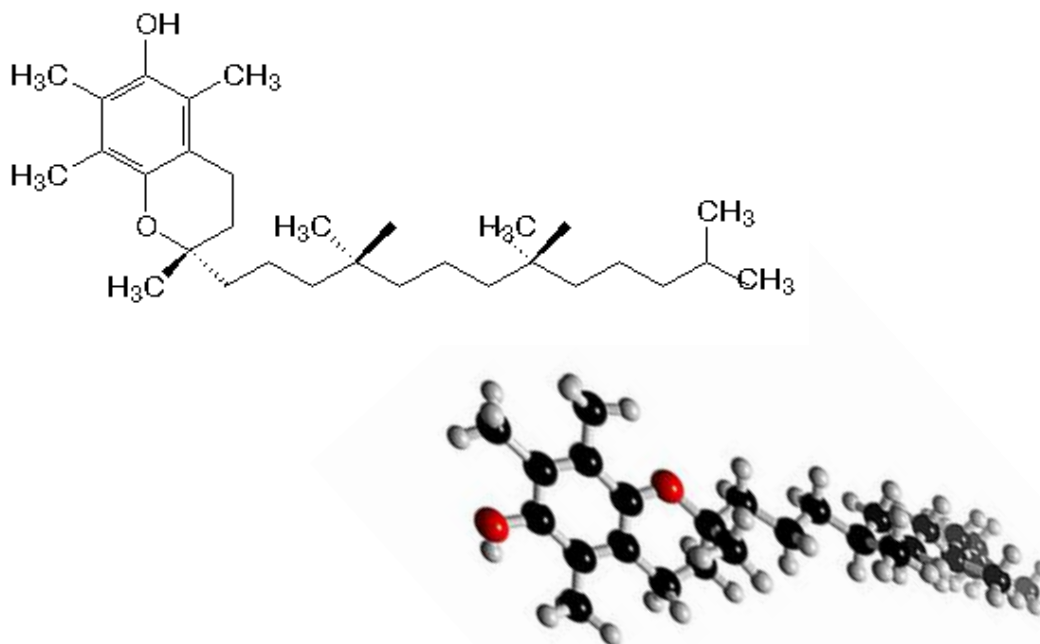
Methionine is one of two sulfur containing proteinogenic amino acids with potent antioxidant properties. It is an essential amino acid that is not synthesized in the human body. MET protects both the auditory hair cells and auditory neurons from various types of ototoxic drugs, including cisplatin, ionic platinum compounds, and aminoglycosides. The protective effects of MET have been explained by its antioxidant capacity through glutathione formation. In addition, upregulation of HMG1 and iNOS in response to cisplatin chemotherapy could be prevented by systemic delivery of MET [14, 40]. Various animal models (including Wistar and Sprague-Dawley rats) have been tested for D-MET's otoprotection capacity against cisplatin-induced ototoxicity [41, 42]. Data from these and our studies have demonstrated that animals receiving various dosages (150, 300, 350, 400 and 450 mg/kg) of D-MET presented differing degrees of otoprotection, with the best results observed in animals receiving higher dosages of D-MET.



**Figure 4.** Molecular structure of D-methionine

## Vitamin E

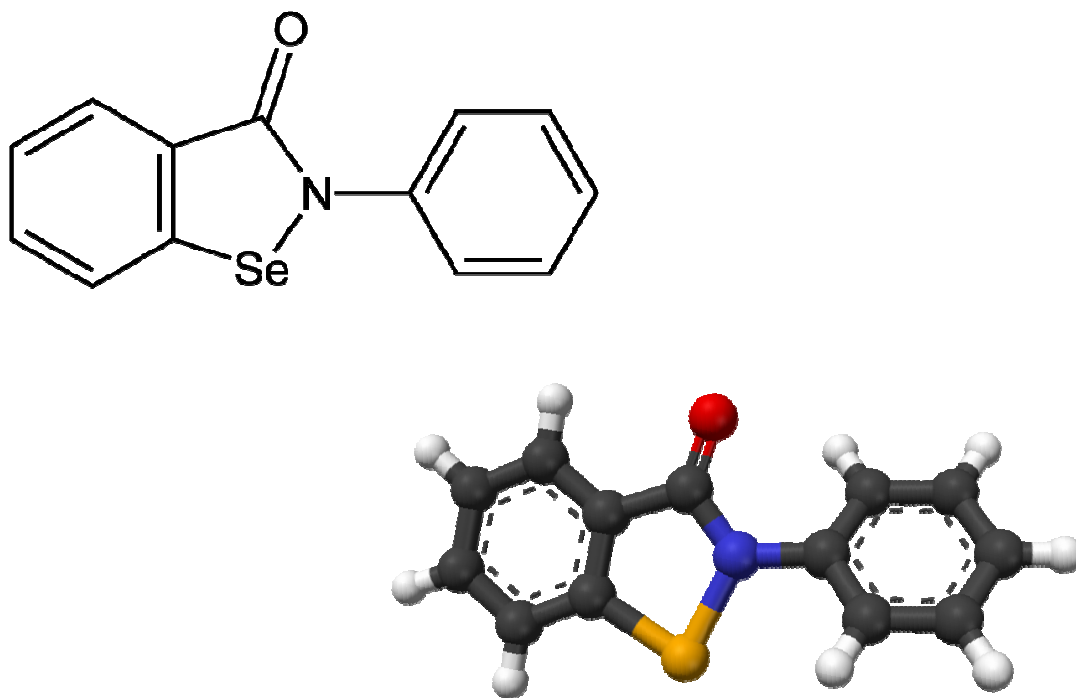
Vitamin E is the main lipid-soluble, chain-breaking antioxidant found in membranes and in plasma.  $\alpha$ -tocopherol has been the most studied form of vitamin E as it has the highest bioavailability, with the body preferentially absorbing and using it. Apart from its free radical scavenger capacity, it also shows potential otoprotection. Therefore, it has been used as an otoprotector against cisplatin-induced ototoxicity [44]. In the study by *Kalkanis J et al.*, 2004, rats showed remarkable preservation of ABR thresholds at 8-, 16-, and 32-kHz when treated with a single dose of vitamin E (4 g/kg) before the 16 mg/kg cisplatin injection. These results were further confirmed by electron microscopic images of the cochlea, where a significant preservation of OHCs was observed in the group injected with vitamin E [45].



**Figure 5.** Molecular structure of Vitamin E

## **Ebselen**

Ebselen is an antioxidant compound with some anti-inflammatory capacity. It acts as glutathione peroxidase mimics, exhibits neuroprotection, and inhibits free radical induced apoptosis. Ebselen treatment has demonstrated an increase in both reduced glutathione (GSH) and oxidized glutathione (GSSG) [46 - 51]. A study by Ryback et al. already confirmed the cytoprotective effect of ebselen after cisplatin administration in rats [52]. In another study, separate (16 mg/kg) and combined formulations (8 mg/kg for both) of ebselen and allopurinol were used in an attempt to reduce the formation of ROS during a 16 mg/kg cisplatin exposure in rats. The results revealed that a combined administration of these two agents gave an improved otoprotective effect at lower doses which was not achieved by either agent alone [53]. These results were supported by OHC preservation and ABR thresholds.



**Figure 6.** Molecular structure of Ebselen

## **Drawbacks of the widely used Otoprotectors against Cisplatin induced Ototoxicity in animal models.....**

The otoprotecting compounds referenced so far have shown some good otoprotection against cisplatin, but additional studies demonstrate that these protectors could reduce the antineoplastic effect of cisplatin and are toxic at high dosages.

### **N-Acetylcysteine and D-Methionine**

These two compounds are known for their complex formation with cisplatin, and these complexes may reduce the anti-tumor effect of cisplatin when administered systemically. According to *Schweitzer et al. 1993*, sulfur containing compounds may prevent cisplatin from interacting with intracellular target molecules due to their nucleophilic oxygen or sulfur atoms interacting with the electrophilic site of the cisplatin [54, 55]. Furthermore, cisplatin is known to react with methionine's sulfhydryl group [56]. Earlier, *Campbell et al., 1999*, demonstrated the protective effect of D-MET against cisplatin-related side effects in animal studies [40]. But *Ekborn et al. 2003* found that I.V. administration of D-MET lowered the systemic exposure of cisplatin. Further, they suggested that even the pre-administration of D-MET does not reduce the ototoxic or nephrotoxic effects of cisplatin in guinea pigs after dose adjustment compared with similar cisplatin exposure in treated and control animals [11,57]. Moreover, L-MET *in vitro* [58] and *in vivo* [59] may reduce the anti-tumor effect of cisplatin when administered systemically.

### **Vitamin E**

The 4g/kg dose of vitamin E given to the animal models is extremely high, as the oral median lethal dose found in several species was 2 g/kg. Furthermore, high doses of vitamin E could cause an increase in human mortality due to subarachnoid hemorrhage [60]. In addition, high dose of vitamin E may depress leukocyte oxidative bactericidal activity and mitogen-induced lymphocyte transformation. This is not preferable for clinical cases where the subjects are undergoing cisplatin treatment.



## **Selenium**

In theory, the selenium group in the ebselen structure is even more nucleophilic than L-NAC and D-MET due to its higher degree of polarization than sulfur. Therefore, ebselen is also suspected in complex formation, and may reduce the anti-tumor effect of cisplatin.

## **The advantages of an acute Noise stimulus in cisplatin induced ototoxicity .....**

Noise, being a physical stimulus, will not directly interact with cisplatin as in the case of most chemical otoprotectors. Apart from this, an acute noise stress could have a number of indirect beneficial effects that may counter the cisplatin ototoxicity. These possible beneficial effects of noise stress will be discussed later in the discussion part of this thesis. Furthermore, studies in gerbils showed that an hour of 85 dB noise exposure did not show any permanent cochlear damage as in the case of an hour of 105 dB noise exposure [61]. Accordingly we believed that a 15–20 minute, 85dB acute noise stress given before cisplatin treatment could have an otoprotective role against cisplatin-induced ototoxicity.

## **Aims**

To find out whether an acute noise stress given before cisplatin treatment (pre noise stress) can promote hair cell survival.

To find out whether a pre noise stress can increase the amount of protection offered by an otoprotecting drug.

To find out the mechanism or mechanisms of the protection offered by noise stress in cisplatin induced ototoxicity.

To find out the possibilities of increasing the efficacy of noise stressing techniques upon understanding the mechanism or mechanisms of the protection offered by noise stress in cisplatin induced ototoxicity.

To find out how a pre noise stressing could be applied in clinical practice

Finally, to find out whether this technique could be used to mobilize the systemically given molecule, drug, or stem cells towards the cochlea.

**Materials and methods.....**

**Animals**

All animals used in our studies were Sprague-Dawley rats (Charles River, Italy) weighing 170-250 g. All rats were screened for middle ear pathology and randomly divided into groups. The animals were housed on sawdust in macrolon cages. They had free access to rat food and water and were maintained on a 12:12 h dark/light cycle. All animals were treated according to the Italian guidelines DL 116/92 with reference to EEC directive no. 86-609.

**Drugs**

**Cisplatin:** Cisplatin 1mg/ml obtained from Ebewe pharma was delivered by slow infusion (0, 1 ml/min) in the caudal vein (I.V.) at a dose of 14mg/kg body weight cisplatin under anaesthesia. Cisplatin was infused either 45 minutes after noise stress or 1 hour after otoprotecting drug administration.

**L-N-Acetylcysteine:** L-NAC (Sigma Chemical Co., St. Louis, USA) was dissolved in saline (100 mg/ml) and adjusted to pH 7.0. L-NAC was injected intraperitoneally an hour before 14mg/kg cisplatin infusion. All animals were divided into groups which received either 275 mg/kg D-MET alone or in combination with noise stress.

**D-Methionine:** D-MET; (Sigma Chemical Co., St. Louis, USA) was dissolved in saline (50 mg/ml) and administered as bolus intraperitoneal injection one hour before the 14mg/kg cisplatin infusion. All animals were divided into groups which received either 300 mg/kg D-MET alone or in combination with noise stress.

**Anaesthesia:** A cocktail prepared by mixing 57mg of Zoletil 100 (Virbac) in 1ml of physiological solution and 0.5ml of 2% Xilazin was given at a dose of 0.1ml for 100g. Anaesthesia was supplemented as needed with half doses throughout testing.

### **Weight**

Each animal's weight was measured before the administration of the anesthetic on the first and the last day of experiments. This was performed in order to analyze the cytotoxic effect of cisplatin, cytoprotective effect of L-NAC and D-MET against cisplatin, and normal weight gain.

### **Preyer's reflex**

The elicitation of startle response to auditory stimuli (a handclap) was used as an optional method for the evaluation of auditory dysfunction at the beginning and at the end of all our animal studies. The reflex was considered positive when a rapid movement of the whole body of the animal was clearly noticed. Thereafter, rats were assessed for the auditory brain stem response under general anesthesia.

### **Sound level calibration**

The levels of noise and other stimuli used during the study were checked by a Bruel and Kjaer type 2209 impulse precision sound level meter coupled with a one-inch Bruel and Kjaer condenser microphone type 4145 for open field use, which had a normal incidence open field linear response from 1 to 2 Hz (-3 dB) to 18 kHz ( $\pm 1.5$  dB) and meets the ANSI (American National Standards Institute) requirements for a laboratory standard type L microphone. In addition, a Bruel and Kjaer 1/3 octave filter set (type 1616 for 1/3 octave analysis in the range of 18 Hz to 44 kHz covered by 34 band-pass filters) was used in conjunction with the 2209 precision sound level meter.

### **Noise Stress**

Animals were exposed to 85dB SPL white noise centered at 8 kHz by 2 speakers placed on the top of a 1 M<sup>3</sup> open field acoustic chamber. Rats underwent noise stress for 15 minutes immediately after the intraperitoneal administration of the otoprotecting drug or underwent 15 minutes noise stress alone.

### **Auditory Brainstem Response (ABR)**

ABR was used to assess auditory threshold. Testing occurred one day prior to the administration of cisplatin (with and without a protective agent and noise stress) and 4 days after (96 hours from cisplatin administration). All ABR tests were performed with the animal in 1 M<sup>3</sup> acoustic chamber.

ABR responses were recorded by three platinum-iridium needle electrodes, placed subdermally over the vertex (positive), mastoid (negative), and dorsum area (reference/ground) of the animal. The sound transducer, a Motorola tweeter (flat response  $\pm$  1.5 dB from 4.0 to 35 kHz), was placed 4 cm away from the animal's ear. ABRs were amplified 20,000 times and filtered from 20 to 5000 Hz. Each recording was the average of 1000 individual responses. ABRs were generated in response to 8, 12, and 16 kHz tone pips (1 ms rise-fall time, 10 ms plateau), in the range intensity 128 to 30 dB SPL. The stimulus sound intensity was varied in 5 dB intervals. Threshold was based on the visibility and reproducibility of the third wave, and at the minimum threshold level two recordings were acquired. Threshold was defined as the lowest intensity at which a measurable ABR wave was seen in two averaged runs. The threshold level of the S.D. rat at frequencies up to 16 kHz was found to be 40 dB SPL. Ear plugs were used to occlude the contra lateral ear in order to avoid a binaural stimulation at highest stimulus intensities (>100 dB SPL).

### **Histological analysis**

#### **Tissue preparation**

Post ABR recordings were obtained on the last day of an experiment (96 after the 14mg/kg cisplatin treatment). The animals were killed by decapitation while under general anesthesia immediately following the recordings. The inferior portion of each bulla was opened and the middle ear cavity was examined to ensure the absence of any middle ear infection. The stapes was carefully removed, a small hole in the otic capsule was hand drilled at the apex, and the round window membrane was perforated. In vitro perfusion was performed with Glyofixx ® (Shandon Glyo-Fixx, Genova, Italy) intermittently within 5 minutes of the decapitation through the small hole drilled at the apex, allowing the fixative to exit through the round and oval windows. Afterwards, the

cochleae were immersed in the same fixative for 5 hours at room temperature and then subjected to decalcification for 2 weeks at 37° C using 10% EDTA in 0.1 M PBS pH 7.4. The solution was changed every two days.

### **Paraffin embedding and staining**

The decalcified cochleae were placed in special containers for serial dehydration in 70%, 80%, 90%, and 100% alcohol. These steps were conducted by an electronic tissue processor (Shandon Citadel 2000 tissue processor). Finally, the cochleae were orientated in mid-modiolar and transverse planes and embedded in 65° C paraffin. Semi-thin sections (5 µm) were cut and stained with Hematoxylin and Eosin.

The structure of hair cell and spiral ganglia neurons were evaluated visually under an immersion microscope. Sections of the middle and basal turn of each sample were photographed with a digital camera (Olympus DP10). The photomicrographs were stored on a personal computer and processed with Adobe Photoshop (Adobe version 8.0).

### **TRITC-conjugated phalloidin staining**

The cochlea samples were collected at the end of the experiments and fixed for 4 hours (4° C) with 10% formaldehyde in 0.1 M phosphate buffer (pH 7.4). The samples were then rinsed in 0.1 M PBS and incubated in 0.25% Triton X-100 (Sigma) for 5 min., immersed for 30 min in TRITC-conjugated phalloidin (Sigma, 1:200) in PBS, rinsed three times in PBS, and mounted as surface preparations on glass slides in glycerin containing fluoromount (Molecular Probes). Specimens were examined with a fluorescence microscope (Zeiss Axioskop) equipped with appropriate filters (absorption: 544 nm, emission: 572 nm; 200· or 400·). Samples were photographed with a digital camera (Olympus DP10). We carried out the TRITC-conjugated phalloidin staining in order to visualize the preservation of stereocilia bundles and circumferential actin ring surrounding the cuticular plate of inner and outer hair cells.

### **Statistical analysis**

The ABR data were analyzed in order to compare all treated groups against Cisplatin alone group. We used a repeated measures model a single within factor (frequency) with post-pre differences as response, rather than the doubly repeated measures model, using frequency and occasion (pre or post) as the two within factors to assess the inference on mean change in threshold.

Following the ANOVA, we conducted Dunnett's multiple comparisons. This was used at the 0.05 level to compare the mean threshold changes under Noise + Cisplatin, L-NAC 275 + Cisplatin, L-NAC 275 + Noise + Cisplatin, D-MET 300 + Cisplatin, and D-MET 300 + Noise + Cisplatin with that under Cisplatin alone.

The model is:  $d_{ijk} = \mu + G_i + S_{j(i)} + F_k + GF_{ik} + \epsilon_{ijk}$ , where  $d_{ijk}$  is the post-pre difference at frequency  $k$  for subject  $j$  in group  $i$ ,  $i = 1, \dots, 6, j = 1, \dots, n_i, k = 1, 2, 3$ .

As tests for sphericity indicated that the Huyhn-Feldt condition were not tenable for the DMET analysis, a model with unstructured within-subject covariance matrix was fit using SAS (Version 9.1.2) proc mixed and the REML estimation method. To keep both analyses comparable, the same model was fit for the L-NAC analysis as well. Finally tests for fixed effects were done to examine main effects.

**Groups and Treatment.....**

**Group A** - Animals received 275 mg/kg L-NAC intra-peritoneally (I.P.) and infused intravenously (I.V.) with 14 mg/kg of cisplatin 60 minutes afterwards.

**Group B** - Animals received 300 mg/kg I.P D-MET and infused with I.V. 14 mg/kg of cisplatin 60 minutes afterwards.

**Group C** - Animals underwent 15 minutes noise stress right after the 275 mg/kg I.P L-NAC injection and infused with I.V. 14 mg/kg of cisplatin 45 minutes after the noise stress.

**Group D** - Animals underwent 15 minutes noise stress right after the 300 mg/kg I.P. D-MET injection and infused with I.V. 14 mg/kg of cisplatin 45 minutes after the noise stress.

**Group E** - Animals underwent 15 minutes noise stress alone and infused with I.V. 14 mg/kg of cisplatin 45 minutes after the noise stress.

**Group F** - Animals were infused with I.V. 14mg/kg of cisplatin alone.

**Group G** - Control group treated with I.V. saline alone.



**Experiment protocol.....**

**Day 0**

1. Animals were checked for the presence of Preyer's reflex, then weighed and anaesthetized
2. Assessment of auditory function by auditory brainstem response thresholds (Pre - ABRs).

**Day 1**

3. All groups except control and cisplatin alone treatment groups underwent 15 minutes noise stress with or without the I.P. protection drug injection (L-NAC and D-MET).
4. Animals were infused with I.V. 14mg/kg of cisplatin 45 minutes after the noise stress.

**Day 4**

5. Reassessment of auditory function by auditory brainstem response thresholds (Post - ABRs) 96 hours following-14mg/kg cisplatin infusion.
6. The animals were killed by decapitation while under general anesthesia immediately following recordings. The middle ear cavity was examined to ensure the absence of any middle ear infection. Cochleae were fixed and processed for further histological analysis.

**Results.....**

**Survival during the experiment**

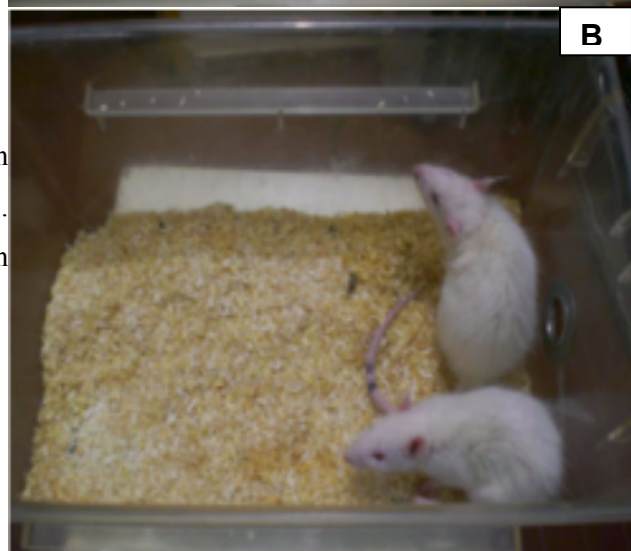
During experiments, a few animals died due to cisplatin toxicity and some did not show a clear pattern of hearing loss. This could be due to interindividual variability with respect to cisplatin-induced ototoxicity. The data from these animals were not included in our studies. When compared among the cisplatin treated groups, animals which received 300mg/kg D-MET showed a better physical status four days after the cisplatin treatment.

**Figure 7.**

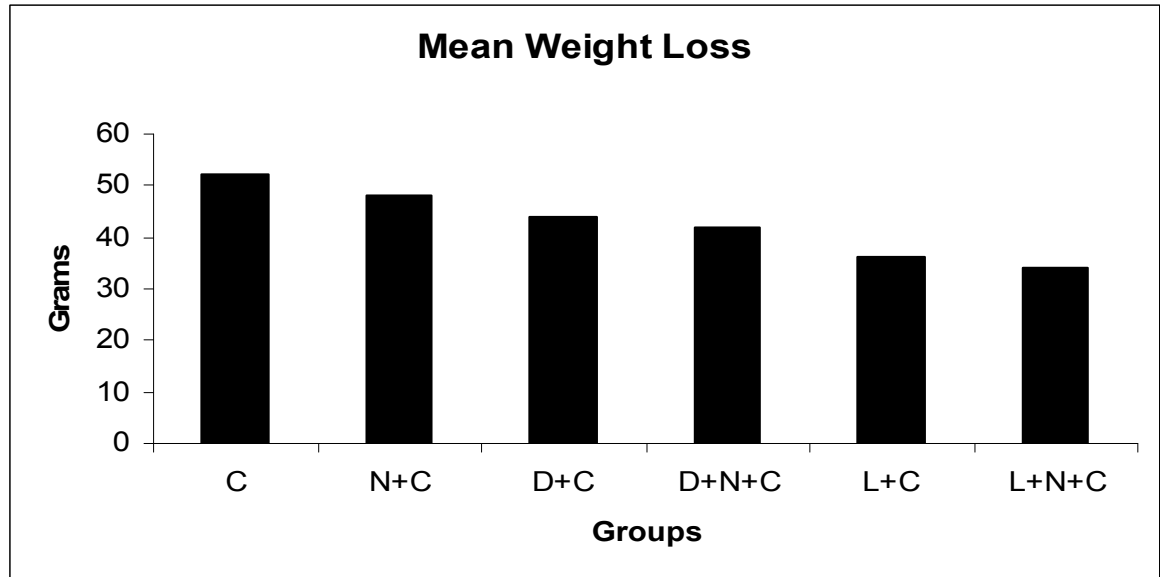
**A** – Untreated control rats showed good physical status and were active.



**B** – 96 hours after 14mg/kg I.V. cisplatin infusion, rats were weak and less active. Fewer rat droppings were observed when compared with the control group.



## Weight loss



**Figure 8.** The data shown is the mean weight loss of each group. Each animal's weight was measured before the administration of the anesthetic on the first and the last day of experiments. Overall L-NAC and D-MET had a partial protection against cisplatin induced weight loss.

**L- NAC:** Groups treated with L-NAC (L + C) alone and L-NAC with noise stress (L+C+N) had less mean weight loss against cisplatin toxicity.

**D-MET:** Groups treated with D-MET (D+C) alone and D-MET with noise stress (D+N+C) showed the minimum mean weight loss when compared with all other cisplatin treated groups.

**Cisplatin + Noise stress and Cisplatin alone Groups:** Groups which underwent noise stress alone (N+C) and groups which received cisplatin alone (C) had the highest weight loss against cisplatin toxicity.

**This suggests that L-NAC and D-MET had a partial protection against cisplatin induced weight loss.**

## **Ototoxicity**

### **Preyer's reflex**

**L- NAC and D-MET:** Groups treated with L-NAC (L + C) alone, L-NAC with noise stress (L+C+N), D-MET (D+C) alone, and D-MET with noise stress (D+N+C) exhibited a clear rapid whole body movement for the handclap 96 hours after the cisplatin infusion.

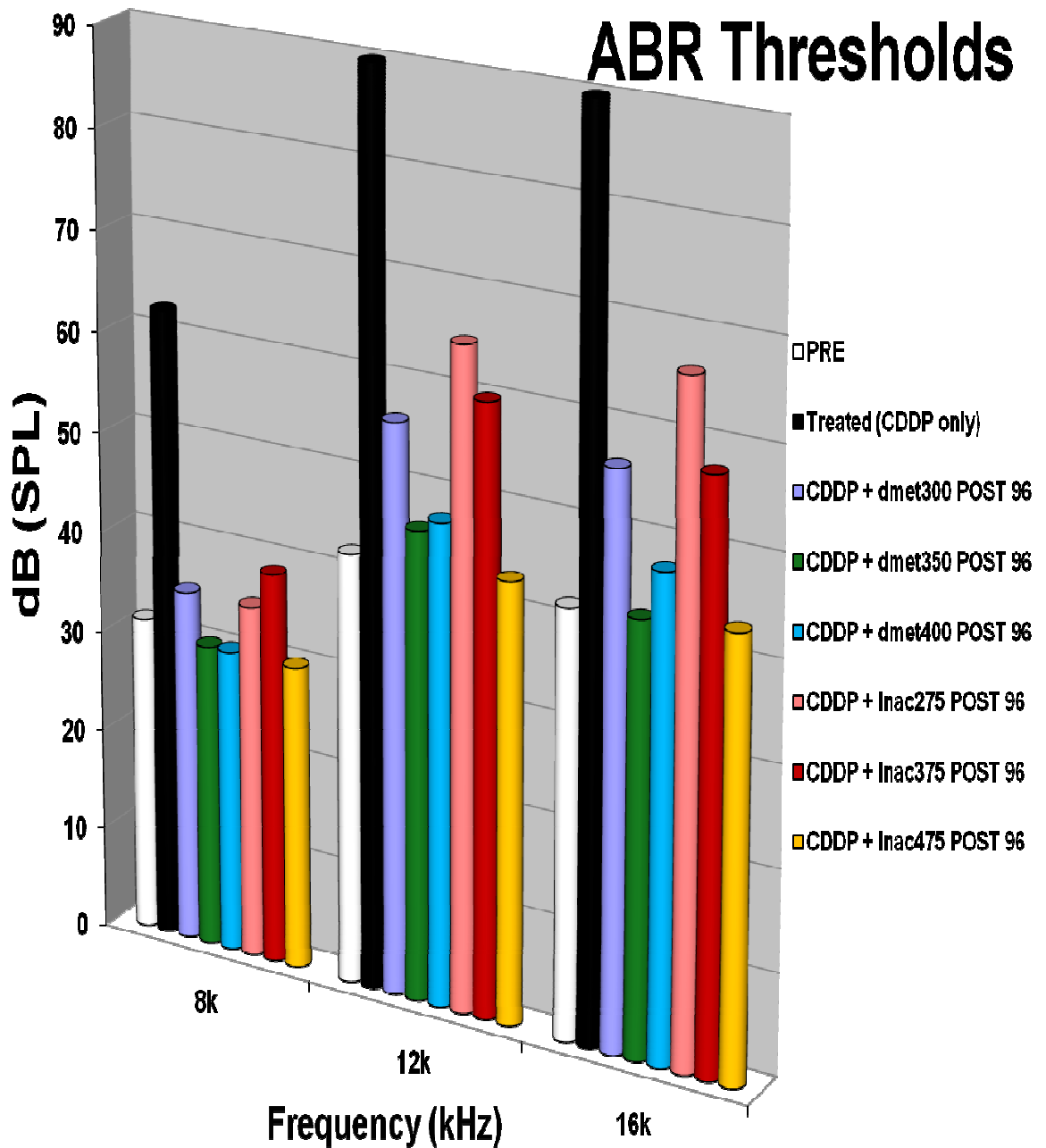
**Cisplatin + Noise stress:** Most of the animals which underwent noise stress alone (N+C) had a positive Preyer's reflex 96 hours after the cisplatin infusion, except a animals few which showed only a head movement.

**Cisplatin alone Groups:** After 96 hours, most of the animals in this group were negative for the Preyer's reflex, but head movement was elicited in few animals.

**Absences of the Preyer's reflex reflected a profound sensorineural hearing loss in those animals.**

### **Auditory Brainstem Response**

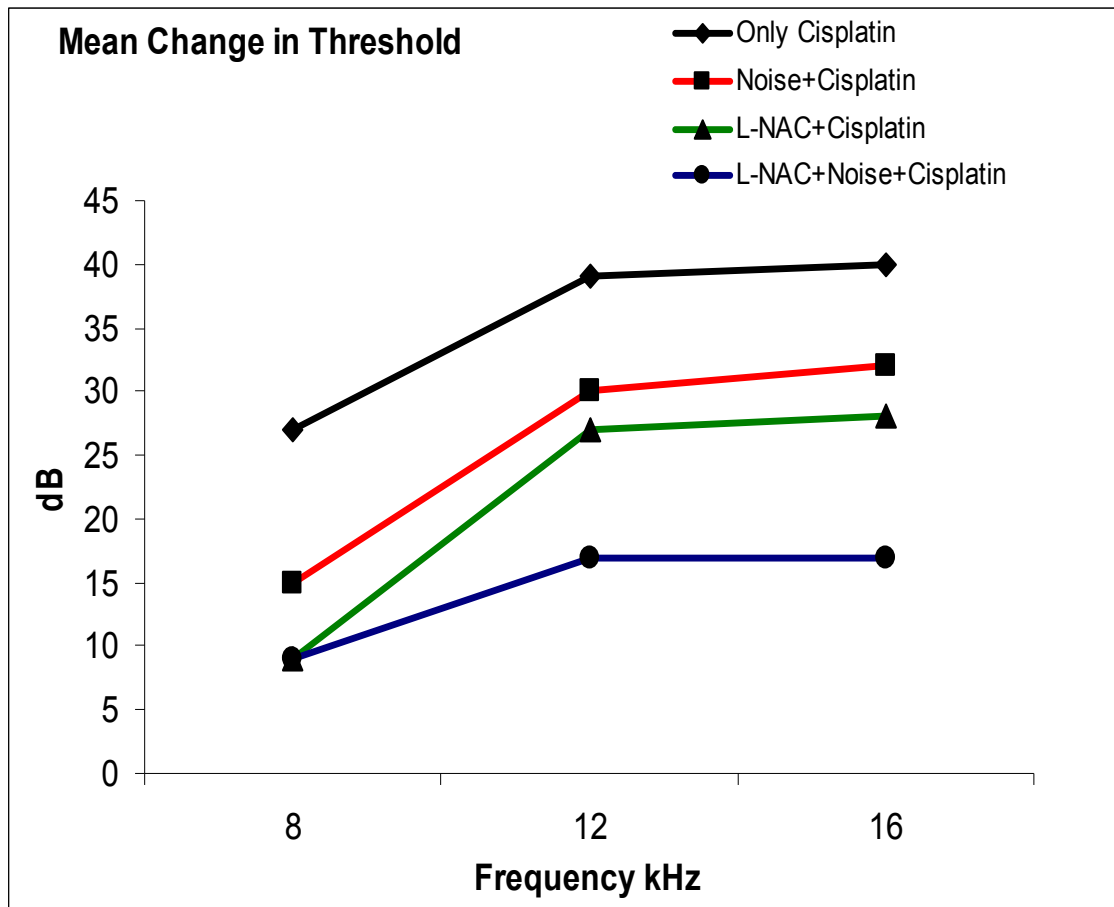
Mean changes in ABR threshold among all different groups following cisplatin treatment during our studies are shown in *Fig. 10* and *Fig. 11*. Groups which received either otoprotectors or noise stress alone or in combination clearly revealed a better mean hearing threshold than the group which received only cisplatin treatment. Animals in the control group which received only saline instead of cisplatin did not show any threshold shifts during our experiments.



**Figure 9.** Mean ABR threshold from our previous studies showing that rats receiving, various dosages of L-NAC and D-MET presented differing degrees of otoprotection 96 hours after 14mg/kg of cisplatin treatment, with the best results observed in animals receiving higher dosages of L-NAC and D-MET.

**L- NAC**

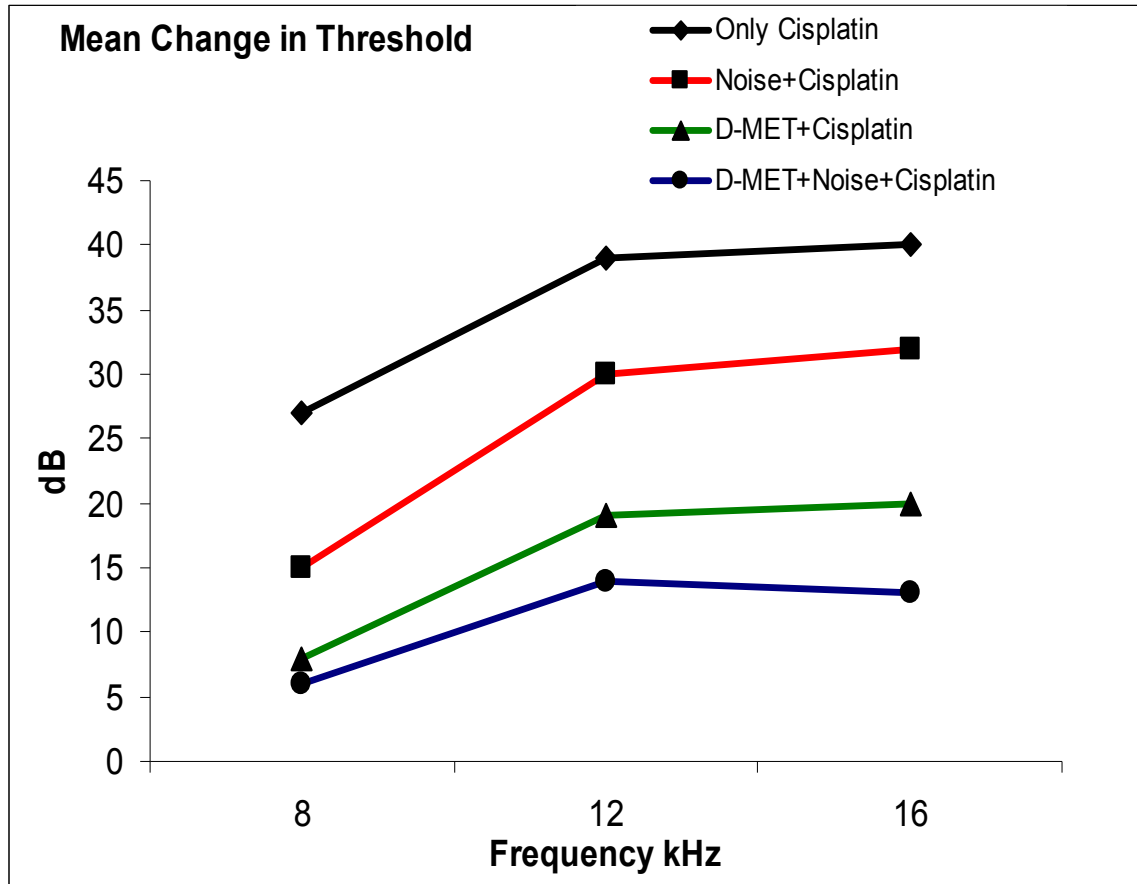
When compared against cisplatin alone treated group, we found that the noise stress technique increased the otoprotecting efficacy of 275mg/kg L-NAC, which showed less otoprotection when given alone (*Fig. 10*). Moreover, 275mg/kg L-NAC + noise stress group was most statistically significant ( $p = 0.0003$ ) against cisplatin alone treated group (*Table 1*).



**Figure 10.** 275mg/kg L-NAC and noise stress alone or in combination were analyzed for their capability to reduce cisplatin-induced ototoxicity. ABR threshold were obtained one day prior to cisplatin treatment (14mg/kg, I.V.) and four days after cisplatin treatment. The data shown are mean change from baseline. Cisplatin alone group show the highest mean threshold shift. L-NAC + Noise + Cisplatin group show low mean threshold shift than that of Noise + Cisplatin and L-NAC + Cisplatin groups.

**D-MET**

The protection level in 300 mg/kg D-MET + noise stress was slightly better than the 300 mg/kg D-MET alone group (*Fig. 11*) and the most statistically significant otoprotection was seen in 300 mg/kg D-MET + noise stress group ( $p < 0.0001$ ) against the cisplatin alone treated group (*Table 1*).



**Figure 11.** 300 mg/kg D-MET and noise stress alone or in combination were analyzed for their capability to reduce cisplatin-induced ototoxicity. ABR threshold were obtained one day prior to cisplatin treatment (14mg/kg, I.V.) and four days after cisplatin treatment. The data shown are mean change from baseline. Cisplatin alone group show the highest mean threshold shift. D-MET + Noise + Cisplatin group show low mean threshold shift than that of Noise + Cisplatin and D-MET + Cisplatin groups.

**Cisplatin + Noise stress**

Most interestingly, we found that noise stress alone showed better mean ABR threshold at all 8, 12, and 16 kHz frequencies against cisplatin ototoxicity (*Fig.10 and Fig. 11*) and that this protection was statistically significant ( $p=0.0477$ , *Table 1*).

Comparison Cisplatin – Treated Groups		Estimate	Standard Error	Dunnett-adjusted p-value	Simultaneous 95% Confidence limits
Cisplatin	Noise+Cisplatin	-9.6667	4.2360	0.0477	-18.6038 -0.7296*
Cisplatin	L-NAC+Cisplatin	-14.0000	4.3546	0.0135	-25.2050 -2.7950*
Cisplatin	L-NAC+Noise+Cisplatin	-20.8889	4.1693	0.0003	-31.6169 -10.1609*
Cisplatin	D-MET+Cisplatin	-19.6667	4.2360	0.0006	-28.6038 -10.7296*
Cisplatin	D-MET+Noise+Cisplatin	-24.2222	4.0556	< 0.0001	-32.7788 -15.6656*

**Table 1:** Dunnett’s multiple comparisons is used at the 0.05 level to compare the mean threshold changes under Noise + Cisplatin, L-NAC 275 + Cisplatin, L-NAC 275 + Noise + Cisplatin, D-MET 300 + Cisplatin, and D-MET 300 + Noise + Cisplatin with that under Cisplatin alone.



The tests for the fixed effects show that the main effects group and frequency are highly significant while the group by frequency interaction was not significant; a result supported by *Table 2* and *3*.

**Type 3 Tests of Fixed Effects**

Effect	Num DF	Den DF	F Value	Pr > F
group	3	17	8.70	0.0010
freq	2	17	27.76	<.0001
group*freq	6	17	0.92	0.5064

*Table 2.* The tests for the fixed effects for L-NAC groups show that the main effects group and frequency are highly significant while the group by frequency interaction is not significant,

**Type 3 Tests of Fixed Effects**

Effect	Num DF	Den DF	F Value	Pr > F
group	3	17	13.83	<.0001
freq	2	17	13.62	0.0003
group*freq	6	17	0.35	0.8988

*Table 3.* The tests for the fixed effects for D-MET groups show that the main effects group and frequency are highly significant while the group by frequency interaction is not significant,

**ABR results clearly suggest that the noise stress had a positive impact in improving the hearing threshold.**

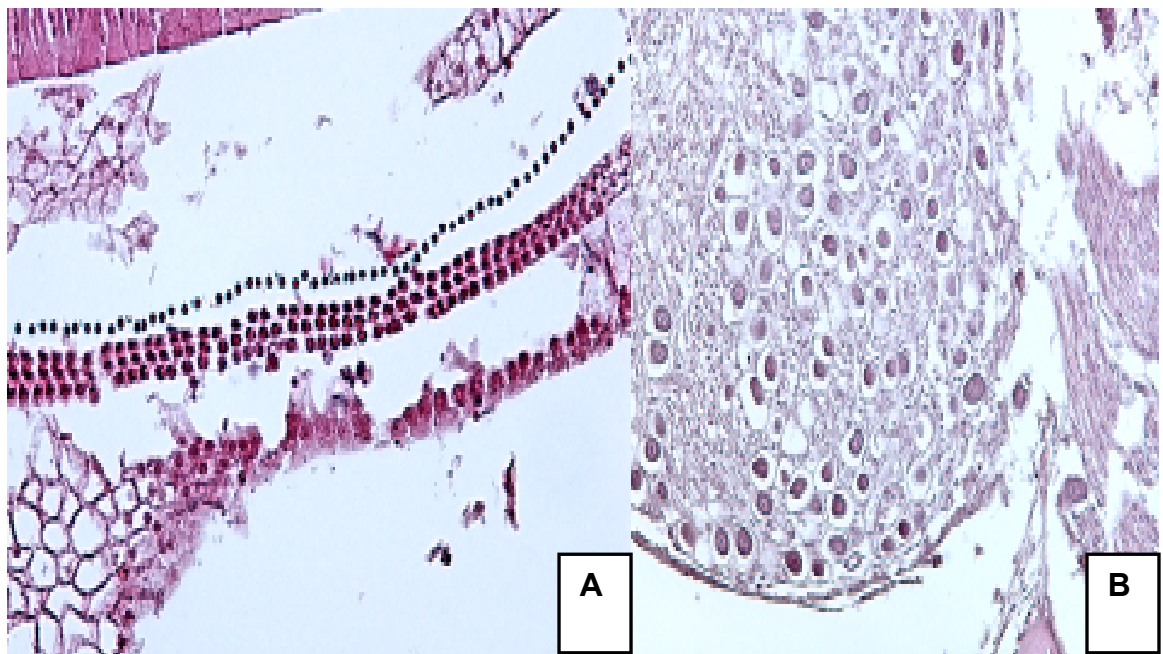
## **Histological analysis**

### **Hematoxylin and Eosin staining**

Examination of mid-modiolar and transverse plane preparations of cochleae revealed that groups which received either otoprotectors or noise stress alone or in combination had better spiral ganglion and hair cell preservation than groups that received only cisplatin treatment. Animals in the control group which received only saline instead of cisplatin showed the typical morphological structure of this organ

### **Control group animals**

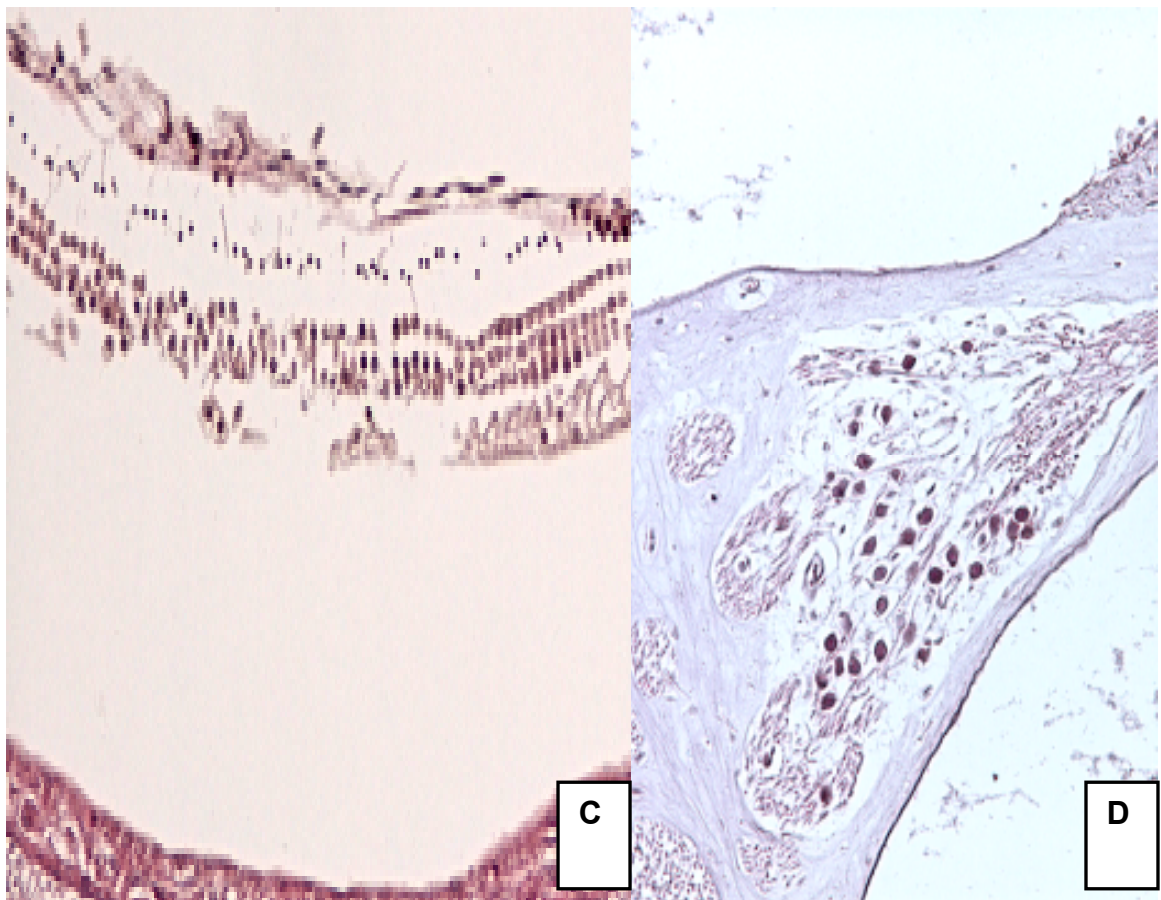
Mid-modiolar and transverse plane preparations of cochlea from control Sprague Dawley rats revealed that nuclei of the OHCs lay in three rows, were monomorphic, and had regular spherical shapes. Nuclei of IHCs were arranged in single rows and had spherical shapes. Most nucleoli occupied a central position. Nuclei of the spiral ganglion cells in the basal segment of cochlear had regular spherical shapes (*Fig. 12 A and B*).



**Figure 12.** **A-** Control animal: Upper basal turn demonstrating a normal number of OHCs and IHCs. **B-** Control animal: Spiral ganglion cells in basal turns demonstrating regular spherical shape.

### **Groups treated with cisplatin**

Different levels of changes in cochlea cell morphology were seen 96 hours after cisplatin treatment. The main targets of cisplatin were the hair cells, with OHCs being more susceptible than IHCs. Hair cell loss began in the basal, high-frequency region of the cochlea and progressed towards the apex. Moreover, spiral ganglion cells showed cell loss and cell shrinkage. Among all groups, the cisplatin alone group exhibited the maximum amount of hair and spiral ganglion cell damage (*Fig. 13 C and D*).



**Figure 13.** C- Cisplatin alone treated animal (14mg/kg): Upper basal turn demonstrating maximum amount of hair cell damage, with OHCs being more susceptible than IHCs. D- Cisplatin alone treated animal (14mg/kg): Spiral ganglion cells in basal turn demonstrating cell loss and cell damage.

**L – NAC**

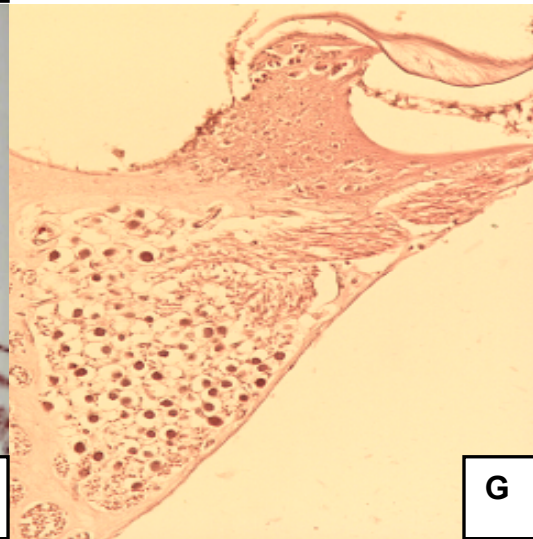
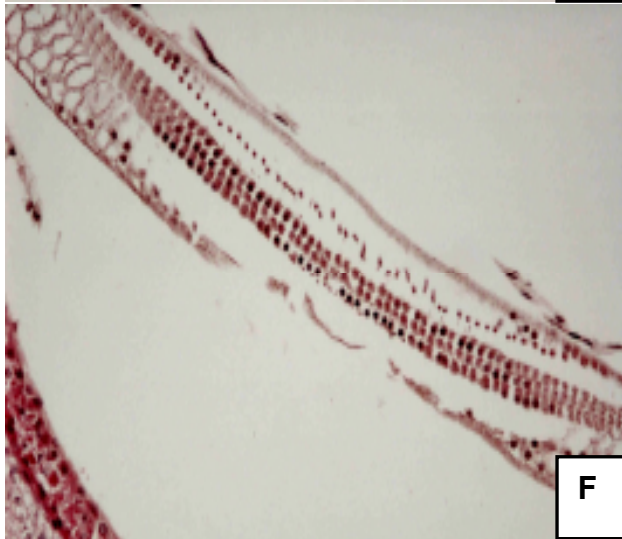
When compared against the cisplatin alone treated group, we found that 275mg/kg L-NAC alone and in combination with noise stress showed better hair cell preservation in the middle and basal segments of the cochlea. The apex segment of the cochlea did not exhibit any significant hair cell loss, since these hair cells are less susceptible for cisplatin toxicity. The spiral ganglion cells of these two groups showed less cell loss and shrinkage and retained their form and nuclei.

The group which received 275mg/kg L-NAC in combination with noise stress always revealed a better morphological picture than the 275mg/kg L-NAC alone group against cisplatin toxicity.



**Figure 14. E-** Upper basal turn of 275mg/kg L-NAC + 14mg/kg cisplatin treated animal. **F-** Upper basal turn of 275mg/kg L-NAC + Noise stress + 14mg/kg cisplatin treated animal showing better OHCs preservation than **E**.

**G-** Spiral ganglion cells in basal turns of 275mg/kg L-NAC + Noise stress + 14mg/kg cisplatin treated animals showing less cell loss and shrinkage than **D**.



**E**

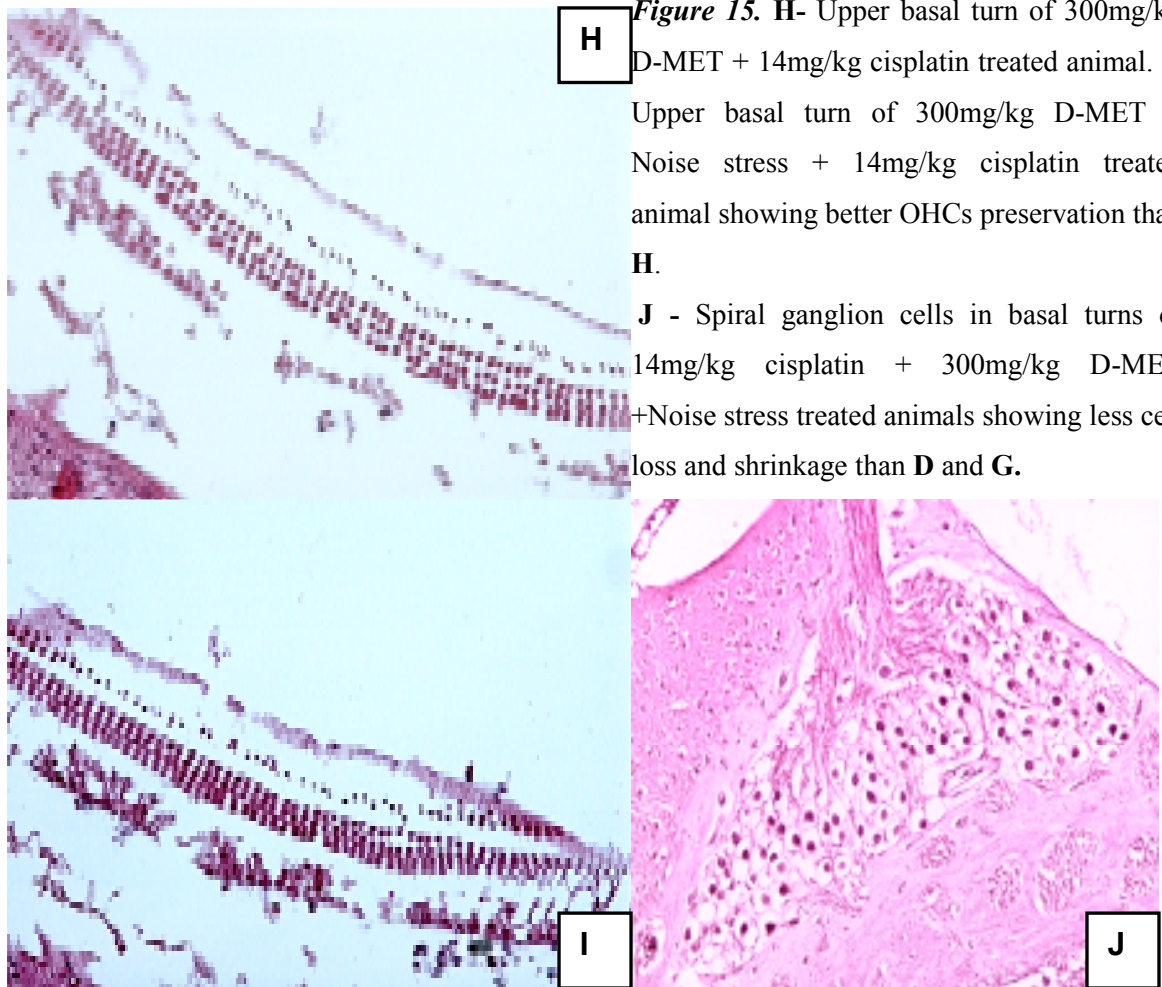
**F**

**G**

## D-MET

When compared against the cisplatin alone treated group, we found that 300 mg/kg D-MET alone and in combination with noise stress showed better hair cell preservation in the middle and basal segments of the cochlea. The hair cells in the apex of the cochlea did not exhibit any significant cell loss since they are less susceptible for cisplatin toxicity. The spiral ganglion cells of these two groups showed less cell loss and shrinkage and retained their form and nuclei.

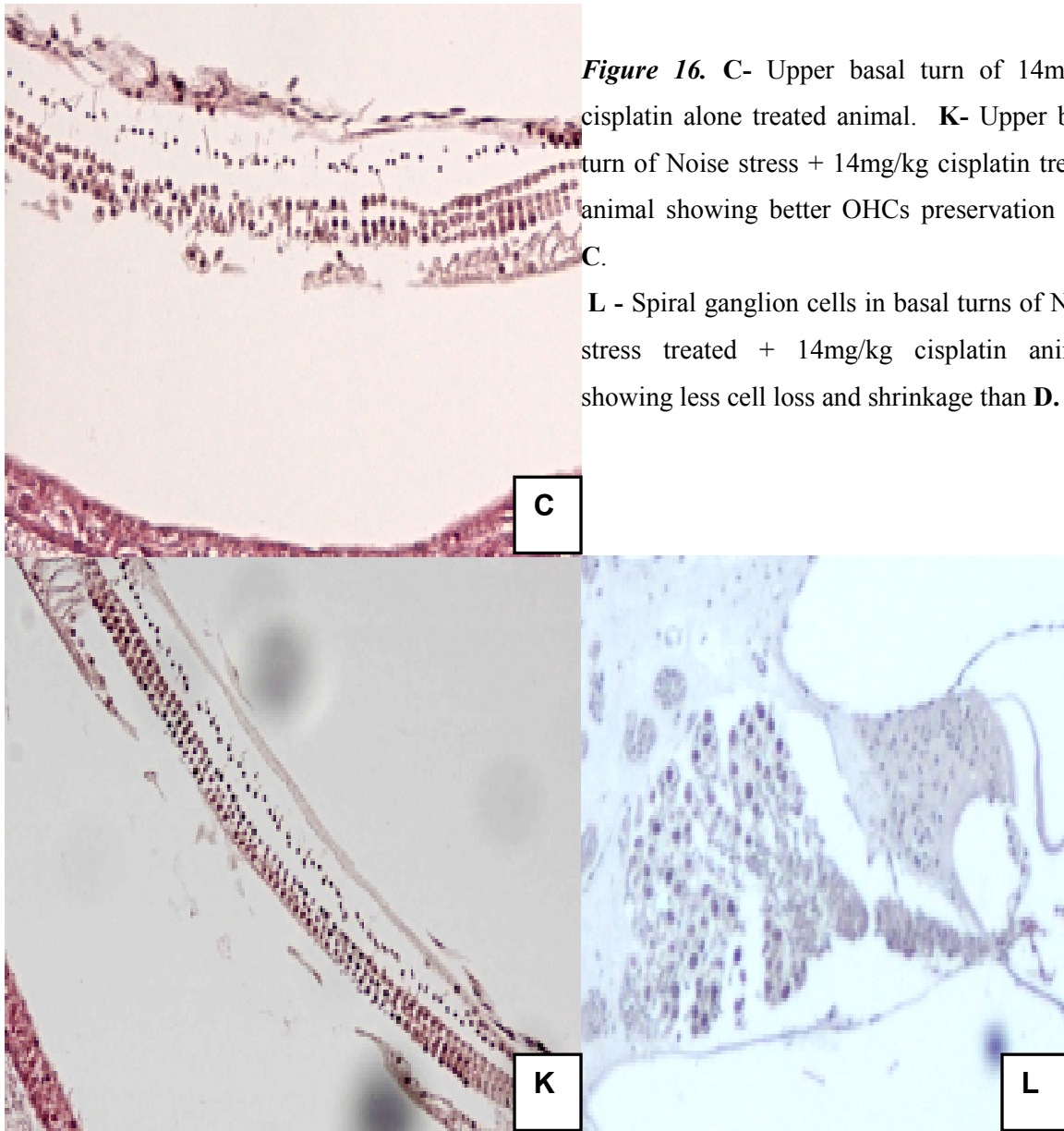
The group which received 300 mg/kg D-MET in combination with noise stress revealed a better morphological picture than the 300 mg/kg D-MET alone group. Moreover, the hair and spiral ganglion cell perseveration against cisplatin toxicity was better in D-MET groups than all other groups.



**Noise stress alone**

Animals from this group reassembled a similar morphological picture with that of the 275mg/kg L-NAC alone group, but the 300 mg/kg D-MET alone group exhibited a better morphological picture than these groups.

Interestingly, we found that animals which underwent noise stress alone against the cisplatin ototoxicity exhibited a considerable hair and ganglion cell preservation when compared with the cisplatin alone treated group.

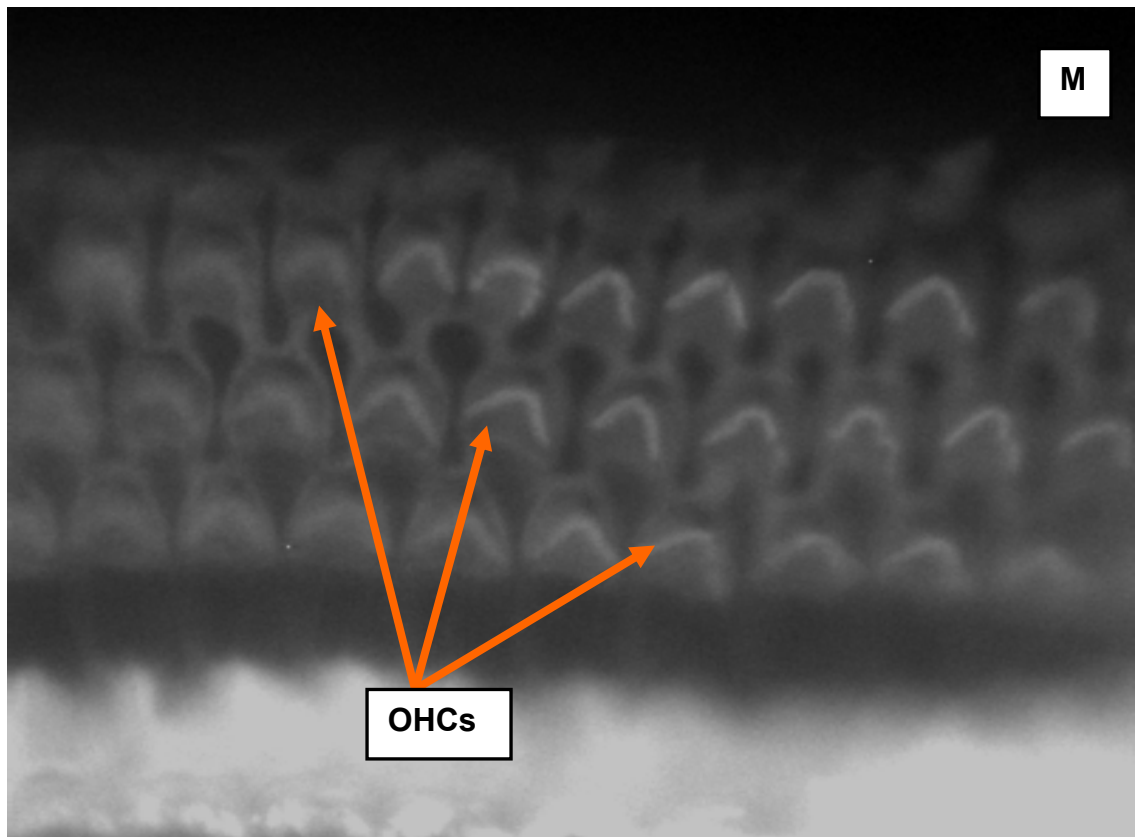


### **TRITC-conjugated phalloidin staining**

The stereocilia bundles and circumferential actin ring surrounding the cuticular plate of the haircells were clearly seen in the TRITC-phalloidin stained specimens. This allowed us to identify the presence of hair cells and their hair bundles

### **Control group animals**

Three rows of OHCs and a single row of IHCs were seen clearly with their hair bundles intact and properly arranged. We did not find any loss of hair bundles or haircells in the control group (*Fig. 17*).

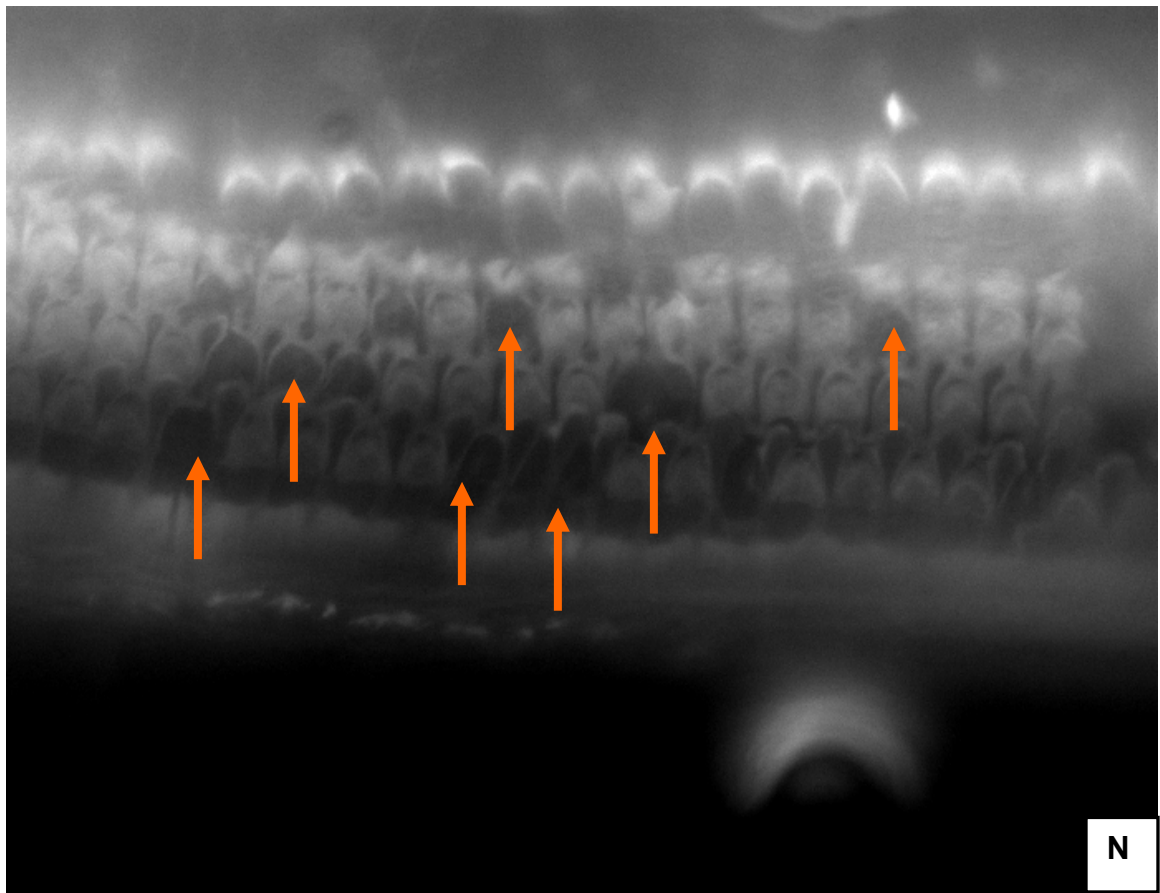


**Figure 17.** M- Basal turn photomicrograph of a control animal clearly demonstrating three rows of normal OHCs and their hair bundles.

### **Groups treated with cisplatin**

Changes in hair bundle arrangements and loss of hair cells were seen 96 hours after cisplatin treatment. When compared with IHCs, damage to cells and hair bundles of OHCs was more severe since OHCs are more susceptible to cisplatin toxicity. The loss and damage began in the basal, high-frequency region of the cochlea and progressed towards the apex.

Among all groups, the cisplatin alone group exhibited the maximum amount of damage. In this group of animals, a considerable number of OHCs completely lost their hair bundles without losing the whole cell (*Fig. 18*).



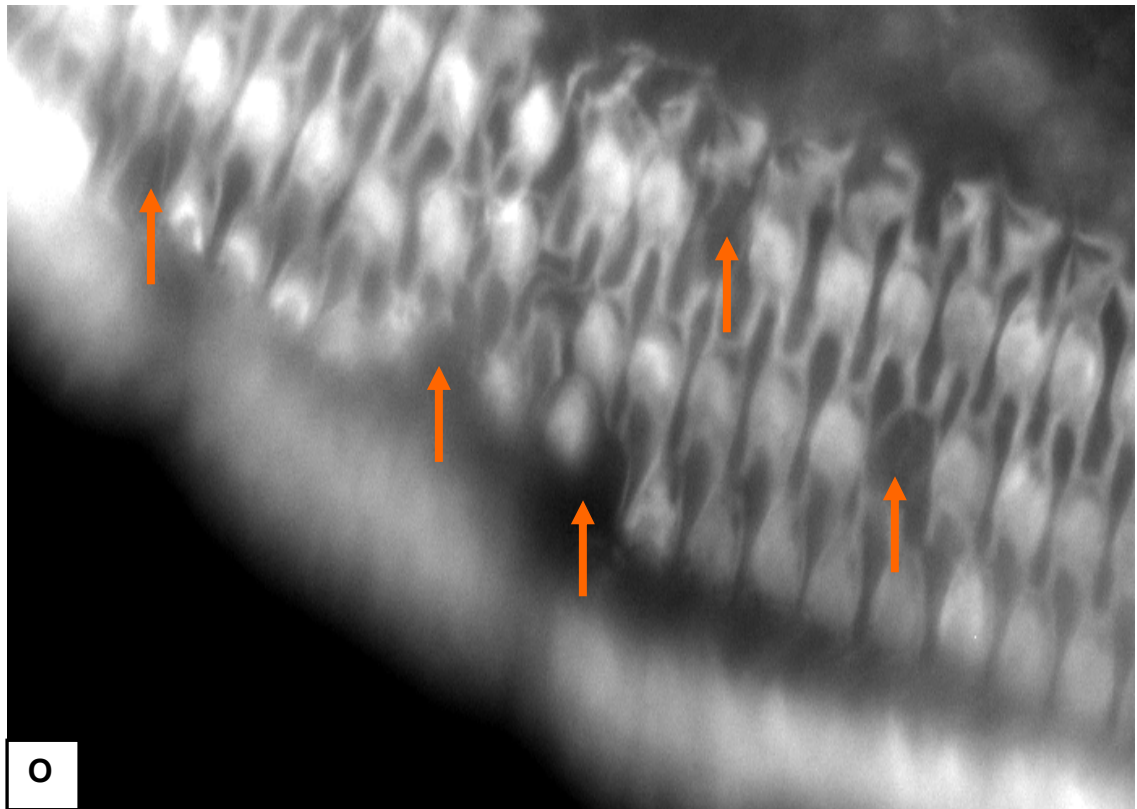
**Figure 18.** N- Basal turn photomicrograph of a 14mg/kg cisplatin alone treated animal clearly demonstrating severe loss of OHCs (arrows) and complete loss of their hair bundles.



### **L – NAC**

When compared against the cisplatin alone treated group, we found that 275mg/kg L-NAC alone and in combination with noise stress showed better hair cell preservation and proper hair bundle arrangement in the middle and basal segments of the cochlea. Only a small number of hairs cells lost their hair bundles without losing the whole cell. The hair cells in the apex segment of the cochlea did not exhibit any considerable changes, as these hair cells are less susceptible for cisplatin toxicity.

The group which received 275mg/kg L-NAC in combination with noise stress always revealed a better morphological picture than the 275mg/kg L-NAC alone group against cisplatin toxicity.

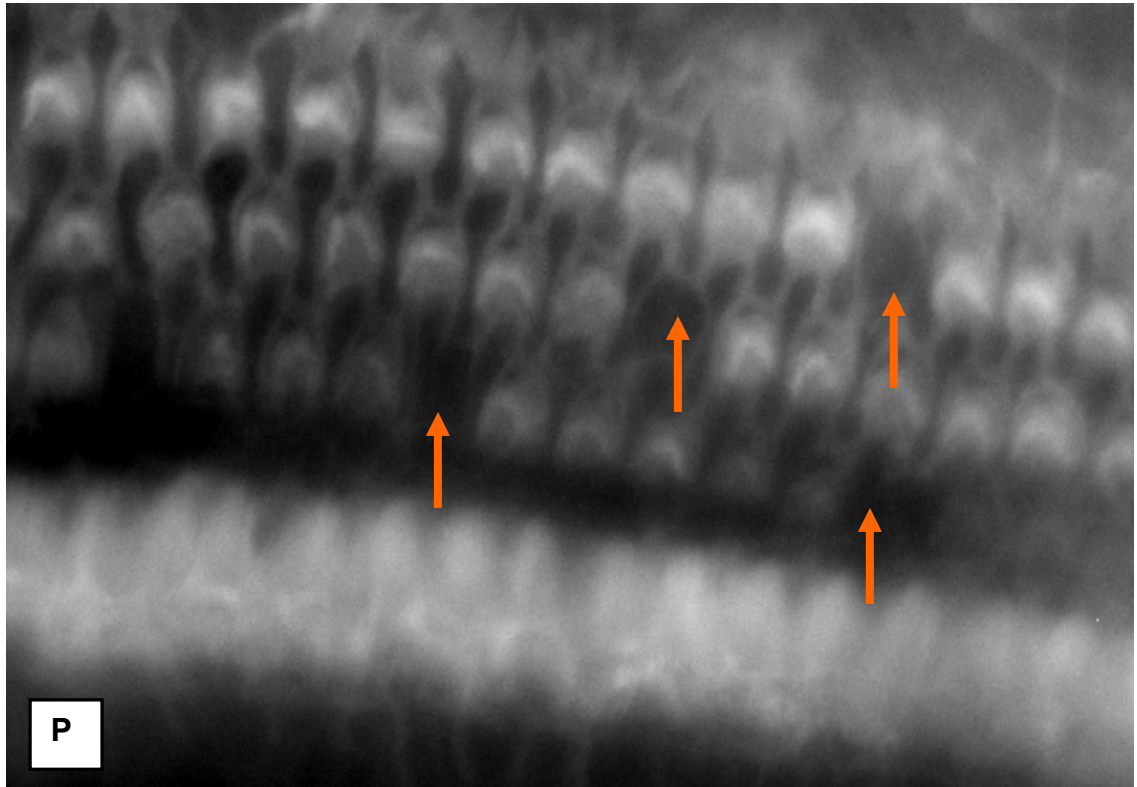


**Figure 19. O** - Basal turn photomicrograph of a 275mg/kg L-NAC + Noise stress + 14mg/kg cisplatin treated animal demonstrating better OHC and their hair bundle preservation than cisplatin alone treated animals (N). Arrows show OHC loss.

### **D – MET**

When compared against the cisplatin alone treated group, we found that 300 mg/kg D-MET alone and in combination with noise stress showed better hair cell preservation and proper hair bundle arrangement in the middle and basal segments of the cochlea. Loss of hair bundles was seen very rarely and hair bundle loss was noticed only in the basal segment of the cochlea. The hair cells in the apex segment of the cochlea did not exhibit any considerable changes, as with other groups.

The group which received 300 mg/kg D-MET in combination with noise stress revealed a better morphological picture than the 300 mg/kg D-MET alone group. Moreover, the preservation of hair cells and proper arrangement of hair bundles against cisplatin toxicity was better in D-MET groups than that of all other groups.

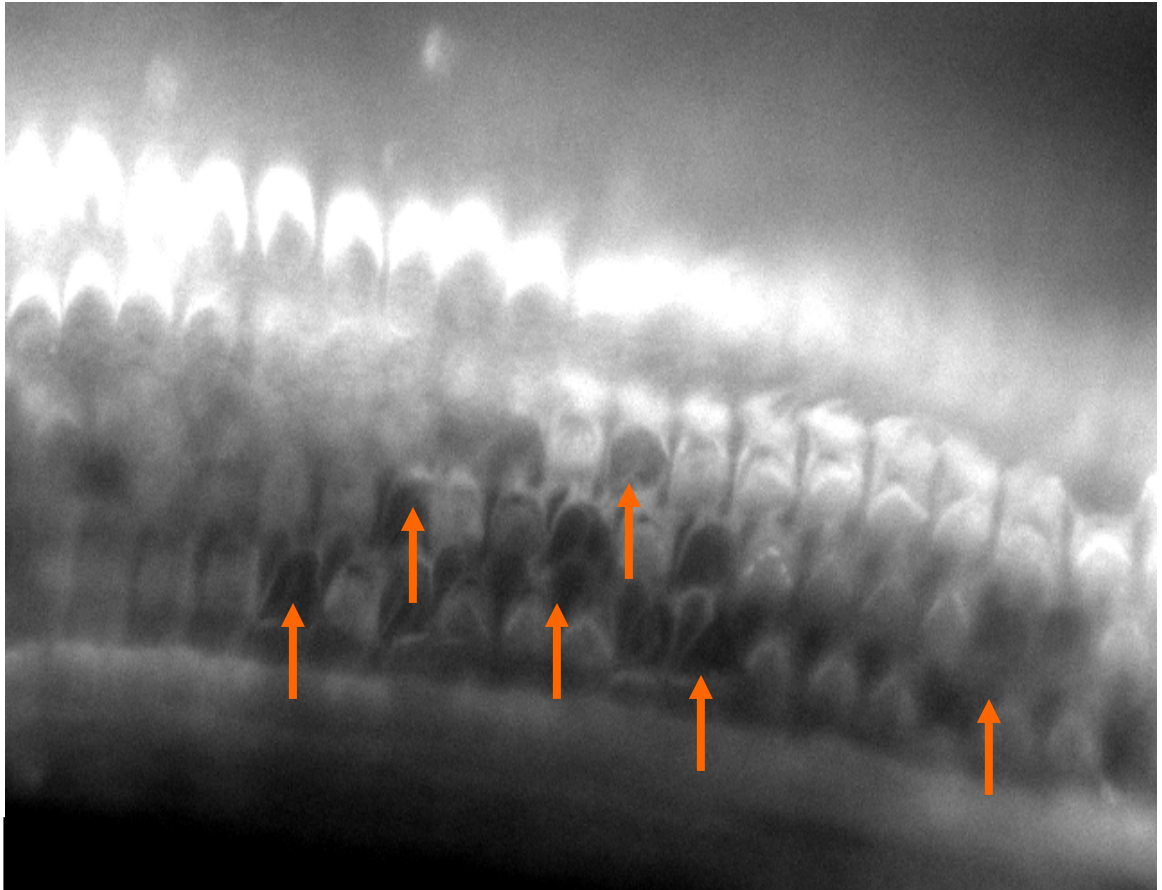


**Figure 20.** P - Basal turn photomicrograph of a 300mg/kg D-MET + Noise stress + 14mg/kg cisplatin treated animal demonstrating better OHC and their hair bundle preservation than N and O. Arrows show OHC loss.

### **Noise stress alone**

Animals from this group reassembled a similar picture than that of the 275mg/kg L-NAC alone group, but the 300 mg/kg D-MET alone group exhibited the best morphological picture compared to these groups.

Consistent with Hematoxylin and Eosin staining results, the animals which underwent noise stress alone against the cisplatin ototoxicity exhibited a considerable OHC preservation when compared with the cisplatin alone treated group.



**Figure 21. Q** - Basal turn photomicrograph of a Noise stress + 14mg/kg cisplatin treated animal demonstrating better OHC and their hair bundle preservation than Fig. N. Arrows show OHC loss.

**Both Hematoxylin and Eosin staining and TRITC-conjugated phalloidin staining were essentially consistent with the ABR findings.**

**Discussion.....**

Currently, cisplatin is the most effective drug among platinum-based chemotherapy for head and neck cancer. Unfortunately, ototoxicity is a common and major dose-limiting side effect of cisplatin therapy, and which decreases post-chemotherapeutic life quality [62, 63, 64].

We planned to study the effect of noise stress against cisplatin ototoxicity alone and in combination with two other thiol based otoprotectors, namely L-NAC and D-MET, at very low dosages which had shown otoprotection against cisplatin ototoxicity. Although these two otoprotectors have shown promising results in animal studies at high dosages [41, 40, 43], there is concern that high dose of L-NAC and D-MET could have negative effects on chemotherapy, leading to reduced chemotherapeutic efficacy [57, 65-68]. In an attempt to avoid this negative chemotherapeutic effect, studies have been conducted administrating the otoprotecting drug at varying times and space [43]. We hypothesised that noise stress may enhance the otoprotective effects of L-NAC and D-MET. Moreover, we were interested in studying the effect of noise stress alone.

Results of our studies have demonstrated that the noise stress technique maximizes the otoprotecting efficacy of 275 mg/kg L-NAC and 300 mg/kg D-MET. The protection level of 300 mg/kg D-MET + noise stress is the best among all treated groups. More interestingly, we found that noise stress alone showed better results against cisplatin ototoxicity when compared with the cisplatin only group. Both Hematoxylin and Eosin staining and TRITC-conjugated phalloidin staining were essentially consistent with the ABR findings.

As this is the first time that noise stress has been used against cisplatin induced ototoxicity, we have postulated a few mechanisms which may be core reasons for the observed otoprotection.

## **Possible mechanisms underlying noise conditioning**

### **Noise stress in combination with Otoprotective drugs**

Cochlear blood flow during noise stress could have played an important role in our experiments. Differing levels of noise have been shown to affect cochlear microcirculation and its cellular metabolisms.

Using laser doppler flowmetry, *Scheibe et al., 1993*, demonstrated that a 85 dB SPL wide-band noise or a 10 kHz tone at 105 dB SPL increase cochlear blood flow, while a 125dB SPL loud noise exposure decreased cochlear blood flow [69]. *Ryan et al., 1988*, observed increased cochlear blood flow in the spiral ganglion, VIII nerve, and spiral lamina during 85 dB SPL noise exposure [70]. Moreover, in a previous study they showed an increase in local cellular metabolism during one hour exposure to 85 dB SPL wide-band noise [71].

These studies help us–explain the increase in otoprotection observed in groups which received either L-NAC or D-MET just before the noise stress. An increase of blood flow in the organ of Corti during noise stress could increase the distribution of the protection molecule given just before the beginning of the noise stress, which may trigger the hair cells to utilize the protection molecules well before the cisplatin treatment, and which could make the hair cells more tolerant to cisplatin treatment.

### **The role of Noise stress alone**

#### **Expression of C-JNK**

Hair cells undergoing adaptation to the acute noise stress could be another reason for noise stress only induced otoprotection. This could be better explained by the activation of stress activated protein kinases known as c-Jun N-terminal kinase (JNK), a group of MAP kinases (MAPKs), which in turn phosphorylates the transcription factor c-Jun [72]. This environmental stress activated MAPK–JNK signal transduction pathway plays a main role in the apoptosis of stressed cells that have been damaged by ROS [73].

A strong phosphorylation of JNK has been documented in high dosage cisplatin treatment [74], and this activation of the JNK signaling transduction pathway, which phosphorylates the NH<sub>2</sub>-terminal region of c-Jun, has a protective role against DNA damage-induced apoptosis [75, 76]. This was further evident in the study by Wang *J et al.*, 2004, where they found that inhibition of JNK potentiates the ototoxic effect of cisplatin. In consideration of these previous works, we suggest that noise stress could be the stress which activates the MAPK–JNK signal transduction pathway, and which in turn has a protective effect on cisplatin-induced ototoxicity by repairing the DNA [74].

### **Antioxidants**

As discussed previously, the generation of excess free radicals within hair cells during cisplatin treatment leads to the damage or death of those hair cells. Procedures and agents that scavenge ROS or enhance the production of natural free radical scavenger systems within the hair cells have been shown to increase hair cell survival after cisplatin treatment [37-53]. Studies on sound conditioning shown increase in the antioxidant enzyme glutathione reductase during sound conditioning against noise induced trauma [77, 78]. Being a major antioxidant, Glutathione reductase plays a major role in protecting the hair cells from cisplatin insult. Therefore, an increase in the antioxidant enzyme glutathione reductase during noise stress could be one mechanism behind the protection offered by our noise stress technique.

Even though several studies had been conducted to investigate the effect of sound conditioning against noise induced trauma, no studies have been done to evaluate the effect of acute noise stress on cisplatin induced ototoxicity yet.

### **Glucocorticoids**

Glucocorticoids are stress hormones secreted from the adrenal gland. The main biological roles of glucocorticoids are to protect the body from stress by regulating glucose metabolism and blood pressure, as well as controlling behavior and neural function. Varying levels of glucocorticoid receptors are expressed in the inner ear

tissues including the organ of Corti [79, 80]. The biological reaction to glucocorticoids depends on the number of glucocorticoid receptors presented on a tissue.

Activation of the hypothalamus–pituitary–adrenal axis and release of glucocorticoid from adrenal glands were documented during different stressors. Stressors which are found to affect the auditory function through the release of glucocorticoids include restraint stress [81–82]; acoustic trauma [80, 82–85] and heat shock [84, 85]. Studies have found that acoustic stress increases serum glucocorticoid levels and decreases glucocorticoid receptor expression in the organ of Corti [81, 86, 87]. On the other hand, Dexamethasone, a synthetic glucocorticoid, is already used to treat conditions such as Ménière’s disease, noise-induced hearing loss, sudden hearing loss, salicylate ototoxicity, aminoglycoside ototoxicity, and has shown a significant protective effect against cisplatin induced ototoxicity when administered intratympanically before exposure to toxic doses of cisplatin [88–91]. The protection offered by glucocorticoid has been explained by the ability of glucocorticoid to increase antioxidant enzymes and in restraining both the increase of NOS mRNA and the release of reactive nitrogen intermediates inside the cells.

Therefore, we postulate that increases in glucocorticoid levels during noise stress could confer protection against the cisplatin induced ototoxicity [90, 92, 93].

### **Heat Shock Proteins**

The Heat shock protein (HSP) family includes both functional and stress-activated members. The functional members are important for protein folding, trafficking, and metabolism. Expression of stress-activated HSPs occurs in response to a variety of stressors which could change the cellular environment. [94]. HSPs, which are found in bacteria, yeast, plants, and all other eukaryotes, are named according to their molecular weights, such as: HSP-90, which supports the cytoskeleton and maintains steroid receptors and transcription factors; HSP-70, which are the commonly expressed and highly conserved stress-inducible HSPs in mammals that constitute the protein folding and unfolding mechanisms and provides thermotolerance to cells on exposure to heat stress; small HSPs, such as ubiquitin and HSP-27, which marks proteins that are produced in response to ischemia for degradation. Stress-induced HSP

expression promotes cellular survival in a large number of systems, including the organ of Corti [94, 95]. This stress resistance and survival is explained by the chaperone function of HSPs, where HSPs prevent the irreversible aggregation of heat-denatured proteins [96]. In addition, HSPs are capable of controlling both the signaling and execution of major cell death pathways [97].

In a study by *Lim et al. 1993*, it was demonstrated that noise stimulation in the rat cochlea causes increases in levels of Hsp72 in the outer hair cells of the rat cochlea [98]. In addition, *Karlseder et al. 1996*, found that overproduction of Hsp72 protected cells from a DNA-damaging anticancer drug, doxorubicin [99]. In another study, it was found that hsp90, which is involved in the activation of the glucocorticoid receptor response, increases in inner and outer hair cells in response to noise [100]. Finally, *Lisa L. Cunningham et al., 2006*, demonstrated that a robust expression of HSP-70 mRNA and protein and a modest expression of HSP-90 and HSP-27 showed significant protection against both cisplatin- and aminoglycoside-induced hair cell death in the utricle preparation *in vitro* [95]. This indicates that up-regulation of HSPs can inhibit ototoxic drug-induced hair cell death.

Therefore, we postulate that up-regulation of HSPs during acute noise stress inhibits cisplatin-induced hair cell death.



**Conclusion.....**

We see noise stress as an effective new otoprotecting technique against cisplatin-induced ototoxicity. However, absence of data regarding the influence of noise stress against cisplatin-induced ototoxicity makes it hard to explain the exact underlying mechanisms of noise stress induced otoprotection. Therefore, further studies on the underlying molecular mechanisms are required to improve our understanding of this type of otoprotection.

**Future benefits.....**

If studied accurately, noise stress could be used in patients who undergo cisplatin treatment to protect at least the ability to perceive the speech perception frequencies.

Future studies on noise stress could help us to apply this technique not only as an otoprotection method, but also to use this method to mobilize a systemically given molecule, drug, or stem cells toward the cochlea.

Good otoprotection by noise stress could change the present course of drug-only otoprotection approaches against cisplatin ototoxicity. In clinic, this may allow increases in the use of cisplatin, thereby improving the efficacy of cancer treatments and the life quality of young cancer patients, which in turn will have a positive impact on their family, community, and country.

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