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Project Title: The Effect of Delayed Systemically Administered n-Acetylcysteine on the Cochlear Oxidative State and on Cisplatin Induced Ototoxicity In Vivo in Guinea Pigs

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None

SUMMARY: (no more than 250 words single spaced)

Cisplatin is an effective antineoplastic drug which is often limited in it's use by an ototoxic side effect. After cisplatin enters the cochlea, a large amount reactive oxygen species (ROS) is generated. This large production of ROS appears to overwhelm the cells' mechanisms of ROS regulation and is thought to mediate damage to cells within the cochlea. Past studies have shown that the ototoxicity of cisplatin can be diminished when it is administered concurrently with an antioxidant such as n-acetylcysteine (NAC). It is thought that the mechanism of NAC's action is through it's ability to counteract the overwhelming ROS production. Unfortunately, a concern exists that the efficacy of cisplatin is reduced by the NAC when the two agents are administered concurrently. Our study was designed to circumvent this concern by administering NAC long after the cisplatin treatments have ended. Thus we could determine if the delayed administration of NAC could reverse the ototoxic effect of cisplatin, possibly without affecting the efficacy of cisplatin. In our study, guinea pigs were administered a course of cisplatin treatments. NAC was then administered 30 days after the completion of the cisplatin treatments. Auditory brainstem responses were used to determine hearing loss. A redox probe was used to measure the intracochlear oxidative states in vivo. NAC did not reverse the ototoxicity of cisplatin and was found to not change the intracochlear oxidative state. The results of this study suggest the possibility of a tolerance to the effect of systemically administered antioxidants.

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The Effect of Delayed Systemically Administered n-Acetylcysteine on the Cochlear Oxidative State and on Cisplatin Induced Ototoxicity In Vivo in Guinea Pigs

Introduction & Background

Cisplatin is an effective antineoplastic drug used for the treatment of solid tumors including the treatment of head and neck tumors (1). A limiting side effect is an irreversible ototoxicity with an accompanying hearing loss (13, 45). The hearing loss is typically bilateral, initially affecting high frequencies, with a potential for progression to involve the lower frequencies. The hearing loss within patients has been found to be variable and the extent of the hearing loss is related to factors such as age, dose, preexisting morbidity, genetic factors and concomitant drugs (13, 14).

The biology of the inner ear has been well described (5). Sound waves enter the ear and eventually travel through the fluid-filled cochlea from the base to the apex. The traveling pressure waves within the cochlea cause a vibration of the basilar membrane (7) which in turn mechanically moves the stereo cilia within the hair cells. The movement of the stereo cilia creates a current along the lateral wall and base of the hair cells (8). The current causes hair cells to release glutamate which is the neurotransmitter that stimulates the afferent nerves cells (9). The stimulation of the afferent nerves cells leads to the experience of hearing.

In evolutionary terms, the development of the use of aerobic respiration by organisms has provided for large gains in the efficiency of energy extraction when compared to anaerobic metabolism. However, a drawback to the use of anaerobic metabolism has been the toxic reactive oxygen species that are produced during the process of energy extraction. Complex cellular systems have evolved to manage these byproducts. In addition, these oxygen byproducts have evolved to become an integral part of normal cellular functioning and have been shown to be involved in the control of a myriad of complex cellular systems including apoptotic pathways (4, 6). Reactive oxygen species (ROS) molecules which participate in metabolic processes in the cochlea include hydroxyl radicals, superoxide anions, hydrogen peroxide and singlet oxygen (5).

Cisplatin is administered intravenously. After administration, the cisplatin molecule undergoes hydrolysis to form an active metabolite (13, 15, 16). Studies have shown that cisplatin is actively transported into the cochlea via various possible protein transporters (17). One of the primary properties of cisplatin is its ability to cross-link DNA which in turn can interfere with DNA replication (2). However, the cells of the inner ear are mostly post-mitotic therefore other mechanisms of toxicity appear to be of greater significance. It has been established that after cisplatin enters the cochlea, a large amount of reactive oxygen species (ROS) is generated (13, 18, 19, 20, 21, 22). This large flood of ROS appears to overwhelm the cells' homeostatic mechanisms of ROS regulation (13). ROS have been found to cause damage to cells by reacting with various proteins, DNA and lipids (34). This damage presumably leads to a state of cellular dysfunction. Beyond cellular dysfunction some cells undergo apoptosis. ROS participate directly as signaling molecules in various apoptotic processes as well as working indirectly through up regulating the genes that are involved in apoptosis.

Histological studies of cisplatin induced cell damage has revealed that cisplatin can destroy cells. Primarily, the outer hair cells are targeted with diminishing damage in a basal to apical direction (1). To a lesser extent, the inner hair cells, marginal cells of the stria vascularis, cells of the spiral ganglion and cells of the spiral ligament can be affected (3, 10, 11). In addition to cell death, cisplatin has been seen to cause stria vascularis edema and damage to the cells of the

spiral ganglion (5, 10). Damage has also been described with light microscopy. Specifically, architectural changes have been noted to the tunnel of Corti as well as changes to the stereo cilia of the hair cells (10, 12).

Thus, it seems that cisplatin mediated damage is not an all or nothing phenomenon. The damage likely occurs in gradations and ranges from a temporary disruption of cell membrane currents (35) all the way to outright death of the cell.

Past studies have shown that the ototoxicity of cisplatin can be diminished when it is administered concurrently with systemic n-acetylcysteine (NAC) (40, 41, 42). Unfortunately, a concern exists that the efficacy of cisplatin is reduced by the NAC when the two agents are administered concurrently (1). This concern is based on the fact that the tumoricidal action and the ototoxic action of cisplatin share similar mechanisms (43). Our study attempts to circumvent this concern by administering the rescue agent long after the cisplatin treatments have ended. We have hypothesized the existence of a therapeutic window which can be exploited to ameliorate the delayed development of cisplatin induced ototoxicity. To this end, the aim of this study was to focus on the damaged but potentially viable cells which remain alive after cisplatin has completed it's tumorocidal actions. Given that these potentially viable cells arrive to their state of dysfunction in part because of an overload of ROS, we tested the hypothesis that the damage could be at least partially reversed or slowed by improving the oxidative state of the cells.

In support of our approach, there are already reports in the literature that some of the toxic effects of cisplatin are delayed, temporary and/or reversible. In a study using guinea pigs, a statistically significant spontaneous recovery of outer hair cells was found during the first to fourth weeks after cisplatin administration. The histological findings were in accord with the electrophysiological measurements (23). Others have observed some spontaneous recovery of ototoxicity in guinea pigs after receiving cisplatin for 8 days (24). In another study, a significant spontaneous recovery in ototoxicity was noted in guinea pigs for up to 3 weeks after cessation of cisplatin administration (25). In an associated investigation, a spontaneous improvement in cochlear transduction occurred over an 8 week period after the cessation of cisplatin. An example of how cisplatin induced ototoxicity can be reversed long after the cisplatin treatments are completed was shown in a pediatric case study that documented the recovery of hearing loss one year after the cessation of therapy (27). The observation of these spontaneously reversing ototoxicities provides for a possible therapeutic window of opportunity. It seems that some cells that are injured, but not dead, may be capable of repair.

The potential for reversing some of the ototoxicity long after cisplatin treatments are finished may be quite large as was seen in a study where the majority of the hearing loss developed over an extended period of time. In that study involving children, a 5% rate of hearing loss was measured at the end of cisplatin therapy but this grew to 44% after 2 years. The median time for the first significant decrease in hearing was 135 days (28). The majority of the morbidity occurred well after the completion of the treatment with cisplatin so in this case a treatment that induces cell repair could potentially provide for a large benefit.

In our experiments, systemic n-acetylcysteine was chosen as the agent to modify the oxidative state of the cochlea. NAC is an antioxidant which acts by chemically reducing hydroxyl radicals. NAC is also metabolized within the body to form glutathione which itself is an antioxidant (31). The terminal half-life of NAC is approximately 6 hours in humans (30) which would permit for a reasonable dosing schedule.

Our choice of NAC as the otoprotective agent is in accord with some of the criteria of the ideal otoprotective agent as described by others (13). Firstly, the agent should be non-toxic. NAC fulfills this criterion as it has been used safely as an antidote in acute acetaminophen poisoning (29).

Secondly, the agent should be capable of being transported into the cochlea. Previously, it was shown that a radiolabeled component of a single dose of intra-peritoneally administered NAC can enter into the cochlea and remain detectable for up to 48 hours (32). Furthermore, previous unpublished studies from this lab have shown that systemically administered NAC can reduce the oxidative state of the cochlea.

Thirdly, the otoprotective agent should not interfere with the anti-tumor efficacy of cisplatin. As stated above, there have been reports that NAC can reduce the efficacy of cisplatin when both compounds are administered together (33). Our study circumvents this potential problem by delaying the administration of NAC until after the cisplatin treatments are finished. At least one study has indicated that an antioxidant would not interfere with the anti-tumor efficacy of cisplatin when administered more than 8 hours after the last dose of cisplatin (44).

Methods & Materials

Animals

Adult albino guinea pigs weighing approximately 300 to 500 grams were used. All procedures were followed under The University of Manitoba's requirements for animal use and care. The animals were assessed upon arrival and monitored on a daily basis by trained staff. The animals were given an acclimation period of at least 1 week. They were maintained with a normal day/night cycle and were provided with free access to food and water.

Measurements of Intra-cochlear Oxidative State

The intra-cochlear oxidative state was measured with a Lazar Labs Jenco model 6230N millivolt/pH/temperature meter mated to either a model ORP-146C micro-oxidation reduction electrode or a PHR-146 micro-combination pH electrode. Guinea pigs were anesthetized with 60 mg/kg ketamine/7 mg/kg xylazine. Exposure of the round window, through which a probe was inserted to measure the redox potential of the perilymph, was achieved through a terminal microsurgical procedure. The duration of elapsed time between making an opening in the round window and taking the redox potential measurement was also recorded for later analysis.

Auditory evoked potentials

Prior to ABR testing, each guinea pig was sedated with a mixture of Ketamine 60mg/kg and Xylazine. Animals were placed in a copper shielded sound suite during all recording sessions. Body temperature was maintained at 37 C. by a Cincinati Subzero Microtemp Pump. Subdermal electrodes were inserted at the temporal bones of the skull (ipsilateral and contralateral) and the lower right quadrant of the abdomen (ground). Sound stimuli were pure tones of 3, 6, 12, 24 kHz with low pass filtering at the lower frequencies. High pass filtering was used for 24 kHz. Responses were averaged at 10 dB SPL increments from 100 dB to 20 or 30 dB SPL depending on the frequency. Two tracings were recorded at each dB level. The threshold was determined to be the lowest dB level at which no response was detected. Or, if the lowest dB level was determined to have a response, 10 dB lower than that was stated as the threshold. The software used was SmartEP by Intelligent Hearing Systems.

Experimental Design

Three groups of 12 guinea pigs were randomly assigned.

Group 1 served as the negative control group. They received intra-peritoneal saline injections. They underwent ABR testing at baseline, two weeks after 3 doses of saline injections on alternate days, and then after 30 once-daily saline injections. Group 1 also underwent terminal REDOX measurements with the probe. This group mimicked the time course of the other groups.

Group 2 was the positive control group. They received intra-peritoneal ciplatin, 4 mg/kg for 3 doses on alternate days. At two weeks after the cisplatin administration was completed, ABR testing was performed in order to confirm the expected hearing loss. They then received daily saline injections only for 30 days after which time a final ABR assessment and REDOX measurements were made.

Group 3 received intra-peritoneal cisplatin, 4 mg/kg for 3 doses on alternate days as with Group 2. At two weeks and at one month after the cisplatin administration was completed, ABR testing was performed to confirm the expected hearing loss. Then daily intra-peritoneal doses of NAC were administered at 400mg/kg for 30 days. After the NAC doses were completed, a final ABR was performed and REDOX measurements were made.

Table 1: Timeline of Protocol

Negative control group n=12	ABR	14 days after saline injections	ABR	30 days	ABR	*In vivo* redox potential measurements
Positive control group n=12	ABR	Cisplatin injections, then 14 day rest	ABR	30 days	ABR	*In vivo* redox potential measurements
Treatment group n=12	ABR	Cisplatin injections, then 14 day rest	ABR	Daily N-acetylcysteine injections for 30 days	ABR	*In vivo* redox potential measurements

Table 1: A tabular representation of the timeline of the protocol and the differences between experimental groups.

Results

At the beginning of the study, all three groups of animals had approximately the same ABR thresholds and there were no statistically significant differences between the ABR thresholds within the three groups (Fig. 1).

After the animals in Group 2 and Group 3 were administered the cisplatin treatments, the ABR thresholds were found to increase in these animals, as expected. The ABR thresholds were raised by approximately 10 to 20 decibels in these two groups. The animals in Group 1 which received no cisplatin had unchanged ABR thresholds.

After receiving the cisplatin treatments, Group 3 animals received a course of NAC treatments for 30 days. When ABR thresholds were measured at the end of the NAC treatments, no improvement in ABR thresholds were seen these animals.

Figure 1: Effect of Treatments on Mean ABR Thresholds

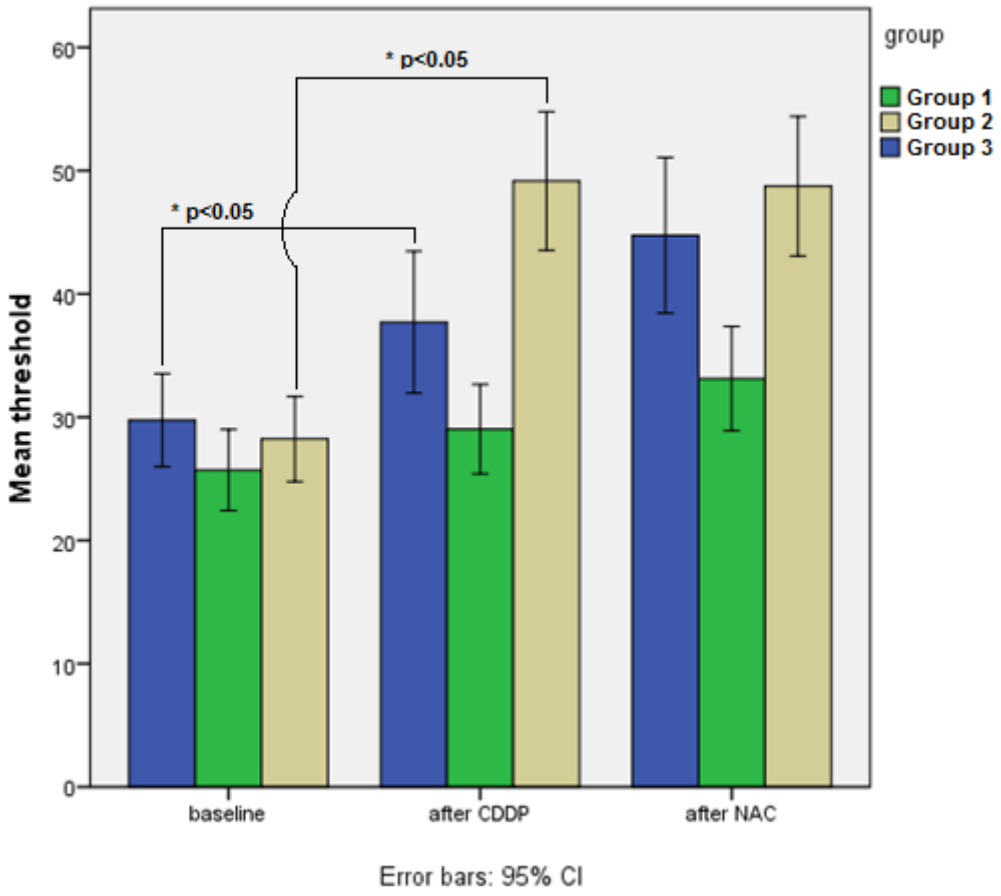

Figure 1: "Baseline bars" show the initial mean ABR thresholds of each group at the beginning.

"After CDDP" bars show the mean ABR thresholds of each group after the cisplatin or sham cisplatin injections.

"After NAC" bars show the mean ABR thresholds of each group after the NAC or sham NAC injections.

Figure 2 shows the oxidative states of the perilymph of each experimental group. The oxidative state of the perilymph was not statistically significantly different between any experimental groups.

Figure 2: The Effect of NAC on the Oxidative State of the Perilymph

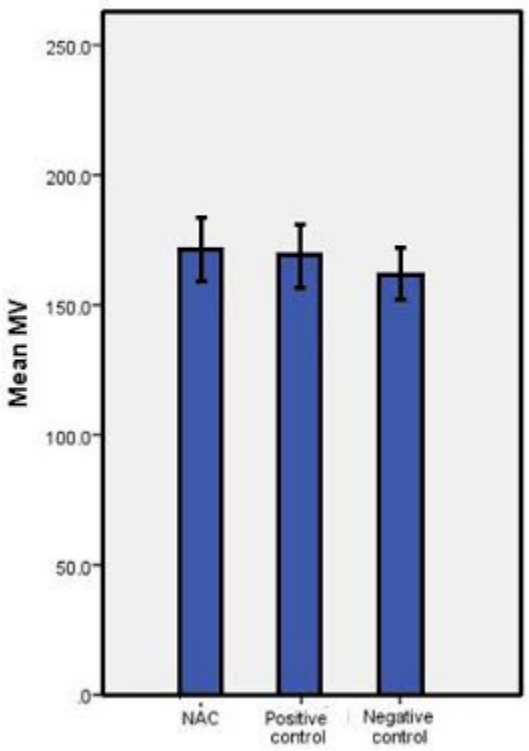

Figure 2: The oxidative state of the perilymph in the three groups of animals at the end of the protocol is graphically represented. There are no significant differences between any of the groups.

Discussion

There is presently no accepted therapy for the prevention of cisplatin induced hearing loss. As a result of the ototoxicity, the usefulness of cisplatin can be limited (39). The exact mechanisms of ototoxicity remain to be elucidated but it is known that ROS plays an important role.

One of the main concerns that our study attempts to address is the possibility that if the treatment to prevent ototoxicity is given concurrently with cisplatin, then the treatment could possibly neutralize the anti-tumor effect of the cisplatin. In order to circumvent this concern, our approach was to try to reverse the ototoxicity with NAC after the therapeutic effect of cisplatin was finished, that is, at a time when the cisplatin has completed it's tumoricidal actions and thus the efficacy of the cisplatin would be unaffected.

Previously unpublished studies from this lab have shown that in guinea pigs, an acute intra-peritoneal dose of the antioxidant NAC will change the oxidative state of the cochlea, as measured by a redox probe. Since at least part of the ototoxicity of cisplatin is mediated via the generation of ROS, we hypothesized that after the cisplatin therapy is finished, if the oxidative state of the cochlea is modified with systemic NAC then this could possibly be a pathway to preventing or even reversing the hearing loss. Unfortunately, in our study, the long-term systemic administration of the antioxidant, NAC, did not reverse cisplatin induced hearing loss.

One possible conclusion from our study is that although the cisplatin induced damage may be initiated or mediated by ROS, that once the chain of ototoxic events has begun, too many other unrelated factors come into play for the outcome to be controlled with an antioxidant.

Another possible conclusion from our findings is that, unlike acutely administered NAC, the long term administration of NAC does not change the oxidation state of the cochlea. It is possible that a tolerance to the effect of NAC develops over time. Many redundant homeostatic systems exist to control the oxidative state of the cell and we can hypothesize that the administered NAC was unable to overcome these control mechanisms. Thus, it is possible that there was no improvement in cisplatin induced hearing loss because NAC failed to improve the oxidation state of the cochlea due to a tolerance effect.

Our finding of a possible tolerance to the effect of the long term administration of the antioxidant NAC has implications in general for the use of antioxidants to treat other chronic conditions. For example, antioxidants have been proposed for the treatment of coronary artery disease, diabetes and a host of other diseases as well as for aging (36, 37, 38). If a tolerance to antioxidants can develop, then it would be necessary to reconsider the approach to long term antioxidant therapy.

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