Antioxidant Enzyme Levels Inversely Covary with Hearing Loss After Amikacin Treatment

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Abstract
This study’s purpose was to determine if a correlation exists between cochlear antioxidant activity changes and auditory function after induction of aminoglycoside (AG) ototoxicity. Two groups of five 250-350 g albino guinea pigs served as subjects. For 28 days, albino guinea pigs were administered either 200 mg/kg/day amikacin, or saline subcutaneously. Auditory brainstem response testing was performed prior to the first injection and again before sacrifice, 28 days later. Cochleae were harvested and superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase activities and malondialdehyde levels were measured. All antioxidant enzymes had significantly lower activity in the amikacin group (p ≤ 0.05) than in the control group. The difference in cochlear antioxidant enzyme activity between groups inversely correlated significantly with the change in ABR thresholds. The greatest correlation was for the high frequencies, which are most affected by aminoglycosides. This study demonstrates that antioxidant enzyme activity and amikacin-induced hearing loss significantly covary.

Key Words: Amikacin, aminoglycosides, antioxidants, hearing loss, ototoxicity

Abbreviations: ABR = auditory brainstem response; AG = aminoglycoside; CAT = catalase; EDTA = ethylenediaminetetraacetic acid; GR = glutathione reductase; GSH = glutathione; GSH-Px = glutathione peroxidase; GST = glutathione S-transferase; GSSG = oxidized form of glutathione; MDA = malondialdehyde; NADPH = reduced form of nicotinamide adenine dinucleotide phosphate; OHC = outer hair cell; peSPL = peak equivalent sound pressure level; s.c. = subcutaneous; SPL = sound pressure level; SOD = superoxide dismutase; TBAR = thiobarbituric acid reactive

Sumario:
El propósito de este estudio fue determinar si existe una correlación entre los cambios de actividad antioxidante coclear y la función auditiva, luego de una inducción de ototoxicidad por aminoglucósidos. Los sujetos del estudio fueron dos grupos de cinco cuyos albinos con pesos entre 250 y 350 gr. Durante 28 días los cuyos recibieron amikacin intramuscular a 200 mg/kg/día o solución salina subcutánea. Se realizaron evaluaciones de respuestas auditivas del tallo cerebral previas a la primera inyección y de nuevo, antes de sacrificarlos 28 días después. Las cócleas fueron cultivadas y se midieron los niveles de dialdehído malónico, y la actividad de dismutasa de superóxido, catalasa,
Aminoglycosides (AGs) are prescribed routinely to infants and children for sepsis and meningitis. In other age groups they are commonly used to treat intra-abdominal sepsis, complicated urinary tract infections, respiratory exacerbations in patients with cystic fibrosis, and other gram-negative infections (McCracken, 1986). They are among the most frequently prescribed drugs in the United States owing to their low incidence of resistance.

The toxicity of amikacin and the other AGs has been widely studied. The most clinically important adverse reactions are nephrotoxicity and ototoxicity (Begg and Barclay, 1995). Whereas nephrotoxicity is usually medically reversible, the ototoxicity may result in permanent hearing loss and vestibular dysfunction (Nishida and Takumida, 1996). The incidence of ototoxicity secondary to amikacin ranges from 6 to 41 percent (Brummett and Fox, 1989; Matz, 1993). In a recent study of 370 hospitalized patients by Fausti and colleagues (1999), a 33 percent incidence of AG ototoxicity was reported. Concomitant administration of loop diuretics, and previous AG use predispose patients to ototoxic effects (Black et al, 1976; Lane et al, 1977). Apnea, prolonged perinatal hypoxemia, hyperbilirubinemia, and familial predisposition are associated with increased risk of AG-related ototoxicity (McCracken, 1986). Otoxicity is especially common in patients with gastrointestinal disease. Patients with osteomyelitis are at increased risk for AG-induced ototoxicity owing to the duration and high doses of drug required for this therapy (Matz, 1993).

It has been known for decades that AGs can damage both the vestibular and cochlear systems (Schuknecht, 1957). It has long been established that the various AG molecules vary in their relative degrees of cochlear and vestibular toxicity and, in some cases, by species (Brummett et al, 1978; Hawkins and Johnsson, 1981; Nadol, 1981; Stebbins et al, 1981; Weiner and Schacht, 1981).

Cochlear changes consist of outer hair cell (OHC) damage beginning at the base and progressing toward the apex (Tange et al, 1982). Inner hair cell damage and degeneration of the supporting cells follows OHC damage. Strial damage occurs less systematically within the membranous labyrinth, but the degree of destruction is related to severity of hair cell damage. Nerve fiber degeneration is generally thought to be secondary to inner hair cell damage (Huizing and deGroot, 1987), although cases of selective spiral ganglion cell loss have been reported (Sone et al, 1998). For a more comprehensive review of AG ototoxicity, the reader is referred to Govaerts and colleagues (1990) and Forge and Schacht (2000). For an update on recent findings, the reader is referred to Campbell and colleagues (2000). For a review of clinical issues in AG ototoxicity, the reader is referred to Edson and Terrell (1999).

Despite our knowledge of the lesions characteristic of AG-induced ototoxicity, the mechanisms of these changes are complex and continue to be an active area of research.
Some aspects of the mechanisms are not fully understood. For example, the exact relationship between the structure of each amino-glycoside and the preferred site of attack has not yet been elucidated (Schacht, 1993). In fact the structural features of AGs that underlie ototoxicity remain to be discovered (Forge and Schacht, 2000).

There are two stages of AG ototoxicity. There is an early and reversible component owing to competitive inhibition of Ca$^{2+}$ binding and an irreversible intracellular mechanism that is less well understood. Schacht and Weiner (1986) proposed a molecular hypothesis in which the AGs disrupt the signal transduction pathway and increase cellular permeability by acting on membrane phospholipids. They proposed that this site of action is responsible for the long-term and irreversible ototoxicity inside the cochlear cells. Schacht (1993) concluded that cochlear susceptibility to AGs is attributable to a combination of the presence of specific polyphosphoinositides and an unknown transport mechanism for AG uptake.

It has long been postulated that free radical activity may also play an important role in the ototoxicity induced by AGs. Most support for this hypothesis has been from ototoxicity protection studies that show attenuation of AG ototoxicity by free radical scavengers (Huizing and deGroot, 1987; Song and Schacht, 1996; Song et al, 1997). However, the idea that the protection was secondary to free radical scavenging was not universally accepted as other results did not link free radicals to ototoxicity (Bock et al, 1983). Recently, Sha and Schacht (1999) showed that AGs cause free radical formation in vitro and that the free radical formation was inhibited by iron binding.

Further support for a free radical mechanism is provided by dietary modulation. Semi-starvation increases AG ototoxicity and decreases survival in guinea pigs (Prazma et al, 1983). Decreasing hepatic and cochlear glutathione (GSH) levels by a low-protein diet, even without caloric restriction, also exacerbates AG ototoxicity (Lauterman et al, 1995). GSH ester administration can ameliorate this exacerbation but does not decrease AG ototoxicity in animals receiving normal dietary protein levels (Lauterman et al, 1995).

The fact that antioxidants, such as D-methionine, can protect against AG ototoxicity further supports free radicals as an underlying cause of AG ototoxicity (Sha and Schacht, 2000). The differential susceptibility of basal versus apical hair cells to AG ototoxicity also correlates to the intrinsic GSH level (Sha et al, 2001), again supporting the role of oxidative processes in AG-induced hearing loss. Protection against AG ototoxicity by antioxidants has been demonstrated both in vivo (Sha and Schacht, 2000) and in vitro (Sha et al, 2001).

To our knowledge, no study has been done to correlate antioxidant enzyme status of the cochlea to auditory threshold shift after AG administration.

The purpose of this research is to determine if cochlear antioxidant enzyme activity is altered by amikacin administration and to correlate any alteration with amikacin-induced hearing loss.

**METHODS AND MATERIAL**

**Animal Preparation and Auditory Brainstem Response Thresholds**

Southern Illinois University institutional guidelines regarding animal experimentation were followed. Ten male Hartley white guinea pigs (250-350 g) comprised two groups of 5 animals each. Both groups received a daily injection for 28 days according to the following protocol: (1) an experimental group receiving 200 mg/kg/day amikacin s.c. which has been previously shown to cause ototoxicity (Nishida and Takumida, 1996); and 2) a control group receiving equivalent volume saline injection s.c.

Auditory brainstem response (ABR) testing was performed immediately prior to the first injection and again at 28 days, just prior to sacrifice. Subcutaneous electrodes were placed at the vertex (noninverting), to a point directly below the ipsilateral pinna (inverting) with a ground electrode in the hind leg.

ABR data collection was obtained with a customized Biologic Navigator system with an additional custom-made high frequency stimulator for 14 kHz. ABR thresholds were measured in response to 100-msec clicks and to tone bursts centered at the frequencies of 1, 4, 8, and 14 kHz presented at 10/sec. Toneburst stimuli were gated by a Blackman envelope with 2 msec rise-fall for 1 kHz and 1 msec
rise-fall for 4.8, and 14 kHz and a 0 sec plateau. An intensity series was obtained for each animal from 100 dB peak equivalent sound pressure level (peSPL) for click stimuli and SPL for tone-bursts, proceeding in 10 dB decrements to below threshold. The term peSPL means that the amplitude of the click stimulus from the prestimulus baseline to the first peak is equivalent to the SPL of a pure-tone stimulus having the same prestimulus baseline to peak amplitude. Threshold was defined as the lowest intensity capable of eliciting a replicable, visually detectable response.

A total of 512 sweeps constituted each average. The recording epochs were 15 msec following stimulus onset. Responses were analog filtered with a 30 to 3000 Hz bandpass. Rectal temperatures were monitored throughout recordings with animal temperature maintained by a warming pad.

All animals were fully anesthetized throughout all ABR testing and prior to sacrifice with a 1 mL/kg intramuscular injection of Rompun cocktail (a solution containing 86.21 mg/mL ketamine and 2.76 mg/mL xylazine), which was supplemented as needed with half doses.

Measures of Cochlear Antioxidant Enzymes

After 28 days of administration, the animals were euthanized by decapitation immediately after the final ABR measurements while still under general anesthesia. Both cochleae were dissected out and placed into liquid nitrogen within 5 minutes of sacrifice. Cochlear tissue extracts were tested for antioxidant enzyme activity, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and glutathione S-transferase (GST).

All tissue was homogenized using a manual Con Torque homogenizer. All enzyme activity was measured on a Hitachi 2000 Spectrophotometer. Protein concentration was estimated according to the method of Read and Northcole (1981) using bovine serum albumin as a standard and Coomassie protein assay as the reagent.

SOD activity was determined at room temperature according to the method of Misra and Fridovich (1972). One hundred ml of tissue extract was added to 880 µl (0.05 M, pH 10.2, 0.1 mM EDTA) sodium carbonate buffer. Twenty µl of 30 mM epinephrine (dissolved in 0.05% acetic acid) was added to the mixture and SOD was measured at 480 nm for 4 min on a Hitachi U2000 Spectrophotometer. The rate of the reaction was calculated where linearity occurred, usually between 90-100 seconds. One unit of SOD activity was expressed as the amount of enzyme that inhibited the oxidation of epinephrine by 50 percent.

CAT activity was determined at room temperature by a slight modification of the method of Aebi (1984). Ten µl of ethanol was added per 100 ml of tissue extract (dissolved in 0.05 M, pH 7.0, 0.1 mM EDTA, phosphate buffer), and then placed in an ice bath for 30 minutes. Then 10 µl of Triton X-100 RS was added. Ten µl of tissue extract was added to a cuvette containing 240 ml phosphate buffer and 250 ml (0.066 M) H2O2 (dissolved in sodium phosphate buffer) and measured spectrophotometer at 240 nm for 30 seconds. The molar extinction coefficient of 43.6 M–1 cm–1 was used to determine CAT activity. One unit of CAT activity was defined as the mmoles of H2O2 degraded/min/mg protein.

GSH-Px activity was determined by a method of Flohe and Gunzler (1984) at 37°C. All reaction mixtures were dissolved in 0.05 M, pH 7.0, 0.1 mM EDTA phosphate buffer. A reaction mixture consisted of 550 ml phosphate buffer, 100 µl 0.01 M GSH, 100 µl 1.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 100 µl GR (0.24 units). One hundred µl of the tissue extract was added to the reaction mixture and incubated at 37°C for 10 minutes. Then 50 µl of 12 mM t-butyl hydroperoxide was added to the tissue reaction mixture and measured at 340 nm for 180 seconds. The millimolar extinction coefficient of 6.22 mM–1 cm–1 was used to determine the activity of GSH-Px. One unit of activity was equal to the millimoles of NADPH oxidized/min/mg protein.

GR activity was determined by the method of Carlberg and Mannervick (1985) at 37°C. Fifty microliters of NADPH (2 mM) in 10 mM Tris-HCl buffer (pH 7.0) added in a cuvette containing 50 µl of GSSG (20 mM) in phosphate buffer (0.5 M, pH 7.0, 0.1 mM EDTA), and 800 µl of phosphate buffer were incubated at 37°C for 10 minutes. One-hun-
dried µl of tissue extract was added to the NADPH-GSSG buffered solution and measured at 340 nm for 3 minutes. The extinction coefficient 6.22 mM⁻¹ cm⁻¹ was used to determine the activity of GR. One unit of GR activity was equal to the millimoles of NADPH oxidized/min/mg protein.

GST was determined at room temperature by the method of Habig and colleagues (1974). Fifty microliters of tissue extract was added to 750 µL (0.1M, pH 6.5, 0.1mM EDTA) sodium phosphate buffer and 100 µL (10mM) GSH. One hundred microliters of 10mM 1-chloro-2,4-dinitrobenzene were added to start the reaction. GST was measured at 340 nm for 3 minutes. Activity was calculated using the extension coefficient 9.6 mM⁻¹ cm⁻¹, and expressed as mmoles substrate used/min/mg protein.

**Lipid Peroxidation Assay**

The extent of lipid peroxidation was estimated by the concentration of thiobarbituric acid reactive products (TBAR), which were measured by the method used by Ohkawa and colleagues (1979). Concentrations of TBAR (malondialdehyde [MDA] levels) were determined using 1,1,3,3-tetraethoxypropane as the standard, and the results were expressed as nmoles of MDA/mg protein.

**Statistical Analysis**

An analysis of variance (ANOVA) with a between subjects factor consisting of groups (control and experimental groups) and a within subjects factor consisting of time (pre vs post) was conducted on the ABR data. The antioxidant enzymes (SOD, CAT, GSH-Px, GR, GST, and MDA) were treated as separate dependent variables in a univariate ANOVA. Additionally, a correlational analysis was carried out between change in enzyme activity and pre/post differences in the ABR. Probability of a type I error was set at .05 or less for all analyses.
RESULTS

ABR Thresholds

Post-test ABR hearing thresholds are presented in figure 1. As expected, there was no threshold shift in the control group, and significant threshold shifts occurred in the experimental group for all stimuli at the .05 level. Further, the ABR threshold shift was also significant at p ≤ .005 for toneburst stimuli of 8 and 14 kHz.

Antioxidant Enzymes and Lipid Peroxidation

Quantitative enzyme activity data are presented in Figures 2, 3, 4, 5, and 6. The SOD activity (see Fig 2) in the amikacin group cochleae was 56.3 percent lower than in the control group (p ≤ .005). Amikacin group CAT activity (see Fig 3) was 34.3 percent lower than control activity (p ≤ .01). GSH-Px activity (see Fig 4) in the amikacin group was 51.8 percent less than control activity (p ≤ .005). The GR activity (see Fig 5) was 37.2 percent lower in the amikacin group than in the control group (p ≤ .005). GST activity (see Fig 6) was 49.2 percent less than control activity (p ≤ .01). The amikacin group MDA levels (Fig 7), indicative of lipid peroxidation levels, were 21.9 percent greater than control group levels, although this difference was not significant.

Figure 4. Antioxidant enzyme activity (means ± 1 SD) for the control and experimental animal groups of glutathione peroxidase. ** Indicates significant difference from the saline-treated controls at p ≤ .01. NADPH = reduced nicotinamide adenine dinucleotide phosphate.

Figure 5. Antioxidant enzyme activity (means ± 1 SD) for the control and experimental animal groups of glutathione reductase. *** Indicates significant difference from the saline-treated controls at p ≤ .005.

Figure 6. Antioxidant enzyme activity (means ± 1 SD) for the control and experimental animal groups of glutathione S-transferase. ** Indicates significant difference from the saline-treated controls at p ≤ .01.

Figure 7. Malondialdehyde levels (means ± 1 SD) for the control and experimental animal groups.
Correlational Analysis

The correlation between antioxidant enzyme activity and the ABR threshold shift is presented in Table 1. The ABR threshold shift at 8 and 14 kHz for each animal correlated significantly (p ≤ .005) with each of the antioxidant enzymes (SOD, CAT, GSH-Px, GR, and GST). In addition, differences between the two groups in SOD and GSH-Px, and in CAT correlated significantly (p ≤ .01 and p ≤ .05, respectively) with the ABR threshold shift in response to click stimuli. MDA levels correlated significantly (p ≤ .01) with the threshold shift in the 4 kHz toneburst. Some of the differences in antioxidant enzyme activity between the two groups also correlated significantly.

DISCUSSION

This study demonstrates that amikacin produces not only hearing loss, but also changes in the cochlear antioxidant system. Our findings are in keeping with the known AG-induced hearing loss studies that indicate a cytotoxic form of AG exists as suggested by the often delayed or progressive onset of aminoglycoside-induced hearing loss (Huizing and deGroot, 1987; Beaubien et al, 1990) and more direct evidence of a metabolite (Priuska and Schacht, 1995, 1997). All antioxidant enzyme activity was significantly reduced in the experimental compared with the control group.

Most importantly, this study demonstrates that the degree of hearing loss and differences in cochlear antioxidant systems between the two groups significantly covary following amikacin administration. Further, the statistical correlation is strongest between the antioxidant enzymes and the frequencies most susceptible to amikacin ototoxicity, 8 and 14 kHz. Although a causative conclusion cannot be drawn from these results, the high rate of correlation between the decreased antioxidant enzyme activity and the degree

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Table shows the correlation between changes in auditory brainstem response (ABR) thresholds and the cochlear oxidation enzyme activity. * Indicates significant correlation at p ≤ .05. ** Indicates significant correlation at p ≤ .01. *** Indicates significant correlation at p ≤ .005. CAT = catalase; GR = glutathione reductase; GSH-Px = glutathione peroxidase; GST = glutathione S-transferase; MDA = malondialdehyde; SOD = superoxide dismutase; CCHANGE = ABR threshold change for click stimuli; V1CHANGE = ABR threshold change for 1 kHz stimuli; V4CHANGE = ABR threshold change for 4 kHz stimuli; V8CHANGE = ABR threshold change for 8 kHz stimuli; V14CHANGE = ABR threshold change for 14 kHz stimuli.
of hearing loss further supports the hypothesis that AG-induced ototoxicity is antioxidant mediated.

Hearing loss can progress after discontinuation of AG administration (Beaubien et al, 1990). Aran and colleagues (1995) found that AGs are retained in the cochlea for a prolonged period of time. Further, this delayed hearing loss suggests that AG-induced ototoxicity is not a direct effect of the AGs but a response to an alteration induced by their activity. Huang and Schacht (1990) directly demonstrated that metabolism is required for AG cytotoxicity. Sha and Schacht (1999) found that a cytotoxic metabolite produced after initial exposure to AG generates free radicals in a chain reaction. Crann and Schacht (1996) found that AGs form a secondary toxic compound within the cochlea itself. The combined effects of prolonged AG retention in the cochlea and cochlear conversion of AGs into a toxic metabolite contribute to the delayed component of AG-induced hearing loss. This ototoxic metabolite may also have the effect of reducing antioxidant enzyme activity.

Documenting the relationship between AG-induced ototoxicity and cochlear antioxidant enzyme activity further substantiates that free radicals are a factor in the development of this ototoxicity. A large body of evidence indicates that a cytotoxic form of AG induces free radicals. In vitro studies have shown that AGs induce free radical formation in hepatic cytosol (Crann et al, 1992), as well as hair cells and inner ear tissue explants (Clerici et al, 1996; Hirose et al, 1997). Song and Schacht (1996) concluded, based on a number of recent studies, that a gentamicin-iron complex also plays a role in gentamicin ototoxicity. Reducing iron levels using iron-chelating agents decreases this ototoxicity (Song et al, 1997). These authors suggest that iron may also be a factor in the hearing loss induced by other AGs. Iron’s major role in tissue damage is secondary to its propensity to form highly reactive oxygen species (Aust et al, 1985). Our data correlate these cochlear antioxidant system changes with the degree of hearing loss.

In this study, the antioxidant enzyme activity is markedly reduced after 28 days of amikacin administration. Further, the reduction is not specific to one enzyme, rather, it is a more generalized response involving SOD, CAT, GSH-Px, GR, and GST indicating impaired function at several steps of the antioxidant pathway. The reduction in antioxidant enzyme activity we observed should decrease the ability of the system to detoxify the free radicals formed in response to AG administration, possibly exacerbating the toxicity. In a healthy system, one might expect up-regulation of these same enzymes; however, that is not what we found. It is possible that up-regulation occurred earlier in the time course of AG administration, but after 28 days, antioxidant enzyme activity was consistently reduced. Schacht (1993) indicated that the regulatory mechanisms of a cell can be altered by the interaction of AGs with phosphoinositides. This combination of simultaneously depleting or otherwise reducing antioxidant enzyme activity while increasing free radical generation may underlie the frequent hearing loss seen with AGs.

As expected, the amikacin-induced hearing loss in this study was greatest for the high frequencies. AGs’ preferential damage of the hair cells in the basal portion of the cochlea, and thus high-frequency hearing loss, has long been documented (Tange et al, 1982). Immunohistochemical studies by Hayashida (1989) showed localization of amikacin in the OHCs of the cochlea occurring in a pattern of decreasing intensity towards the apex. Other studies by Hiel and colleagues (1992) using tritiated gentamicin in combination with ethacrynic acid also found this pattern of localization in OHCs, which correlated well with ABR threshold changes at the higher frequencies. Collection of labeled gentamicin was denser in lysosomes below the cuticular plate, important sites of oxidative-enzymatic activity (Hiel et al, 1992). Thus, if cytotoxic AGs induce free radical formation, cellular localization of these drugs in the OHCs of the basal turn with decreasing intensities towards the apex would explain our findings.

In summary, our data document a significant and widespread reduction in cochlear antioxidant enzyme activity following 28 days of amikacin administration, ranging from a 34.3 percent decrease in activity to a 56.3 percent decrease. More importantly, these reductions significantly correlate with the degree of hearing threshold shift observed after amikacin administration. The high rate of correlation between the decreased antioxidant enzyme activity and the degree of hearing loss further supports the hypothesis that AG-
induced ototoxicity is antioxidant mediated. Our findings are in keeping with earlier work suggesting a role of free radicals in AG-induced ototoxicity. These data support the hypothesis that the depression in antioxidant enzyme activity may be instrumental in the development of AG-induced hearing loss. The decreased activity of these specific enzymes may mirror a generalized decrease in enzymatic activity secondary to cell death, but other evidence does not support a direct toxic effect of AGs. Inhibiting this depletion may lead to preservation of hearing during AG administration; therefore, mechanisms for countering these antioxidant enzyme changes should be investigated.

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