Protection from ototoxicity of intraperitoneal gentamicin in guinea pig

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Background. Aminoglycoside antibiotics are common to treat peritonitis and exit-site infections in patients on peritoneal dialysis. Ototoxicity (loss of hearing or balance) is a well-documented adverse effect of aminoglycosides, and severe ototoxic reactions have been noted in patients receiving these drugs by intraperitoneal lavage. We have proposed a free-radical hypothesis for the mechanism of aminoglycoside ototoxicity and suggested a therapeutic prevention by the concomitant administration of antioxidants or iron chelators. Here we investigate whether 2,3-dihydroxybenzoate can prevent the ototoxicity of intraperitoneal gentamicin.

Methods. Two strains of pigmented guinea pigs received daily intraperitoneal injections of gentamicin. Both strains developed ototoxicity, although different dosages were needed to produce similar auditory deficits (120 mg gentamicin base/kg body weight daily for 19 days vs. 135 mg/kg for 14 days). Dihydroxybenzoate was administered intraperitoneally once or twice daily. Auditory thresholds were measured by evoked brain stem response. Pathology was assessed as a loss of sensory cells in surface preparations of the organ of Corti.

Results. The auditory threshold shifts and hair cell loss were similar to the pathology observed following subcutaneous injections of gentamicin. Animals sustained almost complete loss of outer hair cells in the basal cochlea and a progressive hearing loss with threshold shifts of 60 dB at 18 kHz. The concomitant administration of dihydroxybenzoate significantly attenuated the threshold shift to less than 30 dB and reduced the loss of hair cells. The treatment with dihydroxybenzoate did not affect serum gentamicin levels.

Conclusions. Antioxidant therapy is a promising approach to prevent aminoglycoside-induced hearing loss following intraperitoneal application.

Aminoglycoside antibiotics are indispensable for the treatment of infections, especially those caused by gram-negative bacteria, including Pseudomonas aeruginosa, Klebsiella, and Serratia marcescens. They are not significantly absorbed after oral dosing, and their most frequent application is by intravenous or intramuscular routes. In addition, cystic fibrosis patients may receive aminoglycosides by inhalation [1, 2], and their intraperitoneal administration is common to treat peritonitis and exit-site infections in chronic renal failure patients [3–5].

The potential for cochlear and vestibular side effects (loss of hearing or balance) is associated with all forms of aminoglycoside treatment. For the two rather divergent routes of systemic injection and inhalation, an incidence of ototoxicity of around 20% has been documented [2, 6]. Comprehensive data for intraperitoneal application are, to the best of our knowledge, not available, and some studies suggest a minimal risk of acute ototoxicity [7]. However, systemic absorption of aminoglycosides across the peritoneal membrane is rapid and can lead to significant serum levels [8–10]. Consequently, both hearing loss and vestibular toxicity have been observed after chronic peritoneal dosing of aminoglycosides [11–14] and even after a single dose of peritoneal neomycin [15].

We have recently proposed a novel hypothesis for the mechanism of aminoglycoside toxicity and developed a successful prophylactic treatment in guinea pigs. Aminoglycosides can chelate iron, and the resulting iron complex is redox active, catalyzing the formation of free radicals [16–19]. Accordingly, ototoxicity of systemically injected aminoglycosides could be prevented through the concomitant administration of antioxidants or iron chelators. Both hearing loss and vestibular dysfunction induced by gentamicin, kanamycin, or streptomycin were significantly attenuated without compromising the antibacterial efficacy of the drug [20, 21]. The effectiveness of antioxidant treatment was subsequently confirmed and extended to neomycin and amikacin [22, 23].

In the present study, we investigated whether iron chelators can also prevent the ototoxicity of gentamicin administered intraperitoneally in guinea pigs. The intraperitoneal...
route is seldom used in experimental aminoglycoside research, and therefore, little is known about pharmacokinetics and the development of ototoxicity in this form of application. We chose 2,3-dihydroxybenzoate (DHB) as a potential prophylactic agent because of its previously established protective qualities against aminoglycoside-induced loss of hearing and vestibular function [20, 21, 24], its beneficial effects on gentamicin-induced nephrotoxicity [25], and general low toxicity [26].

METHODS

Materials

Gentamicin sulfate was purchased from Spectrum Chemical (Gardena, CA, USA), DHB from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA), Metofane from Schering-Plough Animal Health Corp. (Union, NJ, USA). Ketamine was Ketaset® from Fort Dodge Animal Health (Fort Dodge, IA, USA). Xylazine was Tranqulizer from Vedco Inc. (St. Joseph, MO, USA).

Experimental animals and treatment groups

Pigmented male guinea pigs initially weighing 250 to 300 g (Murphy’s Breeding Laboratories, Plainfield, NJ, USA, or Elm Hill Breeding Laboratory, Chelmsford, MA, USA) had free access to water and a regular guinea pig diet (Purina 5025; Purina, St. Louis, MO, USA). The animals were allowed one week of acclimation before treatment began. All experimental protocols were authorized by the University of Michigan Committee on Use and Care of Animals. Animal care was under the supervision of the University of Michigan’s Unit for Laboratory Animal Medicine.

The ototoxic effects of intraperitoneal injections of gentamicin alone or in combination with DHB were assessed in two strains of guinea pigs, necessitated by a change to a specific pathogen-free strain (Elm Hill) in the course of the study. Both strains were susceptible to aminoglycoside toxicity, although different dosages were needed to produce similar auditory deficits (abstract; Halsey et al., Assoc Res Otolaryngol 23:206, 2000). Both strains also showed protection by DHB. Guinea pigs from Murphy’s Breeding Laboratories were used for pharmacokinetics and an initial protection study in which they received 120 mg gentamicin base/kg body weight daily for 19 days, alone or combined with once-daily 100 mg DHB plus 15 mg mannitol/kg body weight [20]. In all other studies, Elm Hill animals were treated with 135 mg gentamicin base/kg body weight for 14 days, and DHB was administered twice daily (100 mg/kg body weight each time), the first time intraperitoneally in combination with gentamicin and a second time intraperitoneally 8 to 10 hours later. After the end of the gentamicin treatment, twice-daily injections of DHB were continued for one more week. Gentamicin was dissolved in physiological saline, pH 7.4; DHB was dissolved by briefly warming in 5% NaHCO₃ at a concentration of 100 mg DHB/mL. The pH of all solutions was between 4.5 and 7.4. In animals not receiving DHB, saline was substituted. Body weight of animals was monitored daily, and the administered dosages were adjusted accordingly. Gentamicin treatment slowed weight gain during treatment. After the end of treatment, animals resumed a normal rate of weight gain.

Serum values

Blood was obtained by nail clipping after light anesthesia of the animals with Metofane inhalation. For gentamicin pharmacokinetics, two animals each were injected with doses of 60, 100, 120, and 180 mg gentamicin base/kg body weight. Blood samples were taken at intervals between 15 minutes and 3 hours after the gentamicin injections. Blood cells were removed by centrifugation at 1000 g for 15 minutes, and sera were stored at −20°C. Gentamicin was measured using a disk diffusion assay. Escherichia coli (LE392) were suspended in melted 0.7% agar and poured over a tryptone agar bed. Ten microliter aliquots of either a standard solution of gentamicin or a serum sample were pipetted onto 1 cm paper disks, which were placed on the bacterial lawn. The plates were incubated overnight at 37°C, and the inhibition zones were measured.

For the determination of blood urea nitrogen (BUN), serum albumin, creatinine, and gentamicin levels in the study on twice-daily protection, blood was taken from six animals per group one hour after the gentamicin injection on day 14. Samples were analyzed by the Clinical Chemistry Laboratory at the University of Michigan Hospital using a fluorescence polarization immunoassay for gentamicin (Abbott Laboratories, Abbott Park, IL, USA) and VITROS® test methodologies (Johnson & Johnson Clinical Diagnostics, Raritan, NJ, USA) for the other assays.

Evaluation of auditory function

Auditory thresholds were measured as evoked auditory brainstem response (ABR). Thresholds were determined for each animal prior to the beginning of the study, again at day 14, and then weekly up to week 7, as stated in the figure legends.

For ABR recordings, animals were anesthetized with an intramuscular injection of 60 mg ketamine/kg body weight and 8 mg xylazine/kg. Measurements were performed in a sound-proof booth at 3, 8, and 18 kHz. In brief, tone bursts of 3, 8, and 18 kHz (10 msec duration, 1 msec rise and fall time) were generated using a SigGen software package (Tucker-Davis Technologies, Gainesville, FL, USA) and presented to the left external auditory meatus in a closed acoustic system through an ear bar connected to a Beyer DT-48 transducer (Beyer Dynamic,
Farmingdale, NY, USA). ABRs from the left ears were obtained from the anesthetized animals in a one-channel recording using an in-house constructed amplifier. The active electrode was placed at the vertex, in the midline of the scalp between the external auditory canals. The reference electrode was placed subcutaneously below the pinna of the right ear, and the ground electrode was inserted contralaterally. The output was fed to an amplifier, viewed on an oscilloscope, and recorded. The average responses from 512 stimuli were obtained at 5 dB intervals near threshold. Threshold was defined as the lowest stimulus level at which a positive waveform in the evoked response trace was evident. Thresholds at each frequency were verified at least twice, and threshold shifts were calculated for individual animals by comparison to their threshold before treatments. The final ABR of each animal was taken and interpreted without knowledge of the treatment history.

**Histopathology of the cochlea**

Seven weeks after the start of drug administration, three animals randomly chosen from each group in the study of twice-daily protection were deeply anesthetized in a CO₂ chamber and decapitated, and their auditory bullae were removed. The round and oval windows and the apex of the cochlea were opened. The cochlea was perfused at 4°C with 4% paraformaldehyde in 10 mmol/L phosphate-buffered saline (PBS), pH 7.4, and kept in this solution overnight. The cochlea was then washed with cold PBS three times for 10 minutes each. Following the fixation, preparations of the organ of Corti were stained with rhodamine phalloidine for 45 minutes [27], microdissected into individual turns, and mounted on glass slides (GVA Mounting Solution; Zymed Laboratories, Inc., San Francisco, CA, USA). Hair cells were counted using fluorescence microscopy.

**Statistical analysis**

Data were statistically evaluated by analysis of variance with Newman-Keuls post hoc test for significance ($P < 0.05$) using Primer of Biostatistics software (McGraw-Hill Software, New York, NY, USA).

**RESULTS**

**Serum kinetics of gentamicin**

For a pharmacokinetic study, guinea pigs received different doses of gentamicin (60, 100, 120, or 180 mg gentamicin base/kg body weight). The uptake of gentamicin into serum was rapid, and serum levels essentially reflected the injected dose (Fig. 1).

**Ototoxicity of intraperitoneal gentamicin**

Significant threshold shifts developed within two weeks of intraperitoneal gentamicin treatment. For comparison, a parallel study was conducted assessing auditory deficits following subcutaneous injections of gentamicin (Fig. 2). The time course was essentially identical with damage progressing after the end of the two-week treatment and maximal threshold shifts reached by three weeks. A frequency gradient was also evident in both routes of application with the highest threshold shift of over 60 dB found at 18 kHz. The effects of intraperitoneal and subcutaneous application were similar at 18 and 8 kHz, but animals receiving gentamicin intraperitoneally sustained a significantly greater permanent hearing loss at 3 kHz ($P < 0.05$). Control animals on saline only maintained stable thresholds (data not shown).

**Protection from ototoxicity**

**Once-daily protection.** A single daily intraperitoneal injection of DHB and mannitol combined with gentamicin was partially effective in attenuating the gentamicin-induced threshold shift (Fig. 3). Protection was significant at 18 kHz ($P = 0.03$) but not at 3 kHz and 9 kHz ($P = 0.1$).

A study using α-lipoic acid as a protectant against ototoxicity was prematurely terminated. Intramuscular injections of α-lipoic acid had been successfully tested as a protectant against intramuscular amikacin [23]. However, combining gentamicin with intraperitoneal injections of 100 mg lipoic acid/kg body weight resulted in enhanced weight loss and death of several animals.
Fig. 2. Auditory deficits after intraperitoneal and subcutaneous injections. Guinea pigs from Elm Hill Breeding Laboratory were treated with 135 mg gentamicin/kg body wt daily for 14 days either by intraperitoneal (●) or subcutaneous (□) injection (N = 6 per group). Animals developed significant threshold shifts at 8 and 18 kHz (P < 0.05), which did not differ between the groups. At 3 kHz, however, animals receiving gentamicin intraperitoneally showed a significantly greater permanent threshold shift than animals treated by subcutaneous injection (P < 0.05).

Twice-daily protection. This study increased the daily antioxidant level and prolonged the period of protective treatment (Fig. 4). The result was a significant reduction of gentamicin-induced threshold shifts at all tested frequencies. At the end of the study (7 weeks), hearing loss was reduced from 56 to 28 dB at 18 kHz (P < 0.01), from 47 to 13 dB at 8 kHz (P < 0.01), and from 36 to 10 dB at 3 kHz (P < 0.01).
Animals treated with gentamicin only exhibited severe to almost complete loss of outer hair cells in all three rows of cells in the basal turn of the cochlea, with lesser damage extending into the middle and some damage into the apical turns (Fig. 6). This pattern of damage corresponds well to the more severe threshold shift at high frequencies (18 kHz) and the lesser effect of gentamicin on the physiological response at low frequencies (3 kHz). Coadministration of DHB with gentamicin significantly reduced damage to outer hair cells in all rows. Inner hair cells did not sustain significant damage with gentamicin treatment.

Renal function and serum levels of gentamicin

Blood samples from animals in the study on twice-daily protection (Figs. 4 to 6) were analyzed on day 14 for BUN, serum albumin, creatinine, and gentamicin levels. Drug treatment showed a marginally significant tendency to increase BUN (0.1 > P > 0.05; Table 1). There were no other significant changes in renal parameters, but all values from animals receiving gentamicin plus DHB were closer to controls than were the corresponding data from animals treated with gentamicin only.

Increased BUN correlated somewhat with serum gentamicin levels ($R^2 = 0.46$, gentamicin group, and $R^2 = 0.65$, gentamicin plus DHB). However, gentamicin levels overlapped between the groups (119 to 350 μg/mL, gentamicin only; 112 to 260 μg/mL, gentamicin plus DHB), and there was no significant difference in the mean values.

There was also no correlation between individual serum levels and resulting ototoxicity in the two treatment groups. At 18 kHz, five out of six animals receiving DHB...
coadministration had lower threshold shifts than the least affected animal in the gentamicin-only group despite the overlap of serum gentamicin values. Even the animals with the highest gentamicin levels in the “protected” gentamicin plus DHB group (241 and 260 μg/mL) had lower threshold shifts (29 and 30 dB at 18 kHz) than the animals with the lowest gentamicin levels in the gentamicin-only group (119 and 151 μg/mL; 44 and 63 dB). At 3 kHz, all threshold shifts in the DHB group were lower than in the gentamicin-only group.

DISCUSSION

Two salient points arise from this study. First, gentamicin has similar ototoxic potential whether given intraperitoneally or subcutaneously. Second, protection from ototoxicity caused by intraperitoneal injection of gentamicin can be achieved. This protection agrees with and extends earlier observations that DHB attenuates cochlear and vestibular toxicity of subcutaneously applied aminoglycosides [20, 21].

The first conclusion becomes evident by comparing the time course and pattern of hearing damage presented here to the well-documented pathology following systemic application of the drug [28]. Hearing loss usually

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**Table 1. Renal functional parameters and serum gentamicin levels**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BUN (mg/dL)</th>
<th>Creatinine (g/dL)</th>
<th>Albumin (mg/dL)</th>
<th>Gentamicin (μg/mL)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20 ± 3</td>
<td>0.53 ± 0.08</td>
<td>2.2 ± 0.1</td>
<td>—</td>
<td>6</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>32 ± 14*</td>
<td>0.58 ± 0.23</td>
<td>2.7 ± 1.0</td>
<td>243 ± 96</td>
<td>6</td>
</tr>
<tr>
<td>Gentamicin plus DHB</td>
<td>26 ± 12</td>
<td>0.50 ± 0.13</td>
<td>2.1 ± 0.3</td>
<td>191 ± 61</td>
<td>6</td>
</tr>
</tbody>
</table>

Blood was sampled from animals in the study of “twice-daily protection” (Figs. 4-6) on day 14 of treatment and analyzed as described in the Methods section. Data are means ± SD for samples from 6 animals per group.

*Different from controls, 0.1 > P > 0.05
develops in the course of chronic aminoglycoside administration requiring days to weeks to reach its maximal threshold shift. High frequencies, represented in the basal coils of the cochlea, are affected more than the low frequencies represented in the apical coils of the cochlea. The morphological basis of the hearing loss is primarily a destruction of outer hair cells in the organ of Corti.

It is not surprising that intraperitoneal application of gentamicin causes a pathology comparable to other systemic applications. Gentamicin uptake into serum is rapid from the peritoneum, and the resulting serum levels appear even higher than after similar doses injected subcutaneously [24, 29]. Consistent with this observation, both the functional deficit at 3 kHz and the corresponding hair cell loss near the apex are significantly greater following intraperitoneal injection.

The similar pattern of ototoxicity after subcutaneous and intraperitoneal injections in the guinea pig agrees well with clinical observations. In humans, gentamicin predominantly affects vestibular function, and the intraperitoneal route is not safer than intravenous or intramuscular administration [30]. However, treatment with DHB also protects from the vestibular side effects of aminoglycosides, as shown for gentamicin [20] and streptomycin [21] in guinea pigs. While ototoxicity may develop regardless of the route of administration, the actual incidence of loss of hearing or balance in patients will depend on a number of additional parameters. Generally, the amount of drug and the duration of treatment are determining factors. Metabolic stress resulting from infections or malnutrition may predispose toward the adverse effects of aminoglycosides [31]. Nutritional supplements may therefore be indicated to reduce the risk of ototoxicity, but the addition of iron would be detrimental. Iron supplementation in experimental animals enhances both aminoglycoside nephrotoxicity and ototoxicity [32–34].

Aminoglycoside antibiotics have both ototoxic and nephrotoxic potential. However, nephrotoxicity and ototoxicity are not necessarily expressed together in experimental animals or in patients [35]. In guinea pigs, the ototoxic effects of gentamicin are far more pronounced than the nephrotoxic side effects. It is important to consider that impaired renal function may lead to higher drug serum levels, potentially resulting in increased ototoxicity. A therapeutic intervention might reduce ototoxicity under such circumstances by restoring renal function and lowering drug serum levels. In our study, parameters of renal function are only slightly elevated, and therefore, it is difficult to evaluate any beneficial effects of DHB co-treatment on the kidney. However, DHB may attenuate aminoglycoside-induced nephrotoxicity in the rat [25].

Most significant for an interpretation of the action of DHB on ototoxicity is the fact that serum gentamicin levels in the two treatment groups did not correlate with the resulting ototoxicity. The protective action of DHB is thus not based on an indirect effect through decreasing drug serum levels, but is consistent with the hypothesis that gentamicin exerts its toxicity via the formation of an iron complex catalyzing superoxide production [16, 18]. DHB, as an iron chelator and antioxidant, would lower available free iron, reducing the formation of the redox active iron-gentamicin complex and possibly also suppressing subsequent Fenton reactions [29]. It remains to be established whether these interactions occur locally in the cochlea or in other compartments. It is also unresolved whether antioxidants could attenuate hearing loss in cases of hypersensitivity to aminoglycosides based on a mutation in location 1555 of the mitochondrial ribosomal RNA [36]. In patients carrying this mutation, a single injection may cause profound hearing loss, although the mutation does not enhance vestibular sensitivity to aminoglycosides. Little is known about the underlying mechanisms, and it is impossible to predict the efficacy of any prophylactic treatment.

While a single daily administration of DHB provided limited protection, a twice-daily DHB regimen was highly effective at all tested frequencies. The reduction of a 60 dB threshold shift (at 18 kHz) to less than 30 dB corresponds to a reduction from a “severe” to a “moderate” hearing loss. It also speaks to the efficacy of the treatment that protection is achieved at drug concentrations (135 mg/kg body weight × 14 days) far exceeding the human clinical usage. Moreover, DHB and other antioxidants do not interfere with the antibacterial activity or the serum levels of aminoglycosides [20, 21, 28], critical points in considering a clinical application.

2,3-Dihydroxybenzoate was initially intended as an aspirin analogue [37] and later as an iron-chelating agent [38]. It was tested clinically in patients suffering from thalassemia major. At a dose of 100 mg/kg/day for one year, no significant side effects were apparent, and patient tolerance to this drug was good [26]. Likewise, DHB had negligible or no toxicity in experimental animals [38, 39], and only very high doses (500 mg/kg) were associated with renal toxicity in rats [40]. We tested up to 300 mg DHB/kg in guinea pigs and saw no detrimental effects on hearing thresholds or histopathology [21]. Moderate doses (100 mg/kg) even reduced gentamicin-induced damage to the kidney [25], suggesting that the mechanisms of ototoxicity and nephrotoxicity are similar. Consequently, DHB appears to have an almost ideal potential as a protective agent in patients receiving aminoglycosides. However, it is currently not a clinically approved drug.

In summary, the principle of an antioxidant therapy, developed from systematically injected gentamicin, is also applicable to intraperitoneally administered gentamicin. Other recently confirmed antioxidant antidotes to aminoglycoside ototoxicity include salicylate [29] and D-methionine [41]. Since antioxidant therapy is a well-established
clinical treatment, it should be possible to find protective agents that are suitable for peritoneal application combining safety with efficacious uptake from the peritoneum.

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Reprint requests to Dr. Jochen Schacht, Kresge Hearing Research Institute, 1301 East Ann Street, Ann Arbor, Michigan 48109-0506, USA.

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