Tanshinone (Salviae miltiorrhizae Extract) Preparations Attenuate Aminoglycoside-Induced Free Radical Formation In Vitro and Ototoxicity In Vivo

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Antioxidant therapy protects against aminoglycoside-induced ototoxicity in animal models. A clinically suitable antioxidant must not affect the therapeutic efficacy of aminoglycosides or exhibit any side effects of its own. In addition, the treatment should be inexpensive and convenient in order to be implemented in developing countries where the use of aminoglycosides is most common. Standardized Salviae miltiorrhizae extracts (Danshen) are used clinically in China and contain diterpene quinones and phenolic acids with antioxidant properties. We combined in vitro and in vivo approaches to investigate the effect of a clinically approved injectable Danshen solution on aminoglycoside-induced free radical generation and ototoxicity. In vitro, Danshen inhibited gentamicin-dependent lipid peroxidation (formation of conjugated dienes from arachidonic acid), as well as the gentamicin-catalyzed formation of superoxide (in a lucigenin-based chemiluminescence assay) and hydroxyl radicals (oxidation of N,N-dimethyl-p-nitrosoaniline). Danshen extracts were then administered to adult CBA mice receiving concurrent treatment with kanamycin (700 mg/kg of body weight twice daily for 15 days). Auditory threshold shifts induced by kanamycin (approximately 50 dB) were significantly attenuated. Danshen did not reduce the levels in serum or antibacterial efficacy of kanamycin. These results suggest that herbal medications may be a significantly underexplored source of antidotes for aminoglycoside ototoxicity. Such traditional medicines are widely used in many developing countries and could become an easily accepted and inexpensive protective therapy.

The side effects of aminoglycoside therapy on the kidneys (nephrotoxicity) and the inner ear (ototoxicity) have largely limited the use of these drugs in a trend promoted in industrialized countries by the development of new, less toxic, alternative antibiotics. In developing countries, however, aminoglycosides remain the antibiotics of choice because of their efficacy, low cost, and easy availability as over-the-counter drugs. Furthermore, the World Health Organization recommends treatment of the rapidly increasing cases of multidrug-resistant tuberculosis with a regimen that includes aminoglycosides, primarily amikacin or streptomycin. This extensive use makes aminoglycosides probably the most commonly used antibiotics worldwide. Ototoxicity associated with aminoglycoside therapy is the major cause of preventable drug-induced hearing loss today.

Aminoglycosides have the ability to catalyze the formation of free radicals in vitro and in intact cells as well as in explants of the inner ear by a mechanism that may involve metal chelation. Free-radical formation as an underlying cause of ototoxicity has received strong support from the fact that antioxidants attenuate aminoglycoside-induced loss of hearing and balance in guinea pigs and mice in vivo. Effective protective agents include iron chelators and antioxidants, such as 2,3-dihydroxybenzoate, deferoxamine, d-methionine, and salicylate.

In recent years, drug development has rediscovered the potential value of phytopharmaceuticals, and their incorporation into medical care has been encouraged by the World Health Organization’s Traditional Medicines Strategy. At the same time, folk remedies in many developing countries have undergone a change from unspecified herbal extracts (or the actual herbs) to well controlled and chemically analyzed preparations. The traditional Chinese medicine Danshen, derived from the dried root or rhizome of Salviae miltiorrhizae Bge, is an example of such a standardized medication. Approved for clinical use in China, indications for Danshen include treatment of angina pectoris and cerebrovascular disorders. Danshen extracts contain diterpene quinone and phenolic acid derivatives, including tanshinone (I, IIA, and IIB), cryptotanshinone, isocryptotanshinone, miltirone, tanshinol (I and II), and salviol. These compounds have antioxidant properties and protect against lipid peroxidation in vitro and in vivo.

In an effort to improve on prophylaxis of aminoglycoside-induced hearing loss without jeopardizing acceptability and affordability in developing countries, we investigated the effect of Danshen on aminoglycoside-induced free radical generation in vitro and ototoxicity in vivo. The CBA mouse was used because this strain is well established in auditory research and does not carry genes (e.g., ahl) that may predispose to premature hearing loss or sensitivity to stress. Furthermore, the response of CBA mice to ototoxic aminoglycoside antibiotics has been well characterized.

MATERIALS AND METHODS

Materials. Injectable Salvia miltiorrhiza was obtained from Shanghai 1st Pharmaceutical Factory (Shanghai, China). This is a standardized injectable preparation approved for clinical use (approval number 001065; 1995; Department of Drug Administration, Shanghai, China) and widely available over the counter. Lot number 011101 from 20 November 2001 was used in our experiments.
Kanamycin sulfate was purchased from USB Corporation (Cleveland, Ohio) (catalog no. 17924, lot no. 110755), gentamicin sulfate was purchased from Spectrum Chemical Mfg. Corp. (Gardena, Calif.), ketamine (Ketaset) was purchased from Fort Dodge Animal Health (Fort Dodge, Iowa), xylazine (Tranqyl-V) was previously described in detail (15). Sodium phalloidin was purchased from Molecular Probes Inc. (Eugene, Ore.). All other reagents came from Sigma Chemical Co. (St. Louis, Mo.).

In vitro experiments. (i) Superoxide formation. The antioxidant properties of Danshen against gentamicin-induced superoxide formation were tested in a chemiluminescence assay according to the method of Gyllenhammar (6) with modifications. Purified superoxide anion was generated using lucigenin (20 μM) dissolved in dimethyl sulfoxide in antifade mounting medium. The reaction mixture (20 μM) contained 50 mM sodium phosphate buffer (pH 7.4), 0.05 mM CuCl2 with or without 0.1 mM gentamicin sulfate and various concentrations of Danshen. The absorption measurements were recorded at 25°C on a Perkin-Elmer Lambda 9 spectrophotometer at the characteristic wavelength of NDMA, 440 nm. Hydroxyl radical formation was calculated from the change in NDMA absorbance using an extinction coefficient of 34 × 10⁻⁴ M⁻¹ cm⁻¹.

(ii) Lipid peroxidation. Arachidonic acid peroxidation was monitored by measuring conjugated diene levels spectrophotometrically according to the method of Buege and Aust (1). The reaction mixtures (200 μM) contained 50 mM sodium phosphate buffer (pH 7.4), 0.25% arachidonic acid (vol/vol), 0.5 mM H2O2, and 0.05 mM CuCl2 with or without 0.1 mM gentamicin and various concentrations of Danshen. After incubation for 60 min at 37°C, the reaction was stopped by the addition of 1 ml of chloroform-methanol (2:1 [vol/vol]). Following mixing and centrifugation, the lower organic phase material was removed and dried at 45°C overnight and the residue, and absorbance was read at 235 nm. Concentration of conjugated dienes was calculated from an extinction coefficient of 2.52 × 10⁻³ M⁻¹ cm⁻¹.

In vivo experiments. (i) Experimental animals and drug administration. Male CBA/J (CBA) mice, at an initial age of 4 weeks, were purchased from Harlan Sprague-Dawley Co. (Indianapolis, Ind.). The animals were given free access to water and a regular mouse diet (Purina, St. Louis, Mo.), and were allowed 1 week to acclimate before treatment. All experimental protocols involving animal use were approved by the University of Michigan Committee on Use and Care of Animals. Animal care was supervised by the University of Michigan’s Unit for Laboratory Animal Medicine.

Kanamycin (700 mg of kanamycin base/kg of body weight twice daily) was tested alone or in combination with various concentrations of Danshen. It should be noted that the concentration of Danshen is given by the manufacturer as the equivalent to the original herb: “1 g of Danshen” is the amount of extract derived from 1 g of herb. The study to evaluate auditory effects was comprised of one group serving as the saline control group (n = 11), one group receiving kanamycin injections only (n = 14), five groups (n = 5 each) receiving kanamycin plus Danshen at 1, 4, 6, 10, or 20 g/kg twice daily, and three groups (n = 3 each) receiving Danshen only at 6, 10, or 20 g/kg twice daily. In a separate study, levels of kanamycin in serum were determined for animals receiving either kanamycin alone (700 mg of kanamycin base/kg twice daily) or kanamycin plus Danshen (6 or 10 g/kg twice daily). Kanamycin and Danshen were each dissolved in 0.9% sterile saline, and subcutaneous injections were given separately but simultaneously twice daily for 15 days. Control mice received an equivalent volume of saline.

(ii) Evaluation of auditory function. Auditory thresholds were measured by evoked auditory brainstem responses (ABR). Thresholds were taken for each animal at the beginning of the study, 2 weeks after the start of drug treatment, and then at 3 weeks and 5 weeks. ABR measurements were performed as described previously (22). In brief, the mice were anesthetized with an intramuscular injection of 100 mg of ketamine and 5 mg of xylazine/kg and kept warm with a heating pad. The positive needle electrode was subdermally inserted at the vertex, the midline of the scalp between the external auditory canals. The negative electrode was placed below the pinna of the left ear, and the ground electrode was inserted contralaterally. Tone bursts of 12 and 24 kHz (10 ms duration, 10 ms rise and fall time) were presented at a rate of 10 Hz (full time) with a 90% duty cycle in a closed acoustic system through an ear bar con- nected to a DT-48 transducer (Beyer Dynamic, Farmingdale, N.Y.). Responses from 1020 stimuli were averaged for each frequency, fed to an amplifier, viewed with an oscilloscope, and recorded. Threshold was determined by reducing the sound intensity in 5 dB steps until threshold. Thresholds were defined as the lowest intensity that yielded a reproducible detectable response. Both peak and trough deflection in the ABR were measured and visualized twice at each threshold. All thresholds were bracketed by kanamycin standards which served to normalize the data.

Antimicrobial activity. Efficacy of kanamycin alone and in the presence of Danshen was tested against Escherichia coli (ATCC no. 25922) in a standardized microbiological assay (2). Ten microdrops of kanamycin (100 μg/ml) was dispensed onto 1-cm filter paper disks that had been placed on circular 100-mm agar plates previously inoculated with E. coli. Subsequently, 10-μl aliquots of different concentrations of Danshen (0.15 mg/ml, 1.5 mg/ml, and 15 mg/ml) were added to the disks. The concentration of kanamycin was derived from a dose-response curve relating concentration to inhibition zone size; the concentrations of Danshen were chosen to yield the same ratio to kanamycin as administered in the in vivo study, flanked by a concentration 10-fold higher and another 10-fold lower. Kanamycin only was assayed directly or with 10 μl of saline instead of Danshen; since the resulting inhibition zones were not significantly different (P > 0.05), these two groups were collapsed for later statistical comparison to kanamycin plus Danshen. Additionally, kanamycin (100 μg/ml) and Danshen (15 mg/ml) were added together (full time) with a 90% duty cycle in a closed system through a DT-48 transducer. Control disks with Danshen only were also plated. The inoculated plates were incubated overnight at 37°C in an incubator. The diameter of the...
inhibition zones was measured with a caliper to the nearest 0.01 mm across each disk. Statistical analysis. Data were statistically evaluated by Student’s t test and by analyses of variance with a Student-Newman-Keuls posthoc test for significance (P < 0.05) using Primer of Biostatistics software (McGraw-Hill Software, New York, N.Y.).

RESULTS

Danshen inhibits gentamicin-catalyzed free radical formation and lipid peroxidation in vitro. For the in vitro experiments, gentamicin concentrations were selected based on previous studies of gentamicin-stimulated metal-catalyzed free radical formation (9, 10, 13). Gentamicin binds Cu(II), forming 1:1 complexes which catalyze hydrogen peroxide disproportionation at pH 7.4 in a reaction involving hydroxyl radical formation. We used a 1:2 molar ratio of Cu(II)-gentamicin to maintain Cu(II) ions in the form of the Cu(II)-gentamicin complex and an excess of H₂O₂ to promote efficient hydroxyl radical formation.

Gentamicin (1 mM) stimulated the formation of lucigenin luminosity, presumably an indicator of superoxide, consistent with our earlier observations (16). Danshen at concentrations ranging from 0.1 to 0.25% significantly suppressed gentamicin-dependent luminosity (P < 0.05). A 50% inhibition of luminosity was observed with 0.15% Dasen (Fig. 1A).

Hydroxyl radical formation was monitored by the oxidation of the indicator NDMA, which depended on the presence of gentamicin: 0.1 mM gentamicin increased hydroxyl radical formation from 0 to 3.23 nM/min. Danshen inhibited this gentamicin-catalyzed formation of hydroxyl radicals at each concentration (P < 0.05). Danshen decreases gentamicin-induced lipid peroxidation. Lipid peroxidation was measured as described in Materials and Methods. The concentration of Danshen is given as the final concentration of the commercial extract in the assay. Data are means ± standard deviations for five experiments per condition. All values in the presence of Danshen are significantly different from gentamicin alone (P < 0.05).

Conjugated diene formation from arachidonic acid was significantly increased by the addition of 0.1 mM gentamicin to the H₂O₂-Cu(II) system. At concentrations ranging from 0.25 to 5%, Danshen effectively suppressed gentamicin-induced lipid peroxidation (P < 0.05), achieving a 50% inhibition around 0.2% (Fig. 1C).

Danshen attenuates kanamycin-induced threshold shifts. Auditory thresholds (hearing sensitivity) as assessed by ABR were comparable for all animals at the beginning of the study. Saline-injected animals maintained stable thresholds throughout the course of treatment. In contrast, animals receiving kanamycin (700 mg/kg twice daily) developed a progressive
hearing loss (Fig. 2). This dosage had previously been established as well tolerated in CBA mice while producing a consistent threshold shift and hair cell loss (22). Auditory thresholds were significantly elevated after 15 days of treatment and remained elevated after cessation of treatment. Following the common pattern of aminoglycoside ototoxicity, the functional deficit was always greater at the higher frequency, and by the end of the fifth week, threshold shifts averaged about 45 dB at 24 kHz and 36 dB at 12 kHz.

The effect of Danshen on the kanamycin-induced threshold shifts was dose dependent. The dosing range had been selected based on experiments with several animal species showing protective effects of 1 to 4 g of extract/kg on myocardial infarct size or aflatoxin-induced hepatocarcinogenesis (11, 25). No effect on kanamycin-induced threshold shifts was observed at 1 g/kg twice daily, while 4 and 6 g/kg twice daily provided a small but significant ($P < 0.05$) reduction of the threshold shift at 24 kHz only. A consistent attenuation at both 12 and 24 kHz was seen with 10 g or 20 g of Danshen/kg twice daily. Concurrent treatment with $2 \times 10$ g of Danshen lowered the final kanamycin-induced threshold shifts to $25 \pm 11$ dB at 24 kHz and to $17 \pm 10$ dB at 12 kHz ($P < 0.05$; Fig. 2). A concentration of $2 \times 20$ g of Danshen/kg likewise yielded a significant protection at both frequencies ($27 \pm 13$ dB at 24 kHz, $13 \pm 7$ dB at 12 kHz; $P < 0.05$) but no improvement over the treatment with $2 \times 10$ g of

**FIG. 2.** Danshen reduces kanamycin-induced threshold shifts. Kanamycin (700 mg/kg twice daily) and Danshen (10 g/kg twice daily) were administered, and ABR thresholds were measured at 24 kHz (A) and 12 kHz (B) as described in Materials and Methods. Data are means ± standard deviations. Filled circles (●), saline controls, $n = 11$; filled squares (■), kanamycin alone, $n = 14$; filled triangles (▲), kanamycin plus Danshen, $n = 5$; cross (x), Danshen alone, $n = 3$. Cotreatment with Danshen significantly attenuated kanamycin-induced threshold shifts at all times ($P < 0.05$).

**FIG. 3.** Danshen reduces kanamycin-induced hair cell loss. Cytocochleograms were obtained from the surface preparations of the organ of Corti as described in Materials and Methods. The percentage of missing hair cells is plotted for the entire length of the cochlea. In the saline-treated CBA mice, outer hair cells in the basal turn were almost completely present (A). After treatment with kanamycin (700 mg/kg twice daily), most of the outer hair cells in the basal turn of the cochlea disappeared (B). Animals receiving kanamycin plus Danshen (10 g/kg twice daily) demonstrated less outer hair cell loss (C) than animals receiving kanamycin alone. Heavy solid line, inner hair cells; light solid line, outer hair cells of row 1; dashed line, outer hair cells of row 2; solid line with dots, outer hair cells of row 3.
Danshen (10 versus 20 g; \( P > 0.05 \)). Danshen alone up to 20 g/kg twice daily had no effect on auditory thresholds.

**Histopathology.** Cochlear pathology (Fig. 3) reflected the functional results obtained from the ABR measurements. Control animals receiving saline injections had a normal complement of outer hair cells with a loss of less than 10% scattered throughout the length of the cochlea (Fig. 3A). Kanamycin-treated animals (700 mg of kanamycin/kg twice daily) exhibited severe to complete hair cell loss in all three rows of outer hair cells in the basal turns of the cochlea, while cells in the apex remained intact. Inner hair cells appeared to be preserved (Fig. 3B). Coadministration of Danshen (10 g/kg twice daily) significantly reduced damage to hair cells, although a 30 to 50% loss was still apparent at the very base of the cochlea (Fig. 3C).

**Serum kanamycin levels.** Levels of kanamycin in serum were measured at 20 min and at 1 h in animals receiving kanamycin (700 mg/kg twice daily) or kanamycin plus Danshen (6 or 10 g/kg twice daily). The selected times correspond to peak levels in serum (20 min) and the approximate half-life (1 h) of kanamycin in CBA mice (22). The level of kanamycin in serum at 20 min was 271 ± 61 μg/ml (mean ± standard deviation; \( n = 5 \)) in the absence of Danshen and remained unchanged in its presence (280 ± 6 μg/ml; \( n = 3 \)). At one h, the values were 108 ± 86 μg/ml in the kanamycin group (\( n = 10 \)) and 175 ± 27 μg/ml in the group receiving kanamycin plus Danshen (10 g/kg twice daily; \( n = 5 \)). Although the values at 1 h were not statistically different, a trend towards higher serum levels in the presence of Danshen seemed supported by somewhat elevated levels in a small group of mice cotreated with 6 g of Danshen/kg twice daily, 137 ± 9 μg/ml (\( n = 3 \)).

**Antimicrobial efficacy.** Growth inhibition zones created by kanamycin on a lawn of *E. coli* were determined as a measure of antimicrobial efficacy. The concentration of kanamycin for this assay had been derived from a dose-response curve indicating that a 20% reduction of the effective kanamycin concentration would lead to a reduction in zone size of 1 mm. Ten microliters of kanamycin (100 μg/ml) created an inhibition zone of 14.3 ± 0.9 mm. The additional presence of 10 μl of different concentrations of Danshen (0.15, 1.5, and 15 mg/ml) gave inhibition zones of 14.6 ± 0.14 mm, 15.0 ± 0.8 mm, and 14.6 ± 1.0 mm, respectively (all values are mean ± standard deviation; \( n = 18 \) each). These zone sizes were not different from those with kanamycin alone (\( P > 0.05 \)), indicating that there was no effect of Danshen on the antimicrobial efficacy of kanamycin. Incubation of kanamycin with Danshen for 30 min prior to plating likewise did not affect the inhibition zone size. Danshen plated in the absence of kanamycin had no effect on the growth of the bacterial culture.

**DISCUSSION**

The results of this study suggest that traditional medicines hold promise as pharmacological protectants against aminoglycoside-induced hearing loss. Both the physiological measurement (ABR) and the morphological assessment of hair cell loss showed significant protection by Danshen against the ototoxicity of kanamycin at both the structural and functional levels. Kanamycin induced a massive destruction of outer hair cells at the base of the cochlea where high frequencies are being processed. The apical region was less affected, consistent with a lesser threshold shift at 12 kHz than at 24 kHz. The reduction of hair cell loss in the middle and the apex of the cochlea by cotreatment with Danshen was consistent with the observed protection against functional loss at both 12 and 24 kHz. The preferential loss of outer hair cells at the base and the apparent sparing of inner hair cells is a general pattern of aminoglycoside-induced ototoxicity. While a correlation between preservation of hair cells and function along the length of the cochlea is obvious in our experiments, a quantitative relationship cannot be expected. The histology (cytocochleogram) assesses the presence of sensory cells without insight into possible structural changes that might affect function. Conversely, a small and scattered loss of hair cells as seen in control animals or in the apex of treated animals may not lead to a noticeable threshold shift. Hence, both morphological and functional measures have to be combined to determine ototoxic actions and protection.

Danshen has been widely used clinically in China since its introduction in the 1960s as an effective remedy for cerebrovascular disorders, angina pectoris, and hypertension with minimal side effects (25). Its components provide a wide spectrum of antioxidant activity. Tanshione and tanshinol derivatives protect against lipid peroxidation in vitro and in vivo (12, 24). Three of the water-soluble components, salvianolic acid A, salvianolic acid B, and rosmarinic acid, inhibited NADPH- vitamin C- and Fe(II)-cysteine-induced lipid peroxidation in microsomes and the production of superoxide in a xanthine-xanthine oxidase system (8). Another ingredient, magnesium lithospermate B, is a hydroxyl radical scavenger (23). In addition to a direct antioxidant action, Danshen may protect against oxidant stress in vivo by regulating the activities of antioxidant enzymes. Sodium tanshinone IIA sulfonate protected against doxorubicin hydrochloride (Adriamycin)-induced lipid peroxidation in mouse hearts by increasing the activities of endogenous antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, and catalase (24). As we show here, Danshen acts as an antioxidant against aminoglycoside-induced free-radical formation and lipid peroxidation, showing similar efficacy in three different in vitro assay systems. Whether Danshen also influences gene activation in the inner ear remains to be established. In any case, the correlation between antioxidant actions in vitro and the attenuation of hearing loss in vivo reinforces the connection between free radical formation and ototoxicity (5).

The dosage of kanamycin used here by far exceeds dosages that produce ototoxicity in other animals, such as guinea pigs, or that are commonly administered to patients in antimicrobial therapy. In fact, on a body weight basis, the kanamycin dose for CBA mice is 100 times the human dose. These high doses, however, are well tolerated by the mice without signs of nephrotoxicity, and the necessity for such high concentrations to achieve auditory damage in mice has been discussed at length elsewhere (22). The reasons may include different pharmacokinetics in small animals and species differences in susceptibility. The dose of Danshen (per kilogram of body weight) used in our experimental animals is also higher than in humans but to a lesser degree than in the case of kanamycin. In clinical applications, Danshen is administered intramuscularly at 9 to 15 g per day (25) or intravenously at up to 24 g/day, corre-
sponding to 0.2 to 0.3 g/kg. A salient point to consider here is the fact that the efficacy of protection depends on the relative concentrations of the aminoglycoside and the protectant (22). Since Danshen is effective in the presence of high levels of kanamycin in animals, it should be equally or more effective at clinical concentrations of aminoglycosides. The clinical equivalent of the model studied here (kanamycin injections for 2 weeks) would be a treatment for acute infections. Tuberculosis patients, however, may receive aminoglycosides for up to 6 months, and it is an important question whether Danshen could be useful in such a protracted regimen. An extension of our experiments would be interesting, but at the moment, no animal model exists that mimics extremely long-term, low-dose application of aminoglycosides.

A crucial question in view of a possible clinical application of Danshen is potential interference with the pharmacological activity of kanamycin. No such interference seems to exist. First, Danshen does not lower levels of kanamycin in serum, which would be a confounding factor in establishing its protective potential. In fact, the trend towards higher serum levels in its presence rules out kanamycin pharmacokinetics as an explanation of the attenuation of auditory damage. Second, Danshen does not adversely affect the antibacterial efficacy of kanamycin even when present at a 10-fold-higher ratio than is administered in vivo. Since traditional medicines are frequently used in many developing countries, they could easily become an accepted and inexpensive therapeutic prophylaxis in many developing countries, they could easily become an accepted and inexpensive therapeutic prophylaxis in many developing countries. Tuberculosis patients, however, may receive aminoglycosides for up to 6 months, and it is an important question whether Danshen could be useful in such a protracted regimen. An extension of our experiments would be interesting, but at the moment, no animal model exists that mimics extremely long-term, low-dose application of aminoglycosides.

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