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Main Article

Protective effect of melatonin against gentamicin ototoxicity

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Abstract

Objective: To research the protective effect of melatonin against gentamicin ototoxicity.

Methods: Guinea pigs were randomly divided into four groups. The first group received intramuscular gentamicin (120 mg/kg body weight/day) for 17 days. Over the same time period, a second group simultaneously received intramuscular gentamicin (120 mg/kg body weight/day) plus (on the other side) intramuscular melatonin (0.3 ml kg body weight/day). Two groups of controls were treated for 17 days with either intramuscular melatonin or intramuscular saline. After the 17 days, each animal underwent distortion product otoacoustic emission testing (both ears). The guinea pigs were sacrificed by decapitation just after the final injection. Their cochleae were used to produce a tissue section, surface preparation and scanning electron microscope preparation.

Results: Distortion product otoacoustic emission testing indicated gentamicin-induced hearing loss at 3, 4, 6 and 8 kHz in gentamicin-treated animals. Animals receiving melatonin co-therapy had significantly attenuated hearing loss and their cochleae showed lower rates of outer hair cell loss (comparing the same cochlear turns), compared with gentamicin-treated animals ($p < 0.01$).

Conclusion: These findings confirm the occurrence of outer hair cell loss after gentamicin treatment, and the attenuation of such loss following simultaneous melatonin injection, using the method of morphological evaluation. These results suggest that melatonin protects against gentamicin ototoxicity by interfering with cytotoxic mechanisms.

Key words: Gentamicin; Toxicity; Inner Ear; Melatonin

Introduction

Gentamicin is widely used in the treatment of infections by Gram-negative bacteria.[1] The use of gentamicin has been limited by its toxic effect on both the cochlea and the vestibular apparatus. However, it remains indispensable, even today when many new antibiotics with fewer side effects are available; in fact, its high efficacy, broad spectrum of activity and low cost probably make it the most commonly used antibiotic in developing countries.

Attempts to reduce the severity and incidence of aminoglycoside antibiotic side effects have been unsuccessful, and studies frequently report contradictory findings.[2,3] Recent studies have demonstrated that gentamicin can chelate iron and form a compound with oxidative properties. As a consequence of this binding to iron, gentamicin becomes a redox-active compound which can promote the formation of free radicals.[2] Free radicals are highly reactive compounds, as they have an unpaired electron which can oxidise many important molecules such as lipids, proteins and deoxyribonucleic acid (DNA).[4] In cases of aminoglycoside ototoxicity, a variety of free radical species (including both oxygen and nitrogen types) have been detected in the inner ear, and these are believed to initiate the apoptotic cascade.[5]

Melatonin is considered the principal secretory product produced by the pineal gland. It is a direct free radical scavenger and an indirect antioxidant. Regarding its scavenging activity, melatonin has been shown to quench the hydroxyl radical, superoxide anion radical, singlet oxygen, peroxyl radical and peroxynitrite anion. In addition, melatonin's antioxidant actions probably derive from its stimulatory effect on superoxide dimutase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase, as well as its inhibitory action on nitric oxide synthase. Melatonin also acts to stabilise cell membranes, thereby making them more resistant to oxidative attack.[6]

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The purpose of this study was to investigate whether melatonin could protect against gentamicin ototoxicity in guinea pigs, by determining distortion product otoacoustic emissions and by observing tissue morphology via light and scanning electron microscopy.

Materials and methods

Experimental groups

Adult pigmented guinea pigs (supplied by the Academy of Science in China, Wuhan, Hubei, PR China) weighing 250–300 g were used. The guinea pigs were randomly divided into four groups, to receive: gentamicin only (group one), gentamicin plus melatonin (group two), melatonin only (group three) or saline (group four). Group one animals were injected with intramuscular gentamicin sulphate (120 mg gentamicin base/kg body weight). Group two animals were injected with intramuscular gentamicin (120 mg gentamicin base/kg body weight) and simultaneously on the other side with intramuscular melatonin (0.3 ml melatonin base/kg body weight; Sigma, Louis, USA). Group three animals were injected with intramuscular melatonin only (0.3 ml melatonin base/kg body weight). Control group animals (group four) were injected with a volume of intramuscular saline equivalent to the volume of intramuscular gentamicin received by group one animals. All animals were injected daily for 17 days.

In group one, 13 animals survived and five died. In group two, 13 animals survived and two died. In group three, 11 survived and no animal died. In group four, 11 survived and no animal died.

Distortion product otoacoustic emissions

After 17 days of injections, each animal underwent distortion product otoacoustic emission testing, for both ears, while conscious. Stimulus presentation, data recording, averaging and spectrum analysis were conducted using Madsen Celesta 503 hardware and software (Madsen, Copenhagen, Danmark). The primary parameter for the distortion product otoacoustic emission protocol (denoted as f_1 and f_2) were set in order to achieve an f_2/f_1 ratio of 1.22 at intensity levels of 70 dB SPL for f_1 and 65 dB SPL for f_2. A probe fit routine specified by the manufacturer was used to ensure proper placement of the probe tip in the ear canal. The distortion product otoacoustic emission amplitude was recorded at the following frequencies: 0.75, 1, 1.5, 2, 3, 4, 6 and 8 kHz. Distortion product otoacoustic emission test results were plotted graphically; Celesta 503 software was used to plot the cubic difference frequency ($2f_1 - f_2$) as a function of the geometric mean of the two primary parameter. The geometric mean of the primary parameter has been reported to be a possible site for generation of the distortion product on the basilar membrane.[7]

Cochlear section

At the end of testing, the guinea pigs were deeply anaesthetised with sodium pentobarbital and decapitated. An apical opening was made in the extirpated left cochlea and the organ was perfused with 0.1 M sodium cacodylate, 2 per cent paraformaldehyde, 2 per cent glutaraldehyde and 2 mM calcium chloride. After 24 hours of gentle rotation in the same fixative at 4°C, cochleae were rinsed in a phosphate buffer at 4°C for two days. Excess bone was removed and the cochleae were then decalcified in 3 per cent ethylene diamine triacetic acid at 4°C with gentle rotation for two to three weeks. Specimens were then dehydrated via graded series of alcohol and embedded in 812 resin (6 mm) in a para-modiolar plane. Every third mid-modiolar section was mounted on a glass slide and stained with Richardson's stain. Four left cochleae (two from group one animals and two from group two animals) were processed under double-blind conditions. Two comparable mid-modiolar sections from each animal were assessed using bright-field and differential interference contrast optics on an Olympus light photomicroscope (Olympus, Tokyo, Japan).

Surface preparation

The right cochleae were quickly removed and perfused through the oval window with a silver nitrate (0.5 per cent 10 ml) and formaldehyde (10 per cent 10 ml) staining solution. Cochleae were then post-fixed with 10 per cent formalin and stored in a fixative. Stained cochleae were dissected from the apex to the base, mounted in sections in glycerine and examined under light microscopy ($\times 400$ magnification).

Scanning electron microscopic preparation

Two organ of Corti specimens were observed by scanning electron microscopy. These specimens were dissected out and placed in 7 per cent ethanol. After dehydration in graded alcohols, the specimens were dried by the critical point method (CO_2; Balzers CPD 010) (Bothde, Wuhan, China), mounted on stubs with double-coated adhesive tape (Fullam, Wuhan, Hubei) and covered with a thin layer of gold-palladium (7–8 nm thick) in an anion sputter coating unit (Balzers SCD 030) (Bothde, Wuhan, China). A Philips 505 scanning electron microscope (Philips, Best, Netherlands) was used to examine the specimens.

Statistical analysis

Groups were compared using the one-way Kruskal–Wallis analysis of variance (ANOVA) and the post-ANOVA Tukey-B test. Differences at the level of 5 per cent were considered statistically significant.

Results

Distortion product otoacoustic emissions

Animals injected with melatonin or saline maintained essentially stable hearing at all of the distortion product otoacoustic emission test frequencies (Figure 1). Animals receiving gentamicin showed the expected hearing loss; decreased

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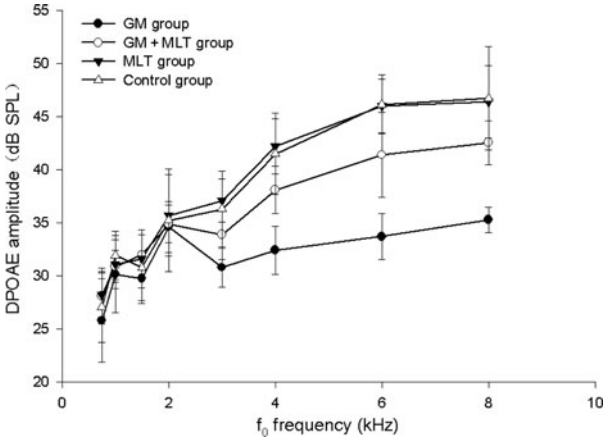

FIG. 1

Distortion product otoacoustic emission (DPOAE) amplitudes at the test frequencies, for the four groups.

distortion product otoacoustic emission amplitudes were detected between 3 and 8 kHz. Animals treated with gentamicin plus melatonin showed reduced lower hearing loss at all frequencies, compared with the gentamicin group; this difference was statistically significant at 3, 4, 6 and 8 KHz ($p < 0.01$).

Cochlear histopathology

Cochleae from two group one animals and two group two animals were randomly examined histopathologically under light microscopy. The observed morphology supported the electrophysiological findings, confirming that melatonin treatment exerted a protective effect on outer hair cells. Cochlear sections from the gentamicin group showed almost complete loss of outer hair cells (Figure 2a), consistent with the documented pattern of aminoglycoside-induced pathology.[8] In contrast, less outer hair cell loss was apparent in animals treated with both gentamicin and melatonin (Figure 2b).

Surface preparation and outer hair cell loss

All cochleae were examined under light microscopy for outer hair cell loss. The rates of cochlear outer hair cell loss, for different groups and for different cochlear turns, were statistically analysed; the results are shown in Table I. Little outer hair cell loss was seen in the gentamicin plus melatonin group. The rate of outer hair cell loss in animals treated with gentamicin plus melatonin was lower than that in the gentamicin group, for the same cochlear turn ($p < 0.01$). Rates of outer hair cell loss in animals treated with melatonin alone were similar to those in the control group.

Scanning electron microscopy

Outer hair cells each with a W-shape patern of stereocilia bundles protruding from the cuticular plate show normal under scanning electron microsope as shown in Figure 3. In the gentamicin plus melatonin group, there was little outer hair cell loss, with outer hair cell stereocilia resembling the classic 'W' shape.

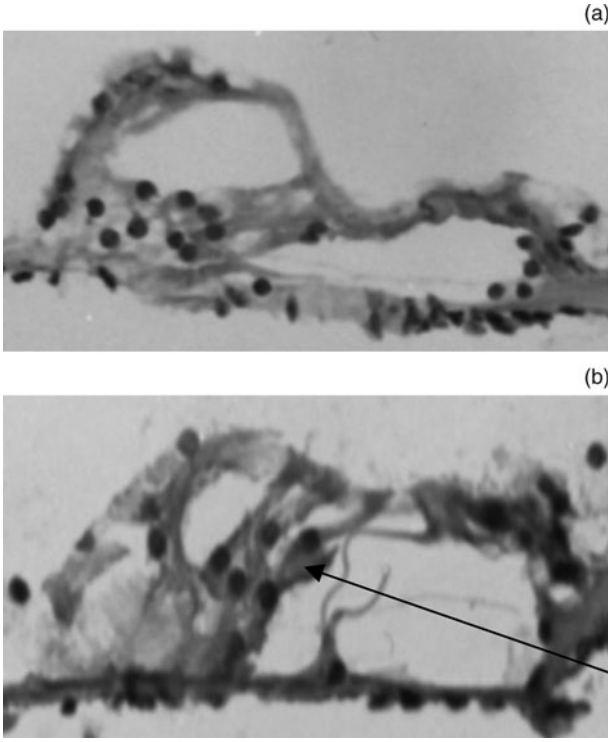

FIG. 2

(a) Photomicrograph of cochlea from the gentamicin group, showing loss of outer hair cell over three rows. (b) Photomicrograph of cochlea from the gentamicin plus melatonin group, showing three rows of normal outer hair cells (arrow) (Hematoxylin and eosin (HE); ×400).

Discussion

In our study, guinea pigs injected with saline or melatonin maintained essentially stable hearing at all of the distortion product otoacoustic emission test frequencies. In the gentamicin group, reduced distortion product otoacoustic emission amplitudes were detected between 3 and 8 kHz. Combined administration of melatonin and gentamicin significantly reduced this gentamicin-induced hearing loss (Figure 1). There was a statistically significant difference in distortion product otoacoustic emission amplitudes between the gentamicin group and the gentamicin plus melatonin group at 3, 4, 6 and 8 kHz, indicating that gentamicin ototoxicity was

TABLE I

RATES OF COCHLEAR OUTER HAIR CELL LOSS*, BY GROUP AND COCHLEAR TURN

Group	1st turn (%)	2nd turn (%)	3rd turn (%)
I	47.59 ± 9.87	39.10 ± 9.52	23.46 ± 4.21
II	$12.43 \pm 3.44^{\dagger}$	$6.78 \pm 2.24^{\dagger}$	$2.32 \pm 0.41^{\dagger}$
III	0.54 ± 0.12	0.42 ± 0.08	0.37 ± 0.04
IV	0.82 ± 0.10	0.63 ± 0.18	0.53 ± 0.11
$p^{\ddagger}$	<0.01	<0.01	<0.01

*$\bar{x} \pm S$; $n = 46$. †group I vs group II ($p < 0.01$). ‡One-way Kruskal-Wallis Variance Analysis (ANOVA).

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completely prevented at 0.75, 1, 1.5, 2, 3, 4, 6 and 8 kHz.

Histopathological results indicated that gentamicin-induced degeneration was worse in the cochlear basal turn than the apex. This agrees with the findings of Kalkandelen *et al.*[9] There was little outer hair cell loss in the gentamicin plus melatonin group. Animals treated with gentamicin plus melatonin were found to have lower rates of outer hair cell loss compared with the gentamicin group, for the corresponding cochlear turn ($p < 0.01$). The morphological results of light microscopy and scanning electron microscopy confirmed the electrophysiological findings, that is, gentamicin induced significant loss of outer hair cells, while melatonin co-therapy attenuated such loss (Figures 1 to 3).

Schacht demonstrated that aminoglycosides can interact fairly efficiently with metals, including iron and copper, which can then form free radicals.[2] Aminoglycosides also have a very high affinity for binding to various types of phosphate lipids, and this affinity correlates fairly well with their potential ototoxicity.[5] Both *in vivo* and *in vitro* evidence suggests that aminoglycosides can interact with iron and lipids to form reactive oxygen species, with toxic effect.[2,10] *In vitro* observations indicate that aminoglycosides can catalyse the formation of reactive oxygen species[4,11] in the presence of transition metals such as iron and copper.[12] Arachidonic acid can serve as an electron donor in the formation of reactive oxygen species by gentamicin and iron cell-free systems, and lipid peroxidation occurs in the early stages of aminoglycoside-induced hearing loss in inner-ear tissues.[4,11]

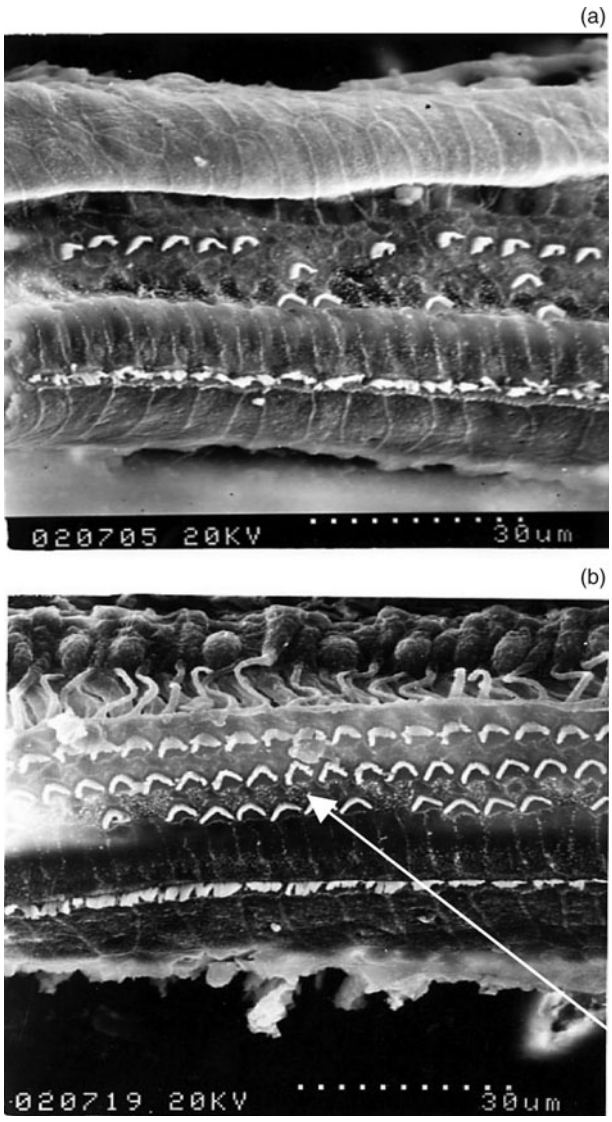

FIG. 3

(a) Scanning electron photomicrograph of cochlea from the gentamicin group, showing loss of outer hair cells. (b) Scanning electron photomicrograph of cochlea from the gentamicin plus melatonin group, showing reduced loss of outer hair cells (arrow).

The basal cochlear region is more metabolically active than the apical region, which may in part explain why the basal turn is more easily damaged by oxidative stress.[13] Under normal conditions, reactive oxygen species (ROS (reactive oxygen species) means molecules or ions formed by the incomplete one-electron reduction of oxygen. These reactive oxygen intermediates induce singlet oxygen, superoxides, peroxides, hydroxyl adical. etc.) produced by the mitochondria are easily metabolised or scavenged by endogenous antioxidant mechanisms. However, in the face of the quantity of reactive oxygen species and free radicals produced by toxic challenge, the cochlea's intrinsic defences may be inadequate to prevent oxidative injury. Gentamicin and kanamycin have been found to enhance the production of hydroxyl radicals in cochlear explant tissue.[14]

Melatonin, an electron-rich molecule, may interact with free radicals via an additive reaction to form several stable end-products which are excreted in the urine. Melatonin does not undergo redox cycling; thus, it does not promote oxidation, as shown under a variety of experimental conditions. Melatonin can be considered a 'suicidal' or terminal antioxidant, as distinct from the opportunistic antioxidant. The ability of melatonin to scavenge free radicals does not occur in a mole-to-mole ratio; indeed, one melatonin molecule scavenges two •OH molecules. Also, its secondary and tertiary metabolites (e.g. N(1)-acetyl-N(2)-formyl-5-methoxykynuramine and 6-hydroxymelatonin), which are believed to be generated when melatonin interacts with free radicals, are also regarded as effective free radical scavengers. The continuous free radical scavenging potential of the original melatonin molecule and its metabolites may be defined as a scavenging cascade reaction.[15] Melatonin detoxifies a variety of free radicals and reactive oxygen intermediates, including the hydroxyl radical, peroxynitrite anion, singlet oxygen and nitrite oxide. In addition, melatonin reportedly stimulates several antioxidative enzymes, including glutathione peroxidase and glutathione reductase. Melatonin also crosses all morphophysiological barriers, which increases its efficacy as an antioxidant. Melatonin has been shown to have a significant protective effect against oxidative damage for both membrane lipids and nuclear DNA.[16]

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In the present study, surface preparations indicated a significant increase in outer hair cell loss in the gentamicin group, compared with the gentamicin plus melatonin group ($p < 0.01$; Table I). On the basis of distortion product otoacoustic emission testing and histopathological findings, we conclude that melatonin may be effective in reducing gentamicin-induced cochlear damage.

- **The use of gentamicin has been limited by its toxic effect on both the cochlea and the vestibular apparatus**
- **However, gentamicin's high efficacy, broad spectrum of activity and low cost probably makes it the most commonly used parenteral antibiotic in developing countries**
- **This animal study investigated a possible protective effect of melatonin on gentamicin ototoxicity**
- **Melatonin appears to protect against gentamicin-induced ototoxicity, possibly by interfering with cytotoxic mechanisms**

Conclusion

The co-administration of melatonin protects against gentamicin-induced ototoxicity by interfering with cytotoxic mechanisms, and does not damage the cochlea itself. Melatonin may represent a novel, effective and reliable treatment for the reduction of gentamicin-induced ototoxicity.

References

1 Williams SE, Zenner HP, Schacht J. Three molecular steps of aminoglycoside ototoxicity demonstrated in outer hair cells. *Hear Res* 1987;**30**:11–18
2 Schacht J. Antioxidant therapy attenuates aminoglycoside induced hearing loss. *Ann N Y Acad Sci* 1999;**884**:125–30
3 Sha SH, Schacht J. Prevention of aminoglycoside induced hearing loss. *Keio J Med* 1997;**46**:115–19
4 Priuska EM, Schacht J. Formation of free radicals by gentamicin and iron and evidence for an iron/gentamicin complex. *Biochemical Pharmacology* 1995;**50**:1749–52
5 Roland PS. New developments in our understanding of ototoxicity. *Ear Nose Throat J* 2004;**83**:15–16
6 Reiter RJ, Guerrero JM, Garcia JJ, Acuna-Castroviejo D. Reactive oxygen intemediates, molecular damage, and aging, Relation to melatonin. *Ann N Y Sci* 1998;**854**:410–24
7 Ye L, Xiao B, Hua Q, Tao Z, Huang Z, Zhou X. Research on DPOAE of guinea pigs under awake and anesthetic conditions [in Chinese]. *Sheng Wu Yi Xue Gong Cheng Xue Za Zhi* 2004;**21**:625–7
8 Garetz SL, Altschuler RA, Schacht J. Attenuation of gentamicin ototoxicity by glutathione in the guinea pig in vivo. *Hear Res* 1994;**77**:81–7
9 Kalkandelen S, Selimoglu E, Erdogan F, Ucuncu H, Alats E. Comparative cochlear toxicities of streptomycin, gentamicin, amikacin and netilmicin in guinea-pigs. *J Int Med Res* 2002;**30**:406–12
10 Lesniak W, Pecoraro VL, Schacht J. Ternary complexes of gentamicin with iron and lipid catalyze formation of reactive oxygen species. *Chem Res Toxicol* 2005;**18**:357–64
11 Sha SH, Schacht J. Formation of reactive oxygen species following bioactivation of gentamicin. *Free Radical Biol Med* 1999;**26**:341–7
12 Lesniak W, Harris WR, Yudenfreud-Kraviz J, Schacht J, Pecoraro VL. Solution chemistry of copper(II)-gentamicin complexes. Relevance to metal-related aminoglycoside toxicity. *Inorg Chem* 2003;**42**:1420–9
13 Thalmann R, Kusakari I, Miyoshi T. Dysfunction of energy releasing and consuming processes of the cochlea. *Laryngoscope* 1973;**83**:1690–712
14 Clerici WJ, Hensley K, Dimartino DL, Butterfield DA. Direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants. *Hear Res* 1996;**98**:116–24
15 Tan DX, Manchester LC, Reiter RJ, Qi WB, Karbownik M, Calvo JR. Significance of melatonin in antioxidative defence system: reactions and products. *Biol Signals Recept* 2000;**9**:137–59
16 Reiter RJ, Tan DX, Cabrera J, D'Arpa D, Sainz RM, Mayo JC, Ramos S. The oxidant/antioxidant network: role of melatonin. *Biol Signals Recept* 1999;**8**:56–63

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