Antioxidant treatment with coenzyme Q-ter in prevention of gentamycin ototoxicity in an animal model

Il trattamento antiossidante con coenzima Q-ter previene il danno ototossico da gentamicina nel modello animale

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SUMMARY
Aminoglycosides, such as gentamycin, are well known ototoxic agents. Toxicity occurs via an activation process involving the formation of an iron-gentamycin complex with free radical production. Antioxidants like Q-ter (a soluble formulation of coenzyme Q₁₀, CoQ_{₁₀}), can limit or prevent cellular ototoxic damage. The present study was designed to investigate the possible protective effects of Q-ter on gentamycin ototoxicity in albino guinea pigs (250-300 g). Animals were divided into five experimental groups: I, a sham control group given an intra-peritoneal (I.P.) injection of 0.5 ml saline (SHAM); II, gentamycin group (GM), treated with an injection of gentamycin (100 mg/kg); III, gentamycin + Q-ter group (GM+Q-ter), treated with gentamycin (same dose as group II) and an I.P. injection of coenzyme Q₁₀ terclatrate (Q-ter) at 100 mg/kg body weight; IV, injected with gentamycin (100 mg/kg) plus saline; V, treated with Q-ter alone (100 mg/kg). All animals were treated for 14 consecutive days. Auditory function was evaluated by recording auditory brainstem responses (ABR) at 15 and 30 days from the beginning of treatment. Morphological changes were analyzed by rhodamine-phalloidine staining. Gentamycin-induced progressive high-frequency hearing loss of 45-55 dB SPL. Q-ter therapy slowed and attenuated the progression of hearing loss, yielding a threshold shift of 20 dB. The significant loss of outer hair cells (OHCs) in the cochlear medio-basal turn in gentamycin-treated animals was not observed in the cochleae of animals protected with Q-ter. This study supports the hypothesis that Q-ter interferes with gentamycin-induced free radical formation, and suggests that it may be useful in protecting OHC function from aminoglycoside ototoxicity, thus reducing hearing loss.

KEY WORDS: Aminoglycoside antibiotic • Gentamycin • Ototoxicity • Antioxidant therapy • Coenzyme Q₁₀ • Outer hair cells

RIASSUNTO
È ben noto che gli antibiotici aminoglicosidi, tra cui la gentamicina, hanno una spiccata ototossicità che si verifica attraverso il legame ferro-gentamicina con l’attivazione di una cascata intracellulare e mitocondriale che porta alla produzione di radicali liberi. Gli antiossidanti come il Q-ter (forma idrosolubile del coenzima Q_{₁₀}, CoQ_{₁₀}), potrebbero limitare o prevenire il danno ototossico. Questo studio ha come obiettivo quello di indagare il possibile ruolo protettivo del Q-ter sull’ototossicità indotta da gentamicina. Come modello sperimentale sono state impiegate cavie albine di peso compreso tra 250-300 g. Gli animali sono stati divisi in modo casuale in cinque gruppi: gruppo I (Sham, controllo), animali trattati con una dose di soluzione fisiologica 0,5 ml somministrata per via intraperitoneale (I.P.): gruppo II GM, animali trattati con gentamicina (GM) somministrata con una iniezione intramuscolare (IM) ad una dose di 100 mg/kg; gruppo III GM+Q-ter, animali trattati con gentamicina (IM100 mg/kg) + Coenzima Q_{₁₀} Terclatrato (Q-ter) ad una dose di 100 mg/kg I.P.; gruppo IV (controllo farmaco), animali trattati con gentamicina IM+ la soluzione fisiologica I.P.; gruppo V (controllo antiossidante), animali trattati con il solo Q-ter 100 mg/kg I.P. Tutti gli animali sono stati trattati per 14 giorni consecutivi. La funzione uditiva è stata valutata mediante le registrazioni dei potenziali evocati audiitivi del tronco dell’encefalo (ABR) a 15 e 30 giorni dall’inizio del trattamento. La tecnica di marcatura con Rodamina-Falloidina è stata impiegata per studiare le modificazioni morfologiche delle cellule. La gentamicina induce un innalzamento di soglia uditiva di circa 45-55 dB nel range delle alte frequenze che il trattamento con il Q-ter attua di circa 20 dB. Inoltre, negli animali trattati con gentamicina si osserva, dopo 30 giorni dal trattamento, una perdita di cellule ciliate esterne che procede dalla prima alla terza fila; la prevenzione farmacologica con Q-ter previene in modo significativo la morte cellulare nel giro mediobasale della coclea. Questo studio dimostra l’ipotesi secondo cui il Q-ter è in grado di prevenire la formazione di radicali liberi indotta da gentamicina protegendo, in questo modo, le cellule ciliate esterne dall’ototossicità indotta da aminoglicosidici e riducendone così la perdita uditiva.

PAROLE CHIAVE: Antibiotici aminoglicosidici • Gentamicina • Ototoxicità • Terapia antiossidante • Coenzima Q₁₀ • Cellule ciliate esterne

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Introduction

Numerous external agents, including high-intensity noise and various drugs such as aminoglycoside antibiotics, damage hair cells and/or supporting cells of the inner ear. However, the aminoglycoside antibiotic gentamicin provides benefits that often outweigh the risks: its ototoxicity is a dose-limiting side effect, and the trans-tympanic injection of gentamicin is widely used for the treatment of Ménière’s disease. Although the mechanisms underlying gentamicin-induced cytotoxicity have not been understood in detail, drug-induced apoptosis in the cochlea often contributes to ototoxicity. Despite differences in the initial targeted compartments of the inner ear by the interactions of ototoxic drugs and damaging noise, these agents ultimately seem to produce similar cellular pathologies and likely have overlapping mechanisms of action, perhaps including activation of the same cellular signalling pathways that lead to cell death. Exposure to aminoglycosides or acoustic trauma leads to oxidative stress and production of reactive oxygen species (ROS) which trigger an apoptotic signalling pathway.

Taken together, there is substantial evidence that oxidative damage and mitochondrial dysfunction may play a key role in sensorineural hearing loss caused by aminoglycoside ototoxicity and noise. The mitochondrial respiratory chain is a powerful source of ROS, and oxidative stress triggers the opening of the mitochondrial permeability transition pores that causes the collapse of inner mitochondrial membrane potential and release of pro-apoptotic factors. Ubiquinone 50 or coenzyme Q_{10} (CoQ_{10}) is a mobile electron carrier in the mitochondrial electron transport chain (ETC) that is the major source of ATP in the mitochondria. It participates in the ETC by carrying electrons from complex I (succinate-ubiquinone oxidoreductase) to complex III (ubiquinone-cytochrome C oxidoreductase). Within mitochondria, ubiquinone is reduced by the respiratory chain to its active ubiquinol form, which is an effective antioxidant that prevents lipid peroxidation and mitochondrial damage. In a previous report, we demonstrated that idebenone, a synthetic analogue of CoQ_{10}, attenuates hearing loss in a guinea pig model of acoustic trauma by virtue of its antioxidant properties. It has been suggested that its efficacy depends on the ability to intercept free radicals in both aqueous phases and lipid-water interfaces. In fact, the mobility of the agent in membranes and lipoproteins plays a key role in determining its antioxidant activity. We have further addressed the protective role of CoQ_{10} in the same acoustic trauma model by comparing the efficacy of the native lipophilic CoQ_{10} molecule with that of a multi-composite formulation of CoQ_{10} with high water solubility and oral bioavailability, namely, coenzyme Q_{10} terclatrate (Q-ter), and we have provided functional and morphological evidence on the potential efficacy of Q-ter in preventing oxidative injuries that result from mitochondrial dysfunction. Considering that CoQ_{10} serves as an important cofactor in the electron transport chain, and serves as an important antioxidant in both mitochondria and lipid membranes, the objective of this study was to assess the efficacy of the enhanced formulation of CoQ_{10} (Q-ter) in the prevention or slowdown of gentamicin-induced cochlear damage.

Materials and methods

Animals

Adult Hartley albino guinea pigs (3 months old), weighing 250-300 g, with normal Preyer’s reflex, were used in the study. All procedures on animal use and care were conducted in accordance with the Laboratory of Animal Care and Use Committee of the Catholic University, School of Medicine of Rome, and of the European Communities Council Directive (86/609/EEC) and were approved by the Italian Department of Health (Ministero della Salute).

Eighteen guinea pigs were randomly separated into five groups: I) sham control group undergoing intra-peritoneal (LP) injection with 0.5 ml saline (SHAM; n = 4); II) gentamicin group (GM; n = 4), treated with an injection of gentamicin (100 mg/kg body weight [b.w]); III) gentamicin + Q-ter group (GM+Q-ter; n = 4), treated with gentamicin (same dose as group II) and with i.P. injection of Q-ter alone (n = 3) at the same dose as group II; V) control animals treated with Q-ter alone (n = 3) at the same dose of group III. All animals were treated daily for 14 consecutive days, the administration of Q-ter was given 1 hour before gentamicin.

The animals were housed in separate cages in temperature-controlled rooms, with a 12-hour light-dark cycle, and had free access to commercial food and water.

Drug preparation

Gentamicin was used as a sulphate salt dissolved in saline at a dose of 127 mg per 2 ml corresponding to 40 mg/ml of gentamicin (Gentaryl® Schering Plough SpA, Milan, Italy). Q-ter is a terclatrate substance, obtained by mechano-physical activation: a solid-state procedure that brings different substances into supramolecular contact through the administration of energy, and turning a simple mixture into a multi-composite material (Applicant, Asoltech Srl, Trieste, Italy). Q-ter consists of an outer case (an inactive pharmaceutical grade excipient) that entraps CoQ_{10}.
moieties (10% w/w) and an amino acid that serves as a catalyst to enable the formation of the multicomposite. Q-ter was provided by Scharper Therapeutics, Milan, Italy, and was manufactured using an industrially available native CoQ₁₀ (Kaneka Pharma Europe, Brussels, Belgium). For the purposes of treatment, Q-ter was dissolved in saline and injected intraperitoneally at a dose of 100 mg/kg body weight, corresponding to 10 mg/kg CoQ₁₀. The Q-ter saline solution was prepared fresh daily as previously described ¹².

Electrophysiological measurements of auditory function
Hearing function was evaluated in all animals by recording auditory brainstem responses (ABR) at low (2-4 kHz), mid (6, 8, 12 kHz) and high (16, 20 kHz) frequencies. ABRs were measured before the first drug administration and 15 and 30 days afterwards. Animals were mildly anaesthetized (ketamine hydrochloride 12.5 mg/kg, xylazine 2.5 mg/kg and acepromazine maleate 0.75 mg/kg body weight) and placed in a sound-proof room. Three electrodes were subcutaneously inserted into the right mastoid (active), vertex (reference) and left mastoid (ground). A PC-controlled TDT System 3 (Tucker-Davis Technologies, Alachua, Florida, USA) data acquisition system with real time digital signal processing was used to generate the auditory stimulus and ABR recording. Tone bursts from 2 to 20 kHz (1 msec rise/fall time, 10 msec total duration, 20/sec repetition rate) were presented under free field conditions. Responses were filtered (0.3-3 kHz), digitized and averaged across 500 discrete samples at each frequency-level combination. Thresholds were determined by increasing the intensity of the 5 dB tone, starting at 0 dB and moving up to 100 dB, until ABR response was detected. Next, the stimulus intensity was decreased in 5 dB steps until the latency-appropriate response disappeared. The threshold value was defined as the lowest intensity able to evoke an appropriate ABR response ¹².

Quantitative assessment of hair cell survival and cochleogram
After ABR testing all animals were killed by a lethal injection of anaesthetic (ketamine hydrochloride 25 mg/kg, xylazine 5 mg/kg and acepromazine maleate 1.5 mg/kg body weight). Cochleae were processed for the quantitative assessment of hair cell survival and cochleogram. The right cochleae were quickly removed from the skull and fixed in 10% buffered formalin for 4 hours. Cochleae were then dissected in 0.1 M PBS, and the organs of Corti were incubated with a solution containing 0.5% Triton X-100 and rhodamine-conjugated phalloidin (1:100 dilution; R-415, Molecular Probes) for 1 hour at room temperature and protected from light. At the end of incubation, all specimens were washed twice in PBS. Next, the specimens were mounted on slides containing an antifade medium (ProLong Gold, Invitrogen P36930). Quantification of the remaining number of hair cells and calculation of hair cell loss was done with the aid of the Leica confocal microscope (TCS-SP2; Leica Microsystems, GmbH, Wetzlar, Germany). Hair cells were considered missing if the stereocilia bundles were absent in rhodamine-phalloidin-stained and/or the profiles of outer hair cells (OHCs) or inner hair cells (IHCs) were not detectable. The results were expressed as a percentage of remaining hair cells in IHC layer and the three rows of OHCs over the entire length of the cochlear duct.

Data analysis
Statistical significance of ABR and cochleogram data was calculated by analysis of variance (ABR: group × frequency × day, three-way ANOVA with repeated measures; cochleogram: group × HCs type × cells survived along 20 mm, three-way ANOVA with repeated measures). When significant differences were found with the overall analysis, post-hoc comparisons were assessed with Tukey’s test (Statistica, Statsoft, Tulsa, USA). A p value < 0.05 was considered significant.

Results
Auditory function evaluation
ABRs were recorded in each animal before, and at 15 and 30 days from the beginning of treatment (Fig. 1). Pre-treatment baseline ABR thresholds did not differ among the five groups and were consistent with data previously obtained in our laboratory ¹². The administration of Q-ter alone did not modify auditory threshold at each time point compared to group I. No differences were observed between group II and the group of animals treated with gentamycin plus saline (data not shown), and threshold values remained stable throughout the course of treatment. Consistent with data obtained previously by recording compound action potentials ¹⁴ ¹⁵, gentamycin treatment in our guinea pig model (group II, GM) determined, at day 15 upon the end of treatment, the greatest hearing loss at frequencies of 6-16 kHz. The average hearing threshold shifts with standard deviation, are shown in Figure 1C. In gentamycin-treated animals, at the end of treatment (day 15), the threshold shift was about 15-25 dB at 2 and 4 kHz, 35-45 dB at 6-16 kHz and 25-30 dB at 20 kHz (Fig. 1C). At day 30, the average threshold shift further increased by about 5-10 dB at low frequencies, 5-10 dB at 6-16 kHz and 10 dB at 20 kHz (Fig. 1D).

In the animals treated with gentamycin plus Q-ter (group III), the gentamycin-induced hearing loss was greatly attenuated. Namely, the threshold shift was about 5-15 dB at low frequencies and 15-20 at mid and high frequencies (Fig. 1C). At day 30, the trend of threshold shift increase was similar to group II (GM). However, in group III (GM+ Q-ter) the threshold shift increased by only about 5 dB for all frequencies (Fig. 1D).
A comparison between group GM and group GM+ Q-ter (Figs. 1C-D) showed that the ABR threshold shift was significantly attenuated at all frequencies in the Q-ter group (post hoc analysis p < 0.03) compared to the gentamycin group at days 15 and 30. Specifically, at day 15 (Fig. 1C) there was a difference of about 10 dB at low frequencies (2-6 kHz), 14 dB at 8 kHz, 17-20 dB at 12-16 kHz and 10 dB at 20 kHz. At day 30 (Fig. 1D), the difference between the two groups was about 8-10 dB at 2-8 kHz, 20 dB at 12-16 kHz and 15 dB at 20 kHz.

Taken together, the frequencies most affected were 12 and 16 kHz, and the ability of Q-ter to protect from gentamycin ototoxic injury reached, at day 15, about 55% of protection at 12 kHz (21.25 dB vs. 38.75 dB) and about 53% of protection at 16 kHz (22.50 dB vs. 42.50 dB). A further 6% of protection was detected at day 30 at 16 kHz, leading to a total of 59% protection (23.75 dB vs. 47.50 dB). Remarkably, Q-ter administered in parallel with the ototoxic gentamycin diminished hearing loss by about 50%; the protection was maintained for 15 days and more pronounced attenuation was observed at 16 kHz.

**Hair cell loss**

The functional data were paralleled by histological findings. Normal cochleograms were observed in untreated animals (control animals, data not shown) [15], and no significant differences were observed between the gentamycin group and the one treated with vehicle, suggesting that saline did not interfere with cochlear damage and protection. Missing hair cells were counted in rhodamine-phalloidin labelled surface preparations, and the percentage loss of IHC and OHC was quantitatively evaluated and expressed as the mean for each treatment group (Fig. 2).

In group II (GM) (Fig. 2A), massive OHC loss and minor IHC disappearance were detected in the medio-basal turn of the cochlea. In fact, the damaged region extended from approximately 11-19 mm from the apex with a decreasing pattern in the transitional area and a typical grading from the first (inner row) to the third row of OHCs. In contrast, animals receiving Q-ter treatment group III (GM+Q-ter) (Fig. 2B) had only moderate hair cell loss and narrower gentamycin-induced lesions, with cell damage visible between 13 and 19 mm from the apex. Specifically, in the first row of the basal turn of the gentamycin group (II, GM) and gentamycin plus Q-ter group (III, GM+Q-ter), the surviving stereocilia bundles were 59% and 74%, respectively; in the second row, OHCs were missing only in the gentamycin group, whereas only a few cells were lost in gentamycin plus Q-ter group (survival values of 62.5% and 80%, respectively). OHCs of the third row were occasionally affected in both groups (survival values of 84% for gentamycin vs. 95% for gentamycin plus Q-ter) (Figs. 2C-D). Concerning IHCs, the damage showed a trend similar to OHC loss (Figs. 2C-D). In the gentamycin-treated group, 73% of OHCs survived in the basal turn, whereas 87% of OHCs remained in the Q-ter treated group (Fig. 2E). IHC survival was no significantly different between Sham and Q-ter treated group. The recovery of damage by concomitant Q-ter treatment is illustrated in Figure 2F, where it can be seen that the area of greatest cell loss is around 16 mm from the apex in both groups. Taken together, OHCs and IHCs in the basal turn of the cochlea were mostly affected in the gentamycin group, whereas hair cell loss extension and width decreased with concomitant administration of the antioxidant Q-ter (post hoc analysis p < 0.005).
Discussion

In this study, we investigated the protective effects of a CoQ<sub>10</sub> analogue, namely Q-ter, against gentamycin-induced ototoxicity. Our results showed that the concomitant administration of Q-ter reduced the extent of gentamycin-induced auditory impairment. The cochlear histopathological findings correlated with the functional results obtained by ABR recordings. In gentamycin-treated animals, the increase in ABR threshold shift at high and medium frequencies was consistent with a high degree (60%) of OHC loss in the basal and middle turns, as shown by the rhodamine-phalloidin hair cell count along the organ of Corti. The inner row of OHCs was mostly affected and damage to IHCs was also detected. Both functional and morphological results are consistent with our previous investigations on gentamycin toxicity and literature data (see for review 2). In the Q-ter protected group, the reduced ABR threshold shift in the high and medium frequencies was coupled with a small degree (20%) of OHC and IHC loss in the basal and middle turns. Taken together, Q-ter reduces hearing loss and the extent and distribution of hair cell damage.

A first question to be discussed concerns the distribution of gentamycin toxicity and the cochlear inner row and IHCs. The cochlea OHCs are substantially more vulnerable to insult than IHCs, whether the insult is from aminoglycoside ototoxicity, excessive noise or presbycusis. Similarly, basal turn OHCs are more vulnerable than more apically located OHCs to the
same insults\textsuperscript{19}. However, by comparing gentamycin damage with noise damage, a difference in the order of affected OHC rows can be observed. Noise first damages the outermost row with grading towards the inner row; gentamycin affects the inner row with grading to the second and third outer rows\textsuperscript{17} \textsuperscript{19} \textsuperscript{20}. This histopathological data may provide an indication on the distribution of systemic gentamycin to OHCs. Recent experimental studies suggest that, in vivo, systemically-administered aminoglycosides enter cochlear hair cells from the endolymph prior to inducing auditory dysfunction\textsuperscript{21} \textsuperscript{22}. As detailed by other authors\textsuperscript{22}, there are two routes by which systemically-administered aminoglycosides could enter the endolymph: first by a trans-strial trafficking route from strial capillaries to marginal cells, and secondly by traversing the blood-labyrinth barrier into perilymph and then within (white arrow heads). Alternatively, aminoglycosides pass to the scala tympani through the basilar membrane into extracellular fluids within the organ of Corti by traversing the basolateral membranes (red arrow head). Gentamycin enters the hair cell through mechano-electrical transducer channels; it forms a complex with iron (Fe), a transition metal. These redox active complexes generate ROS/RNS including the superoxide anion radical, hydrogen peroxide, hydroxyl radical and peroxynitrite anion. Reactive species activate JNK (c-Jun N-terminal kinase), which then translocate to the nucleus to activate genes in the cell death pathway by transfer to the mitochondria where they promote the release of cytochrome c (cyt c). In the cytosol, cyt c triggers the activation of a series of caspases followed by apoptosis (programmed cell death) via what is referred to as caspase-dependent cell death. In addition to this caspase-dependent apoptotic pathway, gentamycin may also kill cells via caspase-independent mechanisms.

The second and main point of discussion concerns the mechanism of Q-ter protection against gentamycin ototoxicity. Q-ter is a water-soluble coenzyme $\text{Q}_{10}$ formulation. A therapeutic approach with native $\text{CoQ}_{10}$ is limited by its poor bioavailability in aqueous media and stability problems. In contrast, the Q-ter formulation is more
soluble than the native molecule, the chemical moieties of the starting materials are preserved and physicochemical properties such as stability and antioxidant capacity are improved. In a previous publication, we demonstrated the antioxidant efficacy of the water soluble Q-ter in a guinea pig model of acoustic trauma. Thus, considering that the mechanism of cochlear damage by noise and aminoglycosides is reported to be analogous, Q-ter protection against gentamycin toxicity, as described in the present paper, is not surprising (see suggested intracellular activated pathway in the inset box of Figure 3). Furthermore, the role of oxidative damage and possible mitochondrial dysfunction in gentamycin ototoxicity can be indirectly hypothesized by the efficacy of our CoQ_{10} formulation. CoQ_{10} blocks apoptosis by inhibiting activation of the mitochondrial permeability transition independently of its free radical scavenging activity. Another potential protective mechanism of CoQ_{10} concerns its role as an obligatory cofactor of mitochondrial uncoupling proteins and the activation of these proteins reduces mitochondrial-free radical generation.

There is increasing interest in the utility of CoQ_{10} to treat neurodegenerative diseases. CoQ_{10} induces mitochondrial uncoupling in the substantia nigra of primates, and this is associated with marked neuroprotection. Increased expression of mitochondrial uncoupling proteins protects against brain damage associated with both experimental stroke and epilepsy. CoQ_{10} diminishes ischemia-induced neuronal injury in the hippocampus, and protects cultured cerebellar neurons against excitoxin-induced degeneration. The potential efficacy of CoQ_{10} in the treatment of both Parkinson’s and Alzheimer’s disease has also been described.

The data in the present paper may hold additional indication on the novel therapeutic design of Q-ter and its potential use in combating oxidative damage induced by exposure to ototoxic compounds.

Acknowledgements

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