



Antioxidative stress-induced damage in cochlear explants

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ABSTRACT

The imbalance of reactive oxygen species and antioxidants is considered to be an important factor in the cellular injury of the inner ear. At present, great attention has been placed on oxidative stress. However, little is known about fighting oxidative stress. In the current study, we evaluated antioxidant-induced cochlear damage by applying several different additional antioxidants. To determine whether excessive antioxidants can cause damage to cochlear cells, we treated cochlear explants with 50 μ M M40403, a superoxide dismutase mimetic, 50 μ M coenzyme Q-10, a vitamin-like antioxidant, or 50 μ M d-methionine, an essential amino acid and the important antioxidant glutathione for 48 h. Control cochlear explants without the antioxidant treatment maintained their normal structures after incubation in the standard serum-free medium for 48 h, indicating the maintenance of the inherent oxidative and antioxidant balance in these cochlear explants. In contrast, M40403 and coenzyme Q-10-treated cochlear explants displayed significant hair cell damage together with slight damage to the auditory nerve fibers. Moreover, d-methionine-treated explants exhibited severe damage to the surface structure of hair cells and the complete loss of the spiral ganglion neurons and their peripheral fibers. These results indicate that excessive antioxidants are detrimental to cochlear cells, suggesting that inappropriate dosages of antioxidant treatments can interrupt the balance of the inherent oxidative and antioxidant capacity in the cell.

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1. Introduction

The balance of intracellular oxidation and antioxidant is an important prerequisite for maintaining cell survival and activity. Both excessive oxidation and excessive antioxidantation can cause damage to cells.

The cochlea can be damaged by many stressors, including blast wave, intense noise, various ototoxic drugs, heavy metals,

pesticides, and aging (Chen et al., 2013; McFadden et al., 2001; Lesniak et al., 2005; Ding et al., 2012; Ding and Salvi, 2005; Ding et al., 2014; McFadden et al., 2003; Nicotera et al., ; Prakash Krishnan Muthaiah et al., 2017; McFadden et al., 1999; Ding et al., 2013). Experimental studies have confirmed that, regardless of the causes, the development of intracellular oxidative stress often precedes cell damage. For example, the iron-containing complex composed of a combination of gentamicin and iron ions in hair cells can react with an electron donor unsaturated fatty acid to produce a large amount of superoxide and hydroxyl radicals (Lesniak et al., 2005; Priuska and Schacht, 1995). In another example, upon entering a cell through a copper transport channel, cisplatin undergoes a structural change with the chlorine atom being replaced by active oxygen and the formation of a cisplatin glutathione dimer. This structural change allows cisplatin to exert its cytotoxic effect,

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causing intracellular oxidative stress (Hanigan et al., 2001; Ding et al., 2008). Therefore, increasing the antioxidant level is considered to be one of the important strategies to prevent or treat inner ear disease (McFadden et al., 2003; Nicotera et al., ; Ding et al., 2008). However, there is a lack of proper understanding or insufficient understanding of tissue damage caused by excessive antioxidant treatments.

To understand whether excessive antioxidants can cause damage to the cochlea, we used several antioxidants to treat cochlear explants. The experimental results show that over-treatment with the antioxidant itself is sufficient to damage cochlear sensory hair cells and/or spiral ganglion neurons. Our study raises a concern on the side effects of the high-dosage treatment of antioxidants and warrants vigilance for excessive antioxidant hazards.

2. Materials and methods

2.1. Experimental animals

12 Sprague Dawley rat pups at postnatal day 3 were used for this study. Six cochlear explants were cultured in a standard serum-free medium for 48 h as the normal control group. Another 18 cochlear explants were divided into three groups, with 6 cochleas in each group. These groups served as experimental groups. The cochlear explants in the M40403-treated group was incubated in a serum-free medium containing 50 μ M M40403 (superoxide dismutase analog) for 48 h, the cochlear cultures in the coenzyme Q-10 group was treated with 50 μ M coenzyme Q-10 (CoQ-10) for 48 h, and the cochlear organs in the d-methionine-treated group were incubated with 50 μ M of d-methionine for 48 h.

2.2. Preparation of serum-free medium

The serum-free medium contains of 190.8 ml of 1 \times Basal Medium Eagle (Sigma B-1522), 2 g bovine serum albumin (Bovine Serum Albumin, Sigma A-1949), 4.8 ml of 20% glucose (Sigma G-2020), 0.4 ml of penicillin G (Sigma P-3414), 2 ml of 200 mM glutamine (Glutamine, SigmaG-6392), and 2 ml of Serum-free supplement (Sigma I-1884) (McFadden et al., 2003; Nicotera et al., ; Prakash Krishnan Muthaiah et al., 2017; Ding et al., 2013; Ding et al., 2018; Ding et al., 2011).

2.3. Preparation of rat tail collagen gel matrix

The collagen gel matrix is freshly prepared with 9 parts of collagen I, rat tail containing 0.02 N acetic acid and 50 \times collagen (Collaborate Biomedical Products Cat #40236) and 1 part of 10 \times Basal Medium Eagle solution (Sigma B-9638) and 1 part of 2% sodium carbonate. A drop of 10 μ l of the collagen gel matrix was placed in the center of a 35 mm culture dish (Falcon 1008, Becton Dickinson) for about 20 min to allow the gel to become solid. Then, 1.3 ml of serum-free medium was added into the dish to the level of the top of the collagen gel matrix. The culture dish was placed in a carbon dioxide incubator at 37 $^{\circ}$ C with 5% carbon dioxide (McFadden et al., 2003; Nicotera et al., ; Prakash Krishnan Muthaiah et al., 2017; Ding et al., 2013; Ding et al., 2018; Ding et al., 2011).

2.4. Microdissection of the cochlear basilar membrane

The preparation of cochlear explants has been described in detail in our previous publications (McFadden et al., 2003; Nicotera et al., ; Prakash Krishnan Muthaiah et al., 2017; Ding et al., 2013; Ding et al., 2018; Ding et al., 2011). Briefly, the temporal bone was removed and immersed in Hank's Balanced Salt Solution (1 \times GIBCO, 14175, Invitrogen, Carlsbad, CA). Under a dissection

microscope, the cochlear basilar membrane containing the organ of Corti and spiral ganglion neurons was dissected out and placed on the top of the collagen gel matrix. Then, the culture dish was placed in an incubator at 37 $^{\circ}$ C with 5% CO₂.

2.5. Treatment of cochlear explants

Six cochlear basilar membranes were cultured with the standard serum-free medium without any antioxidants for 48 h as the control group. A total of 18 cochlear explants were equally divided into three experimental groups to test toxic effects of three antioxidants (M40403, coenzyme Q-10, and d-Methionine). 6 cochlear explants were cultured with 50 μ M M40403 in the serum-free medium for 48 h; 6 cochlear explants were cultured with 50 μ M coenzyme Q-10 in the serum-free medium for 48 h; and the remaining 6 cochlear explants were cultured with 50 μ M d-methionine in the serum-free medium for 48 h.

2.6. Histology

Upon completion of the drug treatment, cochlear explants in each group were fixed with 10% formalin in PBS for 2 h. The collagen gel matrix carrying cochlear explants was removed from the culture dish, and rinsed with PBS. The cochlear explants were immersed in a staining solution containing 20 μ l of a mouse IgG2a anti-acetylated tubulin antibody (BioLegend 801213), 20 μ l Triton X-100 (10%), 6 μ l normal goat serum, and 154 μ l of 0.01 M PBS overnight. After adequate tissue rinsing, the tissues were immersed in a secondary antibody solution containing 2 μ l of Alexa Fluor 488 conjugated secondary goat anti-mouse IgG2a antibody (ThermoFisher A-21131), 2 μ l of Actin-stain 555 conjugated phalloidin (Cytoskeleton, Inc. Cat.# PHDH1-A), 2 μ l of ToPro-3 (ThermoFisher T3605), 12 μ l of normal goat serum, 40 μ l of Triton X-100 (10%), and 341 μ l of 0.01 M PBS for 2 h. Cochlear explants were placed in glycerin on glass slides and coverslipped. Under a confocal microscope (Zeiss LSM-510), the tissues were observed with the filter for Alexa Fluor 488 (excitation 495 nm, emission 519 nm) to visualize the auditory nerve fibers and spiral ganglion neurons with green fluorescence, Actin-stain 555 (absorption: 544 nm, emission: 572 nm) to visualize the cilia bundles and cuticular plates of hair cells with red fluorescence, and ToPro-3 (absorption: 5642 nm, emission: 661 nm) to visualize the nuclei with blue fluorescence. Images were evaluated with a Zeiss LSM Image Examiner, and further processed with Adobe Photoshop software (Ding et al., 2011, 2013).

To quantify cochlear sensory hair cells, the number of missing hair cells for each 0.24-mm segment was counted over the entire length of the basilar membrane as described previously (Ding et al., 2011, 2013). The cochleogram was constructed by plotting the percentages of inner (IHC) and outer hair cell (OHC) loss as the function of the distance in percentage from the apex of the cochlea. Individual data were then averaged to generate the mean cochleogram for each treatment condition using custom software.

To quantify the degree of spiral ganglion neuron degeneration, the number of ganglion neurons and the number of the SGN with condensed or fragmented nuclei were counted from six cochlear explants per condition. For each condition, the number of SGN in a 141.1 \times 141.1 μ m square area (optical section thickness 1 μ m) was counted from 2 to 3 locations in the middle turn of each cochlear explants. 2–3 optical sections were separated by 20 μ m. The data of spiral ganglion counts were statistically analyzed by one-way ANOVA followed by the Newman-Keuls post hoc analysis (GraphPad Prism 5 software) (Deng et al., 2013).

3. Results

In the normal control group, cochlear hair cells and auditory nerve fibers displayed a normal morphology. Neither cell loss nor detectable pathological changes were observed (Fig. 1A and B). After the treatment with 50 μ M M40403 for 48 h, cochlear explants exhibited a large number of missing hair cells (Fig. 1C and D). The auditory nerve fibers under the organ of Corti appeared to be slightly thinner (Fig. 1C). 48 h after the incubation with 50 μ M coenzyme Q-10, the inner and outer hair cells were largely depleted (Fig. 1E and F). The auditory nerve fibers and terminals beneath sensory hair cells were slightly reduced (Fig. 1F). 48 h treatment with 50 μ M d-methionine caused stereocilia to become scattered or lodging (Fig. 1G). However, the number of hair cells was largely maintained (Fig. 1H). This may indicate that the soma of hair cells were not completely destroyed by 48 h post-50 μ M d-methionine treatment (Fig. 1G). Noticeably, the auditory nerve fibers and terminals were completely destroyed 48 h after the 50 μ M d-methionine treatment (Fig. 1G).

The spiral ganglion neurons in the control group exhibited large, oval somas containing a round uniformly-stained nucleus (Fig. 2A). 48 h after the 50 μ M M40403 treatment, although many spiral ganglion neurons appeared intact, some nuclear condensation or fragmentation were detected in few neurons (Fig. 2B and E). The 48 h-treatment with 50 μ M coenzyme Q-10 also caused nuclear shrinkage in some spiral ganglion neurons (Fig. 2C and E). 48 h after the 50 μ M d-methionine treatment, all spiral ganglion neurons showed apoptotic features of nuclear fragmentation (Fig. 2D and E).

4. Discussion

Free radicals are mainly derived from intermediate metabolites of aerobic metabolism. Although excessive unpaired free radicals can cause damage to the body, free radicals are also considered to be an important cellular defense system that maintains normal cellular metabolism and clears intracellular pathogens, if oxidation and antioxidation are maintained in a good balance⁽¹⁹⁾.

Oxidative stress-induced cell damage has been the focus of research^(1–15), but little is known about cellular damage caused by antioxidative stress. If the level of free radicals in the cells is too low or the level of antioxidants in the cells is too high, the imbalance could pose a hazard effect on cells, which is called excessive antioxidant-induced cell damage. Just as free radicals are not always the “root of all evil”, antioxidants are not always “the more the better”. Since the abuse of antioxidants actually causes excessive antioxidant damage, Dundar proposed the term “antioxidative stress” for the intracellular oxidation/antioxidant imbalance caused by excessive antioxidants (Dundar and Aslan, 2000).

According to the literature, therapeutic effects of antioxidants including coenzyme Q10, vitamin E and β -carotene have been questioned by meta-analysis (Peterneil and Coombes, 2011; Suksumboon et al., 2011; Arain and Abdul, 2010; Bjelakovic et al., 2004; Myung et al., 2010). Villanueva analyzed the efficacy of anti-oxidation therapy in 749,000 cases over the past 15 years and found that more than 65% of antioxidant treatments are ineffective or even harmful (Villanueva and Kross, 2012). It has established that free radicals can induce endogenous reactions against cell damage in the body caused by foreign toxic substances (Tapia,

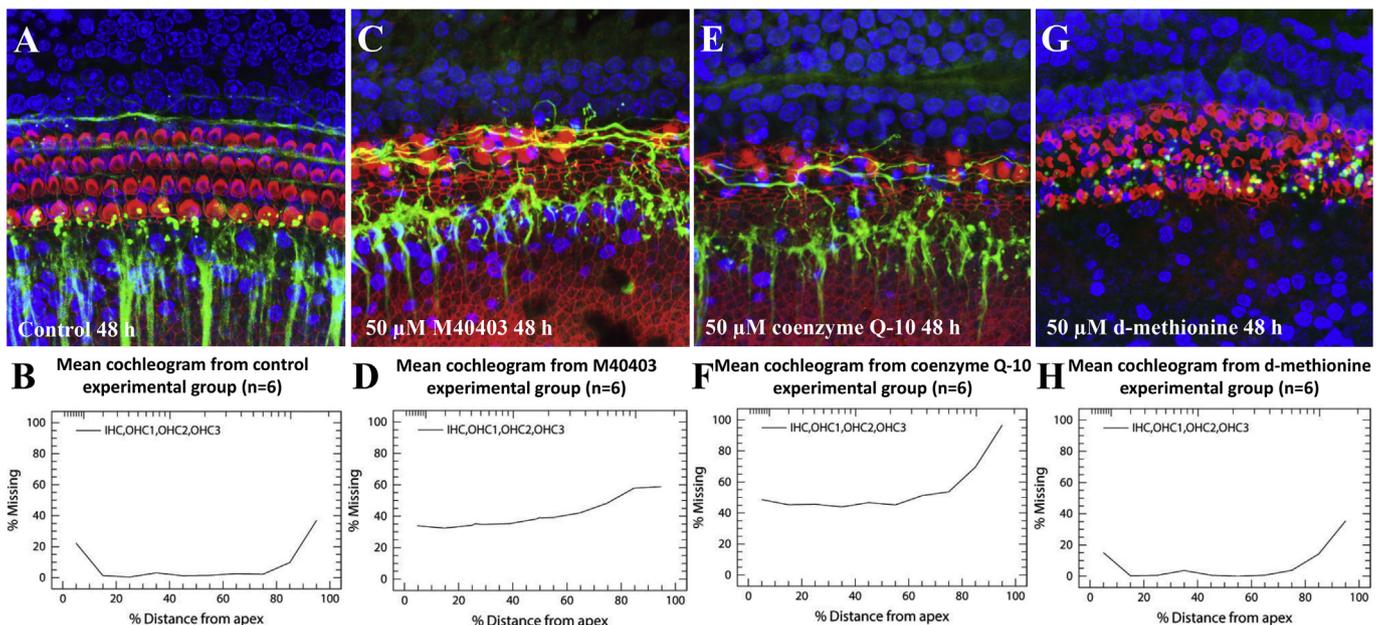


Fig. 1. Photomicrographs and cochleograms showing the surface preparation of cochlear basilar membrane and percentage of hair cell missing 48 h after cultures in standard serum-free medium without antioxidant (A, B), or 48 h after 50 μ M M40402 treatment (C, D), or 48 h post-50 μ M coenzyme Q-10 cultures (E, F), or 48 h after 50 μ M d-methionine treatment (G, H). Specimens were triple stained with Alexa Fluor 555 conjugated phalloidin (red, arrow) to label the stereocilia bundles and cuticular plate of sensory hair cells, with Alexa Fluor 488-conjugated beta-tubulin antibody (green) to stain the auditory nerve fibers, and with ToPro-3 (blue) to label the nuclei. (A) The sensory hair cells and nerve endings present normal 48 h after culture in control condition. (B) The averaged cochleogram showed that most of the hair cells were intact except for the partial hair cell missing at the hook region that might be induced by dissection-induced mechanical damage in control group. (C) 48 h after 50 μ M M40402 treatment, massive hair cell missing and mild damage in auditory nerve fibers were detected. (D) In the mean cochleogram from M40403 treated group, more than 50% hair cell missing throughout entire length of the cochlear basilar membrane. (E) 48 h treatment with 50 μ M coenzyme Q-10 caused a large number of hair cell loss and mild damage in auditory nerve fibers. (F) More than 65% hair cell missing were found as shown in the averaged cochleogram in the experimental condition of 50 μ M coenzyme Q-10 treatment for 48 h. (G) Although the cochlear hair cells were visible in cochlear explants 48 h after 50 μ M d-methionine treatment. However, the damaged and disarrayed stereocilia were detected on all sensory hair cells. (H) The mean cochleogram of 50 μ M d-methionine treated group displayed no obvious hair cell missing.

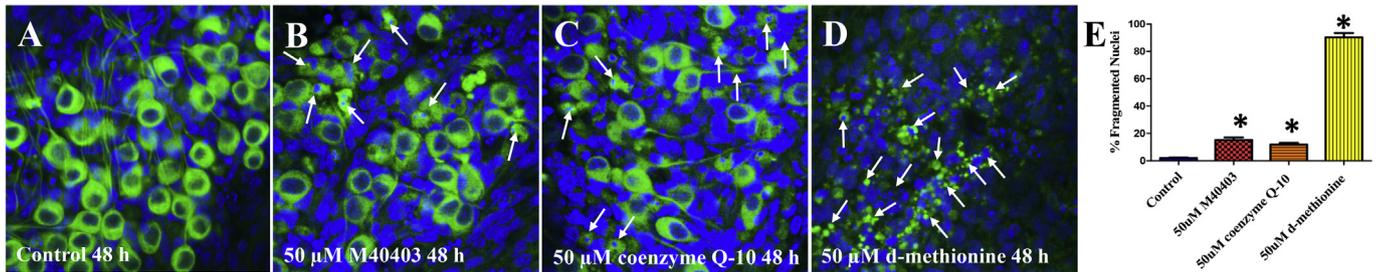


Fig. 2. Photomicrographs and histogram showing the spiral ganglion neuron damage and percentage of degenerating ganglion neurons 48 h after cultures in standard serum-free medium without antioxidant (A), 48 h after 50 μ M M40403 treatment (B), 48 h post-50 μ M coenzyme Q-10 cultures (C), or 48 h after 50 μ M d-methionine treatment (D). Specimens were double stained with Alexa Fluor 488 conjugated beta-tubulin antibody (green) to stain the soma of spiral ganglion neurons, and with ToPro-3 (blue) to label the nuclei. (A) The somas and nuclei of spiral ganglion neurons in normal control cochleae 48 h after culture have a large, oval-shaped soma with a large, round, homogeneously labeled nucleus. (B) 48 h after 50 μ M M40403 cultures, the nuclear condensation were detected (white arrows) in some spiral ganglion neurons. (C) Nuclear shrinkage were detected in few spiral ganglion neurons (white arrows) 48 h after 50 μ M coenzyme Q-10 treatment. (D) 48 h after d-methionine treatment, all spiral ganglion neurons were greatly shrunken with nuclei fragmentation (white arrows). (E) The histogram showing the percentage of cell shrinkage with nuclear fragmentation. *Significantly different from control ($P < 0.05$).

2006). However, inappropriate supplementation of antioxidants is likely to annihilate free radicals that play a positive role in health, which in turn can induce a variety of injuries and diseases (Ristow and Zarse, 2010; Bjelakovic et al., 2007).

Free radicals have a transient high-efficiency reactivity. Once it formed, free radicals can be rapidly catalyzed to the next free radical and transferred along the oxidation reaction chain. Since the conversion of free radicals is a series of rapid changes that occur in the oxidation/antioxidant chain, the detection of one type of free radicals may not reflect the actual situation of the oxidation and antioxidant balance throughout the oxidation chain. Therefore, catalyzing one type of free radicals by supplementing a corresponding antioxidant may not completely solve the problem of the cell damage caused by all other free radicals produced during the chain reaction of free radical metabolism.

There are many technical methods for detecting free radicals or antioxidants in cells. Methods for detecting free radicals include fluorescent probes, electron paramagnetic resonance spectroscopy, chemiluminescence detection, spectrophotometric detection, chromatography, electrochemical methods. Methods for detecting intracellular antioxidants include classical ortho-benzene, tri-sphenol auto-oxidation method, nitrite formation method, polarographic oxygen electrode method, potassium permanganate titration method, polarographic oxygen electrode method, Hafman method and its modified method, etc. However, most of the above techniques can only detect one type of free radicals or one type of antioxidants. Moreover, these assays can only be used at the cellular level of experimental animals but are not suitable for clinical examinations at the cell level or organ level. Since it is difficult to accurately determine the instantaneous levels of various free radicals and antioxidants in a cell, certain antioxidant treatments may have a certain degree of blindness. In addition, oxidative stress sometimes occurs only in certain cells or certain organs without interfering with other cells in the body. Does this mean that antioxidant therapy should only target cells that are in oxidative stress? If excessive anti-oxidation treatment is applied to the whole body, will it cause excessive anti-oxidation of healthy cells? These questions warrant further investigation.

The techniques of the primary culture of cochlear organs can be used to solve some problems that are difficult to address in *in vivo* studies. These techniques offer the opportunity to assess and analyze the results of single-factor experiments (McFadden et al., 2003; Nicotera et al., ; Prakash Krishnan Muthaiah et al., 2017; Ding et al., 2013; Ding et al., 2018; Ding et al., 2011).

Since the purpose in current experiment was to evaluate if excessive antioxidants can damage the normal tissue in the

cochlea, we deliberately applied the antioxidant concentration higher than the conventional concentration to the cochlear explants. In the current experiment, we found that the cochlear cells in the control group maintained a healthy condition, which may be due to the maintenance of cellular dynamic equilibrium state of oxidative-antioxidative balance. However, in the medium, the addition of either M40403, coenzyme Q10 or d-methionine, inevitably caused severe cell damage. Since the experimental conditions are identical except for the presence of additional antioxidants in the culture medium, the cause of damage to cochlear cells can only be attributed to the antioxidative stress by the antioxidants.

One of the antioxidants tested in the current experiment is M40403, a compound that mimics the superoxide dismutase (SOD), which catalyzes the conversion of superoxide to hydrogen peroxide by disproportionation. Our previous studies have confirmed that in cochlear organotypic culture condition for 24 h, once the concentration of M40403 exceeded 30 μ M, all cochlear hair cells show significant pathological changes accompanied by severe cellular swollen. The cause of hair cell damage is probably due to excessive peroxide dismutase mimic producing excessive hydrogen peroxide (McFadden et al., 2003; Nicotera et al., ; Wang et al., 2013). The results of this experiment provide further evidence for the M40403-induced damage to cochlear hair cells (Fig. 1C), suggesting that inappropriate exogenous antioxidants may cause cell damage by disrupting the balance between inherent oxidants and antioxidants. This should be considered as excessive antioxidant damage.

The second antioxidant reagent tested in the current experiment is coenzyme Q-10, which is also called ubiquinone. Coenzyme Q-10 is one of the important components of the mitochondrial respiratory chain. The alcoholic structure of Q-10 is believed to have an anti-lipid peroxidation effect, because it can transfer hydrogen atoms from hydroxyl groups to lipid peroxidation radicals. This reaction leads to the reduction in the lipid peroxide production in the mitochondrial inner membrane. During this chemical reaction, a disproportionate free radical ubiquinone occurs between the coenzyme Q-10 alcohol structure and coenzyme Q-10. The oxime structure in the coenzyme Q-10 molecule makes the ubiquinone oxidized with two different forms, the reaction of free radical ubiquinone with oxygen can lead to the formation of more peroxide anions. Although the process of free radical ubiquinone involved in the transport of free radicals in the continued action of peroxide anions and hydrogen peroxide is believed to contribute to "detoxification", coenzyme Q-10 has roles in both antioxidative and pro-oxidative activities. In the situation where the concentration of coenzyme Q-10 is too high, whether or not more peroxide anions

are formed and the hydrogen peroxide immediately following them constitutes damage to the cells is a problem that needs careful considerations. Our current experiment, for the first time, reports that increasing the concentration of coenzyme Q-10 in the culture medium is able to cause significant damage to cochlear hair cells (Fig. 1E). Understanding of the biological basis for coenzyme Q-10-induced damage to hair cells requires further investigation.

The third antioxidant reagent tested in this experiment is d-methionine. d-methionine is an essential amino acid, and also important antioxidant glutathione, which plays a critical role in metabolism. According to reports in the literature, d-methionine acts as a sulfhydryl antioxidant that neutralizes oxygen free radicals by reversible oxidation and enhances the antioxidant effects of the glutathione pathway (Lu, 1998; Vogt, 1995). However, the methionine residue can be rapidly oxidized to a methionine sulfoxide residue by the action of reactive oxygen species, and the exposed methionine residue can continue to be oxidized by hydrogen peroxide, resulting in misfolding of the amino acid and denaturation of the protein. Whether the methionine sulfoxide residue can be reduced to methionine depends entirely on the methionine sulfoxide reductase. Once the activity of methionine sulfoxide reductase is reduced, the methionine sulfoxide residue will directly damage the cell (Zhou et al., 2009). Therefore, although d-methionine has the function of neutralizing oxygen radicals as an antioxidant, it also poses a risk for provoking cell damage by hydrogen peroxide oxidation.

We found in the experiment that after cochlear explants were incubated in 50 μ M d-methionine medium for 48 h, the surface structure of all hair cells showed significant pathological changes (Fig. 1G), although these cells were present. It is particularly noteworthy that all cochlear spiral ganglion neurons were completely destroyed by the treatment of d-methionine (Fig. 1H), suggesting that excessive d-methionine may preferentially damage the spiral ganglion neurons. These results raise a question that if the intrinsic methionine sulfoxide reductase is originally matched to the endogenous methionine sulfoxide residue, when a large amount of methionine sulfoxide residue is produced by supplementing excess d-methionine, is the intrinsic methionine sulfoxide reductase insufficient? This question warrants further investigation.

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Appendix A. Supplementary data

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