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Free radical scavengers, vitamins A, C, and E, plus magnesium reduces noise trauma

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Abstract

Free radical formation in the cochlea plays a key role in the development of noise-induced hearing loss (NIHL). The amount, distribution, and time course of free radical formation have been defined, including a clinically significant formation of both reactive oxygen species and reactive nitrogen species 7–10 days following noise exposure. Reduction in cochlear blood flow as a result of free radical formation has also been described. Here we report that the antioxidant agents, vitamins A, C, and E, act in synergy with magnesium to effectively prevent noise-induced trauma. Neither the antioxidant agents nor magnesium reliably reduced NIHL or sensory cell death with the doses we used when these agents were delivered alone. In combination, however, they were highly effective in reducing both hearing loss and cell death even with treatment initiated just one hour prior to noise exposure. This study supports roles for both free radical formation and noise-induced vasoconstriction in the onset and progression of NIHL. Identification of this safe and effective antioxidant intervention that attenuates NIHL provides a compelling rationale for human trials in which free radical scavengers are used to eliminate this single major cause of acquired hearing loss.

Keywords

cochlea; free radical; noise; hearing; antioxidant; vasodilation

Mechanical destruction of cells in the organ of Corti was once assumed to be the primary cause of noise-induced hearing loss (NIHL) [1–8], with perhaps some effect of reduced blood flow to the inner ear [9–18]. We now know that another key factor is intense metabolic activity that results in production of excess free radicals [19–23] and lipid peroxidation products [24]. Noise-induced production of reactive oxygen species (ROS) in the cochlea has now been well characterized, and several recent reviews are available [25–27]. Mitochodrial dysfunction and ROS production have been implicated in numerous neurodegenerative syndromes and diseases [28–34, see 35,36]. The use of antioxidant agents holds significant therapeutic promise for many neurodegenerative processes [32,33,37–42], and there is some suggestion that

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combinations of antioxidants are more effective than single agents [43]. The similarity of free radical production across multiple neurodegenerative diseases, including NIHL, and the putative efficacy of antioxidants in reducing neurodegenerative processes, provides a compelling rationale for study of antioxidants to prevent NIHL.

Consistent with antioxidant protection in neurodegenerative-cell death, many antioxidants are well known to partially protect sensory cells in the organ of Corti from stress-induced destruction. Upregulation of the endogenous antioxidant glutathione reduces NIHL and cell pathology [44,45], whereas the opposite is observed with reduced endogenous antioxidants [20,22,23,44]. That exogenous antioxidant agents reduce sensory cell death and NIHL has been well demonstrated in animal studies using a variety of free radical scavengers [24,44–56], including several studies with dietary antioxidants [50,57–60]. Magnesium supplements also reduce NIHL [61–69].

Given the potential for synergy of multiple agents in protection from neurodegenerative-cell death, in this investigation, we evaluated protection from NIHL and noise-induced sensory cell death with the combination of vitamins A, C, and E, or magnesium, or vitamins A, C, and E, plus magnesium. Each of these agents has a distinct mechanism of action. The primary antioxidant action of β-carotene (metabolized to vitamin A *in vivo*) is to scavenge singlet oxygen; because singlet oxygen reacts with lipids to form lipid hydroperoxides, the removal of singlet oxygen prevents lipid peroxidation [for review, see 70]. Vitamin E, present in lipids in cells [see 71], is a donor antioxidant that reacts with and reduces peroxyl radicals and, thus, inhibits the propagation cycle of lipid peroxidation [for review, see 70]. Vitamin C detoxifies free radicals by reducing them [for review, see 72]. Scavenging of oxygen radicals by vitamin C occurs in the aqueous phase [73,74], which is in contrast to the site of action of vitamin E, within membranes. Given differences in mechanism and site of action, there are differences in antioxidant free radical scavenging ability [70,75] as well as synergistic interaction of hydrophilic and lipophilic antioxidants [73,74,76–78]. Antioxidant scavenging of ROS also potentially reduces vasoconstriction that occurs with ROS production [46, for review see 27]. One effect of magnesium is reduction of noise-induced vasoconstriction [67,79–81].

Demonstration of an additive efficacy of these agents would provide greater insight into their mechanisms of action. A treatment that prevents, or more effectively reduces, NIHL would be of significant clinical benefit to the millions of workers exposed to high levels of noise, military personnel, and millions of others exposed to high levels of noise during recreational activities.

Methods

Animals

Pigmented male guinea pigs (250–300g; Elm Hill Breeding Labs, Chelmsford, MA) with a normal Preyer's reflex were used. Male guinea pigs were selected based on description of sex differences in ROS detoxification [82], activity of glutathione S-transferase in the cochlea [83], and susceptibility to NIHL [84]. The experimental protocol was reviewed and approved by the University Committee for the Care and Use of Animals (UCUCA) at the University of Michigan; all procedures conform to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Experimental groups

Guinea pigs were divided into four groups. All groups received once daily treatments beginning one-hour prior to noise exposure and continuing once daily at 24-hour intervals until day 5 post-noise, for a total of 6 daily treatments. Control animals (N=9) received saline injections (1 cc, i.p.). The second group was treated with vitamins A (2.1 mg/kg beta-carotene, p.o.), C

(71.4 mg/kg L-threoascorbic acid, s.c.), and E (26 mg/kg (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, "trolox", s.c.) ("ACE", N=8). Trolox is a cell-permeable, water-soluble derivative of vitamin E. The third group was treated with magnesium sulfate ("Mg", 2.85 mmol/kg, equivalent to 343 mg/kg, s.c., N=6). The fourth group received a combination of ACE and Mg (at the same doses as groups 2 and 3, "ACEMg", N=6). All test substances were purchased from Sigma-Aldrich (St. Louis, MO) (beta-carotene, #C9750, CAS 7235-40-7; L-threoascorbic acid, #A5960, CAS 50-81-7; Trolox, Fluka Chemika #56510, CAS 53188-07-1; magnesium sulfate, #M7506, CAS 7487-88-9).

Noise exposure

All subjects were exposed to octave-band noise (centered at 4 kHz, 120 dB SPL, 5 hours). This noise exposure is routinely used in our laboratory [85,86]. As in those investigations animals were exposed, two at a time in separate cages, in a ventilated sound exposure chamber. The sound chamber was fitted with speakers (Model 2450H, JBL, Salt Lake City, UT) driven by a noise generator (ME 60 graphic equalizer, Rane, Mukilteo, WA) and power amplifier (HCA-1000 high current power amplifier, Parasound Products, San Francisco, CA). Sound levels were calibrated (Type 2203 precision sound level meter, Type 4134 microphone, Bruel and Kjar Instruments, Norcross, GA) at multiple locations within the sound chamber to ensure uniformity of the stimulus, using a fast Fourier transform network analyzer with a linear scale. The stimulus intensity varied by a maximum of 3 dB across measured sites within the exposure chamber. During noise exposure, noise levels were monitored using a sound level meter, a pre-amplifier, and a condenser microphone positioned in the center of the chamber at the level of the animal's head.

Auditory brainstem responses

Auditory brainstem response (ABR) thresholds at 4, 8, and 16 kHz were measured for both right and left ears at two time points. Baseline ABR thresholds were established within 7 days prior to experimental day 1 (the first day of saline or micronutrient treatment, delivered on the day of noise exposure 1 hour prior to noise onset). Post-noise thresholds were established on day 10 post noise exposure. There is some recovery of ABR thresholds initially post-noise; both ABR threshold shift and loss of outer hair cells (OHCs) stabilize within 10 days of noise exposure [86].

Prior to ABR tests, animals were anesthetized with xylazine (10 mg/kg i.m.) and ketamine (40 mg/kg i.m.) and placed on a heating pad in one of two sound-isolated chambers (Industrial Acoustics Company, Bronx, New York, or C-A Tegnér, Bromma, Sweden). The external ear canals and tympanic membranes were inspected using an operating microscope to assure the ear canal was free of wax, there was no canal deformity, no inflammation of tympanic membrane, and no effusion of the middle ear [as in 85], then needle electrodes were placed subcutaneously below the test ear, at the vertex, and below the contralateral ear.

Acoustic stimuli were 4, 8, and 16 kHz tone bursts (15-ms duration; 1-ms rise-fall; 10/s) generated using Tucker-Davis Technology (TDT, Alachua, FL) software (SigGen 3.2, or SigGenRP) and TDT System II/III hardware (DA1, FT6-2, PA5, or RP2.1, PA5, HB7). Signals were presented to the external auditory meatus in a closed acoustic system through a tube connected to a transducer (Beyer DT-48, Beyer Dynamic, Farmingdale, NY). Starting levels were 80–85 dB during baseline tests, and 100–105 dB SPL during post-noise threshold tests. Sound intensity was initially decreased in 10 to 20 dB steps with 5-dB decrements presented near threshold. Evoked responses to 1024 tone presentations were amplified (100,000x) and filtered (300–3,000 Hz) (DB4 BioAmp, or RA4PA/RA4L1 Medusa) then digitized (AD1, or RA16 Medusa) and averaged (BioSig 3.2, or BioSigRP). Threshold, tested separately for each ear, was defined as the lowest intensity of stimulation that yielded a repeatable waveform based

on an identifiable ABR wave III or IV. ABR wave III is the most robust component of the guinea pig waveform [87,88].

Histological examinations

On day 14, after ABR measurement, the deeply anesthetized animals were decapitated and the cochleae were immediately removed for immunohistochemical staining with rhodamine phalloidin and hair cell counts. Upon removal, cochleas were transferred into 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS, pH 7.4). Under a dissecting microscope, the bone nearest the apex and the round and oval windows was opened, followed by gentle local perfusion from the apex. The tissue was kept in fixative for 12 hours, then the bony capsule and the lateral wall tissues were removed, and the modiolar core was carefully removed from the temporal bone. Following permeabilization with Triton X-100 (0.3%, 30 min), the organ of Corti was stained for f-actin using rhodamine phalloidin (1%, 60–120 min) to outline hair cells and their stereocilia [89]. After washing the tissues with PBS, the organ of Corti was dissected and surface preparations were mounted on glass slides. The tissues were observed under fluorescence microscopy, and the number of missing inner hair cells and outer hair cells were counted from the apex to the base in 0.19 mm segments [as described in 86]. Counting was begun approximately 0.76–1.14 mm from the apex, thus omitting the initial irregular most-apical part of the cochlear spiral. Percentages of hair cell loss in each 0.19 mm length of tissue were plotted along the cochlear length.

Statistical analysis

All data values in the text and figures are mean ± S.E.M.; all statistical comparisons were performed using SPSS. Statistical reliability of group differences in threshold and threshold shift were via ANOVA; frequency (4, 8, and 16 kHz) and ear (left, right) were treated as within-subject factors and treatment (saline, ACE, Mg, and ACEMg) was the between-subjects factor. Adjustment for multiple comparisons was accomplished using the Bonferroni correction. Inner and outer hair cell loss in different treatment groups were also compared via ANOVA; cochlear place (0–4.99 mm, 5–9.99 mm, 10–14.99 mm, and 15–20 mm from the apex) and ear (left, right) were treated as within-subject factors and treatment (saline, ACE, Mg, and ACEMg) was the between-subjects factor. Specific analyses are described below.

Results

Noise-induced threshold deficits were significantly smaller in animals treated with a combination of antioxidant agents and magnesium (see Figure 1). ABR threshold shift measured 10-days post-noise varied with treatment group ($F=15.289$; $df=3,25$; $p<0.001$) as well as frequency ($F=43.844$; $df=1.854, 46.338$; $p<0.001$); there was no effect of side (right/left), and there were no significant interactions of frequency x treatment, side x treatment, frequency x side, or frequency x treatment x side (all p's > 0.12). Adjusted pair-wise comparisons revealed that ABR thresholds were reliably lower in the group treated with vitamins A, C, E and magnesium compared to all other groups (all p's<0.001); lack of statistically reliable interactions among factors indicates that protection was equivalent across all test frequencies.

The significant reduction in noise-induced hearing loss in animals treated with both dietary antioxidants and magnesium reflects synergistic effects of these agents. ABR thresholds for this group of animals were lower post-noise, with no systematic group differences in threshold sensitivity prior to noise exposure. To verify that the group differences in noise-induced threshold shift were not a consequence of systematic group differences other than assigned treatment, threshold data used to calculate shift measures were also compared. Baseline ABR thresholds varied with frequency ($F=427.011$; $df=1.763, 44.071$; $p<0.001$) but were not reliably

different based on side (right/left) ($F=0.217$; $df=1, 25$; $p=0.645$) and did not vary as a function of group ($F=1.430$; $df=3, 25$; $p=0.258$). There were no statistically reliable interactions for frequency x group, side x group, or frequency x side x group (all p's > 0.15). In contrast, ABR thresholds measured 10-days post-noise varied with treatment group ($F=15.383$; $df=1,25$; $p<0.001$) as well as frequency ($F=111.660$; $df=1.380, 34.506$; $p<0.001$), and there were significant interactions of frequency x treatment ($F=2.840$; $df=4.141, 34.506$; $p=0.037$) and frequency x treatment x side ($F=2.456$; $df=5.7, 47.499$; $p=0.40$) but no reliable differences associated with side, or interactions of side x group or frequency x side (all p's > 0.2). Adjusted pair-wise comparisons revealed that post-noise ABR thresholds were reliably lower in the group treated with vitamins A, C, E and magnesium compared to all other groups (all p's<0.01). There were no statistically reliable differences between post-noise ABR thresholds in control animals and animals treated with either the antioxidant agents or magnesium (all p's > 0.4). Additional analysis revealed that the threshold differences between animals treated with vitamins A, C, and magnesium, and the other groups, were statistically significant at all test frequencies (all p's ≤ 0.011), with no effects of side and no interactions for side x group comparisons (all p's >0.13).

Sensory cell death was observed primarily within the basal half of the cochlea, i.e., 10–20 mm from the apex of the cochlea (see Figure 2), which corresponds to frequencies of approximately 3 kHz and above [90]. Within the basal half of the cochlea, treatment-based differences in outer hair cell survival exceeded the standard criteria limiting the probability that observed results are due to chance alone to 5% or less (rows 1–3, $p=0.111$). However, statistical power for this between-group comparison was 0.511 and, thus, was not sufficient to detect mean differences of this magnitude given the observed variability (see error bars, figure 2). To reduce the number of pair-wise comparisons, and maintain statistical power, we compared outer hair cell loss in the 10–15 and 15–20 mm from the apex segments of the cochlea in the two groups most crucial to testing our hypothesis: saline and combination treatment with antioxidants and magnesium (see Figure 3). Loss of row 1 outer hair cells was location dependant ($F=5.577$; $df=1, 13$; $p=0.034$) and the reliability of group differences approached the accepted definition of statistical significance ($F=4.134$; $df=1, 13$; $p=0.063$). Loss of row 2 outer hair cells was more homogenous across segments ($F=3.057$; $df=1, 13$; $p=0.104$) and the group difference was statistically reliable ($F=4.829$; $df=1, 13$; $p=0.047$). Outer hair cell loss in row 3 was generally equivalent to row 2 (location: $F=3.364$; $df=1, 13$; $p=0.090$; treatment: $F=4.550$; $df=1, 13$; $p=0.053$). Both the mean and the median hair cell loss for all 24 of the 0.19 mm segments of the cochlea in which hair cells were counted were less for the combined treatment group than for the saline control group; the probability of this outcome occurring by chance is less than 0.0001. Taken together, the combination treatment appears to preserve not only threshold sensitivity but also hair cell survival.

Discussion

Synergistic Effects

Treatment with a combination of vitamins A, C, E, and magnesium, initiated 1 hour prior to noise exposure, produced a compelling reduction in NIHL and cell death. Effects of either the antioxidant agents (vitamins A, C, and E), or magnesium, were very small and not statistically reliable. Thus, the combination of agents was clearly more effective than any single category of agents. High oral doses of some nutrients can have adverse effects in some populations; for example, high-dose vitamin A is associated with an increased rate of lung cancer in smokers [91–93]. However, the Institute of Medicine's Food and Nutrition Board has defined the highest level of daily nutrient intake that is likely to pose no risks of adverse health effects to almost all individuals in the healthy population (upper limit). The lack of significant adverse effects with long-term high-dose intake of several of these micronutrients has now been confirmed in

several investigations [94,95]. In addition, the US Food and Drug Administration has established recommended daily allowances that are safe for human use; these are readily available ‘over-the-counter’.

Mechanism of Action

We know that noise induces free radical formation; there is a nearly 4-fold increase in hydroxyl (OH) radicals within 1–2 hours of noise exposure [21], and a similarly significant early increase in superoxide (O_2^-) with reaction products evident at 5 min and 2 hours post noise [19]. There is also a significant late formation of ROS and RNS, occurring 7–10 days post noise [86]. We also know that noise induces lipid peroxidation; significant noise-induced lipid peroxidation and peroxynitrite ($ONOO^-$) formation have been described 15–30 min post-noise [25,96], and lipid peroxidation products increase in level from hour 1 to hour 5 of noise exposure [24]. We assume that pre-treatment with a variety of scavengers (including vitamins A, C, and E) reduced the early formation of free radicals that has been well characterized by Ohinata, Ohlemiller, Yamane, Nicotera, and their colleagues [19,21,24,25,96]. Daily treatment that continued through day 5 post-noise presumably reduced the late forming radicals revealed in the studies of Yamashita et al. [86]. Unlike most tissues, where increased metabolism increases blood flow to provide additional oxygen to stressed cells, reduced blood vessel diameter and red blood cell velocity [97,98] and decreased blood flow [9,10,16,26,99] are observed in the cochlea post-noise. This noise-induced vasoconstriction is a direct consequence of noise-induced formation of 8-isoprostane-$F_{2\alpha}$, a vasoactive by-product of free radicals [46], and thus, antioxidant agents that reduce free radical formation may eliminate noise-induced vasoconstriction. The combination of vitamins A, C, and E, delivered one hour pre-noise presumably did not adequately preserve inner ear blood flow, as prevention of noise-induced deficits was achieved only by the combination of vitamins A, C, E, and magnesium.

Together, the agents used here scavenge singlet oxygen (Vitamin A), react with and reduce peroxyl radicals in cell membranes (Vitamin E), detoxify free radicals by reducing them in the aqueous phase (Vitamin C), and reduce noise-induced vasoconstriction (magnesium). Although there are well-characterized differences in the primary mechanism and site of action of these agents, there is also potential for overlap in their effects. Specifically, antioxidant scavenging of ROS may reduce the vasoconstriction that occurs with ROS production. In addition to well known effects of magnesium on blood flow, other biochemical mechanisms may further contribute to the protective effects of magnesium. For example, magnesium modulates calcium channel permeability, influx of calcium into cochlear hair cells, and glutamate release [100,101], each of which may reduce NIHL. Regardless of the specific mechanism of action of vitamins A, C, and E, and magnesium, a combination of these agents clearly attenuates NIHL even when treatment is initiated very near the time of noise exposure (i.e., 1 hour prior to noise).

Individual Effects

Given that vitamins A, C, E, and magnesium, have been shown to attenuate NIHL and hearing loss from other stressors, it was somewhat surprising that protection was not observed for either the antioxidant (ACE) or magnesium treatments. Deficits were reduced to some small extent, but these effects were not statistically reliable. Stable plasma and tissue levels of vitamin C are obtained (in humans) approximately 3 weeks after beginning dietary treatment [102], and vitamin E levels similarly stabilize over an initial month-long window [for review, see 103]. Thus, it may be the case that dietary treatments must be provided on a daily basis for some longer period of time pre-noise for single-agent therapies to be effective. Indeed, the best protection against NIHL obtained with vitamin C [L--2-pascorbylolyphosphate, delivered as 5,000 mg per kg chow for 35 days, 60] included a 1-month pre-treatment protocol. Treatment initiated shortly before noise exposure failed to prevent cell death [500 mg/kg ascorbic acid,

i.p., 48 h, 24 h, and 5 min prior to noise exposure, 104]. In contrast, vitamin E (alpha-tocopherol, 10–50 mg/kg, i.p.) reduced NIHL with treatment initiated 3 days pre-noise [50], and vitamin A reduced NIHL with treatment initiated two days pre-noise [all-trans retinoic acid, 1 mg/kg p.o., in sesame oil, 105].

Taken together, there may be differences in the pharmacodynamics of different agents, and protection may be observed with antioxidants or magnesium alone if treatment is initiated 48 hours (or longer) prior to noise exposure to produce higher serum levels of the agents in the bloodstream. Agents that are effective shortly post-treatment have greater clinical utility. While the demonstration of significant protection with vitamins A, C, E and magnesium, delivered within 1-hour of noise exposure, suggests a compelling new clinical strategy to prevent NIHL, several of the active agents were injected. Thus, it is possible that the most efficacious treatment window, assuming oral administration for all agents, will be somewhat longer than 1 hour pre-noise exposure. Indeed, treatments based on other antioxidant combinations are the most effective when treatment begins several days prior to noise exposure [85]. Although increasing total daily doses of single agents might reduce the need for lengthy pre-treatment, clinical strategies tested in human subjects must not exceed the upper limits established by the Institute of Medicine's Food and Nutrition Board.

Efficacy of delayed treatment?

The compelling protection observed when treatment with vitamins A, C, and E, and magnesium was initiated shortly prior to noise exposure raises the possibility that treatments initiated post-noise may also be effective. Ohlemiller et al. [21] were among the first to suggest oxidative stress begins early and becomes substantial over time. Free radical insult that builds over time would explain hair cell death that continues up to 14 days post-noise [106]. Using 4-hydroxynonenal and nitryotyrosine, Yamashita et al. [86] confirmed that peak ROS and RNS production in cells in the organ of Corti occurs 7–10 days post-noise, and that noise-induced hair cell death is similarly delayed. As a consequence of delayed ROS production, ROS and RNS scavengers reduce NIHL not only when administered prior to noise, but also when treatment begins on days 1 or 3 post-noise [85]. Treatment delayed 5 days relative to noise insult was not effective, suggesting the therapeutic "window of opportunity" for successful antioxidant-based intervention against NIHL occurs within the first three days post-noise. Determining the efficacy of vitamins A, C, E, and magnesium when treatment is delayed relative to the noise insult is a compelling topic for future research.

Alternative Strategies

Multiple groups have pursued preservation of auditory function using glutathione (GSH) - based therapeutic strategies in recent years. Ebselen is a potent glutathione peroxidase (GPx) mimic; GPx catalyzes the anti-oxidant actions of GSH [107]. Pretreatment with ebselen reduces NIHL [108–111]. Given that one of the major determinants of GSH levels is bioavailable cysteine, and that cysteine can be derived from methionine, other studies have evaluated the potential for protection by pre-treatment with D-methionine [45]. D-methionine specifically alters the cochlear oxidative state; superoxide dismutase, catalase, GPx, and glutathione reductase levels all increase in animals treated with D-Methionine [112].

N-L-Acetylcysteine (L-NAC), which increases intracellular GSH and acts as a ROS scavenger, has also been used singly [47], and in combination with salicylate [113] to effectively attenuate NIHL. The utility of comparisons across investigations is compromised by variation in dose schedule and duration, species, and noise exposure. However, the significant reduction of NIHL (up to 35 dB at 16 kHz, with treatment initiated 1 hour prior to noise exposure), with vitamins A, C, E, and magnesium, suggests that this combination will prove to be one of the most effective therapies when different agents are compared under identical test conditions.

Relatively greater efficacy could be explained by the combination of antioxidants reducing both ROS and RNS production, with additional protection as a consequence of magnesium-mediated reductions in noise-induced vasoconstriction and excitotoxicity.

Identifying treatments to reduce neurodegenerative disease

Mitochondrial dysfunction and ROS production have been implicated in many, if not all, neurodegenerative processes, and also in association with changes in local blood flow, such as stroke [28–36]. The use of antioxidant agents to prevent neurodegeneration has been widely described [32,33,37–42]. As in our investigation, combinations of antioxidants have been shown to be more effective than single agents in at least some cases [43]. Vasoregulators are also clearly of potential benefit given observations of reduced cortical blood flow in both Alzheimer's and Parkinson's disease [114–118]. This similarity of free radical production and reduced blood flow in neurodegenerative processes and NIHL, and the demonstrated efficacy of antioxidants plus magnesium in reducing sensory cell death and neurodegeneration in the cochlea, provides a compelling rationale for study of combinations of antioxidants, with and without magnesium, to ameliorate neurodegeneration from other stresses and disease processes, at other sites of the nervous system, of more heterogeneous origins, such as Alzheimer's disease, Parkinson's disease, and stroke.

Conclusions

NIHL is a significant clinical issue [for recent review, see 26]. We now know many of the molecular pathways leading to apoptotic cell death, which are triggered by noise and other environmentally mediated trauma such as aminoglycoside antibiotics and chemotherapeutics, as well as aging; and there is increasing evidence for their similarity. Free radical formation has been implicated in all. Interventions can be directed at preventing initial ROS formation and maintaining cochlear blood flow (as reported here and by others); alternative therapeutic interventions and strategies include neurotrophic growth factors [119–122], steroids [123, 124], calcineurin inhibitors [125,126], caspase inhibitors [127–132], and Src protein tyrosine kinase (Src-PTK) inhibitors [133]. Each of these are at least partially effective in prevention of hearing loss and hair cell death; none of these strategies have alone been sufficiently effective. Given the various points of intervention along the cell death pathways, there are clearly an abundance of potential therapeutic targets. For clinical purposes, the most effective strategy may include targeting initiating events as well as early molecular processes, thus maintaining a cell in a relatively 'normal' physiological state. Although additional pre-clinical investigation is essential to the task of defining the most effective combination of agents, given that safe and effective interventions are now available, we should no longer delay the initiation of systematic human clinical trials to demonstrate the potential for pharmaceutical treatment of the inner ear in man.

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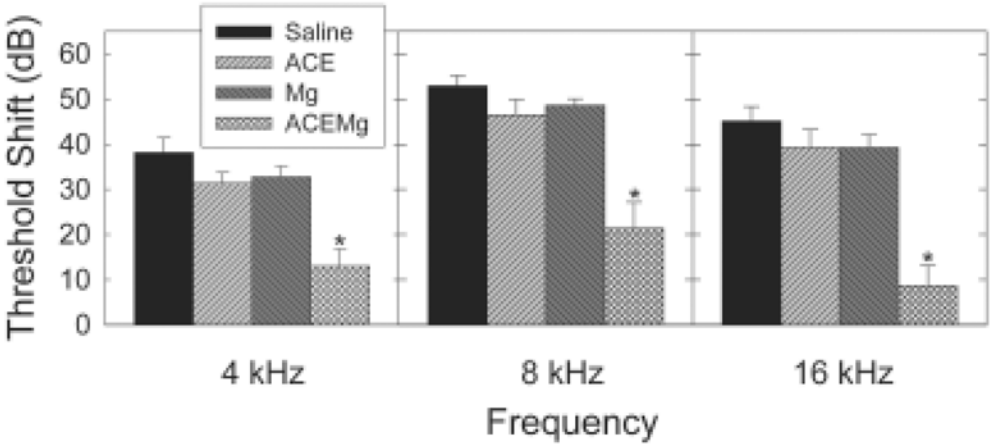

Figure 1.

Noise-induced hearing loss, estimated using auditory brainstem response thresholds prior to and 10 days post noise (octave-band noise centered at 4 kHz, 120-dB SPL, 5 hours), was reduced at 4, 8, and 16 kHz by treatment with a combination of vitamins A, C, E and magnesium (ACEMg), but not by treatment with the antioxidants (ACE) or magnesium (Mg). Asterisks indicate statistically reliable differences (p's < 0.001) between ACEMg and all other groups.

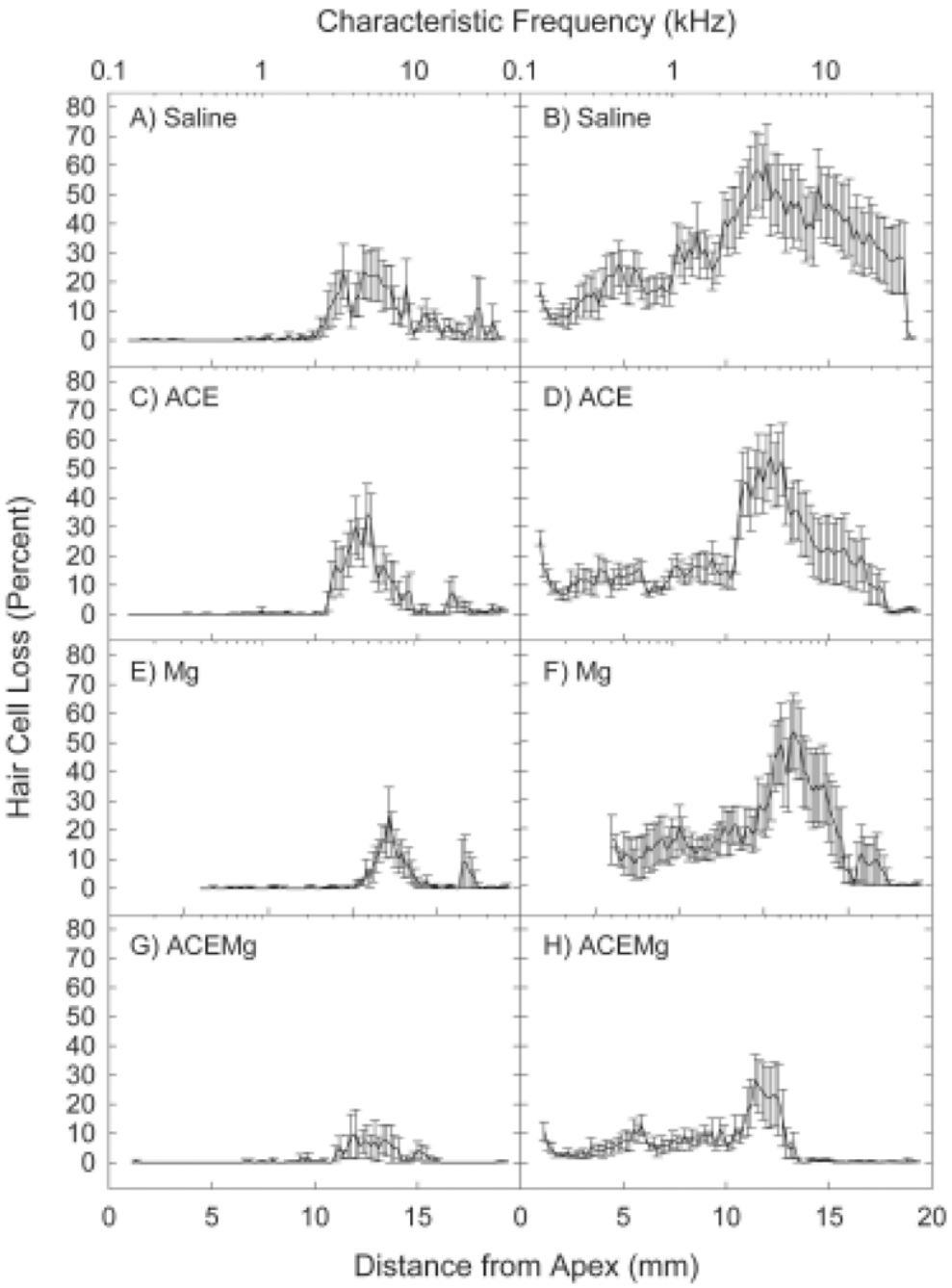

Figure 2.

Sensory cell death was observed primarily within the basal half of the cochlea, i.e., 10–20 mm from the apex of the cochlea, and corresponding to frequencies of approximately 3 kHz and above [90] for both inner hair cells (A, C, E, and G) and outer hair cells (B, D, F, and H). Both the mean and the median hair cell loss for all 24 of the 0.19 mm segments of the cochlea in which hair cells were counted were less for the combined treatment group (ACEMg) than for the saline group; the probability of this outcome occurring by chance is less than 0.0001.

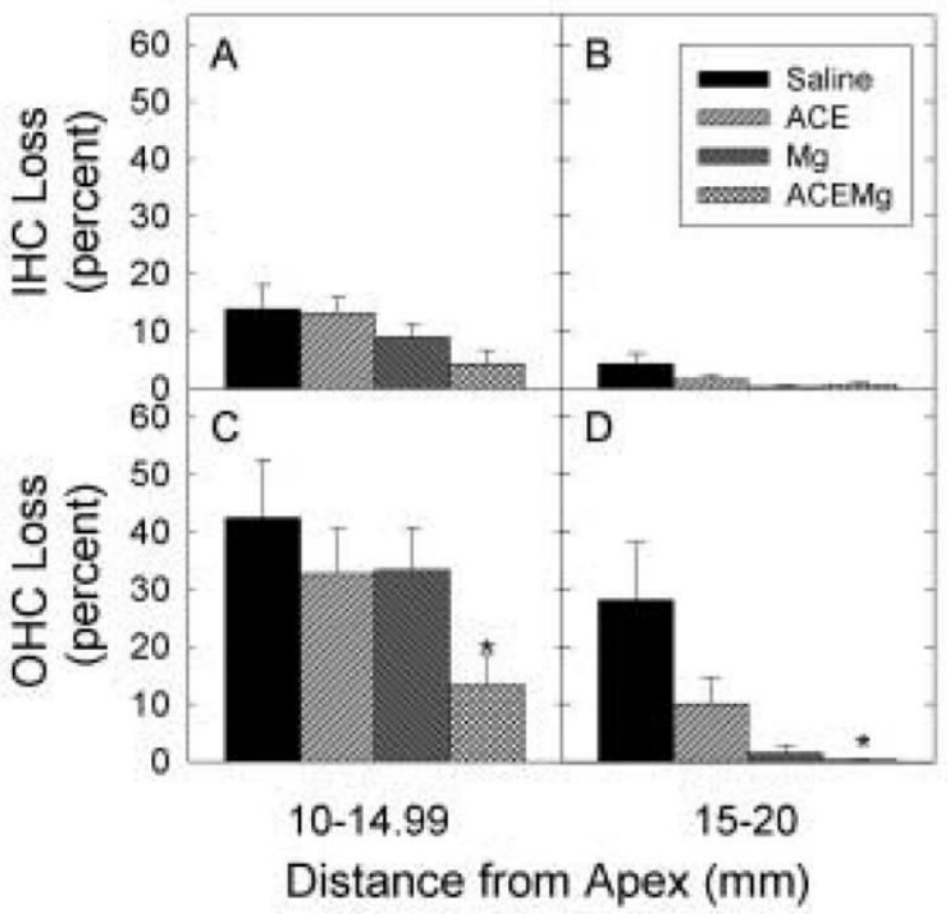

Figure 3.

Percent inner (A, B) and outer (C, D) hair cell loss in the 10–15 and 15–20 mm from the apex segments of the cochlea is illustrated. Treatment with a combination of vitamins and magnesium reduced outer hair cell loss by approximately 30% in both the 10–15 mm region (Saline: 42%; ACEMg: 13%) and the 15–20 mm region (Saline: 28%; ACEMg: <1%). This protection was statistically reliable when group comparisons were limited (to reduce the number of pair-wise comparisons, and increase statistical power) to the two groups most crucial to testing our hypothesis: saline and combination treatment with antioxidants and magnesium.