

# Influence of lecithin on mitochondrial DNA and age-related hearing loss

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**OBJECTIVES:** Lecithin is a polyunsaturated phosphatidylcholine (PPC), which are high energy functional and structural elements of all biologic membranes. PPC play a rate-limiting role in the activation of numerous membrane-located enzymes, including superoxide dismutase and glutathione, which are important antioxidants protecting cell membranes from damage by reactive oxygen species (ROS). ROS-induced damage to mitochondrial DNA may lead to reduced mitochondrial function in the cochlea and resultant hearing loss.

**STUDY DESIGN AND SETTING:** The effects of lecithin on aging and age-associated hearing loss were studied in rats by measuring hearing sensitivities using auditory brainstem responses (ABR). In addition, mitochondrial function as a measure of aging was assessed by determining mitochondrial membrane potentials using flow cytometry and by amplifying mitochondrial DNA deletions associated with aging. Harlan-Fischer rats aged 18 to 20 months ( $n = 14$ ) were divided into 2 groups. The experimental group was supplemented orally for 6 months with lecithin, a purified extract of soybean phospholipid (Nutritional Therapeutics, Allendale, NJ).

**RESULTS:** The data obtained were compared with the control group. ABRs were recorded at 2-month intervals and showed significant preservation of hearing sensitivities in the treated subjects. Flow cytometry revealed significantly higher mitochondrial membrane potentials in the treated subjects, suggesting preserved mitochondrial function. Finally, the common aging mitochondrial DNA deletion (mtDNA<sup>4834</sup>) were amplified from brain and cochlear tissue including stria vascularis and auditory

nerve. This specific deletion was found significantly less frequent in all tissues in the treated group compared with the controls.

**CONCLUSION:** These experiments support our hypothesis and provide evidence that lecithin may preserve cochlear mitochondrial function and protect hearing loss associated with aging. (*Otolaryngol Head Neck Surg* 2002;127:138-44.)

Lecithin is a polyunsaturated phosphatidylcholine, which are high energy, basic, structural, and functional components of all biologic membranes. They are indispensable for cellular differentiation, proliferation, and regeneration. The primary purpose of this study was to determine if lecithin could reduce age-related hearing loss. This premise is based on the membrane hypothesis of aging (MHA), also known as the mitochondrial clock theory of aging. According to the MHA, reactive oxygen species (ROS) are responsible for progressive insults on mitochondria and other cellular structures; and with time, these insults accumulate leading to cellular demise and resultant senescence.

The process of aging is associated with many molecular, biochemical, and physiologic changes. Mitochondrial membranes are considered a probable subcellular locus of the age-linked decline of energy metabolism due to their central role in the energy transduction pathways. Specifically, mitochondrial metabolism occurs within a membrane of highly restrictive permeability properties, and changes in the membrane lipid content, lipid composition, and lipid protein interaction may occur with aging resulting in instability of the mitochondrial membrane and a decrease in the mitochondrial energy production.

Polyunsaturated phosphatidylcholines (PPCs) are highly purified extracts of the semen of soy bean, supplying the organism with nontoxic choline molecules with a high content in polyunsaturated fatty acids, in particular linoleic acid. These PPCs correspond to the body's own phosphatidylcholine molecule. The physiologic functions of

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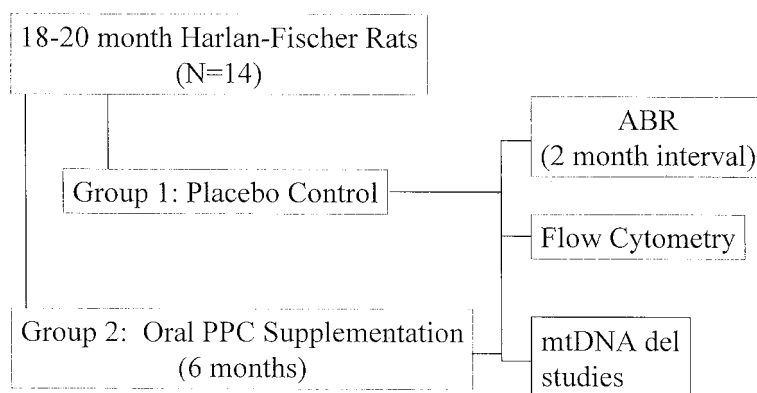
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**Fig 1.** Experimental design: 18- to 20-month-old Harlan-Fisher rats were divided into 2 groups. Oral supplementation of Lecithin was provided to the test group. Auditory brainstem responses, flow cytometry, and mitochondrial DNA deletion studies were done on all subjects.

these phospholipids are related to the morphology of the biologic membranes, the incorporation of these molecules into membranes and thus the effect on the intact structure of cell membranes.<sup>1</sup> PPCs have also been shown to play a rate-limiting role in the activation of various membrane-located enzymes, including superoxide dismutase and glutathione reductase, important antioxidants protecting from ROS-induced membrane damage.

There are several disease processes related to membrane damage for which clinical and pharmacologic trials using PPC have been conducted. Effects of PPC on these various disorders have shown enhancement in cognitive performance of the aging brain, improvement of coronary, peripheral, and cerebral blood flow, activation of liver metabolism, and detoxification and promotion of gastrointestinal function by mucosal restoration.<sup>2-5</sup> This study is a continuum in demonstrating the effects of lecithin on preservation of another important biologic function. It provides evidence regarding the protective effects of lecithin on age-related hearing loss by its ability to preserve mitochondrial function and protect mitochondrial DNA from oxidative damage.

## EXPERIMENTAL DESIGN

Harlan-Fisher 344 rats, 18 to 20 months of age, were used as the experimental subjects. The subjects were caged individually and maintained at 21-22° C in a 12:12 light/dark cycle. The experimental protocols were reviewed and approved by the Care for Experimental Animal Committee

(CEAC) at the Henry Ford Health System. These protocols are in strict compliance with guidelines established by the National Institutes of Health.

The PPC lecithin was obtained from Nutritional Therapeutics (Allendale, NJ). An average dose of 300 mg/kg/d of lecithin was supplemented to each subject by adding the PPC to the oral diet. This dose has been used previously in rodent studies and has not shown any untoward side effects.

## METHODS

The animals were assigned randomly into 2 groups (n = 7 for each group). Group 1 served as the control, and group 2 as the experimental group (Fig 1). These experimental subjects were given oral supplementation of lecithin added to their daily diet. The amount of lecithin administered (300 mg/kg/d) was averaged with their daily oral intake. Group 1 received regular diet without supplemental lecithin.

At the onset of the study, ABRs were obtained to measure baseline hearing thresholds in all subjects. Age-associated changes in hearing sensitivities were then recorded at 2-month intervals for 6 months. To assess age-related changes in mitochondrial function, mitochondrial membrane potentials were studied by flow cytometry. For this purpose, peripheral blood was obtained from each subject at the beginning and at the end of the protocol. At the conclusion, the subjects were euthanized according to NIH protocol, and tissue samples were obtained from brain and cochlea (stria vascularis and auditory nerve) to study mi-

tochondrial DNA deletions associated with aging. This was achieved by amplifying the specific common aging mitochondrial deletion (4834 bp) by polymerase chain reaction (PCR). DNA quantification was performed. The data obtained for each protocol were compared between the 2 groups and analyzed.

### **Auditory Brainstem Responses**

Hearing thresholds were tested every 2 months. Animals were anesthetized using ketamine and xylazine (100 and 10 mg/kg, respectively, intramuscularly) and supplemented with ketamine as required. The subject's head was secured in a head holder, and temperature was maintained with a thermostatically controlled heating blanket and rectal probe. A Bruel & Kjaer condenser microphone with speculum was placed in the external auditory canal and held 2 to 3 mm from the tympanic membrane. Sterile 1/2-inch 26-gauge needles were placed under each pinna and at the vertex. Wires from each needle electrode are led to a Grass P511H amplifier gain  $\times 5000$  (band pass of 0.3 to 3.0 kHz) and then to a signal processing board (Spectrum model TMS320C25). The output of the biologic amplifier was viewed on an oscilloscope (Tektronics Model 5112). The average waveforms were stored on a Pentium computer for offline analysis. A total of 512 samples, 25- $\mu$ sec bin width, and 1024 responses were averaged. Tone bursts (1.0-msec rise-fall time, 15-msec duration) were used to assess the auditory sensitivity. Intensity series were obtained at 3.0, 6.0, 9.0, 12.0, and 18 kHz. The waveforms were recorded and saved for offline analysis.

### **Flow Cytometry**

Mitochondrial membrane potentials were measured by obtaining peripheral blood samples from each subject at the beginning and at the end of the protocol. Flow cytometry was used for this purpose. Lymphocytes were isolated from the peripheral blood samples by the standard Ficoll-Hypaque cell isolation technique. These cells were then incubated with rhodamine 123 (Eastman Organic Chemicals, Rochester, NY), fluorescent activated dye at 37° C. This contrast material is preferentially taken up by the mitochondrial membrane and therefore exclusively measures mito-

chondrial membrane uptake and hence mitochondrial membrane potential. An Argon laser (488 nm) was used and green fluorescence was measured through a 530-nm band-pass filter. FACStar computer software was used for offline analysis. The pretreatment and the posttreatment mitochondrial membrane potentials were compared in the 2 groups.

### **DNA Extraction**

Tissue samples were harvested from brain, auditory nerve, and stria vascularis. These samples were stored at  $-70^{\circ}$  C until the time of mtDNA extraction. The tissue samples were homogenized in 10 mM Tris (pH 8.0), containing 1 mM EDTA buffer and incubated overnight at  $-56^{\circ}$  C with 15  $\mu$ L Proteinase-K (10 mg/mL) in 0.5 mL digestion buffer consisting of 10 mM Tris (pH 8.0), 10 mM EDTA, 50 mM NaCl, and 2% sodium dodecyl sulfate. Standard extraction protocols for DNA were used with phenol, chloroform, and isoamyl alcohol. The proteins are removed from the sample solution with phenol/chloroform (25:24), both of which serve as separate organic solvents and hence deproteinize more efficiently. The tissue extracts are then centrifuged at  $10,000 \times g$  at room temperature to separate mtDNA from cellular debris, proteins, and genomic DNA. The supernatant is drawn off, and the residual phenol removed with equal volumes of chloroform/isoamyl alcohol (24:1). This subsequent extraction with chloroform removes the remaining traces of phenol from the preparation. Volumes of 1/10 of 3 M NaOAc and 1/100 of 1 M  $MgCl_2$  were added and mtDNA was recovered by precipitation with 2.0 volumes of cold ethanol. This preparation was stored at  $-70^{\circ}$  C for 60 minutes, and the precipitate was recovered by centrifugation at  $12,000 \times g$  for 30 minutes ( $4^{\circ}$  C). The supernatant was removed, and the pellet was washed with 70% ethanol, air-dried and redissolved in TE buffer at the desired concentration. PCR was then performed on aliquots of this purified mtDNA.<sup>6</sup>

### **Polymerase Chain Reaction**

Each PCR contained 150 ng of mtDNA from test sample, 200  $\mu$ M of each dNTP, 50 mM HCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM  $MgCl_2$ , 0.01% (wt/vol) gelatin, 1  $\mu$ M of each primer and 5.0 U of

*Taq* polymerase in a final volume of 100  $\mu$ L. The thermal cycling parameters were: initial denaturation at 94° C for 3 minutes, followed by 30 cycles of denaturation at 94° C (30 seconds), annealing at 56° C (30 seconds), and extension at 72° C (60 seconds). Specific primers designed in our laboratory have been synthesized (Operon Technologies, Alameda, CA) to amplify distinct regions of the mtDNA genome. The primer sequences have been published previously. The specific segments amplified include the ND1-16SrRNA genome as well as the mtDNA<sup>4834</sup> common aging deletion.

### Gel Electrophoresis

The amplified PCR products are separated by electrophoresis on 1.5% agarose gel containing ethidium bromide. Gel electrophoresis was performed at 100 V for 3 hours. Gels are then read under ultraviolet light and imaged.

### DNA Quantification

Quantitative PCR was performed using PCR ELISA (DIG Detection) following the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany).

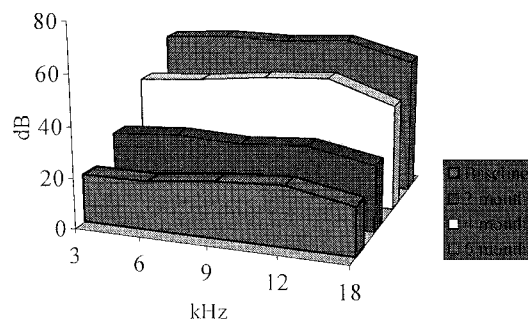
External standards were prepared by gel purifying PCR products of ND1-16S rRNA and the common aging deletion as described above. PCR was performed with standards and the different DNA samples at the exponential phase. The PCR products were labeled with digoxigenin, using DIG-UTP during PCR. An aliquot of the labeled PCR products (2 to 5:1) are then bound to the streptavidin-coated surface of a microtiter plate by the use of a biotin-labeled capture probe. This capture probe has to be designed to hybridize to an internal sequence of the PCR product. The bound DIG-labeled PCR products were then detected with an anti-DIG-peroxidase conjugate and the substrate ABTS. The colorimetric signal at 405 nm allows quantitative determination of the amount of PCR product. The ratio of the deleted mtDNA to the total mtDNA was recorded and compared between the control and treated groups.

## RESULTS

### Hearing Sensitivities

The effects of lecithin on age-related hearing loss and on mitochondrial membrane potential and mito-

*Control Group*



**Fig 2.** Results: auditory brainstem responses. Auditory Thresholds (dB) in the control subjects at 2-month intervals. There was a progressive loss in hearing sensitivities noted with time.

chondrial DNA deletion associated with aging are demonstrated. Figure 2 represents the auditory threshold in the control subjects at 2-month intervals during the study. There was a gradual age-related decline in hearing sensitivities noted at all the frequencies tested (3, 6, 9, 12, and 18 kHz). These results are comparable to previous studies that have shown similar results under similar experimental conditions.<sup>6,7</sup> Comparing these hearing thresholds to the experimental subjects (Fig 3), there was no significant difference noted between the 2 groups at baseline or at the initial 2-month interval. There was, however, a trend for a greater loss in the control subjects at all test frequencies. At 4 months, however, there was statistically significant preservation of hearing noted in the treated subjects at all frequencies. This pattern persisted at 6 months, with a continued decline in hearing in the control subjects and a statistically significant protective effect of lecithin at all frequencies in the experimental subjects.

The hearing threshold shifts from baseline for the tested frequencies are compared between the two groups. Figure 4 demonstrates these findings. Auditory threshold shifts are essentially the changes noted in the hearing level at the end of the protocol. The threshold shifts ranged from 35 to 40 dB in the control subjects at the 4 test frequencies, compared with 13- to 17-dB shifts in the experimental group. Using analysis of variance (ANOVA), these differences were statistically significant at each of the frequencies, demonstrating the protective effect lecithin on age-associated hearing decline.

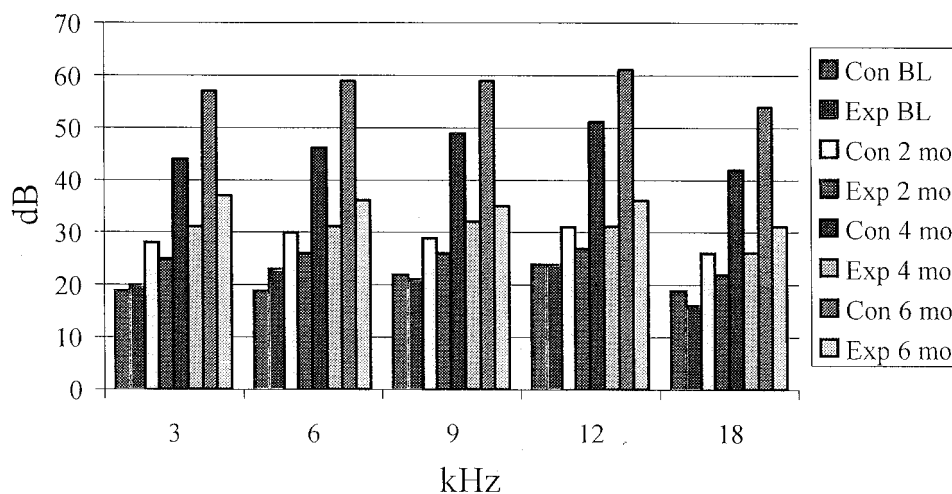


Fig 3. Results: auditory brainstem responses. Auditory Thresholds (dB) in the control group compared with the experimental group. Hearing preservation in the experimental group was statistically significant at 4 and 6 months across all test frequencies.

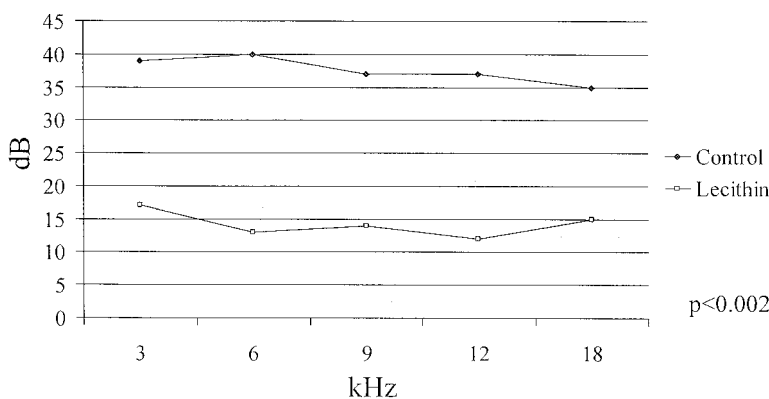


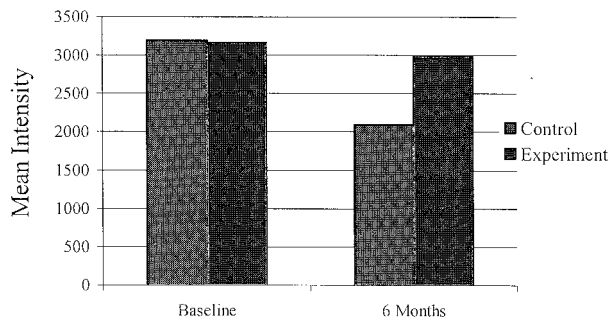
Fig 4. Results: auditory threshold shifts. Hearing threshold shifts at 6 months from baseline values in the control and the experimental (lecithin) group. Statistically significant preservation of thresholds is noted with lecithin use.  $p < 0.002$

### Mitochondrial Membrane Potentials

Mitochondrial membrane potentials were recorded by flow cytometry as a measure of the uptake of rhodamine 123 by mitochondria. This probe is specific for mitochondria as it is taken up preferentially by the mitochondrial membrane. The intensity of this uptake corresponds to the mitochondrial activity and hence membrane potential. The data obtained from the 2 groups were averaged and statistical analysis was performed using ANOVA. These results are demonstrated in Figure 5. The mean fluorescence intensity (MFI) in group 1 subjects measured 3190 and 2100 at the beginning and end of the study, respectively. This decline in membrane potential with time was sta-

tistically significant ( $P = 0.003$ ). On the other hand, the MFI in the experimental group remained essentially unchanged at 3165 at the beginning of the study and 2990 at the conclusion. This change was not statistically significant. However, when comparison is made between the treated versus control subjects, there was a statistically significant better MMP in the treated subjects, thus demonstrating the protective effect of lecithin supplementation on mitochondrial membrane potentials.

For the mtDNA deletion tests, mtDNA from brain, stria vascularis, and auditory nerve were studied. To verify the presence of mtDNA, the ND-1 16S rRNA segment was identified, which is a highly preserved region of the mitochondrial



**Fig 5.** Results: flow cytometry. Mitochondrial membrane potentials, hence mitochondrial function, was measured by the mean intensity of mitochondria-specific rhodamine-123 uptake. Lecithin-treated subjects demonstrated preservation of membrane potentials compared with the control subjects.

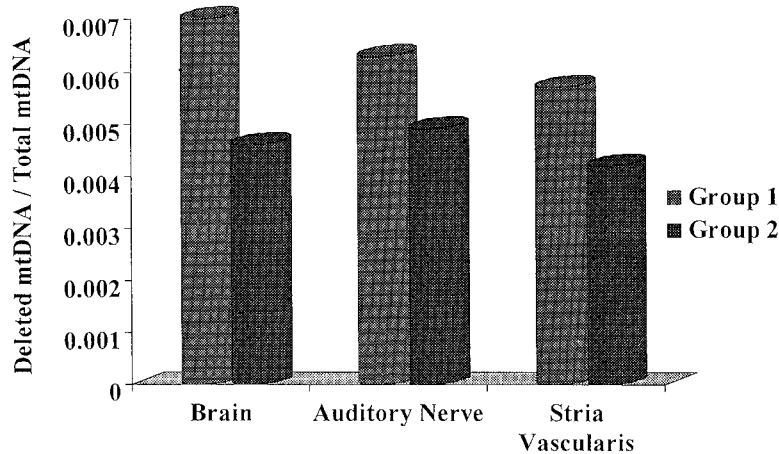
genome. Specific primers for this segment and for the common aging deletion were synthesized, the sequences of which have been previously published.<sup>6</sup> Equal quantities of mtDNA were used in all samples for standardization. The PCR products identified the ND-16S rRNA region by a 601-bp product in all samples and the common aging deletion (4834-bp deletion) by a 598-bp product. This aging deletion was identified in the 5 of the 7 control subjects and 4 of the experimental subjects. Quantitative determination reveals a lower ratio of this common aging deletion to the total mtDNA in the experimental subjects as compared with the control subjects (Fig 6). Based on these findings we conclude that lecithin has a protective

effect on mitochondrial DNA damage and function.

## DISCUSSION

These experiments demonstrate the protective effects of Lecithin on age-related hearing loss and its ability to safeguard mitochondrial function by preserving mitochondrial membrane potential and protect mitochondrial DNA from oxidative damage.

In recent years, the effects of reactive oxygen species (ROS), also known as free radicals, and their metabolites on biologic systems have received much attention. ROS play important roles in many biochemical reactions that maintain normal cell functions. Increasing evidence indicates that ROS are also important mediators of many forms of tissue damage, such as injuries associated with inflammatory responses, ischemic injuries to organs, and injuries resulting from the intracellular metabolism of chemicals and drugs. ROS are increasingly recognized for their contribution to tissue injury during ischemia and, in particular, during the phase of reperfusion and prolonged hypoperfusion. The primary *in vivo* source of ROS is the mitochondrial electron transport system during oxidative phosphorylation. Other sources of ROS include purine catabolism by xanthine oxidase, prostaglandin biosynthesis, infiltration of phagocytes, environmental contaminants,



**Fig 6.** Results: mtDNA quantification. Mitochondrial DNA deletion quantification demonstrates a lower ratio of common aging deletion (mtDNA<sup>4834</sup>) in the experimental group as opposed to the control group.

ionizing radiation, and aging. Many components within the cell are susceptible to attack by ROS.

ROS generation occurs from periods of prolonged relative hypoperfusion such as occurs with aging. It has been demonstrated that in the elderly population there is decreased flow within the circulatory system in general<sup>8-10</sup> and the inner ear in specific.<sup>7,11</sup> Prolonged periods of reduced blood flow such as those accompanying aging lead to the formation of tissue damaging ROS. ROS have been implicated in injury to polyunsaturated fatty acids in cell membranes resulting in the process of auto-oxidation, which is of great importance in the pathogenesis of cell membrane damage.<sup>12</sup> They have also been shown to be mediators of mitochondrial DNA damage including the generation of mitochondrial DNA deletions (mtDNA del). mtDNA del have been associated with cellular and tissue dysfunction, senescence, and death.<sup>13,14</sup> This sequence of events is the foundation of the membrane hypothesis of aging (MHA).

Phospholipids are basic structural components of all biologic membranes with phosphatidylcholine and phosphatidylethanolamine being the predominant types, quantitatively. They constitute the phospholipid bilayer structure of cellular membranes, which is responsible for membrane stability and cellular function. PPCs maintain and promote the activity of several membrane bound proteins and enzymes, including Na<sup>+</sup>, K<sup>+</sup>-AT-Pase, adenylate cyclase, and glutathione reductase. They are also known to be precursors of cytoprotective agents such as eicosanoids, prostaglandins, and antioxidants.

These experiments suggest that lecithin may protect mitochondrial function by preserving the age-related decline in mitochondrial membrane potentials and hence their activity. This may also explain the demonstrated effect of preservation of hearing loss associated with aging, by the ability of lecithin to specifically upregulate cochlear mitochondrial function, and possibly by its cellular protective effects. Recent work studying the effects of mitochondrial metabolites on aging, has shown that acetyl L-carnitine and  $\alpha$ -lipoic acid

delay the progression of age-related hearing loss by protecting cochlear mitochondrial DNA from oxidative damage.<sup>15</sup> These results support the Membrane hypothesis of aging and provide further evidence to support this theory as a possible explanation for age-related hearing loss.

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