

Efficiency of resveratrol in the prevention and treatment of age-related hearing loss

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Abstract. Age-related hearing loss (ARHL) is a major public health concern, which is characterized by gradual, progressive sensorineural hearing loss and deterioration of sound localization, with no effective treatment available to date. The aim of the present study was to evaluate the efficacy of resveratrol to prevent and treat ARHL. For this purpose, 32 male C57BL/6 mice were assigned to four groups: Early treatment, late treatment, control and sham control. The experiment lasted for 15 months. Treatment was started at three months of age in the early treatment group and at sixth months in the late treatment group. The auditory brainstem response test was performed once every three months. At the end of the study period, *inducible nitric oxide synthase (iNOS)*, *cyclooxygenase (COX)-2*, *NF-κB*, *Bcl-2*, *Bcl-xL*, *Bax*, *Bcl-2* homologous antagonist/killer (*Bak*), *caspase-3* and *caspase-9* levels in the cochlear tissues of the animals were analyzed by reverse transcription-quantitative PCR. Hearing thresholds of the mice in the early treatment group were better than those in the other groups ($P<0.001$) at the end of the study. However, hearing levels in the late treatment group were not significantly different from those in the control groups ($P>0.05$), although mean thresholds were lower. The threshold shift in the early treatment group was significantly lower at all frequencies when compared with those in the control groups ($P<0.001$). The mRNA expression levels of pro-apoptotic genes *Bax* and *Bak*

were lower ($P<0.05$), anti-apoptotic genes *Bcl-2* and *Bcl-xL* were higher ($P<0.05$), *NF-κB*, *COX-2* and *iNOS* as genes that have a role in inflammation and *caspase-3* and *caspase-9* as genes with a vital role in apoptosis were lower ($P<0.05$) in the early treatment group when compared with the late treatment and control groups. These results suggested that resveratrol is effective in the prevention of ARHL, particularly when started prior to the beginning of hearing loss.

Introduction

Age-related hearing loss (ARHL), also known as presbycusis, is described as a gradual loss of hearing that is linked to aging. ARHL is characterized by impaired speech discrimination, deteriorated hearing sensitivity, slowed central acoustic signal processing and impaired localization of sound sources. ARHL is the most common sensory disorder in the elderly population (1), with a prevalence of 25-40% of individuals aged 65 years or above. The prevalence increases with age, ranging from 40 to 66% in individuals over 75 years of age and >80% in those over 85 years of age (2).

ARHL is thought to be the result of aging, mitochondrial dysfunction, oxidative damage and environmental factors (3). It has been widely recognized that aging is a process of cumulative oxidative damage caused by free radicals (4,5). Free radical production increases with age and it is recognized that oxidative stress and related mitochondrial dysfunction have an important role in aging and age-related diseases (6,7). Numerous studies have demonstrated that diminishing oxidative stress may extend an organism's life span (8). Therefore, an effective way to slow the progression of age-related damage to the body may be to improve its antioxidant defense against oxidative stress.

Apoptosis, or programmed cell death, is essential for maintaining the balance of cell life and death. It has been known for a long time that apoptosis is involved in aging and age-related diseases (9). In addition, it has been demonstrated that apoptotic cell death occurs earlier in mice with presbycusis (10).

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Resveratrol (3,5,4'-trihydroxystilbene; RSV), which is known to be a potent antioxidant, is a natural phytoalexin and polyphenol that is present in a wide range of plants, such as grapes, berries, mulberries and peanuts (11). The biological effects of RSV include scavenging of free radicals, inhibition of lipid peroxidation, anti-inflammatory effects, copper chelation, modification of eicosanoid synthesis, inhibition of platelet aggregation, vasodilatation, modulation of lipid metabolism, vasorelaxant activity, anticancer activity and estrogenic activity (11,12).

Resveratrol has protective effects against cisplatin-induced (13), aminoglycoside-induced (14) and noise-induced hearing losses (15). Furthermore, it was reported that a mixture of polyphenols, including RSV, have a beneficial effect on presbycusis (16).

The aim of the present study was to evaluate the efficacy of RSV in the prevention and treatment of ARHL as assessed by auditory brainstem response (ABR) testing and evaluation of the changes in the mRNA expression levels of *inducible nitric oxide synthase (iNOS)*, *cyclooxygenase-2 (COX-2)* and *NF- κ B* genes, which are effective in inflammation, and *Bcl-2*, *Bcl-xL*, *Bax*, *Bcl-2* homologous antagonist/killer (*Bak*), *caspase-3* and *caspase-9* genes, which all have a role in apoptosis, in cochlear samples of C57BL/6 mice.

Materials and methods

Animals. A total of 32 male C57BL/6 mice were used for the present study. The animals were obtained from the Gazi University Laboratory Animal Raising and Experimental Research Center at 12 weeks of age (mean weight, 28.6 \pm 1.9 g) and the study was carried out in the same place (Ankara, Turkey). These mice have been extensively studied as a model of ARHL (17). Hearing loss begins at approximately five to six months of age and progresses to near-complete hearing loss by the age of 18 months in this strain (18). The animals were housed in plastic cages at 23 \pm 2°C with a relative humidity of 55% \pm 5, with water and food provided *ad libitum* and maintained under a 12-h light/dark cycle. The Animal Care and Use Committee of Gazi University (Ankara, Turkey) approved the study protocol (approval no. 66332047-604.01.02-7347). The study was performed in accordance with the EU Directive 2010/63/EU.

Anesthesia. Mice were anesthetized by i.p. injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) cocktail during ABR measurements. At the end of the study, animals were painlessly sacrificed by cervical dislocation under the same anesthetic protocol.

Study protocol. Power analysis was used to determine the sample size. A desired power of 0.80 and a desired α of 0.05 were set with an estimated size effect δ of ~1.4, based on preliminary data. Mice were randomized into four groups and treated as follows: Control group (n=8), with no medication; sham control group (n=8), with DMSO administration initiated at three months of age; early treatment group (n=8), with RSV treatment initiated at three months of age; and late treatment group (n=8), with RSV treatment initiated at six months of age. The timeline of the study protocol is presented in Fig. 1.

Trans-resveratrol (Sigma-Aldrich; Merck KGaA), dissolved in 30% DMSO (20 mg/ml) (Sigma-Aldrich; Merck KGaA) and added to the drinking water of mice so that the approximate daily dosage of 500 μ g/kg for each mouse was maintained (16). Drinking water was changed every day to ensure the required dose was taken by the mice. In addition, new solution was prepared weekly during the study period to ensure the stability of RSV. The mice were weighed once every week and the dose of the RSV was adjusted according to the weights of the mice, if necessary. 30% DMSO was also prepared freshly on a weekly basis and added to the drinking water of the sham control group. The sham control group was created to evaluate the possible effects of DMSO, the substance that RSV was dissolved in, on the hearing of the mice.

ABR. ABR measurements were performed with a two-channel Neuro-audio® ABR device (Neurosoft; Ivanova). After anesthesia, ABR measurements were performed for both the right and left ears of every mouse; two traces were recorded for each ear to control repeatability of the response. ER-2, 10 ohm insert earphones with a frequency spectrum of 125-16,000 Hz were used for auditory stimuli and subdermal disposable needle electrodes (Bionen Medical Devices) were used for recording of the ABR. Single-channel ipsilateral recording was performed to prevent a possible short circuit between electrodes. The non-inverting electrode was placed at the vertex, the invert electrode was placed on the ipsilateral mastoid and the ground electrode was placed on the contralateral mastoid. The stimuli consisted of frequency-specific tone bursts over a 4,000-16,000 Hz range, with a 21/sec stimulus rate and 1,024 presentations averaged for each intensity.

Thresholds were determined by reducing the intensity of the stimulus in 5-dB steps starting from 110 decibels sound pressure level (dB SPL). The ABR threshold was defined as the lowest dB SPL level at which waveforms lost their reproducible morphology.

Baseline ABRs measurements were performed when the mice were 3 months of age, prior to the initiation of all treatments, before the start of all treatments. ABRs were recorded every three months till the end of the study, when the mice were 6, 9, 12 and 15 months of age (Fig. 1).

RNA isolation, cDNA synthesis and quantitative PCR (qPCR). At the end of the study period, all mice were sacrificed under anesthesia after the last ABR testing. The cochleae of both ears were removed under a dissecting microscope and crushed. Total RNA was isolated from fresh cochlear tissues by using TRIzol® reagent (Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's instructions and then stored at -80°C until use. The RNA concentration was measured at 260/280 nm, using the NanoDrop® 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA (1 μ g) was used for gene-specific reverse transcription (RT) with a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics), following the manufacturer's protocol.

qPCR reactions were performed using the LightCycler® 480 instrument (Roche Diagnostics), according to the manufacturer's instructions. Gene-specific intron spanning primers and universal probe library (UPL) numbers for each gene were designed using the online UPL Assay Design Center

(<https://www.universalprobe library.com>) and are provided in Table I. The following thermal cycling conditions were applied on the LightCycler® 480 Instrument: Denaturation at 95°C for 10 min, 45 cycles of amplification, 95°C for 10 sec, 60°C for 20 sec and cooling at 40°C for 30 sec. The results obtained were normalized to a housekeeping gene b-actin (ACTB). Fold changes of the gene expression of *iNOS*, *COX-2*, *NF-κB*, *Bcl-2*, *Bcl-xL*, *Bax*, *Bak*, *caspase-3* and *caspase-9* were calculated using the $2^{-\Delta\Delta CT}$ method (19). All experiments were performed in triplicate in 3 independent experiments.

Statistical analysis. The differences in *iNOS*, *COX-2*, *NF-κB*, *Bcl-2*, *Bcl-xL*, *Bax*, *Bak*, *caspase-3* and *caspase-9* expression between groups determined by RT-qPCR analysis were analyzed by Pair Wise Fixed Reallocation Randomization Test® included in the Relative Expression Software Tool (REST®, 2009 v2.0.13) statistical software developed for group-wise comparison and statistical analysis of relative expression results (20). ANOVA was used to determine any statistically significant differences in ABR thresholds across groups. To confirm statistical significance, Tukey's post-hoc test was performed with SPSS v22® (IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

General observations. All mice survived until the end of the study. There were no significant differences in body weight of the mice among groups both at the beginning and at the end of the study ($P = 0.520$, data not shown).

ABR. Baseline ABR measurements that were obtained at the beginning of the study, when the mice were three months of age, were subjected to ANOVA and no significant difference among the groups was obtained (Table II).

Similarly, when the mean ABR thresholds of the mice at the third month of the study (when the early treatment group had received three months of RSV therapy, while no therapy had been provided to the late and control groups) were compared, no significant difference was present among groups at any frequency ($P = 0.825$; data not shown).

The ABR results at the sixth month are presented in Table III. While no significant difference was obtained between the control, sham control and late treatment groups, the average thresholds of the early treatment group were significantly lower than those of the control groups and late treatment group.

Regarding the ABR results at 9 months, while the average thresholds for the early treatment group were determined to be 11.875–19.25 dB lower than those of the control groups ($P < 0.001$), the average thresholds for the late treatment group were 1.875–8.75 dB lower than those of the control groups ($P > 0.05$). The mean thresholds of the early treatment group were also lower when compared to those of the late treatment group (8.125–11.25 dB). This difference was determined to be statistically significant at 12 and 16 kHz ($P = 0.003$ and 0.005 , respectively), while no significant difference was obtained at 4 and 8 kHz ($P > 0.05$; data not shown).

The last ABR threshold data obtained at the end of the study, just before the mice were sacrificed, are presented in

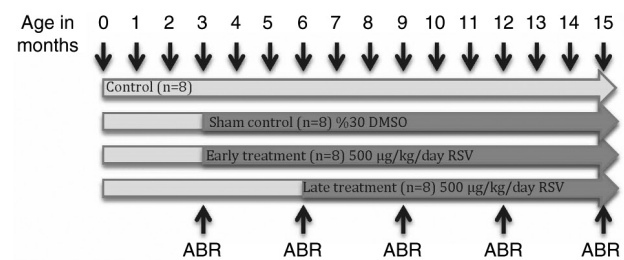


Figure 1. Schematic depicting the study protocol. RSV, resveratrol; ABR, auditory brainstem response test.

Table IV. The mice were 15 months of age and the early treatment group had received twelve months of RSV therapy, while the late treatment group had received nine months of RSV therapy at this time-point. The mean hearing thresholds of the early treatment group were significantly better than those of the late treatment group and control groups ($P < 0.001$). When the thresholds of the late treatment group and control groups were compared, although the hearing thresholds of the late treatment group were lower than those of the controls at all frequencies, the difference was not statistically significant ($P > 0.05$).

The ABR threshold shifts from baseline to 15 months were also analyzed (Fig. 2). Mean ABR threshold shifts for the control and sham control groups ranged between 35.000 and 43.750 dB according to frequencies, with no significant difference between these two groups ($P = 0.895$). While the threshold shifts of the early treatment group ranged between 15.000 and 16.250 dB, the threshold shifts of the late treatment group ranged between 29.375 and 35.625 dB. The threshold shifts for the early treatment group were significantly lower than those of the control groups at all frequencies ($P < 0.001$). The final mean threshold shift differences between the early and the late treatment groups ranged from 14.375 to 20.000 dB; this difference was not statistically significant at 4 kHz ($P = 0.785$). However, at 8, 12 and 16 kHz, the threshold shift was significantly lower in the early treatment group ($P < 0.001$).

Gene expression levels. In the early treatment group, mRNA expression levels of pro-apoptotic genes such as *Bax* and *Bak*, which may be responsible for increased apoptosis in cells, were significantly decreased ($P < 0.001$), mRNA expression levels of anti-apoptotic genes such as *Bcl-2* and *Bcl-xL* were significantly increased ($P < 0.001$), mRNA expression levels of *caspase-3* and *caspase-9* genes, which have a vital role in apoptosis, were significantly decreased ($P < 0.001$) and mRNA expression levels of inflammatory mediators such as *NF-κB*, *COX-2* and *iNOS* genes were significantly decreased ($P < 0.001$), when compared to the control and sham control groups (Fig. 3).

Similarly, in the late treatment group, the mRNA expression levels of the *Bax* and *Bak* genes were decreased, the mRNA expression levels of *Bcl-2* were increased and the mRNA expression levels of *caspase-3*, *caspase-9*, *NF-κB*, *COX-2* and *iNOS* genes were decreased when compared with the control and sham control groups. However, these changes were not statistically significant ($P > 0.05$; Fig. 3). The mRNA levels of the *Bcl-xL* gene were increased when compared

Table I. Gene-specific primer sequences and UPL probe numbers.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	UPL Probe No
<i>ACTB</i>	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA	64
<i>iNos</i>	CTTTGCCACGGACGA	TCATTGTACTCTGAG	13
<i>Cox-2</i>	GATGCTCTTCCGAGC-3'	5'-GGATTGGAACAGCAA-3'	45
<i>NF-κB</i>	CTCTCGACGTCAGTGGGAAT	CTCGTCCTCTCTCGCTCACT	20
<i>Bcl-2</i>	AGTACCTGAACCGGCATCTG	GGGGCCATATAGTTCCACAAA	75
<i>Bcl-xL</i>	TGACCACCTAGAGCCTTGGA	GCTGCATTGTTCCCGTAGA	2
<i>Bax</i>	AGTGTCTCCGGCGAATTG	CCACGTCAGCAATCATCCT	56
<i>Bak</i>	AGAGGGAGCTGGTCATTGC	AAACCTCGCGACTTTGTGAC	33
<i>Caspase-3</i>	ACTCGTGAAGACATTTTGAATTA	TCACCATGGCTTAGAATCACA	68
<i>Caspase-9</i>	AAGAAGACCGGAGTGCAATG	GGCACAATCCCTAACCACAG	27

UPL, universal probe library; ACTB, β-actin.

Table II. Mean auditory brainstem response thresholds by frequency of stimulus at the beginning of the study (values in decibels sound pressure level).

Frequency (kHz)	Control	Sham control	Early	Late	P-value
4	39.375±4.886	40.000±4.978	40.625±4.673	38.750±5.342	0.510
8	16.875±3.578	14.375±3.876	15.000±4.312	16.250±3.632	0.492
12	15.000±3.578	16.250±4.312	15.625±3.876	16.250±3.578	0.476
16	23.125±6.214	25.000±5.983	24.375±5.834	22.500±6.121	0.520

Values are expressed as the mean ± standard deviation.

with the control group; however, they did not change when compared with the sham control group. These changes were not statistically significant ($P>0.05$).

Discussion

ARHL is a complex degenerative disease affecting millions of individuals worldwide. As a major type of sensory impairment, ARHL may cause patients to withdraw from social life and become isolated and depressed (21). The high prevalence of ARHL, its substantial impact on the well-being of elderly individuals and the cost of the rehabilitation by hearing aids or implants, either to the patient or health care system, makes it a major public health concern.

The primary cause of age-related pathology in cell aging is probably oxidative injury caused by free radical damage. It is generally accepted that mitochondria are a major source of free radicals [reactive oxygen species (ROS) and reactive nitrogen species (RNS)] and a major site of ROS/RNS-induced oxidative damage (16).

Several rat studies have demonstrated the favorable effects of RSV on mitochondria. In particular, RSV supplementation causes enhancement of several mitochondrial functions (respiratory enzyme activity, oxygen consumption and activity of lipid-oxidizing enzymes) (22,23). RSV decreases ROS by acting as a strong scavenger of superoxide anions, hydrogen peroxide and hydroxyl radicals (24).

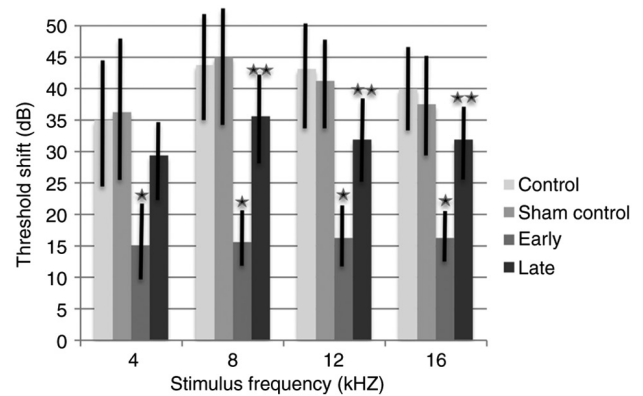


Figure 2. Comparison of baseline auditory brainstem response thresholds and the 15th month thresholds of the mice. Values are expressed as the mean ± standard deviation. * $P<0.001$ early vs. control; ** $P<0.001$ early vs. late treatment group.

Sánchez-Rodríguez *et al* (16) reported that a mixture of polyphenols, of which RSV is also a potent member, exerts a significant protective effect against ARHL by reversing the destructive effects of aging on the integrity of the cochlea in rats. This protection against aging damage in the inner ear was caused by its antioxidant properties. Similarly, Heman-Ackah *et al* (17) determined that a combination of antioxidants (ascorbic acid, vitamin B12, folate, ribose-cysteine, NW-nitro-L-arginine methyl ester and L-cysteine-glutathione

Table III. Mean auditory brainstem response thresholds of the mice at the sixth month of the study by frequency of stimulus (values in decibels sound pressure level).

Frequency (kHz)	Control	Sham control	Early	Late	P-value ^a	P-value ^b
4	51.875±8.421	51.25±9.883	49.375±10.883	42.5±10.033	0.006	0.320
8	25.625±4.323	26.25±3.679	20.625±4.412	24.375±4.873	0.008	0.254
12	24.375±3.362	23.75±4.543	19.375±3.354	25±3.612	0.007	0.465
16	35±5.873	34.375±4.173	30±4.246	35.625±3.644	0.005	0.312

Values are expressed as the mean ± standard deviation. ^aComparison of early treatment group with other groups; ^bComparison of late treatment, control and sham control groups.

Table IV. Mean auditory brainstem response thresholds of the mice at the end of the study by frequency of stimulus (values in decibels sound pressure level).

Frequency (kHz)	Control	Sham control	Early	Late	P-value ^a	P-value ^b
4	74.375±8.766	76.25±10.967	55.625±8.932	68.125±8.463	<0.001	0.090
8	60.625±9.256	59.375±10.112	30.625±9.102	51.875±7.732	0.001	0.112
12	58.125±9.324	57.5±9.646	31.875±8.321	48.125±8.653	<0.001	0.070
16	63.125±8.725	62.5±8.354	40.625±9.627	54.375±9.372	<0.001	0.102

Values are expressed as the mean ± standard deviation. ^aComparison of early treatment group with late treatment and control groups; ^bComparison of late treatment group with control and sham control groups.

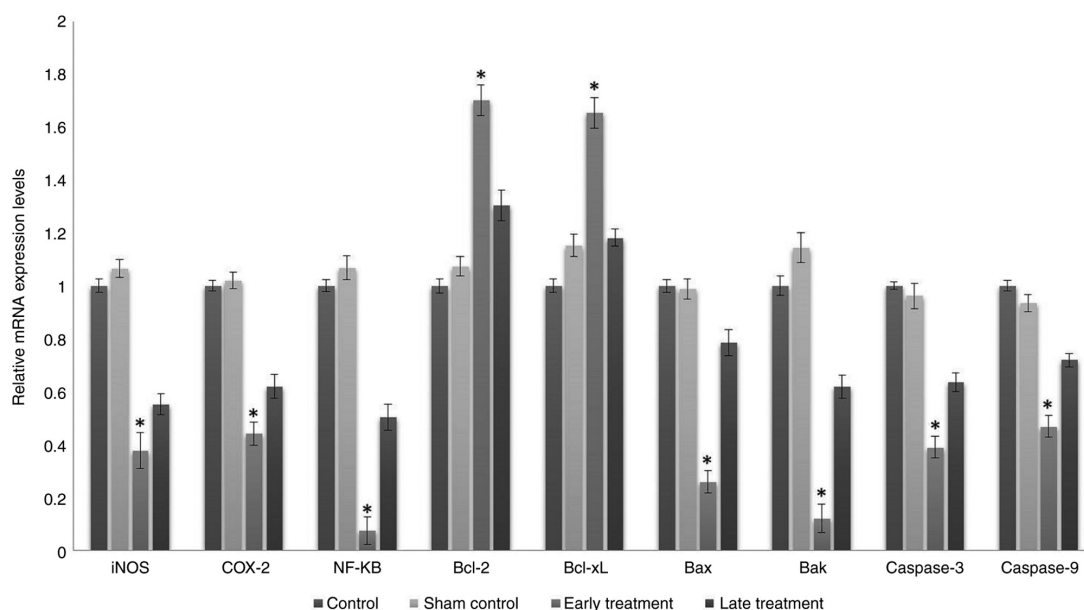


Figure 3. Relative mRNA expression levels of genes. Values are expressed as the mean ± standard deviation. *P<0.001 vs. control. iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; Bak, Bcl-2 homologous antagonist/killer.

mixed disulfide) effectively decreased threshold shifts of ABR in an animal model. The present study indicated that RSV alone also has protective effects against ARHL, particularly when started prior to the onset of the hearing loss. This protective effect is due to its antioxidant properties, which were proven by molecular studies. RSV decreased the mRNA expression levels of the pro-apoptotic genes *Bax* and *Bak*, the

mRNA expression levels of *caspase-3* and *caspase-9*, which have a vital role in apoptosis, and the mRNA expression levels of inflammatory mediators such as *NF-κB*, *COX-2* and *iNOS* genes, while increasing anti-apoptotic genes such as *Bcl-2* and *Bcl-xL*. Similarly, Someya *et al* (18) reported that ARHL in C57BL/6J mice is mediated by Bak-dependent mitochondrial apoptosis and that oral supplementation of mitochondrial

antioxidants decreased *Bak* expression in the cochlea, mitigated cochlear cell death and prevented ARHL.

Chronic inflammation has an important role in age-related intracellular damage in mammalian cells (25). The present study indicated that the expression levels of the *NF-κB*, *COX-2* and *iNOS* genes, which have critical roles on different levels of the inflammatory process, were significantly higher in the cochlear tissues of the mice in the early treatment group. The expression levels of these genes in the late treatment group were also lower than those of the control groups, but these differences were not statistically significant. These results suggested that RSV slows down chronic inflammatory processes, which are responsible for aging, if it is initiated prior to the onset of these processes. It may also suppress the inflammatory processes if it is used after these processes become active, but no significant impact was observed on either the ABR thresholds of the mice or at the molecular level.

Different antioxidant molecules have been studied for the prevention of ARHL, with variable results. In animal studies, when C57BL/6 mice were fed with a diet containing one of 17 antioxidant agents, ARHL was almost entirely prevented by α -lipoic acid and coenzyme Q10 and partially by N-acetyl-L-cysteine, but not by any other agent (26). While addition of vitamin C to the diet did not increase vitamin C levels in the cochlea or decelerate ARHL in C57BL/6 mice (27), Fischer 344 rats given vitamin E, C, melatonin or lazaroid had better auditory sensitivities and fewer mitochondrial DNA deletions in comparison with placebo subjects (28). Similarly, Fischer 344 rats supplemented orally with lecithin for six months had significantly better hearing sensitivities compared with their controls (29).

These results suggested that the prevention and attenuation of ARHL by antioxidant supplements may be affected by numerous factors such as the type and dosage of antioxidant compounds, the timing and duration of the treatment and the species and strains that are studied.

The present study demonstrated that daily oral RSV treatment was able to attenuate the manifestation of ARHL in C57BL/6 mice. This benefit was observed to be significant in the early treatment group, in which the treatment was initiated prior to the beginning of hearing loss. The late treatment group also exhibited slightly improved ABR results, but neither the changes at the molecular level nor the hearing thresholds were significantly better than those in the control groups. These results suggested that antioxidant treatment, in this case RSV, may be more beneficial when initiated prior to the beginning of degenerative changes that are responsible for ARHL. When the treatment is initiated after the onset of ARHL, RSV may be less effective due to the already ongoing apoptotic process and inflammation at the molecular level. These results are compatible with those of previous human studies, which have consensus regarding that the antioxidants are effective in the prevention of ARHL but not as a remedy, since hair cells in the ear do not regenerate and hearing loss may not be restored (30).

ARHL usually begins at high frequencies and most, but not all, of the previous animal studies focusing on ARHL include 32 kHz ABR measurements (17,31). Due to technical limitations, it was not possible to use an ABR device that covers 32 kHz in the present study, and this is its major limitation. In addition, even though the present study was one of the very

few studies on this context to date, only the expression levels of the genes in cochlear tissues were analyzed, providing an investigation of only the peripheral components of ARHL at the molecular level. The lack of pathologic examination and protein expression analysis are also potential limitations to the present study. Advanced histopathological and molecular studies are required to be performed to further elucidate the effects of RSV on the central mechanisms of ARHL. Also, the present study revealed that RSV prevents oxidation-associated aging in the ear to prevent ARHL before it develops. In addition, considering the many beneficial anti-aging effects of RSV, further human studies in elderly patients are needed to evaluate the effects of RSV on hearing loss.

In conclusion, RSV was indicated to have a beneficial role in ARHL, with its potent antioxidant and anti-inflammatory properties, particularly when started prior to the onset of hearing loss. However, even with the promising potential of different preventive drugs, minimizing noise exposure and maintaining a healthy lifestyle remain the most effective accepted routes for limiting ARHL in the human population.

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Availability of the data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TM: Conceptualization, funding acquisition, investigation, writing and original draft preparation. ASYS: Conceptualization, methodology, resources, validation. DU: Data curation, methodology. SM: Software, data curation, resources. ES: Resources, funding acquisition, data curation. HK: Methodology, supervision. TM, DU, SM and ES performed the ABR's. TM, SM and ES removed the cochleae. ASYS prepared the solutions and performed PCR testing. TM, DU and ASYS confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The Gazi University Animal Care and Use Committee (Ankara, Turkey) approved the study protocol (approval no. 66332047-604.01.02-7347).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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