Neuroprotective Effect of Huperzine A on D-Galactose-Induced Hearing Dysfunction

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Abstract
Background: Administration of D-galactose (D-gal) has been used to create animal models of neurodegenerative diseases, and huperzine A has been used to treat the neurodegenerative diseases such as Alzheimer disease. Methods: An animal model of hearing dysfunction was established by administration of D-gal in the rats, and the effect of huperzine A on D-gal-induced abnormal hearing function and cochlear damage was investigated. Senescence of the cochlear tissues was examined by β-galactase staining, and messenger RNA expression of inflammatory cytokines was quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR). Results: It was found that D-gal significantly increased auditory brainstem response (ABR) threshold and cellular senescence and decreased neurofilament in the cochlear tissues. Huperzine A could significantly attenuate D-gal-induced increase of ABR threshold and cellular senescence as well as reduction of neurofilament. Moreover, huperzine A could inhibit D-gal-induced activation of nuclear factor kappa-B (NF-κB) in Schwann cells and significantly blocked D-gal-stimulated gene expression of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, and tumor necrosis factor-α. Conclusion: These findings suggested that D-gal causes hearing dysfunction by inflammatory injury of cochlear neurons and that huperzine A could prevent hearing loss by protecting D-gal-induced physical damage of cochlear tissues.

Keywords
animal model, auditory brainstem response, D-galactose, huperzine A, hearing loss, neurodegenerative disease

Introduction
Degeneration of the central auditory system is an important cause of age-related hearing loss. Animal model of D-galactose (D-gal)-induced neurodegeneration has been used to study aging-associated diseases such as Alzheimer disease and hearing loss.1,2 Oxidative damage to mitochondrial DNA is one of the mechanisms involved in the D-gal-induced neurodegeneration.1,3 In this regard, it has been reported that animals treated with D-gal exhibit a reduction in the activity of antioxidant enzymes,4,5 dysfunctional mitochondria,6,7 increased apoptosis,8 and neurotoxicity.9,10 Consequently, these animals exhibit a shortened life span,11 poor hearing and memory,12,13 and an attenuated immune response.14,15 Previous studies have also suggested that the inflammatory response participates in the pathogenesis of neurodegeneration, and nuclear factor kappa-B (NF-κB) is regarded as an important factor. The activation of NF-κB leads to the release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β.16-18

The auditory brainstem response (ABR) is an auditory-evoked potential extracted from ongoing electrical activity in the brain and recorded through electrodes placed on the scalp. The measured waves are a series of 6 to 7 vertex waves, which generated by the auditory branches of cranial nerve VIII and lower or upper parts of brainstem. The ABR test is often used to determine the hearing lost type and the degree as well as the auditory threshold estimation.19,20

Huperzine A is a reversible and selective acetylcholinesterase (AChE) inhibitor,21 and it has effects of antiaging and anti-

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inflammation in the neural and immune systems. Thus, huperzine A has been used to inhibit immunosenescence and age-related disorders, such as Alzheimer disease. However, it has not been well-understood whether huperzine A can reduce ß-gal-induced hearing dysfunction. This study was, therefore, designed to investigate huperzine A effect on the ß-gal-induced ABR, cellular senescence, and neurofilament damage in cochlear tissues isolated from rat animal models and to explore the effect of huperzine A in association with reduction in NF-κB activity and downregulation of the expression of inflammatory cytokines.

Materials and Methods

Animals and Drug Administration

Two-month-old male Sprague-Dawley rats (200-220 g) were maintained at room temperature (RT) under a 12-hour light/dark cycle with food and water ad libitum in The Laboratory Animal Center, Shanghai Medical College, Fudan University. After acclimatization to the laboratory conditions, the rats were randomly assigned to the following 3 groups (n = 12 per group): the ß-galactose-treated group (ß-gal), the ß-galactose plus huperzine A-treated group (ß-gal + Hup A), and the control group. Based on the previous report, the ß-gal-treated group was subcutaneously injected 300 mg/kg of ß-gal (Sigma, Los Angeles, USA) daily for 8 weeks. In the ß-gal + Hup A group, the rats were subcutaneously co-injected 300 mg/kg ß-gal and 0.1 mg/kg huperzine A (Tauto Biotech, China) daily for 8 weeks. The control group was injected with saline daily for 8 weeks. At the end of the study, the animals were euthanized with CO₂ followed by collecting tissues.

Auditory Brainstem Response Test

Basal threshold of ABR was tested in all rats prior to the use of drugs to confirm normal hearing function in all animals before the experiment. At the end of the experimental treatment, ABR test was conducted again. Briefly, the rats were anesthetized by intraperitoneal injection of pentobarbital with 80 mg/kg and received functional tests in the acoustic and electrical shielding room. During the test, body temperature was maintained with a constant heating pad. Stimulation signal and evoked potential recording were accomplished by TDT system III (RZ6; Tucker-Davis Technology, Washington, USA). The stimulus signal for ABR was 10 ms pure tone, with an ascending/descending time of 0.5 ms, and a repetition rate of 21.1 beats per second. Test frequency range (2-48) kHz was an octave range. At each frequency, the test starts at 90 dB SPL, with the minimum sound intensity of the III wave as the response threshold. Electrodes via hypodermic needle guide, parietal middle ear on both sides of the recording electrodes, reference electrode, and grounding electrode were applied. Bioelectrical signals were introduced by electrodes into RA16PA preamplifier, band pass filtering at 100 to 3000 Hz, and ABR addition of 1000 times.

Table 1. List of the Primers Used for the Quantitative Real-Time PCR.

| Primer       | Sequence                        |
|--------------|---------------------------------|
| TNF-α-F      | 5'-CACCCCTGGAGATGCTTTT-3'       |
| TNF-α-R      | 5'-GGGACCAAGTTGCTCTCT-3'       |
| IL-1β-F      | 5'-CATGTCTGCTGCCGTCAG-3'       |
| IL-1β-R      | 5'-AGAGAGCGGCCTCTCTTTCAA-3'    |
| IL-6-F       | 5'-ATTCTGTCCTGAACCCACCA-3'     |
| IL-6-R       | 5'-CTGGGCGGGTTCTGCGG-3'        |

Abbreviations: IL, interleukin; TNF-α, tumor necrosis factor-α; PCR, polymerase chain reaction.

Tissue Preparation

The rats were culled using an overdose of sodium pentobarbital and perfused through the heart using a 4% paraformaldehyde solution. The cochlea was postfixed in 4% paraformaldehyde overnight, decalcified with 10% EDTA for 2 weeks, and cryo-protected using a gradient sucrose solution (starting with 15%, followed by 30%). Serial sections were sliced at a thickness of 5 mm (Leica, Wetzlar, Germany).

Cellular Senescence Assay

Cellular senescence is believed to be associated with chronic hearing loss and, thus, senescence of cochlear cells was assessed. To accomplish this, a senescence-associated β-galactosidase (SA-β-Gal) staining kit (Beyotime Institute of Biotechnology, Suzhou, China) was used for SA-β-Gal staining following the manufacturer’s instruction. Briefly, the sections were fixed in a SA-β-Gal fixing solution for 15 minutes. After washing 3 times in phosphate-buffered saline (PBS), the sections were incubated overnight at 37°C with the SA-β-Gal working solution followed by photographing under a microscope.

Immunohistochemistry

The cochlea slices of the rats in each group were placed at RT for 30 minutes, fixed with 4% acetone for 10 minutes, and rinsed 3 times for 5 minutes each with PBS containing 1% (wt/vol) bovine serum albumin, 5% heat-inactivated goat serum, and 1% Triton X-100 in 0.1 mmol PBS. The cochlea slices were then incubated with 3% H₂O₂ for 5 minutes followed by rinsing 3 times for 5 minutes each. The primary antibodies, anti-neurofilament 200 antibody (1:100; Abcam, Cambridge, United Kingdom), anti-s-100 antibody (1:100, Abcam), and NF-κB (1:100, anti-p65-antibody) were allowed to react at 4°C overnight. After washing, the following secondary antibodies were allowed to bind for 4 hours at RT: goat anti-mouse immunoglobulin (Ig) G1-fluorescein isothiocyanate (FITC; 1:100; Santa Cruz Biotechnology, California), fluorescein-conjugated rabbit polyclonal secondary antibody to mouse IgG/IgM/IgA (1:100; Abcam), and FITC-conjugated goat anti-rabbit IgG (1:100, Kangchen Biotech, Shanghai, China). The slides were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 15 minutes followed by photographing under a microscope. Number of positively stained...
neurofilament (red) was counted under the microscope by randomly selecting 5 high-power field (200× magnification). Average of the 5 fields between the 3 groups was compared.

**Quantitative RT-PCR**

Total RNA was extracted from the cochlear tissues using Trizol (Invitrogen, Carlsbad, California) followed by reverse transcription into complementary DNA using the reverse transcription system (Invitrogen). Real-time polymerase chain reaction (PCR) was then performed using a SYBR Premix reverse transcription-polymerase chain reaction (RT-PCR) kit (Invitrogen) following the manufacturer’s instructions. The primer sequences are listed in Table 1.

**Image Acquisition and Statistics**

The specific group identity was coded so that all microscopy and computer analyses were performed blinded to the treatment group. The data in all studies were expressed as the means ± standard error of the mean. A one-way analysis of variance was performed to evaluate the differences in the levels of protein and messenger RNA (mRNA). The difference was considered to be significant at \( P < .05 \).

**Results**

**Effect on ABR Threshold**

As shown in Table 2, there was no significant difference in basal frequencies of ABR between the groups. As shown in Table 3 and Figure 1, however, ABR threshold was significantly increased at 4 and 8 kHz sampling after administration of d-gal, it was not significantly altered at 16 kHz (Figure 1A and B, \( P < .05 \) compared to control). Huperzine A could antagonize the effect of d-gal-induced increase of ABR threshold (Figure 1A-C).

**Table 2. Comparison of Basic ABR at Various Frequencies.**

| 1 kHz     | 2 kHz     | 4 kHz     | 8 kHz     | 16 kHz    |
|-----------|-----------|-----------|-----------|-----------|
| Control   | 39.38 ± 4.29 | 35.21 ± 5.29 | 31.34 ± 4.94 | 22.68 ± 5.15 | 16.36 ± 5.38 |
| d-gal     | 39.97 ± 5.24 | 36.17 ± 4.24 | 30.97 ± 4.17 | 22.14 ± 3.44 | 15.96 ± 5.23 |
| d-gal + Hup A | 39.12 ± 4.86 | 35.92 ± 4.82 | 30.72 ± 4.70 | 21.47 ± 4.51 | 16.41 ± 4.74 |

**Table 3. Comparison of ABR at Various Frequencies After Treatment.**

| 1 kHz     | 2 kHz     | 4 kHz     | 8 kHz     | 16 kHz    |
|-----------|-----------|-----------|-----------|-----------|
| Control   | 39.21 ± 3.29 | 34.91 ± 2.29 | 30.06 ± 1.97 | 21.16 ± 2.17 | 15.64 ± 1.71 |
| d-gal     | 48.97 ± 3.24 | 45.17 ± 3.24 | 45.24 ± 2.62 | 35.54 ± 2.02 | 22.86 ± 3.04 |
| d-gal + Hup A | 40.12 ± 2.86 | 36.92 ± 4.17 | 32.25 ± 2.64 | 23.63 ± 1.49 | 17.06 ± 2.18 |

**Effect on the Cochlear Tissue**

To evaluate the effect of d-gal on cellular senescence, SA-β-Gal assay was performed in the cochlear tissues. As shown in Figure 2, cochlear tissues isolated from the animals treated with d-gal revealed higher intensity of SA-β-Gal staining as well as increased cell number of positive staining in comparison with that isolated from control rats. Cochlear tissues isolated from the animals treated with coadministration of huperzine A and d-gal had similar intensity and positivity of SA-β-Gal staining with that of control animals (Figure 2).

Next, the effect of d-gal on number of neurofilament was examined. Consistent with cellular senescence effect of d-gal on cochlear tissues, the number of neurofilament as visualized by immunohistochemistry in the cochlear tissues (red color) was significantly reduced in the animals treated with d-gal compared with that of control animals (Figure 3A and B, \( P < .05 \)). Coadministration of huperzine A could significantly block d-gal-induced reduction of neurofilament in the cochlear tissues (Figure 3A and B).

**Effect on the Pro-Inflammatory Cytokine Synthesis**

Gene expression of pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α was quantified by real-time RT-PCR. As shown in Figure 4, mRNA expression of pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α was significantly upregulated in the animals injected with d-gal (Figure 4, \( P < .05 \)). Huperzine A could completely block d-gal-induced upregulation of IL-1β, IL-6, and TNF-α mRNA in the cochlear tissues (Figure 4, \( P < .05 \)).

Since Schwann cells secrete a variety of active substances including cytokines, and NF-κB signaling regulates pro-inflammatory cytokine production, NF-κB activation in the Schwann cells was evaluated by double staining of S-100 (biomarker of Schwann cell) and p65. As shown in Figure 5, compared with the control group, intensity of Schwann cell staining (red) and NF-κB staining (green) was stronger in the cochlea of
D-gal group. Intensity of Schwann cell staining and NF-κB staining in the cochlea of D-gal plus huperzine A animals was similar to that of control animals (Figure 5).

**Discussion**

In this study, the effect of huperzine A on D-gal-induced cochlear tissue injury was investigated. We found that administration of D-gal resulted in significantly increased ABR threshold and senescent cells and significantly decreased number of neurofilament in the cochlear tissues compared with that of control animals. D-Gal administration further induced significant upregulation of mRNA expression of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α as well as activation of NF-κB. Coadministration of huperzine A could protect cochlear tissues from D-gal-induced increase of ABR and cellular senescence but decrease of neurofilaments. Moreover, huperzine A significantly blocked D-gal-induced upregulation of pro-inflammatory cytokine mRNA expression and NF-κB activation.

Chronic administration of D-gal has been used for studying neural degenerative diseases such as Alzheimer disease and aging. In this regard, studies have shown that chronic administration of D-gal resulted in shortened life span as well as loss of hearing and memory. Here, we report D-gal administration in rats induced abnormal ABR, indicating a rat model of hearing dysfunction was produced in this study. Furthermore,
consistent with previous report, this study demonstrated by histological examination of the cochlear tissues from the animal models that D-gal could induce cellular senescence as well as significant reduction of neurofilament in the cochlear tissues, suggesting D-gal causes hearing dysfunction through physically damaging neurons in the cochlea.

Huperzine A is a reversible and selective AChE inhibitor. Huperzine A has been widely used to improve cognitive and memory deficits in patients with benign senescence forgetfulness, Alzheimer disease, and vascular dementia via non-cholinergic mechanisms, including attenuating oxidative stress, inhibiting apoptosis, and interfering with amyloid precursor protein metabolism. In addition, huperzine A may protect against diverse neurodegenerative diseases by blocking N-methyl-D-aspartate receptors and by inhibiting D-gal-induced neuroinflammation via regulating the NF-κB signaling pathway. Huperzine A can also upregulate the cholinergic anti-inflammatory pathway and inhibit immunosenescence and age-related disorders, such as Alzheimer disease, by inhibiting AChE activity. It has also been demonstrated that huperzine A has the potential to attenuate D-gal-induced inflammation-associated aging in the rat liver by counteracting hepatic inflammation and replicative senescence. Consistent with these reports, this study further extended the findings of the protective effect of huperzine A on neurons in cochlear tissue and demonstrated that huperzine A prevents D-gal-induced increase of ABR threshold and cellular senescence as well as decrease of neurofilaments in cochlear tissues.

While the mechanisms of D-gal-induced hearing dysfunction remains to be further defined, it has been reported that D-gal caused a significant increase in the expression of NADPH oxidase (NOX2) and 8-hydroxy-2-deoxyguanosine, a biomarker of DNA oxidative damage, as well as a decrease in the mitochondrial total antioxidant capabilities in the auditory cortex, as compared with the control rats. Previous studies have also shown that D-gal reacts with the free amines of amino acids in proteins and peptides to form advanced glycation end products, which mediates downstream inflammatory signaling pathways, such as NF-κB, and induce release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Here, we also found that D-gal upregulated pro-inflammatory cytokine gene expression in the cochlear tissues and activation of NF-κB in the Schwann cells, suggesting D-gal-induced chronic inflammation through NK-κB signaling pathway may be involved in the pathogenesis of hearing dysfunction in the rat models.
Several mechanisms may be associated with age-related chronic hearing loss. In this regard, telomerase-associated cellular senescence, abnormal differentiation of the cochlear cells, and membrane hypothesis have been considered to be associated with chronic hearing loss. Therefore, in this study, cellular senescence of cochlear tissues was examined. Consistent with the aforementioned concept, this study demonstrated that D-gal significantly increased cellular senescence, which could be partially blocked by huperzine A. These findings suggested that D-gal could induce senescence of the cochlear tissue, and blockade of senescence may prevent chronic hearing loss.

Huperzine A is an inhibitor of AChE and often used for the treatment of age-related diseases including Alzheimer disease and vascular dementia. However, a systemic review and meta-analysis of 20 randomized clinical trials with 1823 participants indicated that huperzine A appeared to have beneficial effects on improvement of cognitive function, daily living activity, and global clinical assessment in the patients with Alzheimer, the authors suggested that the findings should be interpreted with caution due to the poor methodological quality of the randomized clinical trials. Here, we reported that huperzine A could significantly antagonize D-gal-induced hearing loss, suggesting that huperzine A might be used to treat chronic hearing loss in aging population. We further demonstrated that huperzine A could significantly block D-gal-stimulated gene expression of pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α and that it significantly inhibited NF-κB activation in Schwann cells in response to D-gal exposure. These findings suggested that huperzine A could modulate D-gal-induced inflammation via inhibiting NF-κB activation and thus might be used to prevent aging-associated hearing loss.

There are several limitations in this study. First, only 1 dose of D-gal and huperzine A was examined. Second, protein level alteration of pro-inflammatory cytokines in response to D-gal and huperzine A need to be confirmed. Third, different time windows of huperzine A application following D-gal administration remain to be further investigated. Fourth, other measurements corresponding to the presented frequencies of ABR test, including PCR and immunohistochemistry of the neurofilaments, were not examined in this study.

Conclusion
Taken together, this study demonstrated that administration of D-gal in rats could result in hearing dysfunction and cellular senescence in cochlear tissue, as well as decrease of neurofilaments, a biomarker of neurons. Huperzine A could protect cochlear cells from D-gal-induced cellular senescence and neuron reduction. Inflammation may be involved in mediating D-gal-induced cochlear neuron damage, and the cochlear neuroprotective effect of huperzine A is associated with its inhibition on inflammation via modulating NF-κB signaling.

Authors Contributions
CL and SS contributed to the conception and design of the study, acquisition, and analysis of data. CL wrote the manuscript. All authors
reviewed and approved the final version of the manuscript. All necessary information about this study was presented in the manuscript. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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