Ph.D. Dissertation of Medicine

Protective effects of Dieckol and PFF-A extracted from Ecklonia cava against noise-induced hearing loss in a mouse model

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Chair _____

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Examiner _____

Abstract

Noise is a well-known cause of hearing loss. Approximately 31.1% of Americans between the ages of 20 and 69 years (61.1 million people) have high-frequency hearing loss associated with noise exposure. In addition, recurrent transient or continuous noise exposure can accelerate age-related hearing loss. Dieckol and phlorofucofuroeckol A (PFF-A), polyphenols extracted from Ecklonia cava, are potent antioxidants. In this study, I investigated the protective effect of dieckol and PFF-A against noise exposure in mice. Four hours after noise exposure, dieckol alleviated the noise-induced threshold shift of the auditory brainstem response (ABR) at 4 kHz. Compared to the control group, the high-PFF-A group showed a better ABR threshold 1 day after exposure to click and 16 kHz noise. One day after noise exposure, the high-PFF-A group also showed superior hair cell survival in the apical turn of the organ of Corti compared to controls. These results suggest that dieckol and PFF-A have potential for preventing temporary threshold shifts (TTSs) of the ABR. In addition, as dieckol and PFF-A are the main components of food ingredients approved by the United States Food and Drug Administration, they can be taken by individuals exposed to noise to help prevent TTS, with a low chance of adverse effects.

Keywords: Noise, Hearing loss, Dieckol, PFF-A, Antioxidants
Student Number: 2019-26616

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Chapter 1. Introduction

1.1. Study background

Hearing loss can compromise communication and comprehension, and thus quality of life. Noise is a well-known cause of hearing loss,[1] and noise-induced hearing loss (NIHL) is one of the most common occupational diseases globally.[2,3] According to the National Institute of Deafness and other Communication Disorders, approximately 31.1% of Americans between the ages of 20 and 69 years (61.1 million people) have high-frequency hearing loss associated with noise exposure, including loud occupational noise and noise encountered outside the workplace.[4] Furthermore, NIHL in teenagers has received increasing attention. The Center for Disease Control and Prevention estimates that approximately 16% of American teenagers (12-19 years) are affected by hearing loss caused by loud noise.[5] Considering the substantial associated medical costs, NIHL is an important social, clinical, and economic issue.[6] There are two types of NIHL: that associated with a permanent threshold shift (PTS) and that with a temporary threshold shift (TTS). Permanent hearing loss occurs in individuals with a PTS, whereas hearing loss is recovered after a period of time in those with a TTS. Although the reversibility of hearing loss after a TTS has led researchers to propose that it is less serious than PTS, recent studies have suggested that TTS can induce synaptopathy, and thus accelerate age-related hearing loss.[7-9] As a result, the prevention of TTS has gained increased attention from

researchers, especially those in aging societies.

Previously, mechanical trauma by loud noise was thought to be the main cause of NIHL. Therefore, the recommended precautions for NIHL included avoiding or minimizing exposure to prolonged or loud noise.[10,11] However, such preventive measures may not be applicable to populations that cannot avoid or reduce noise exposure, such as construction workers and soldiers. Recent studies have revealed that reactive oxygen species (ROS) evoked by excessive noise stimulation are a cause of NIHL. Accordingly, various studies have examined the use of antioxidants to prevent NIHL.[12-15] Although preventive treatments must be applied before the development of NIHL, doing so without certainty regarding whether NIHL will develop puts individuals at risk of unnecessary adverse effects of drugs. Thus, compounds with a protective effect but minimal side effects are optimal for preventing NIHL.

Brown algae are commonly used as dietary supplements and herbal remedies in Asian countries. Among the species of brown algae, Ecklonia cava produces unique polyphenols called eckols. Although Ecklonia cava produces various potentially medicinal compounds, from common sterols (fucosterol, cholesterol, and ergosterols) to phlorotannins (eckol, dieckol, and phlorofucofuroeckol A; PFF-A), an increasing number of studies have reported that eckol and its derivatives possess the major medicinal properties of this brown algae.[16-19] Recently, various in vitro and in vivo studies indicated that eckols can elicit a wide spectrum of bioactivities, including matrix metalloproteinase-inhibiting, protease-inhibiting, cytoprotective, anti-inflammatory, and antioxidant effects.[20-22] One study using purified polyphenolic extract from Ecklonia cava (PPEE) reported that it had a

considerable protective effect against TTS. PPEE consists of eckols including dieckol and PFF-A.[23] In this study, I investigated the protective effect of dieckol and PFF-A against TTS in an animal model of NIHL.

1.2. Study objective

Dieckol and PFF-A are well-known and potent antioxidants. Since NIHL is induced by the ROS formation associated with noise exposure, the application of antioxidants may prevent TTS by suppressing ROS formation. I investigated the protective effect of dieckol and PFF-A against TTS in an animal model of NIHL (Fig. 1).

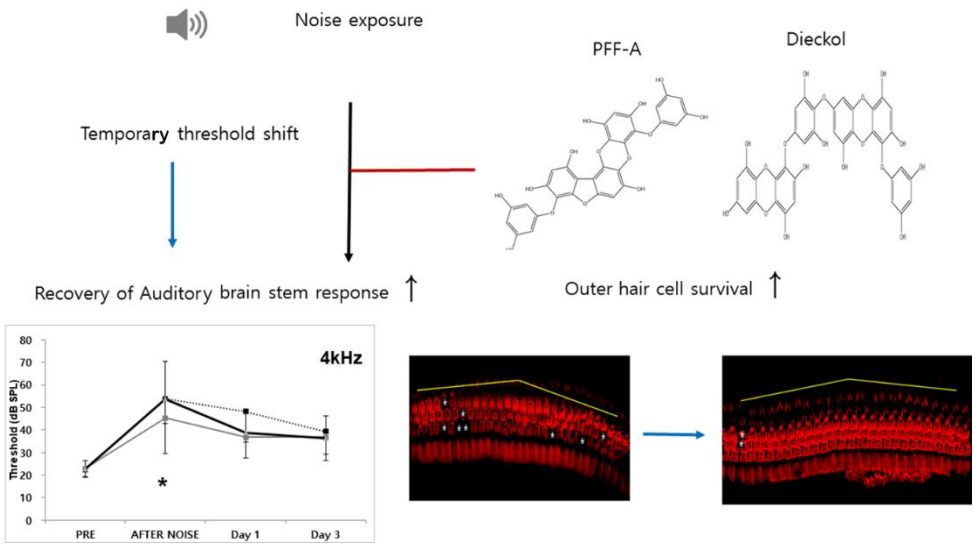


Fig. 1. Schematic diagram of the study, which examined recovery of auditory brain stem response and outer hair cell survival after administration of PFF-A and dieckol.

Chapter 2. Materials and methods

2.1. Preparation of PPF-A and dieckol

Purified dieckol and PFF-A were prepared and kindly supplied by BotaMedi, Inc. (Jeju, Korea). Briefly, the whole Ecklonia cava plant was collected off the coast of Jeju Island, Korea. Dried Ecklonia cava powder was extracted using 70% aqueous ethanol (EtOH) and then partitioned between water and ethyl acetate. The ethyl acetate fraction was subjected to octadecylsilyl (ODS) column chromatography followed by gel filtration on Sephadex LH-20 with methanol. Final purification of individual compounds was accomplished by high-performance liquid chromatography (HPLC; Spherisorb S10 ODS2 column [20 × 250 mm]; eluent, 30% MeOH; flow rate, 3.5 mL/min; Waters, Milford, MA, USA) to isolate dieckol (98.5 wt%) and PFF-A (98.0 wt%).

2.2. Bioinformatics of PFF-A, dieckol, and N-acetylcysteine

The molecular structural formula was drawn using Chem3D (CambridgeSoft, Cambridge, MA, USA) with the reported IUPAC names of dieckol, PFF-A, and N-acetylcysteine (NAC) ((4-[4-[6-(3,5-dihydroxyphenoxy)-4,7,9-trihydroxydibenzo-p-dioxin-2-yl]oxy-3,5-dihydroxyphenoxy]dibenzo-p-dioxin-1,3,6,8-tetrol), (4,9-bis(3,5-dihydroxyphenoxy)-[1]benzofuro[3,2-a]oxanthrene-1,3,6,10,12-pentol), and 2-acetamido-3-sulfanylpropanoic acid, respectively). Bioinformatics

were investigated by inputting the drawn molecular structural formula into SwissADME.[24]

2.3. 1,1-Diphenyl-2-picrylhydrazyl assay

PFF-A and dieckol were diluted in distilled water to obtain the experimental concentrations (0, 12.5, 25, 50, 100, and 200 uM). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) powder (Sigma, St. Louis, MO, USA) was mixed in 95% methanol to create a 1 M stock. PFF-A, dieckol, and 50% methanol were aliquoted into the individual wells of a 96-well plate. The DPPH solution was then added to each well. PFF-A and dieckol were allowed to react with the DPPH solution in a dark area for 30 min at room temperature. Absorbance was measured at 540 nm using a microplate reader. Radical scavenging activity was calculated using the following formula: Scavenging activity = (1- [sample-control]/blank) X 100. Two wells were used for each concentration and the experiment was repeated twice.

2.4. Cell viability assay

2.4.1. Cell culture

Under permissive conditions (33 °C, 10% CO_2), mouse auditory cell lines (House Ear Institute-Organ of Corti 1[HEI-OC1] cells; House Ear Institute, Los Angeles, CA, USA) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; #11965092; Gibco, New York, NY, USA) containing 10% fetal bovine serum (FBS; #10437028; Gibco) without antibiotics.[25]

2.4.2. Cell Counting Kit-8 (CCK-8) cell viability assay

The CCK-8 assay kit (Dojindo Laboratories, Kumamoto, Japan) was used to determine cell viability. After trypsinization and collection, the HEI-OC1 cells were counted using an automated cell counter (Countess; Invitrogen, Bothell, WA, USA). The concentration was adjusted to 1.3×10^5 cells/ml. The cells were then seeded into a 96-well cell culture plate (100 μl per well) and incubated overnight for attachment. The cells were then treated with PFF-A and dieckol in a dose-dependent manner (0, 12.5, 25, 50, 100, and 200 uM). Eight and twenty-four hours after the application of dieckol and PFF-A, a CCK-8 assay was performed according to the manufacturer's protocol. Then, absorbance at 450 nm was measured via a microplate reader using Gene5 software (BioTek Instruments, Winooski, VT, USA). The average optical density (OD) value of the control cells was used as the baseline for viability assessment. [26]

2.5. Animal subjects

Six-week-old male C57BL/6 mice weighing 18–20 g were purchased from Koatech Inc. (Pyeongtaek, Korea). The mice were fed a standard commercial diet, and housed in a facility with an ambient temperature of 20°C–22°C, relative humidity of 50% ± 5%, and 12-h/12-h day/light cycle. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (Seoul, Korea; 18-0025-C1A0), which is endorsed by the International Association for the Assessment and Accreditation of Laboratory Animal Care.

2.6. Experimental groups

A total of 60 C57BL/6 mice were randomly assigned to either the baseline (n = 4), saline with noise (n = 8), PFF-A (10 mg/kg) with noise (n = 8), high-dose PFF-A (100 mg/kg) with noise (n = 8), PFF-A/NAC with noise (n = 8), dieckol (10 mg/kg) with noise (n = 8), High-dose Dieckol (100 mg/kg) with noise (n = 8), or dieckol/NAC with noise (n = 8) group. The baseline group was used as the day 1 and day 3 control. This group was not exposed to noise. Following treatment, the mice from the other seven experimental groups were exposed to noise with a sound pressure level (SPL) of 115 dB for 2 h. Within the seven noise-exposed groups, mice were divided into two equal subgroups (n = 4). A hearing evaluation was performed for one subgroup 1 day after noise exposure (Day 1 group), and that for the other subgroup took place 3 days after noise exposure (Day 3 group). The mice were sacrificed on the day of their hearing evaluation.

2.7. Noise exposure protocol

Prior to noise exposure, the mice were anesthetized via an intraperitoneal injection of 40 mg/kg Zoletil (Zoletil 50; Virbac, Bogotá, Colombia) mixed with 10 mg/kg xylazine (Rumpun; Bayer-Korea, Seoul, Korea). Each animal was placed in a separate wire cage to avoid differences in noise exposure. Each experiment was performed in a specially designed acrylic box in a sound-attenuating laboratory booth (900 mm × 900 mm × 1720 mm) with

an electromagnetic shield. The animals were exposed to 2 hours of broadband white noise at 115 dB SPL using a 2,446-J compression driver (JBL Professional, Los Angeles, CA, USA) with an MA-620 power amplifier (Inkel, Incheon, Korea) to obtain a bilateral NIHL animal model. The sound intensity inside the acrylic box was measured every hour using a CR152B sound level meter (Cirrus Research, Hunmanby, UK) to ensure that the sound level was consistent during noise exposure. The control animals were anesthetized as above and placed in the sound-attenuating booth for the same period of time, but were not exposed to noise.[27] Audiometry was conducted 4 hours after termination of the noise to obtain the most stable measurements (Fig. 2).

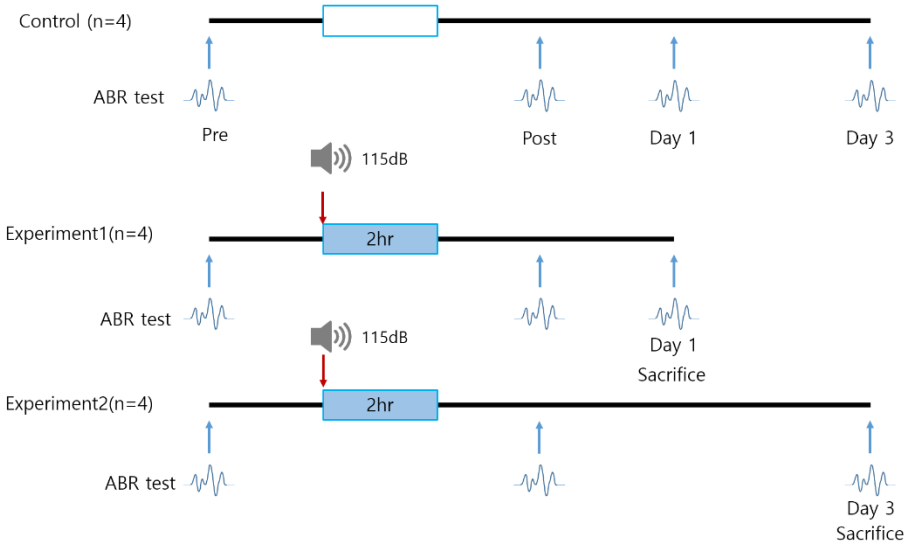


Fig. 2. Noise exposure protocol. The empty box denotes the control condition without noise exposure.

2.8. ABR recordings

Before noise exposure, the auditory function of all animals was

assessed using ABRs. Animals were anesthetized and placed in sound-attenuating booths. Then, three subdermal needle electrodes were positioned, with one at the vertex electrode at the nape of the neck, a negative electrode at the ipsilateral mastoid, and a ground electrode at the contralateral mastoid.[28] The following tone bursts were applied as sound stimuli: a click, a 4-kHz tone, and a 16-kHz tone (duration, 1,562 m; CoS shaping, 21 Hz). High-frequency processing software (ver. 3.30; Intelligent Hearing Systems, Miami, FL, USA) and high-frequency transducers (HFT9911-20-0035; Intelligent Hearing Systems) were used to measure the ABR. Before obtaining the electroencephalography signal, the impedance between the electrodes was evaluated to confirm that it was less than 2 kW. Responses to the signal were amplified approximately 100,000-fold and band-pass filtered (100–1,500 Hz). The intensity of the stimuli ranged from 20 to 90 dB SPL in 5-dB increments. A total of 512 sweeps were averaged at each intensity level. Additional ABRs were measured at 4 h, 1 day, and 3 days after noise exposure. The ABR threshold was defined as the lowest stimulus intensity that produced an evident waveform for wave II or IV. The latencies of waves II–IV, and the amplitudes of waves II and IV, for 90 dB stimuli were also analyzed and compared between the experimental groups.

2.9. Statistical analyses

All data are expressed as the mean ± standard error of the mean, and all data were analyzed using SPSS software (ver. 25.0; IBM Corp., Armonk, NY, USA). An F-test was performed to determine whether the variation within groups was not different. After the F-

test, data were analyzed using Student's t-test to identify significant differences between groups. A p-value of <0.05 was considered statistically significant.

2.10. In silico analysis of target proteins of dieckol, PFF-A, and NAC

Molecular structural formulae, drawn with the Chem3D program using the reported IUPAC names of dieckol, PFF-A, and NAC, were inserted into SwissTargetPrediction. The target proteins of the materials were investigated for humans (Homo sapiens) and mice (Mus musculus).[29]

Chapter 3. Results

3.1. Bioinformatics of dieckol, PFF-A, and NAC

The 3D molecular structures of dieckol, PFF-A, and NAC were drawn using Chem3D (Fig. 3).

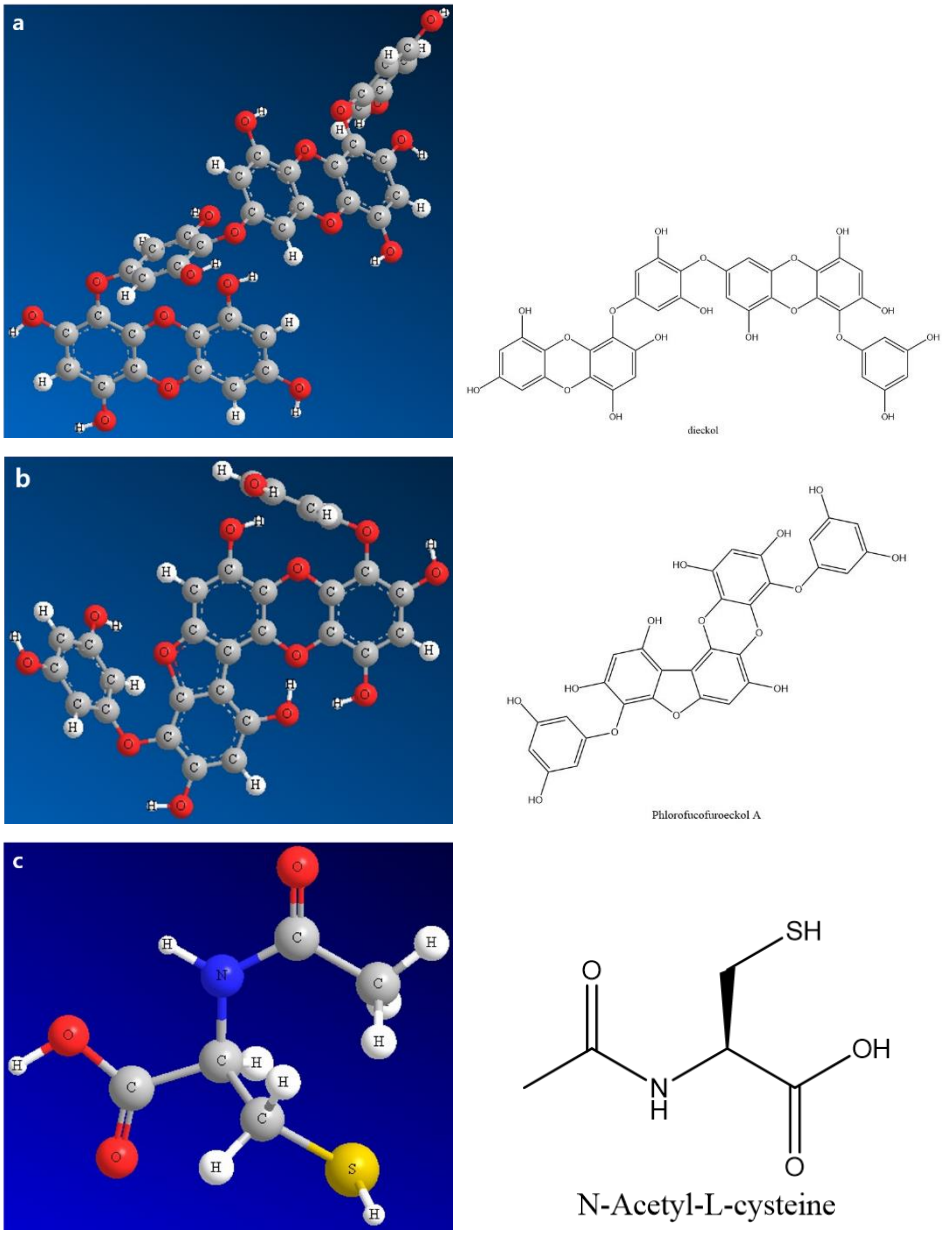


Fig. 3. Molecular 3D and 2D structures of (a) dieckol, (b) phlorofucofuroeckol A, and (c) N-acetyl-L-cysteine.

According to SwissADME, both dieckol and PFF-A have a low probability of passive absorption in the gastrointestinal tract. In addition, both dieckol and PFF-A showed no blood-brain barrier (BBB) permeability, and both compounds showed poor water solubility (Table 1). The gastrointestinal absorption and BBB permeability of the above three molecules can be easily understood through a schematic diagram called a 'boiled egg' plot (Fig. 4).

Molecule	Dieckol	PFF-A	NAC
Formula	C36H22O18	C30H18O14	C5H9NO3S
Molecular weight	742.55	602.46	163.19
Topological polar surface area	287.14	232.13	105.2
WLOGP	7.62	6.42	-0.49
*ESOL class	Poor solubility	Poor solubility	Highly soluble
**Ali class	Insoluble	Poor solubility	Soluble
***Silicos-IT class	Poor solubility	Moderate solubility	Soluble
GI absorption	Low	Low	High
BBB permeable	No	No	No
Bioavailability score	0.17	0.17	0.56
Synthetic accessibility	4.68	4.49	2.08

Table 1. Bioinformatics of dieckol, PFF-A, and NAC. High WLOGP scores indicate good lipid solubility. *ESOL is a topological method

for predicting water solubility **Ali is a topological method for predicting water solubility ***Silicos-IT is a fragmental method for predicting water solubility, calculated using the FILTER-IT program (version 1.0.2) provided by SILICOS-IT (http://www.silicos-it.com). The bioavailability score reflects the probability of a compound having at least 10% oral bioavailability in rats. According to SwissADME, synthetic accessibility is scored from 1 (*very easy*) to 10 (*very difficult*). GI, gastrointestinal; BBB, blood-brain barrier.

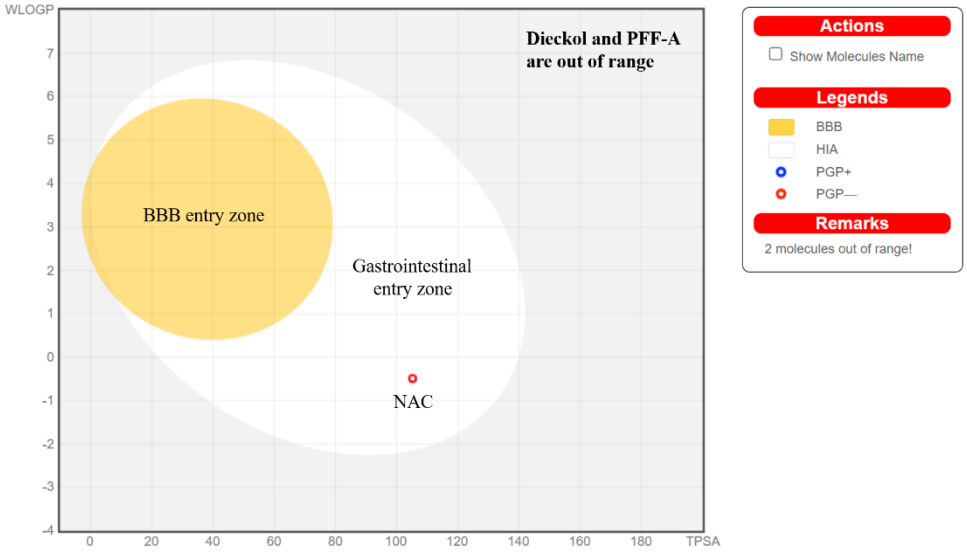


Fig. 4. Boiled egg plot. The white ellipse indicates a high probability of good intestinal absorption and the yellow ellipse indicates a high probability of successful BBB crossing. Dieckol and PFF-A are both outside the plot, suggesting poor intestinal absorption and BBB crossing. NAC (red dot) is also outside the yolk (yellow ellipse), indicating poor BBB permeability. However, NAC (red dot) is within the white ellipse, indicating a high probability of good intestinal absorption. BBB, blood-brain barrier; PFF-A, phlorofucofuroeckol A; NAC, N-acetylcysteine

3.2. DPPH assay

The radical-scavenging activity of dieckol at 12.5, 25, 50, 100, and 200 uM was 15.3, 25, 35.3, 50.6, and 62.1%, respectively. In addition, the radical-scavenging activity of PFF-A at 12.5, 25, 50, 100, and 200 uM was 16.7, 30.9, 44, 59.8, and 70.8%, respectively (Fig. 3). The results suggested that dieckol and PFF-A lowered ROS levels. The effects of dieckol and PFF-A on cell viability in human middle ear epithelial cells were also investigated. Cell viability did not decline below 50% after exposure to 200 uM of either dieckol or PFF-A (Fig. 5).

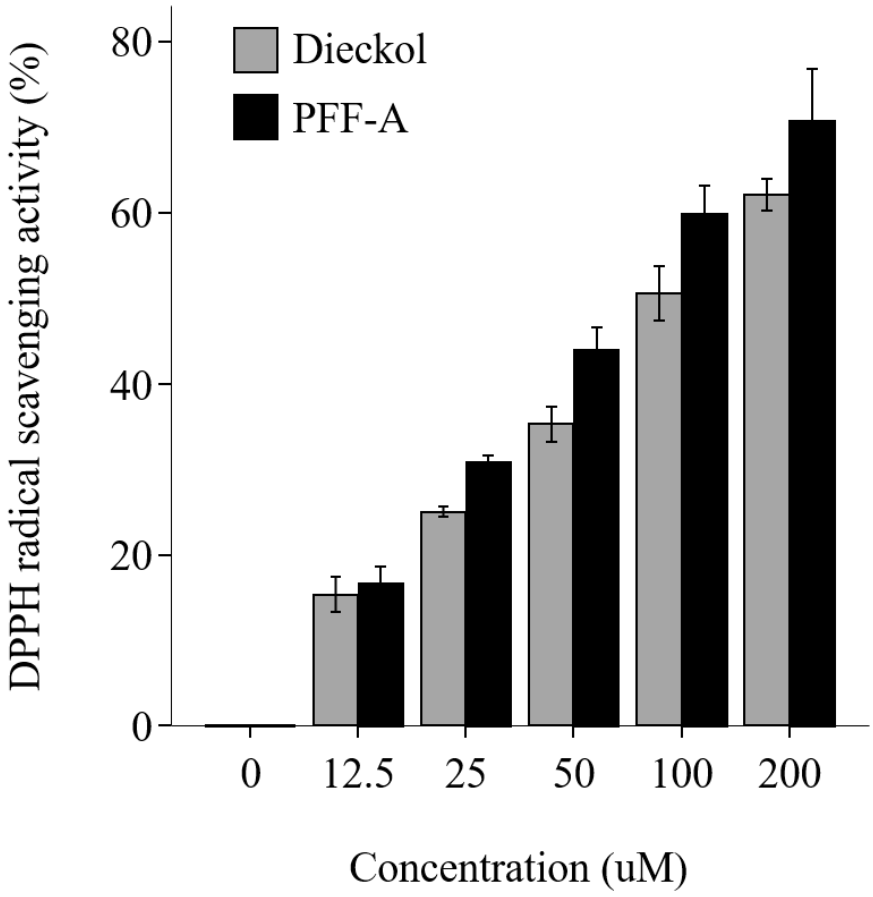


Fig. 5. The antioxidant effect of PFF-A and dieckol, in terms of DPPH radical-scavenging activity. Both PFF-A and dieckol scavenged DPPH radicals in a dose-dependent manner. PFF-A, phlorofucofuroeckol A; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DMSO, dimethyl sulfoxide.

3.3. Cell viability

Cell viability was measured at 12.5, 25, 50, 100, and 200 uM of dieckol and PFF-A. Cell viability decreased in a dose-dependent manner. Cell viability did not fall below 50% at the maximum experimental dose of 200 uM (Fig. 6).

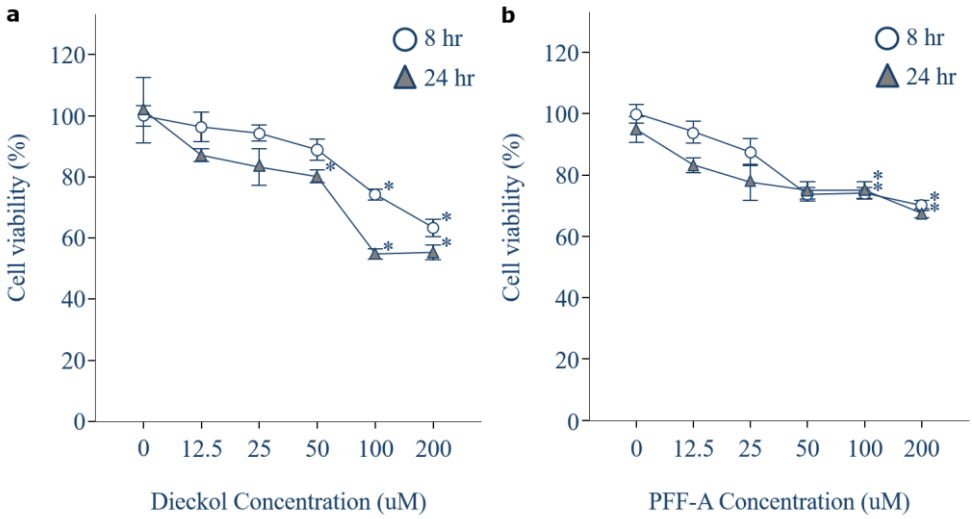


Fig. 6. Effect of dieckol (a) and PFF-A (b) on cell viability in mouse auditory HEI-OC1 cells. PFF-A, phlorofucofuroeckol A.

3.4. ABR threshold shifts after noise exposure

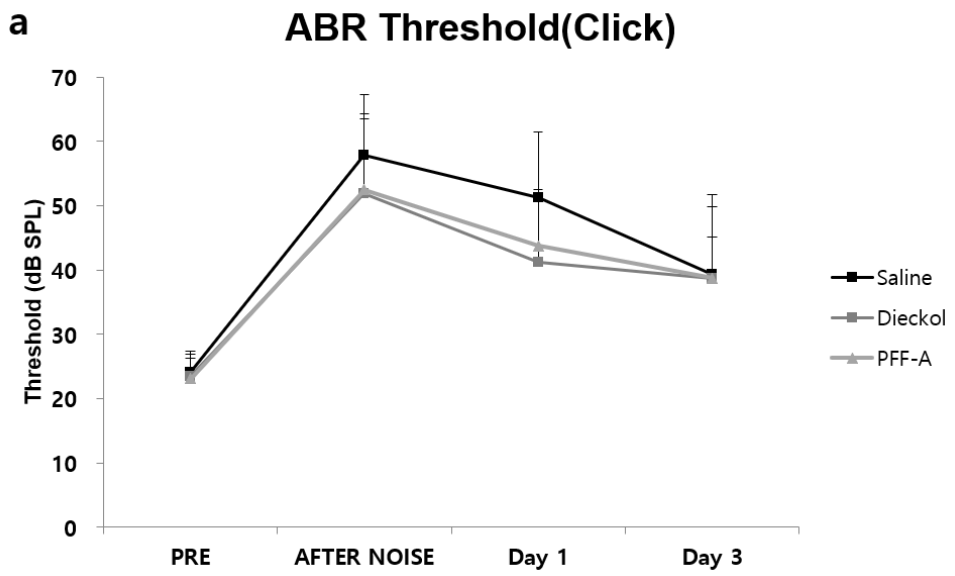
The protective effects of dieckol and PFF-A against TTS were assessed in animals exposed to noise after intraperitoneal injections of saline or treatments. All seven groups (n = 8 each) had a decreased ABR threshold at day 1 after noise exposure.

ABR threshold shifts 4 hours after 4-kHz noise exposure in mice that received dieckol were significantly lower than those in the saline with noise group (p = 0.042). Otherwise, there were no significant differences in ABR thresholds among the saline with noise, dieckol with noise, and PFF-A with noise groups exposed to the click sound, 4 kHz, or 16 kHz noise on day 1, day 3, or 4 hours after noise exposure (Fig. 7).

A high dose of PFF-A (100 mg/kg) led to a significantly smaller ABR threshold shifts 1 day after noise exposure compared to those in the saline with noise group exposed to the click sound (p =

0.035). In addition, ABR threshold shifts 4 hours after 16 kHz noise exposure in mice given a high dose of PFF-A (100 mg/kg) were significantly smaller than those in the saline with noise group (p = 0.018). This suggests that PFF-A had a protective effect against TTS. However, no significant differences in ABR threshold shifts were observed among the saline with noise group, high-dose dieckol (100 mg/kg) group, and high-dose PFF-A (100 mg/kg) group for the 4 kHz sound on day 1, day 3 or 4 hours after noise exposure (Fig. 8).

Furthermore, no significant differences were observed in ABR threshold shifts among the saline with noise, dieckol/NAC with noise, and PFF-A/NAC with noise groups for the click sound, 4 kHz noise, or 16 kHz noise on day 1, day 3, or 4 hours after noise exposure (Fig. 9).



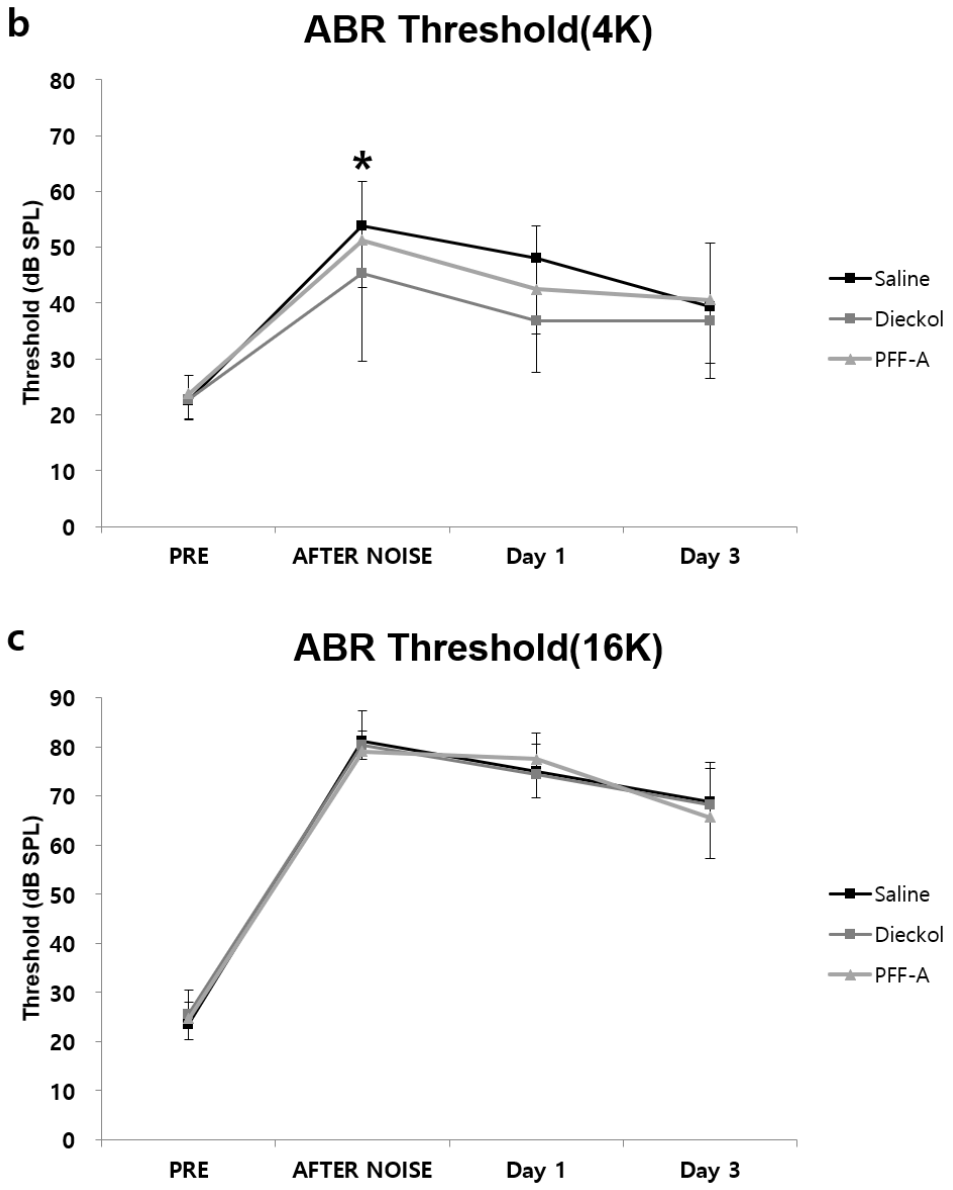
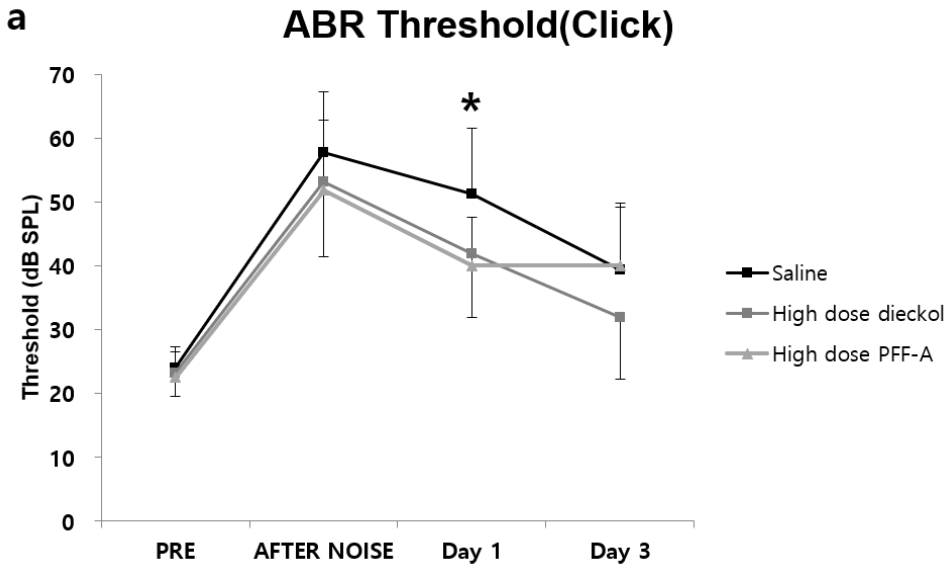
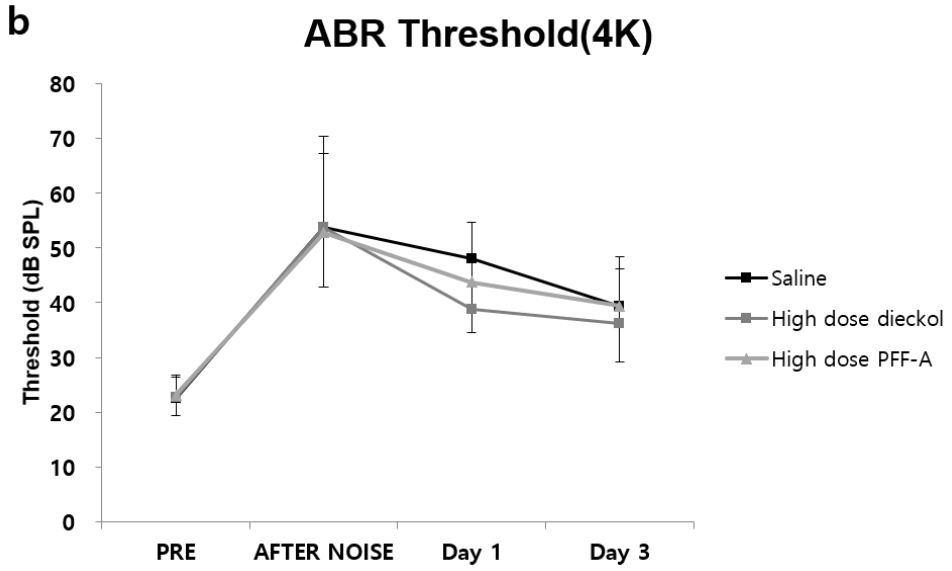


Fig. 7. The effects of protective agents on hearing ability after exposure to noise. Comparison among the saline with noise group, dieckol with noise group, and PFF-A with noise group. (a) ABR threshold shifts in mice exposed to the click sound. No significant group difference was observed. (b) ABR threshold shifts in mice exposed to 4 kHz noise. *The ABR threshold shifts in the dieckol with noise group were significantly smaller than those in the saline with noise group 4 hours after noise exposure. (c) ABR threshold

shifts in mice exposed to 16 kHz noise. No significant group difference was observed. PFF-A, phlorofucofuroeckol A; ABR, auditory brainstem response.





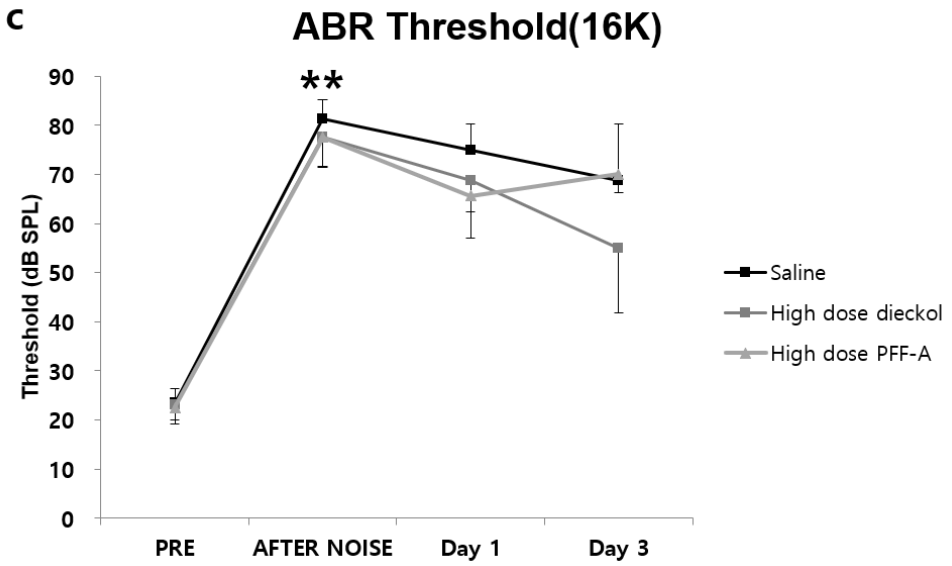
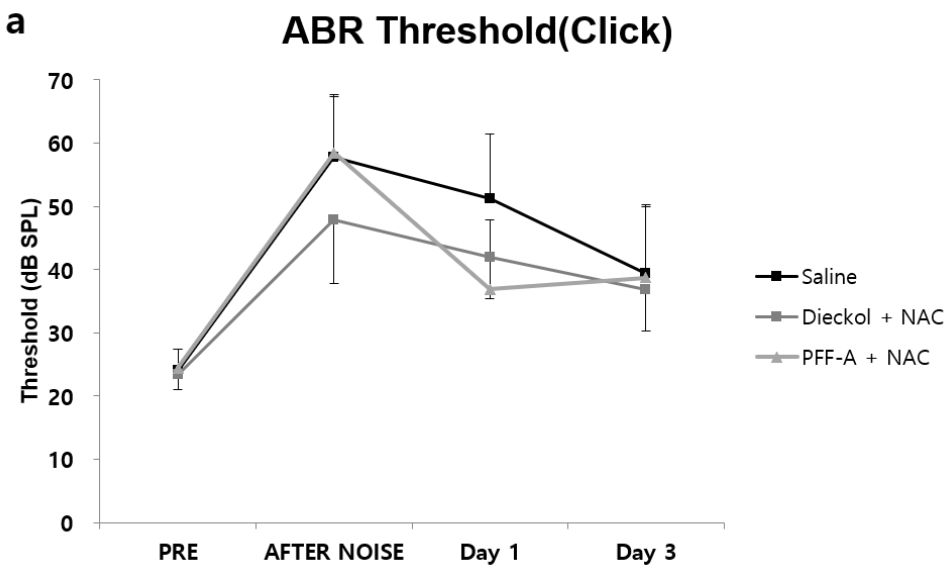
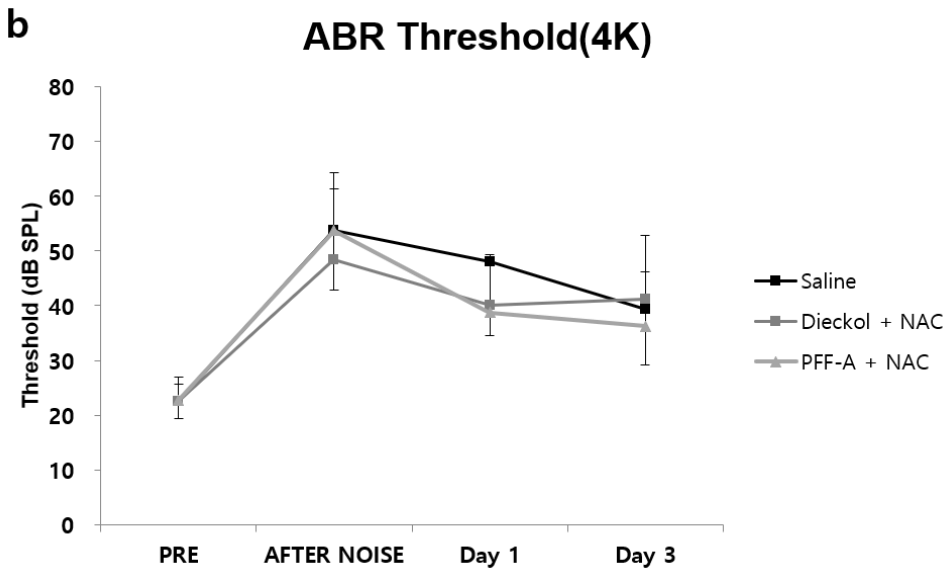


Fig. 8. The effects of protective agents on hearing ability after exposure to noise. Comparison among the saline with noise group, high-dose dieckol (100 mg/kg) with noise group, and high-dose PFF-A (100 mg/kg) with noise group. (a) ABR threshold shifts in mice exposed to the click sound. *The ABR threshold shifts 1 day after noise exposure were significantly smaller in the high-dose PFF-A (100 mg/kg) with noise group than in the saline with noise group. (b) ABR threshold shifts in mice exposed to 4 kHz noise. No significant group difference was observed. (c) ABR threshold shifts in mice exposed to 16 kHz noise. **The ABR threshold shifts 4 hours after noise exposure were significantly smaller in the high-dose PFF-A (100 mg/kg) with noise group than in the saline with noise group. PFF-A, phlorofucofuroeckol A; ABR, auditory brainstem response.


a
ABR Threshold(Click)
Threshold (dB SPL)
70
60
50
40
30
20
10
0
PRE
AFTER NOISE
Day 1
Day 3
Saline
Dieckol + NAC
PFF-A + NAC


b
ABR Threshold(4K)
Threshold (dB SPL)
80
70
60
50
40
30
20
10
0
PRE
AFTER NOISE
Day 1
Day 3
Saline
Dieckol + NAC
PFF-A + NAC

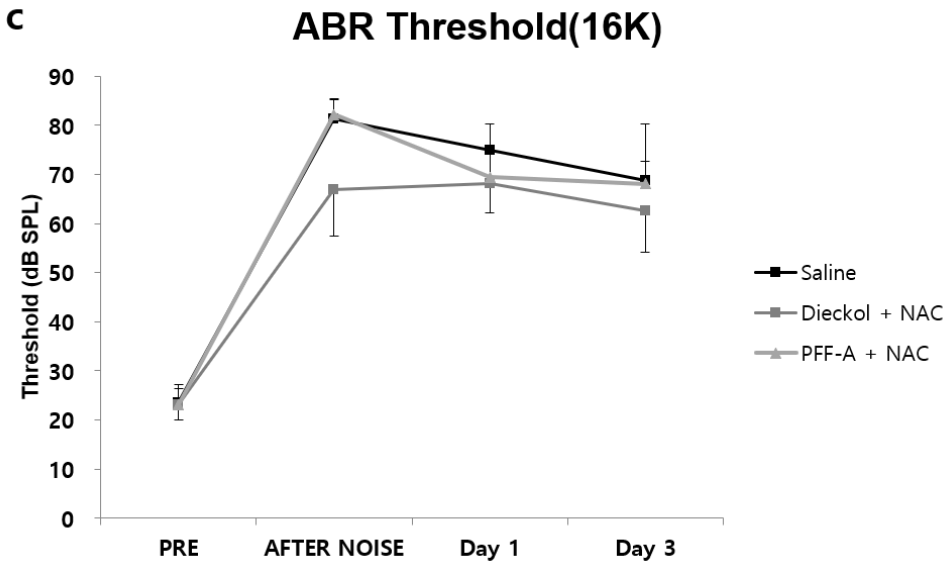


Fig. 9. The effects of protective agents on hearing ability after exposure to noise. ABR threshold shifts in mice exposed to (a) a click, (b) 4 kHz noise, and (c) 16 kHz noise. Comparison among the saline with noise, dieckol/NAC with noise, and PFF-A/NAC with noise groups revealed no significant differences in ABR threshold shifts after exposure to a click sound, 4 kHz noise, or 16 kHz noise, on either day 1, day 3, or 4 hours after noise exposure. PFF-A, phlorofucofuroeckol A; ABR, auditory brainstem response; NAC, N-acetylcysteine.

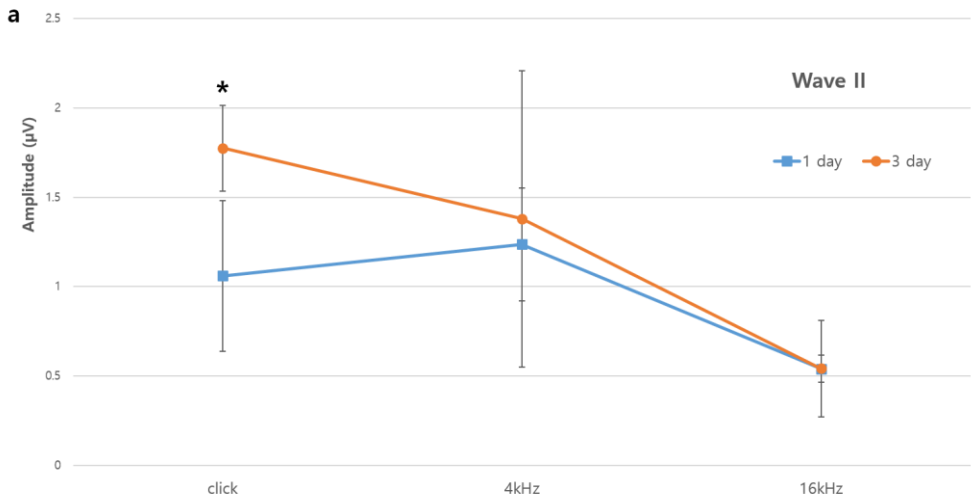
3.5. ABR amplitudes

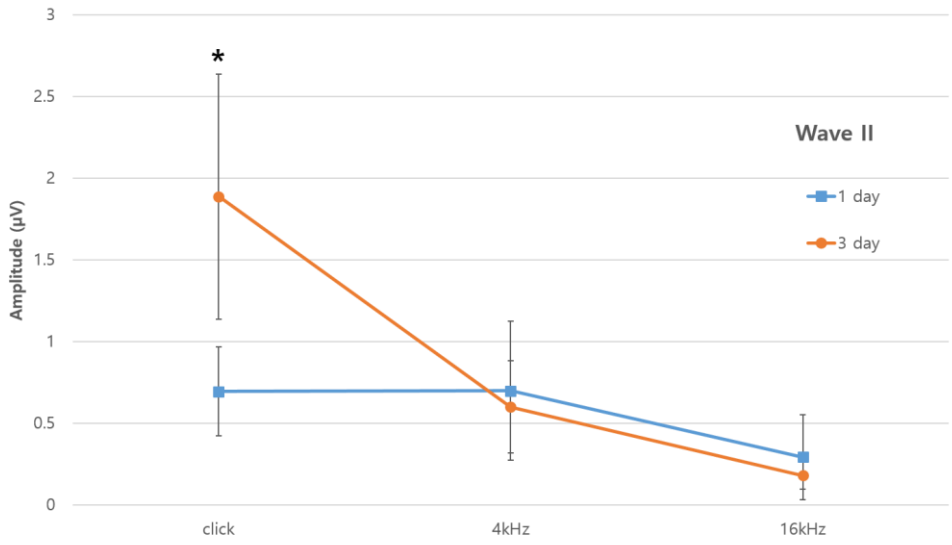
The amplitudes of waves II and IV were calculated based on ABR waveforms. The wave II amplitudes observed on day 1 in the saline with noise group (click, 1.06 ± 0.42 μV) were significantly smaller than those at day 3 (click, 1.78 ± 0.24 μV). The wave II amplitudes observed on day 1 in the dieckol/NAC with noise group (click, 0.695 ± 0.27 μV) were also significantly smaller than those on day

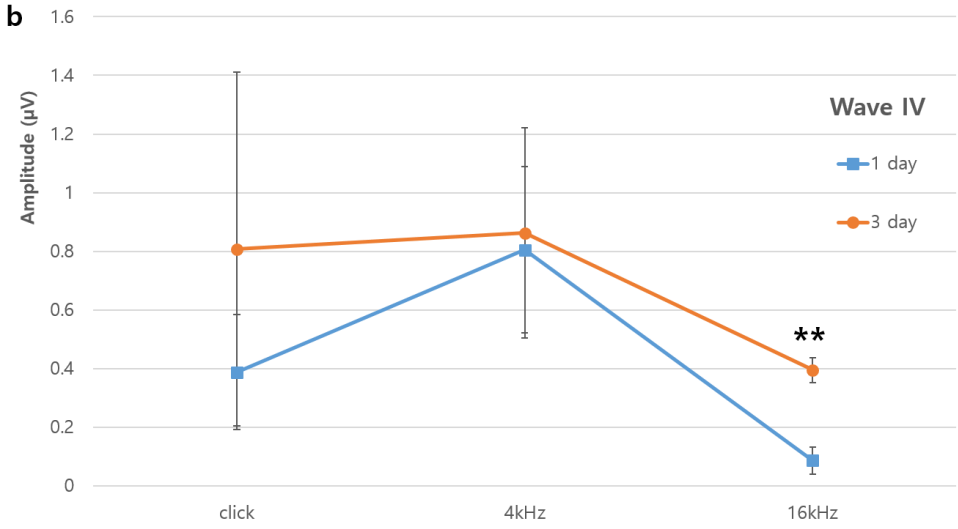
3 (click, 1.89 ± 0.75 μV). There were no significant differences in wave II amplitudes between the other groups at other frequencies. The wave IV amplitudes observed on day 1 in the high-dose dieckol group (16 kHz, 0.087 ± 0.046 μV) were significantly smaller than those measured on day 3 (16 kHz, 0.40 ± 0.04 μV). There were no significant differences in wave IV amplitudes between the other groups at other frequencies (Fig. 8).

3.6. ABR latencies

The latencies between waves IV and II were calculated based on the ABR waveforms. The latency on day 3 in the PFF/NAC with noise group (1.72 ± 0.19 ms) was reduced compared to that on day 1 (2.39 ± 0.16 ms) after exposure to a click sound. There were no differences in latencies between any groups at other frequencies (Fig. 8).




Wave II
1 day
3 day
Amplitude (μV)
3
2.5
2
1.5
1
0.5
0
*
click
4kHz
16kHz


b
Wave IV
1 day
3 day
Amplitude (μV)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
click
4kHz
16kHz

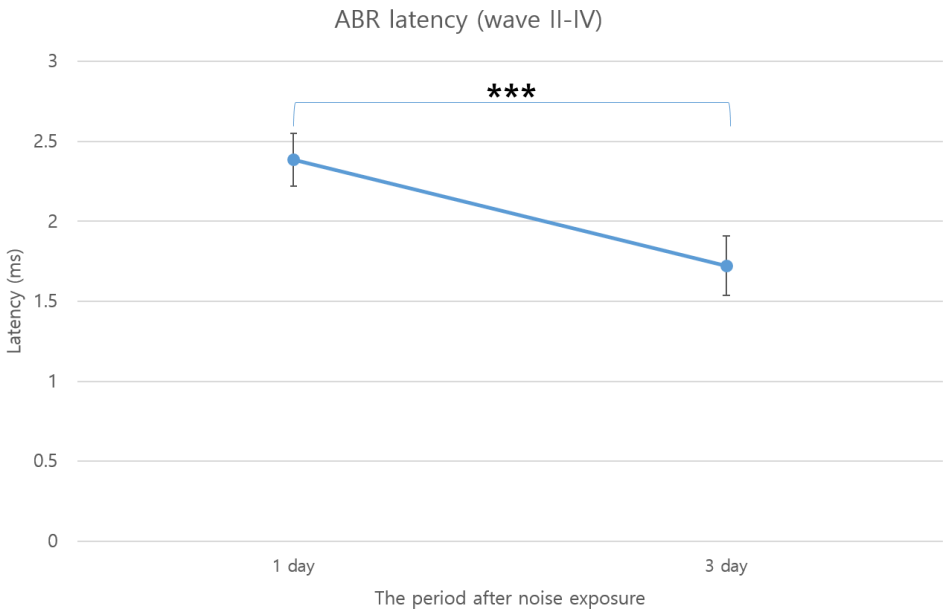


Fig 10. Amplitudes of waves II and IV and latencies of waves IV–II. (a) (Upper) Saline with noise group, (Lower) dieckol/NAC with noise group. Comparison of the amplitude of wave II among frequencies. The wave II amplitude for mice assessed 3 days after noise exposure was significantly larger than that on day 1 in mice exposed to the click sound, saline with noise, and dieckol/NAC with noise ($^{*}p < 0.05$). No significant group differences were observed at the other frequencies. (b) High-dose dieckol (100 mg/kg) with noise group. Comparison of the amplitude of wave IV among frequencies. The wave IV amplitude for mice assessed on day 3 after noise exposure was significantly larger than that on day 1 in mice exposed to 16 kHz and a high dose of dieckol ($^{**}p < 0.001$). No significant differences were observed at the other frequencies. (c) PFF-A/NAC with noise group. The latencies of waves IV–II at each frequency are shown. The wave IV-II latency for mice assessed on day 3 after noise exposure was significantly shorter than that on day 1 in mice exposed to a click sound and PFF-A/NAC ($^{***}p <$

0.05). No significant differences were observed at any other frequency.

3.7. Hair cell phalloidin staining and counts

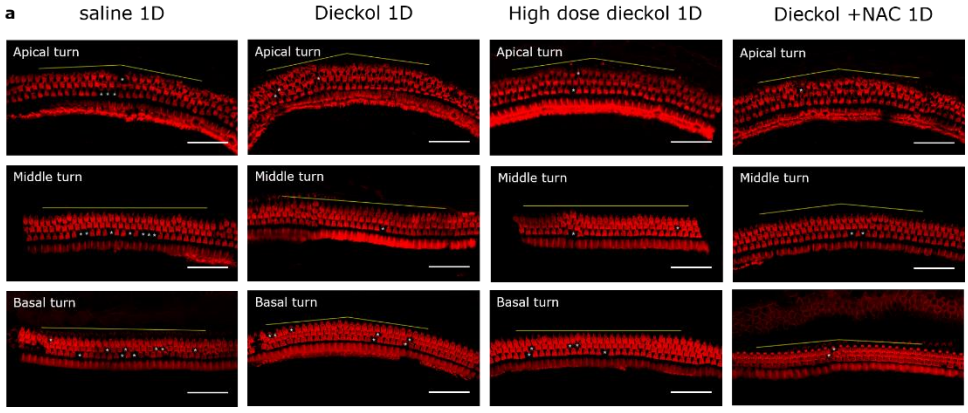
Phalloidin was used to stain outer hair cells (HCs) after whole mount surface preparation. Two 200-μm-long segments were selected from each section of the organ of Corti and used to calculate the mean number of surviving outer HCs. Evaluation of the outer HCs was performed in all eight groups, i.e., the baseline (n = 4), saline with noise (n = 8), PFF-A with noise (n = 8), high-dose PFF-A (100 mg/kg) with noise (n = 8), PFF-A/NAC with noise (n = 8), dieckol with noise (n = 8), high-dose dieckol (100 mg/kg) with noise (n = 8), and dieckol/NAC with noise (n = 8) groups. All three rows of outer HCs from all baseline group samples showed no missing HCs.

Significant outer HC loss was observed in the basal turn sections on days 1 and 3 for all six treatment groups (PFF-A, high-dose PFF-A (100 mg/kg), PFF-A/NAC, dieckol, high-dose dieckol (100mg/kg), and dieckol/NAC) compared to the baseline group.

No statistically significant differences in HC loss were observed between the groups treated with dieckol (dieckol with noise, high-dose dieckol [100 mg/kg] with noise, dieckol/NAC with noise groups) and the saline with noise group in the basal, middle, or apical turn sections on day 1 or 3 (Figs. 9 and 10).

However, significantly less HC loss was observed in the high-dose PFF-A (100 mg/kg) with noise group compared to the saline with noise group on day 1 after noise exposure in the apical turn section (p = 0.019) (Fig. 11). Significantly less HC loss was also

observed in the PFF-A/NAC with noise group compared to the saline with noise group on day 3 after noise exposure in the apical turn section ($p = 0.046$). Statistically significant differences in HC loss were observed in the basal turns of groups treated with PFF-A. For instance, HC loss in the basal turns of the PFF-A/NAC with noise group was significantly less than in the PFF-A with noise group on day 3 after noise exposure ($p < 0.001$). Also, HC loss in the basal turns of the high-dose PFF-A (100 mg/kg) with noise group was significantly lower than in the PFF-A with noise group on day 3 after noise exposure ($p = 0.032$) (Fig. 12). Otherwise, no statistically significant differences in HC loss were observed between the groups treated with PFF-A (PFF-A with noise, high-dose PFF-A [100 mg/kg] with noise, PFF-A/NAC) compared to the saline with noise group in the basal, middle, or apical turn sections on day 1 or 3.



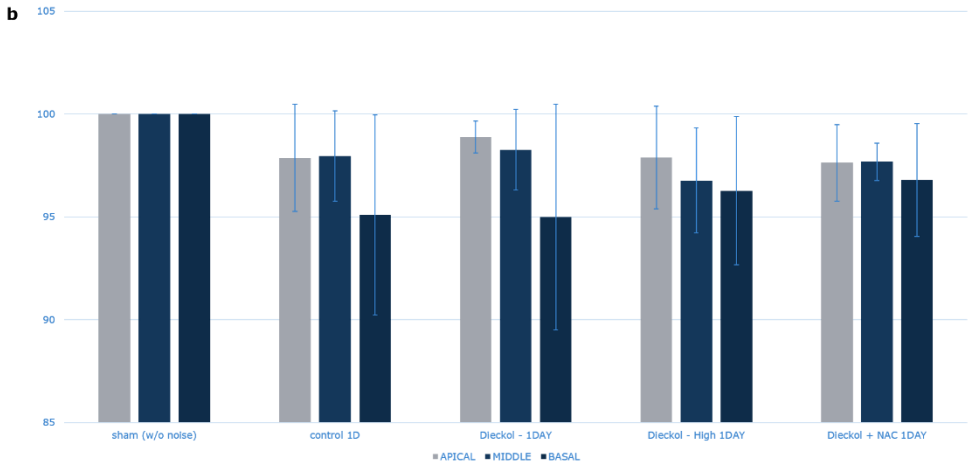
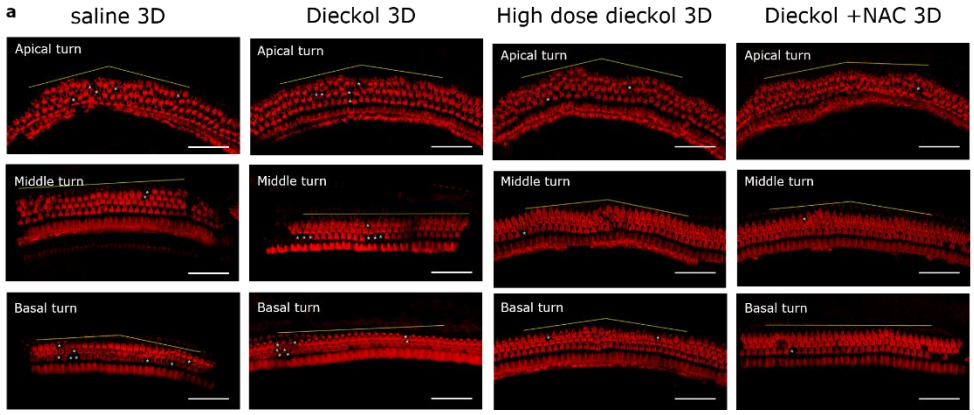


Fig. 11. HC morphology and survival rate of dieckol-treated groups (dieckol with noise, high-dose dieckol [100 mg/kg] with noise, dieckol/NAC with noise) and the saline with noise group on day 1 after noise exposure. The baseline (sham) group was not exposed to noise. The (a) morphology and (b) survival rate of HCs in the apical, middle, and basal turn segments of the organ of Corti. No statistically significant differences in HC loss were observed between the dieckol-treated groups and the saline with noise group. HC, hair cell.



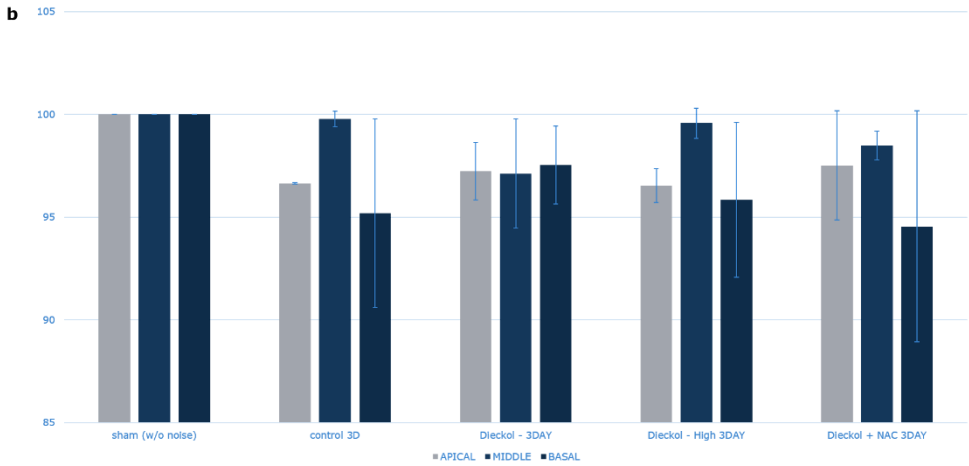
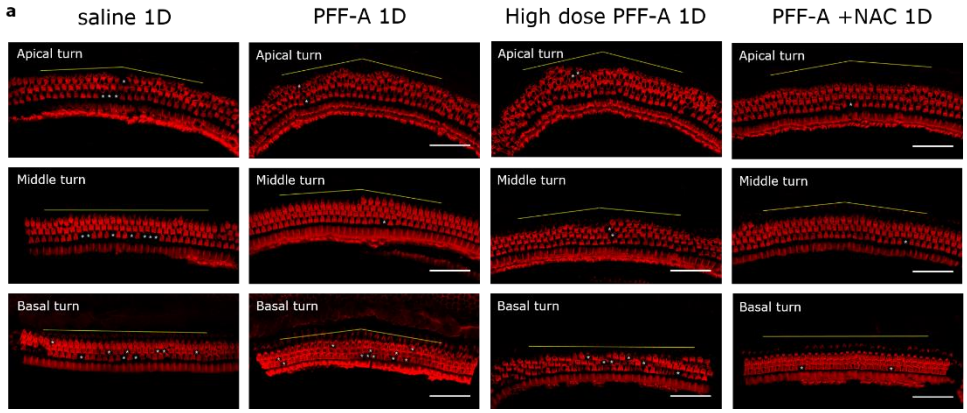


Fig. 12. HC morphology and survival rate of dieckol-treated groups (dieckol with noise, high-dose dieckol [100 mg/kg] with noise, dieckol/NAC with noise) and saline with noise group on day 3 after noise exposure. The baseline (sham) group was not exposed to noise. The (a) morphology and (b) survival rate of HCs in the apical, middle, and basal turn segments of the organ of Corti. No statistically significant differences in HC loss were observed between the dieckol-treated groups and saline with noise group. HC, hair cell.



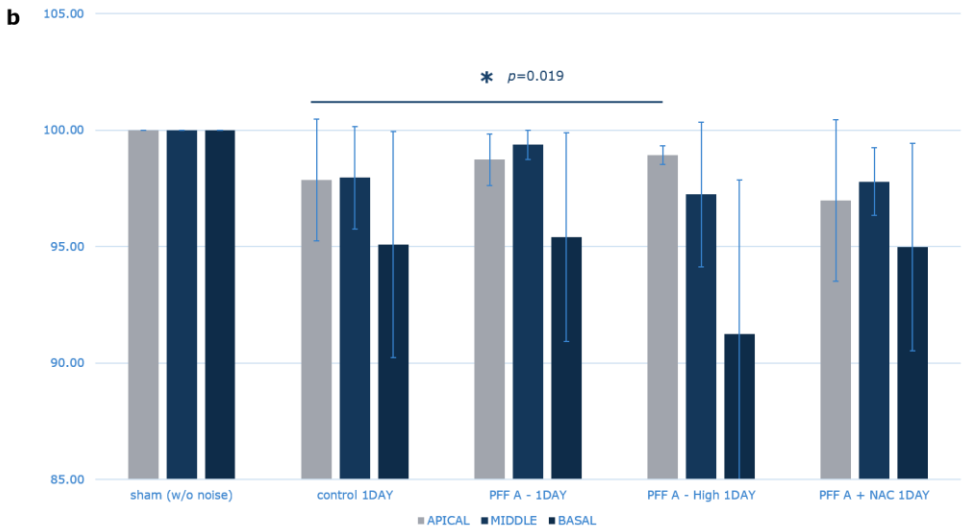
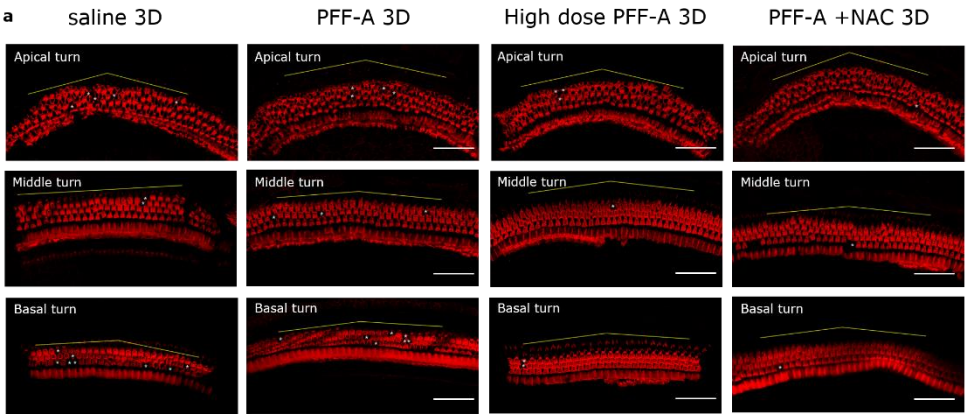


Fig. 13. HC morphology and survival rate of the PFF-A treated groups (PFF-A with noise, high-dose PFF-A [100 mg/kg] with noise, PFF-A/NAC with noise) and saline with noise group on day 1 after noise exposure. The baseline (sham) group was not exposed to noise. The (a) morphology and (b) survival rate of HCs in the apical, middle, and basal turn segments of the organ of Corti. Significantly less HC loss was observed in the high-dose PFF-A (100 mg/kg) with noise groups compared to the saline with noise group in the apical turn section (p = 0.019). HC, hair cell; PFF-A, phlorofucofuroeckol A.



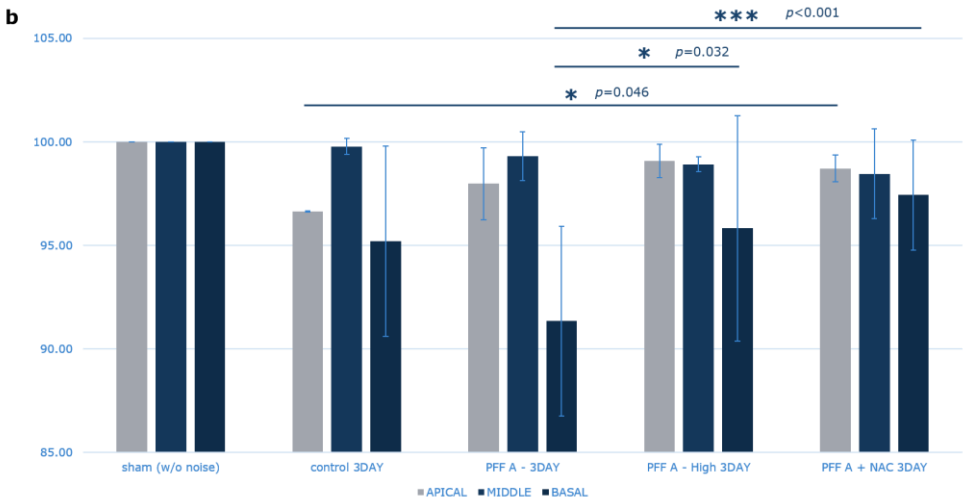


Fig. 14. HC morphology and survival rate of PFF-A-treated groups (PFF-A with noise, high-dose PFF-A [100 mg/kg] with noise, PFF-A/NAC with noise) and the saline with noise group on day 1 after noise exposure. The baseline (sham) group was not exposed to noise. The (a) morphology and (b) survival rate of HCs in the apical, middle, and basal turn segments of the organ of Corti. Significantly less HC loss was observed in the PFF-A/NAC with noise group compared to the saline with noise group in the apical turn section ($p = 0.046$). HC loss in the basal turn segment of the PFF-A/NAC with noise group was significantly less than in the PFF-A with noise group ($p < 0.001$). Also, HC loss in the basal turn of the high-dose PFF-A (100 mg/kg) with noise group was significantly less than in the PFF-A with noise group ($p = 0.032$). HC, hair cell; PFF-A, phlorofucofuroeckol A.

3.8. In silico analysis of target proteins of dieckol, PFF-A, and NAC

Beta-secretase, an aspartic acid protease important in the

formation of myelin sheaths in peripheral nerve cells[30], appeared as the target protein of dieckol and PFF-A in both humans and mice (100% probability). SwissTargetPrediction did not suggest any target molecule for NAC with a probability higher than 5%, in mice or humans.

Chapter 4. Discussion

According to previous studies, noise generates ROS in the inner ear, which produces several compounds via peroxidation of polyunsaturated fatty acids.[31-33] 8-isoprostaglandin F2a (8-iso-PGF2a), one of the compounds formed by ROS, is a powerful vasoconstrictor that reduces cochlear blood flow (CBF) and induces cochlear ischemia.[34,35] Cochlear ischemia causes excessive release and accumulation of glutamate in the inner HCs, leading to glutamate excitotoxicity.[36,37] Cochlear ischemia also reduces the energy supply to the stria vascularis, leading to decreased endocochlear potential[38] and HC swelling.[39] These processes ultimately cause dysfunction in HCs and cochlear afferent neurons, resulting in TTS. To address this, the present study tested potent antioxidants PFF-A and dieckol in terms of ther ability to the prevent TTS.

Various studies have revealed that antioxidant agents have a protective effect against NIHL.[40,41] Diverse antioxidant materials including NAC, acetyl-L-carnitine (ALCAR), 4-hydroxy alpha-phenyl-tert-butylnitrone (4-OHPBN), salicylate, 2,4-disulfonyl a-phenyl tertiary butyl nitrone (HPN-07), HK-2, and others have been found to have a protective effect against noise-induced damage in the cochlea.[42-46] While most studies administered antioxidants orally, one study with rosmarinic acid reported that the protective effect of trans-tympanic application of antioxidants was equal to that of oral administration, with a lower rate of side effects.[47] These studies support the protective effect of antioxidant materials against TTS.

Dieckol, one of the major bioactive components of polyphenols

extracted from Ecklonia cava, has well-documented antioxidant, cytoprotective, and anti-inflammatory effects.[48] According to previous studies, dieckol removes ROS and prevents ROS formation. Dieckol reduces ROS formation through upregulation of antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase[49], and downregulation of pro-inflammatory enzymes such as nitric oxide synthase and cyclooxygenase-2 (COX-2)[50,51]. Previous studies have described dieckol as an antioxidant agent due to its ability to eliminate ROS.[52,53]

PFF-A, another bioactive component of polyphenols extracted from Ecklonia cava, has been reported to have antioxidant [54 55 56 57 58], anti-inflammatory[57,59], anti-allergenic[16], and anti-cancer[60,61] effects. PFF-A exerts an antioxidant effect by scavenging for ROS and reducing ROS production. Furthermore, PFF-A inhibits nitric oxide and prostaglandin E2 (PGE2) via down-regulation of iNOS and COX-2 proteins, resulting in an anti-inflammatory effect. Previous studies have indicated that PFF-A can eliminate and reduce the production of ROS, supporting its use as an antioxidant agent.

Considering the pathophysiology of TTS, an effective preventative treatment must be applied before noise exposure. However, concern regarding potential side effects can limit the administration of medications in the absence of certainty regarding the risk of disease. PFF-A and dieckol are advantageous compared to conventional medications because these natural extracts from Ecklonia cava are as safe as food ingredients. Indeed, a representative Ecklonia cava polyphenol extract that contains PFF-A and dieckol was approved by the United States Food and Drug Administration (USFDA) as a new dietary ingredient in 2008

(FDA-1995-S-0039-0176). As they are considered safe food ingredients, PFF-A and dieckol may be administered to those who are inevitably exposed to noise for the prevention of TTS, with a low possibility of adverse effects.

In this study, mice were given dieckol and PFF-A via intraperitoneal injection. However, dieckol and PFF-A would be administered to humans orally. According to the bioinformatics information obtained during the study, dieckol and PFF-A possess low gastrointestinal absorbability and poor water solubility. Although PFF-A and dieckol are potent antioxidant agents considered safe for consumption, processing may be required before oral administration to achieve effective gastrointestinal absorption and significant bioavailability. For instance, recent studies have suggested that nanoencapsulation of polyphenols could increase gastrointestinal absorption. Reports on the nanoencapsulation of various polyphenols, including ellagitannins, curcumin, oleuropein, and hydroxytyrosol have revealed increased gastrointestinal absorption of these compounds compared to dieckol and PFF-A.[62] A bioinformatics analysis from SwissADME indicated that both dieckol and PFF-A have high WLOGP values, suggesting high lipid solubility and possible BBB permeability. However, both compounds have a large topological polar surface area. Thus, both dieckol and PFF-A show minimal BBB permeability, implying that the effects of both compounds on the inner ear may be negligible after they enter the systemic circulation.[63] To overcome this second obstacle, extra measures to increase BBB permeability are necessary. A study of fluorescein isothiocyanate (FITC)-labeled dieckol and a rhodamine B-labeled dieckol reported effective BBB penetration of dieckols in rats.[64] Further studies are required to

establish an effective delivery method for dieckol and PFF-A into the inner ear.

An in vivo study on the radical-scavenging activity of PFF-A and dieckol would provide evidence regarding the antioxidant effects of these materials. However, in vivo measurement of radical scavenging activity is challenging due to the instability of ROS in the cochlea. Accordingly, in vivo measurement of the radical-scavenging activity of PFF-A and dieckol was not performed in this study. Instead, I performed a DPPH assay to assess the radical scavenging activity of PFF-A and dieckol. Although the present results indicate that PFF-A and dieckol have radical scavenging activity in vivo, further in vivo and human studies are needed to further demonstrate the protective effect of PFF-A and dieckol against TTS.

Chapter 5. Conclusion

This investigation of the prophylactic effect of dieckol and PFF-A against TTS in mice revealed that these compounds, acquired from Ecklonia cava, prevented TTS through antioxidant activity. Because dietary ingredients are approved by the USFDA, they can be used as preventative agents with a low possibility of adverse effects. Our findings suggest that dieckol and PFF-A, as preventative agents, may play an important role in reducing the incidence of TTS.

Bibliography

1. Concha-Barrientos M, Campbell-Lendrum D, Steenland K. Occupational noise: Assessing the burden of disease from work-related hearing impairment at national and local levels: Geneva, World Health Organization, 2004.
2. D H, EC B, KC H, BH H. The role of oxidative stress in noise-induced hearing loss. Ear and hearing 2006; 27.
3. AS N, BA B, GW H. Histopathological differences between temporary and permanent threshold shift. Hearing research 2000; 139.
4. HJ H, RA D, KG L, CL T, GA F. Declining Prevalence of Hearing Loss in US Adults Aged 20 to 69 Years. JAMA otolaryngology--head & neck surgery 2017; 143.
5. J S, SG C, GC C, R E. Change in prevalence of hearing loss in US adolescents. JAMA 2010; 304.
6. H S. Primary structural changes in the organ of Corti after acoustic overstimulation. Acta oto-laryngologica 1971; 71.
7. KA F, PW J, K L, MC L, SG K. Aging after noise exposure: acceleration of cochlear synaptopathy in "recovered" ears. The Journal of neuroscience : the official journal of the Society for Neuroscience 2015; 35.
8. SG K, MC L. Acceleration of age-related hearing loss by early noise exposure: evidence of a misspent youth. The Journal of neuroscience : the official journal of the Society for Neuroscience 2006; 26.
9. SG K, MC L. Synaptopathy in the noise-exposed and aging cochlea: Primary neural degeneration in acquired sensorineural hearing loss. Hearing research 2015; 330.
10. RA D. Prevention of noise-induced hearing loss. Archives of otolaryngology--head & neck surgery 1995; 121.
11. SL L. Noise exposures. Effects on hearing and prevention of noise induced hearing loss. AAOHN journal : official journal of the American Association of Occupational Health Nurses 1997; 45.
12. A D, Y L, A M, E B, AH T, A H. Comparison of the effects of N-acetyl-cysteine and ginseng in prevention of noise induced hearing loss in male textile workers. Noise & health 2014; 16.
13. KK O, JS W, LL D. Early elevation of cochlear reactive oxygen species following noise exposure. Audiology & neuro-otology 1999; 4.
14. Kang WS, Chung JW. Ingestion of Korean Red Ginseng after Noise Exposure Can Potentiate Rapid Recovery of Hearing in Mice. Journal of Ginseng Research 2010; 34:336-341.
15. TN L, LV S, J L, B W. Current insights in noise-induced hearing loss: a literature review of the underlying mechanism, pathophysiology, asymmetry, and management options. Journal of otolaryngology - head & neck surgery = Le Journal d'oto-rhino-laryngologie et de chirurgie cervico-faciale 2017; 46.

16. Y L, SH L, QT L, MM K, SK K. Anti-allergic effects of phlorotannins on histamine release via binding inhibition between IgE and Fc epsilonRI. Journal of agricultural and food chemistry 2008; 56.
17. Y L, ZJ Q, B R, SH L, MM K, SK K. Chemical components and its antioxidant properties in vitro: an edible marine brown alga, Ecklonia cava. Bioorganic & medicinal chemistry 2009; 17.
18. I PS, SB B. Phloroglucinol compounds of natural origin. Natural product reports 2006; 23.
19. MA T, MP B, SC Cet al. Biochemical biomarkers in algae and marine pollution: a review. Ecotoxicology and environmental safety 2008; 71.
20. SJ H, JP K, WK Jet al. Identification of chemical structure and free radical scavenging activity of diphlorethohydroxycarmalol isolated from a brown alga, Ishige okamurae. Journal of microbiology and biotechnology 2008; 18.
21. K I. Antidiabetic and antioxidant effects of polyphenols in brown alga Ecklonia stolonifera in genetically diabetic KK-A(y) mice. Plant foods for human nutrition (Dordrecht, Netherlands) 2008; 63.
22. K K, Y P, HJ H, SH K, JG L, HC S. Antioxidative properties of brown algae polyphenolics and their perspectives as chemopreventive agents against vascular risk factors. Archives of pharmacal research 2003; 26.
23. MY C, SY H, HC S, JY B, YC R, MK P. Protective effect of a purified polyphenolic extract from Ecklonia cava against noise-induced hearing loss: Prevention of temporary threshold shift. International journal of pediatric otorhinolaryngology 2016; 87.
24. A D, O M, V Z. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Scientific reports 2017; 7.
25. G K, P T, C P, F K. HEI-OC1 cells as a model for investigating drug cytotoxicity. Hearing research 2016; 335.
26. T Z, T Z, H Yet al. Autophagy impairment as a key feature for acetaminophen-induced ototoxicity. Cell death & disease 2021; 12.
27. ZT S, Y L, J Wet al. G-CSF attenuates noise-induced hearing loss. Neuroscience letters 2014; 562.
28. MY C, J R, SH K, YH K. The Protective Effect of Egb 761 Against 3-Nitropropionic Acid-Induced Hearing Loss: The Role of Sirtuin 1. Clinical and experimental otorhinolaryngology 2018; 11.
29. A D, O M, V Z. SwissTargetPrediction: updated data and new features for efficient prediction of protein targets of small molecules. Nucleic acids research 2019; 47.
30. Willem M, Garratt AN, Novak Bet al. Control of Peripheral Nerve Myelination by the ß-Secretase BACE1 2006.
31. AW L, LL S, LJ R, JA A, RF B, JD M. Effect of oxygen tension on the generation of F2-isoprostanes and malondialdehyde in peroxidizing rat liver microsomes. Biochemical pharmacology 1994; 47.
32. JD M, JA A, T Ket al. Formation of novel non-cyclooxygenase-derived prostanoids (F2-isoprostanes) in carbon tetrachloride

hepatotoxicity. An animal model of lipid peroxidation. The Journal of clinical investigation 1992; 90.
33. JD M, KE H, RF B, TM N, KF B, LJ R. A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. Proceedings of the National Academy of Sciences of the United States of America 1990; 87.
34. JM M, JN B, J S. 8-iso-prostaglandin F(2alpha), a product of noise exposure, reduces inner ear blood flow. Audiology & neuro-otology 2003; 8.
35. Y O, JM M, RA A, J S. Intense noise induces formation of vasoactive lipid peroxidation products in the cochlea. Brain research 2000; 878.
36. N H, K G, N Y, A M, K K. Efflux of glutamate into the perilymph of the cochlea following transient ischemia in the gerbil. Neuroscience letters 1997; 230.
37. N H, K K, M S, F W, A M, K G. Hearing loss and glutamate efflux in the perilymph following transient hindbrain ischemia in gerbils. The Journal of comparative neurology 2000; 418.
38. HB P, R K, C F. Experiments on temporary obstruction of the internal auditory artery. The Laryngoscope 1959; 69.
39. K T, S T, K F, K O, A H, J K. Outer hair cells functionally and structurally deteriorate during reperfusion. Hearing research 2002; 173.
40. AR F, P DB, SL Eet al. Noise-induced hearing loss (NIHL) as a target of oxidative stress-mediated damage: cochlear and cortical responses after an increase in antioxidant defense. The Journal of neuroscience : the official journal of the Society for Neuroscience 2013; 33.
41. JC A, V F-S, JM J. Antioxidants and Vasodilators for the Treatment of Noise-Induced Hearing Loss: Are They Really Effective? Frontiers in cellular neuroscience 2020; 14.
42. CH C, X D, RA F, RD K. Therapeutic effects of orally administrated antioxidant drugs on acute noise-induced hearing loss. Free radical research 2014; 48.
43. GD C, DM D, D Det al. Novel oral multifunctional antioxidant prevents noise-induced hearing loss and hair cell loss. Hearing research 2020; 388.
44. RD K, PA W, JL Bet al. Reduction of noise-induced hearing loss using L-NAC and salicylate in the chinchilla. Hearing research 2000; 149.
45. DL E, J L, W L, X D, R F, R K. Antioxidant treatment reduces blast-induced cochlear damage and hearing loss. Hearing research 2012; 285.
46. R K, E B, J Let al. Prevention of impulse noise-induced hearing loss with antioxidants. Acta oto-laryngologica 2005; 125.
47. AR F, SLM E, A DPet al. The Antioxidant Effect of Rosmarinic Acid by Different Delivery Routes in the Animal Model of Noise-Induced Hearing Loss. Otology & neurotology : official publication of the

American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology 2018; 39.
48. AG C, LL C, EW R. Mechanisms of hair cell death and protection. Current opinion in otolaryngology & head and neck surgery 2005; 13.
49. MC K, SM K, G Aet al. Protective effect of a marine polyphenol, dieckol against carbon tetrachloride-induced acute liver damage in mouse. Environmental toxicology and pharmacology 2013; 35.
50. MC K, KN K, SM Ket al. Protective effect of dieckol isolated from Ecklonia cava against ethanol caused damage in vitro and in zebrafish model. Environmental toxicology and pharmacology 2013; 36.
51. SH L, JS H, SJ H, JY H, YJ J. Protective effects of dieckol isolated from Ecklonia cava against high glucose-induced oxidative stress in human umbilical vein endothelial cells. Toxicology in vitro : an international journal published in association with BIBRA 2010; 24.
52. SH L, JY K, SY Y, SM K. Cytoprotective effect of dieckol on human endothelial progenitor cells (hEPCs) from oxidative stress-induced apoptosis. Free radical research 2013; 47.
53. MY C, SH B, HC Set al. Protective effects of the seaweed phlorotannin polyphenolic compound dieckol on gentamicin-induced damage in auditory hair cells. International journal of pediatric otorhinolaryngology 2016; 83.
54. Lee J-H, Ko J-Y, Oh J-Y, Kim E-A, Kim C-Y, Jeon Y-J. Evaluation of phlorofucofuroeckol-A isolated from Ecklonia cava (Phaeophyta) on anti-lipid peroxidation in vitro and in vivo. ALGAE 2015; 30:313-323.
55. HS K, HY C, JH J, BW S, JS C. A new phlorotannin from the brown alga Ecklonia stolonifera. Chemical & pharmaceutical bulletin 2003; 51.
56. HS K, HY C, JY K, BW S, HA J, JS C. Inhibitory phlorotannins from the edible brown alga Ecklonia stolonifera on total reactive oxygen species (ROS) generation. Archives of pharmacal research 2004; 27.
57. AR K, TS S, MS Let al. Isolation and identification of phlorotannins from Ecklonia stolonifera with antioxidant and anti-inflammatory properties. Journal of agricultural and food chemistry 2009; 57.
58. AR K, MS L, JW Cet al. Phlorofucofuroeckol A suppresses expression of inducible nitric oxide synthase, cyclooxygenase-2, and pro-inflammatory cytokines via inhibition of nuclear factor-κB, c-Jun NH2-terminal kinases, and Akt in microglial cells. Inflammation 2013; 36.
59. AR K, MS L, TS Set al. Phlorofucofuroeckol A inhibits the LPS-stimulated iNOS and COX-2 expressions in macrophages via inhibition of NF-κB, Akt, and p38 MAPK. Toxicology in vitro : an international journal published in association with BIBRA 2011; 25.
60. N SB, K K. Anticancer drugs from marine flora: an overview. Journal of oncology 2010; 2010.
61. HJ E, TH K, GH Pet al. In Vitro Anticancer Activity of Phlorofucofuroeckol A via Upregulation of Activating Transcription

Factor 3 against Human Colorectal Cancer Cells. Marine drugs 2016; 14.
62. R C, A C, A Net al. Polyphenols Nanoencapsulation for Therapeutic Applications. J Biomol Res Ther 2016; 5.
63. AN S, SK P. Pharmacokinetic principles in the inner ear: Influence of drug properties on intratympanic applications. Hearing research 2018; 368.
64. JH K, Z Y, B Yet al. Blood-brain barrier-permeable fluorone-labeled dieckols acting as neuronal ER stress signaling inhibitors. Biomaterials 2015; 61.

Abstract

난청의 가장 흔한 원인 중 하나는 소음이다. 20세와 69세 사이 미국인들 중 약 31.1%가 소음과 연관된 고음 난청이 있다. 또한 반복적인 소음 노출은 노화와 관련된 난청을 가속시킬 수 있다. 감태에서 추출된 폴리페놀류 성분인 디엑콜과 플로로푸코푸로엑콜 A는 강력한 항산화제들이다. 이번 연구에서 우리는 쥐에서 디엑콜과 플로로푸코푸로엑콜 A의 소음성 난청에 대한 보호 효과를 확인하는 실험을 진행했다. 청성뇌간반응검사에서 지연시간과 진폭은 여러 실험군간 유효한 차이는 없었다. 4kHz에서 디엑콜을 적용한 소음 노출군의 청성뇌간반응검사 역치 변화가 생리식염수를 적용한 소음 노출군보다 소음 노출 4시간 후에 유효하게 적었다. 16kHz와 클릭음에서 청성뇌간반응검사 역치 변화는 고농도 플로로푸코푸로엑콜 A를 적용한 소음 노출군이 생리식염수를 적용한 소음 노출군보다 소음 노출 1일 후에 유효하게 적었다. 이 결과는 일시적 청력 저하에 대해 디엑콜과 플로로푸코푸로엑콜이 강력한 예방약이 될 수 있음을 시사한다. 또한 두 성분 모두 미국 식품의약국 (United States Food and Drug Administration)에서 승인된 식용 성분으로 소음 노출을 피할 수 없는 대상들에게 최소화된 부작용으로 사용할 수 있다.

주요어 : 소음, 난청, 디엑콜, 플로로푸코푸로엑콜 A, 항산화제

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