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Nobiletin Protects Hair Cells against Neomycin-induced Vestibular Hair Cell Death

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Abstract Vestibular hair cells are susceptible to damage from various stimuli such as infections, ischemia, and certain therapeutic drugs, including aminoglycoside antibiotics and the antineoplastic agent cisplatin. In mammals, damage to the vestibular hair cells is permanent. This study aimed to evaluate the protective effects of nobiletin (NOB) against aminoglycoside-induced hair cell death using utricles collected from adult mice. The utricles removed from CBA/N mice were assigned to eight groups according to the dose of NOB and the administration or not of neomycin. Hair cells in the utricles were counted by double labeling with calmodulin and calbindin. NOB inhibited hair cell death in utricles exposed to neomycin. The protective effect of NOB on hair cells in the utricles was also suggested to have resulted from the inhibition of the production and accumulation of 4-hydroxy-2-nonenal, the final product of lipid peroxide aldehyde. NOB suppressed neomycin-induced hair cell death. The principle of hair cell protection from aminoglycoside-induced hair cell death suggests that NOB inhibits reactive oxygen species formation in the utricles exposed to neomycin.

Key words: aminoglycoside, apoptosis, hair cell, nobiletin, ototoxicity

Introduction

Vestibular hair cells are susceptible to damage from various stimuli, such as infections,[1,2] ischemia,[3,4] and certain therapeutic drugs, including aminoglycoside antibiotics[5] and the antineoplastic agent cisplatin.[6,7] Once vestibular hair cells are damaged, the disorder becomes permanent. Therefore, the protection of hair cells is important. Various studies based on morphological and molecular indicators demonstrated that ototoxic drug-induced hair cell death is caused by apoptosis.[8-10] The best evidence supporting this conclusion is that both cisplatin- and aminoglycoside-induced hair cell death are considerably inhibited by broad caspase inhibitors.[8,10] Ototoxic drugs such as aminoglycoside antibiotics and the antineoplastic drug cisplatin cause inner ear damage by increasing reactive oxygen species (ROS) and free radicals.[11,12] Our group has focused on the hair cell-protective effects of antioxidant substances.[13-17]

Citrus fruits contain ascorbic acid and other compounds with antioxidant properties.[18,19] Shikuwasa (*C. depressa*), a citrus fruit, contains high levels of nobiletin (NOB) (5,6,7,8,3′,4′-hexamethyoxflavone).[20] NOB has various pharmacological properties, including anti-inflammatory, antitumor, and antioxidant effects, and improves brain function.[21,22] In this study, we evaluated the antioxidant potential of NOB and validated its antioxidant activity. Therefore, the primary aim of this study was to examine the protective effects of NOB on neomycin (NEO)-induced hair cell death in adult mouse utricles in vitro. We also examined the correlation between the inhibition of NEO-induced hair cell death and NOB concentration.

Materials and methods

Animal use and care

CBA/N mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). All mice (4-6 weeks old) were male and had normal Preyer reflexes. All experimental protocols were reviewed and approved by the Yamaguchi University Animal Use Committee (Animal Experiment Protocol number: 43-004), and the animals were properly maintained at the Science Research Center, Institute of Life Science and Medicine, Yamaguchi University.

Determination of the antioxidant capacity of NOB and NEO using an OXY adsorption test analyzer

The antioxidant capacities of NOB (Fujifilm Wako Pure Chemical, Osaka City, Osaka, Japan) and NEO (Sigma-Aldrich Japan, Meguro, Tokyo) were determined by performing OXY adsorption tests using a FREE Carrio Duo (Wismerll, Bunkyo, Tokyo, Japan). Saline solution was used as a control. Samples were treated by mixing 10 μL of the samples in a tube containing 1 mL of distilled water (1:100 dilution). Then, 10 μL of the diluted sample was added to 1 mL of HClO, mixed, and incubated at 37 ℃ for 10 min. HClO acts as an oxidant and its adsorption depends on the sample's antioxidant capacity. Next, 10 μL of chromogen (aromatic compound) was added, mixed, and the absorbance was measured using a photometer. The oxidative adsorption capacity of the samples was quantified based on the comparative difference in absorbance, and the antioxidant capacity was evaluated. The results are expressed as the amount of HClO eliminated per mL (μM HClO/mL). The normal value for antioxidant capacity was stated in the OXY adsorption test instructions as 350 μM/mL or higher.[23] Two samples were prepared for NOB, NEO, and saline and the average values were calculated.

Organ culture of utricles and induction of hair cell death

All the animals were euthanized by cervical dislocation and immediately decapitated. The temporal bone was quickly removed under aseptic manipulation, and individual vestibular organs were dissociated in Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with Earle's balanced salt solution (Invitrogen) (2:1, v/v). The utricles were immediately transferred to a culture medium supplemented with 5% fetal bovine serum (Cosmo Bio, Koto, Tokyo). The utricles were assigned to eight groups (n=6 utricles in each group) according to the dose of NOB and administration or not of NEO: control group (no NOB and NEO), NOB group (three concentrations of NOB: 1-, 10-, and 100 μM), NEO group (2 mM of NEO), and NEO+NOB group (1-, 10-, and 100 μM of NOB plus 2 mM of NEO) (Table 1). Each utricle was incubated (submerged in water, but not allowed to attach) in 24-well tissue culture plates for 24 h at 37 ℃ in a 5% CO_2 and 95% air environment (Waken Holdings, Sakyo, Kyoto, Japan). NEO solution was added to the culture wells at a final concentration of 2.0 mM, whereas NOB solution was added to the culture wells at final concentrations of 100-, 10-, and 1 μM for both the NOB and NOB+NEO groups. At the end of the culture period, the utricles were fixed in 4% paraformaldehyde at 25 ℃ for 2 h. Otoconia were gently removed from the fixed utricles using a 28-G needle and syringe with flowing phosphate-buffered saline (PBS). Mouse otoliths were cultured as described by Cunningham.[24]

Table 1 Utricles were assigned to eight groups according to the concentrations of nobiletin (NOB) and neomycin (NEO) administered.

		NOB			
		0M	1μM	10μM	100μM
NEO	0M	Control group	NOB 1 μM group	NOB 10 μM group	NOB 100 μM group
	2mM	NEO group	NEO + NOB 1 μM group	NEO + NOB 10 μM group	NEO + NOB 100 μM group

Immunohistochemistry for hair cell labeling

After fixation, the utricles were rinsed with 0.4 M PBS. They were then incubated in a blocking solution (1% bovine serum albumin (Sigma-Aldrich Japan, Meguro, Tokyo), 0.4% normal goat serum (Cosmo Bio, Koto, Tokyo), 0.4% normal horse serum (Cosmo Bio, Koto, Tokyo), and 0.4% Triton X-100 (Katayama Chemical, Chuo, Osaka), PBS) at 4 ℃ for 8 h. To count hair cells, utricles were double-labeled in whole mounts with a mouse monoclonal antibody against calmodulin (C3545-.2ML, Sigma-Aldrich Japan, Meguro, Tokyo) and a rabbit polyclonal antibody against calbindin (ABN2192-100UL, Merck, Meguro, Tokyo). This double-labeled immunohistochemical protocol allows for separate counts of hair cells in the extrastriolar (calmodulin-positive and calbindin-negative) and striolar (calmodulin-positive and calbindin-positive) regions.[8,24] The double-labeling method using calmodulin and calbindin was described in detail by Cunningham et al.[8] The samples were incubated overnight at 4 ℃ in a primary antibody solution (blocking solution of calmodulin 1:1000 and calbindin 1:500). After washing with the blocking solution, the samples were incubated with secondary antibodies diluted in the blocking solution as follows: Alexa 594 goat anti-rabbit IgG (1:1000; Thermo Fisher Scientific Japan Group, Minato, Tokyo) and Alexa 488 goat anti-mouse IgG (1:500; Thermo Fisher Scientific Japan Group, Minato, Tokyo). The utricles were incubated with fluorescent secondary antibodies, washed with a blocking solution, and mounted on Fluoromount-G (Cosmo Bio, Koto, Tokyo). All antibodies used in this study were sensitive to mice.

Hair cell count

Utricles were examined under a fluorescence microscope (Keyence, Higashiyodogawa, Osaka, Japan) to evaluate hair cell survival. In mice, type I and type II hair cells are uniformly distributed in the utricle.[25] Calmodulin-positive and calbindin-positive cells were counted as hair cells in the striolar regions, and calmodulin-positive and calbindin-negative cells were counted as hair cells in the extrastriolar regions. The labeled hair cells were counted in each of the eight randomly selected regions from each utricle (squares with 20 μm on each side in each utricle). The four striolar and four extrastriolar hair cell counts were averaged to calculate the striolar and extrastriolar hair cell densities in each utricle. Six utricles were examined for each experimental condition.

Evaluation of NOB effects on 4-HNE production in hair cells

The amount of 4-hydroxy-2-nonenal (4-HNE) produced was measured to evaluate ROS production. Immunohistochemistry using anti-4-HNE monoclonal antibody (mouse, MHN-100P, 25 μg/mL, Nikken Seil, Bunkyo, Tokyo) was performed to examine the extent of the production and accumulation of 4-HNE. 4-HNE is the final product of lipid peroxide aldehyde. The utricles were assigned to four groups according to the dose of NOB and administration or not of NEO: control group (no NOB and NEO), NOB group (100 μM of NOB), NEO group (2 mM of NEO), and NEO+NOB group (100 μM of NOB + 2 mM of NEO) (Table 2). The incubation time was 12 h. Anti 4-HNE antibody was used as the primary antibody. Alexa 488 goat anti-mouse IgG (1:500) was used as the secondary antibody. Three utricles were examined for each experimental condition. We measured the fluorescence intensity of six randomly selected areas from each utricle (squares

Table 2 To assess 4-hydroxy-2-nonenal (4-HNE) production in hair cells, the utricles were divided into four groups according to the concentrations of nobiletin (NOB) and neomycin (NEO) administered.

		NOB	
		0M	100μM
NEO	0M	Control group	NOB 100 μM group
	2mM	NEO group	NEO + NOB 100 μM group

with 20 μm on each side in each utricle) using ImageJ (version 1.53k, National Institutes of Health, Bethesda, Maryland), an open-source image analysis software. In ImageJ, the fluorescence intensity is expressed as an integer value in 256 steps from 0 to 255, with higher values indicating higher intensity. There are no specific units. The average fluorescence intensity in each of the measured areas was calculated and used for statistical analysis. The mean of the samples from the control group was calculated and used to calculate the relative values of the other groups. Statistical analyses were performed using the calculated relative values.

Evaluation of NOB effects on 3-NT production in hair cells

The amount of 3-nitrotyrosine (3-NT) produced was measured to evaluate the production of reactive nitrogen species (RNS). Immunohistochemistry using anti-3-NT monoclonal antibodies was performed to examine the extent of the production and accumulation of 3-NT. 3-NT is a major modification of protein nitration by peroxynitrite and a marker for protein oxidation. The methods were conducted as defined in the Section "*Evaluation of NOB effects on 4-HNE production in hair cells*"; however, they differed in the following aspects. Anti-3-NT antibody (mouse, 7A12AF6, 1 mg/mL, Abcam plc., Chuo, Tokyo) was used as the primary antibody. Alexa Fluor 594 goat anti-rabbit IgG (1:1000) and 488Phalloidin (1:1000, ab176753, Abcam plc., Chuo, Tokyo) were used as secondary antibodies.

Statistical analyses

All statistical analyses were performed using EZR (XQuartz 2.8.2; xorg-server 1.20.14, Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 4.2.1). EZR is a modified version of the R command, designed to add statistical functions that are frequently used in biostatistics. The Kruskal-Wallis test and the Steel-Dwass method were used to evaluate whether there were:

- significant differences in the number of hair cells in the extrastriolar and striolar regions between groups;
- significant differences in the immunohistochemical fluorescence intensity owing to 4-HNE production between the groups;
- significant differences in the immunohistochemical fluorescence intensity owing to 3-NT production between the groups.

Results

Antioxidant capacity of NOB

The OXY adsorption test was performed using FREE Carrio Duo, a free radical analyzer, to evaluate the antioxidant capacities of NOB and NEO. Two samples were prepared for each type of sample, and the average value was calculated. The results showed that NOB had antioxidant capacity, whereas NEO and saline did not (Table 3).

Effects of NOB on hair cell survival

To evaluate whether NOB causes damage to hair cells, hair cells were assigned to a control group and a NOB group (1, 10, and 100 μM of NOB). Each group was cultured for 24 h, and the utricles were fixed and prepared to count hair cells using a double label for calmodulin and calbindin. The representative results are shown in Figs. 1 and 2. There was no significant decrease in the number of hair cells in the NOB group compared to the control group (Figs. 1A, 1B, 1C, 1D, 2A, 2B, 2C, and 2D).

Table 3 OXY adsorption test results.
Normal antioxidant capacity is ≥ 350 μM/mL.[23]

(μM/mL)	sample1	sample2	Average antioxidant capacity
saline	-35	-43	-39
NOB	722	722	722
NEO	145	182	163.5

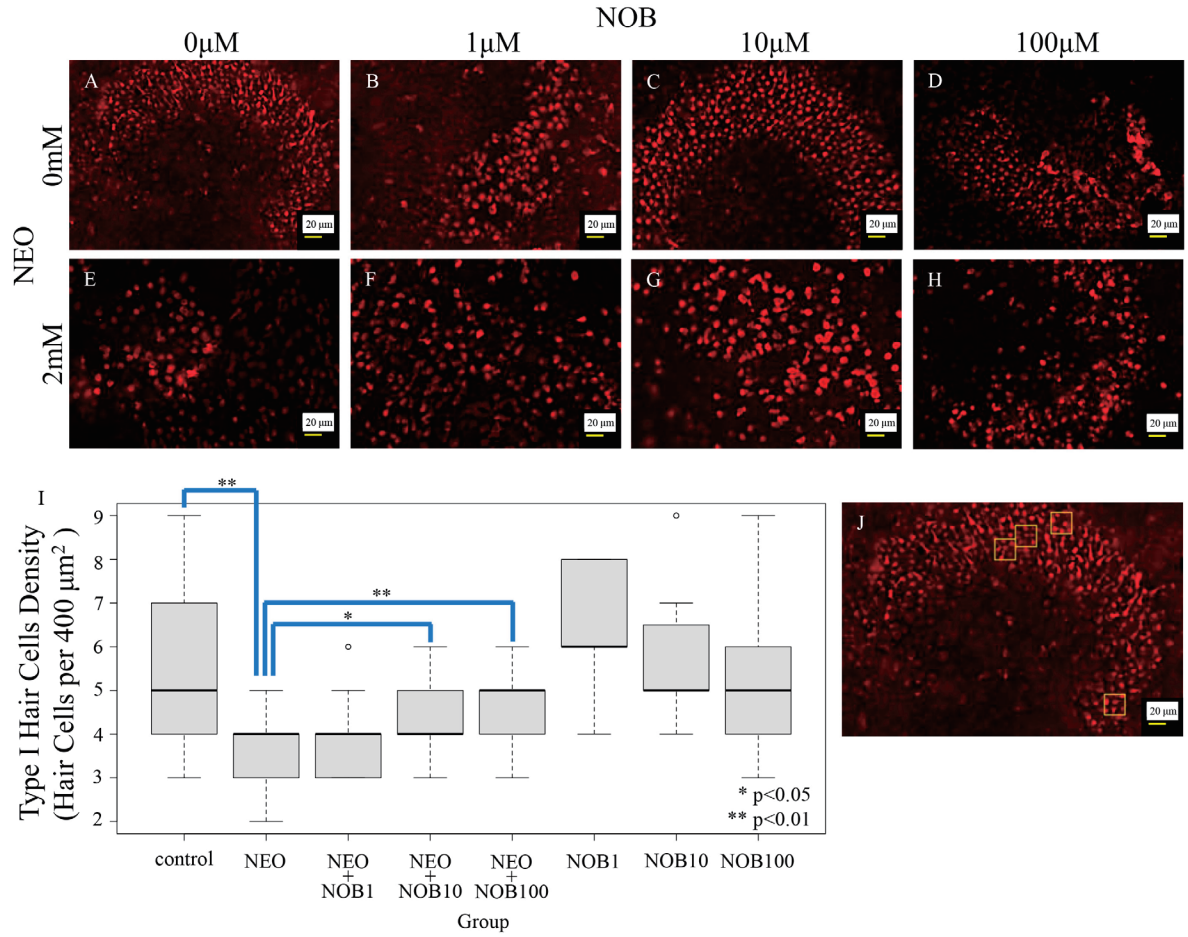

Fig. 1 Microscopic images and statistical analysis of immunostaining using a monoclonal antibody against calbindin.
Nobiletin (NOB) does not cause hair cell damage but prevents it from neomycin (NEO). Hair cells were treated with the control group (A: no NOB and NEO), NOB group (B:1 μmol, C:10 μmol, D:100 μmol of NOB), NEO group (E: 2 mM NEO), and NEO+NOB group (F:1 μM, G:10 μM, H:100 μM of NOB + 2 mM NEO) and cultured for 24 h. Panel I presents the results of the statistical analysis (n=24 regions from 6 utricles for each group). The cells were labeled with anti-calbindin (red) and the striolar regions were evaluated. The labeled hair cells were counted in randomly selected regions from each utricle (squares with 20 μm on each side in each utricle) (J). Exposure to NEO caused a decrease in the striolar regions of hair cells, whereas exposure to NOB did not. The NEO + NOB group showed a significant difference in the number of hair cells compared to the NEO group. There was no significant difference in the number of hair cells between NOB concentrations of 10 μm and 100 μM in the NEO+NOB group. Scale bar, 20 μm. Asterisks indicate statistical significance (Kruskal-Wallis test *p<0.05).

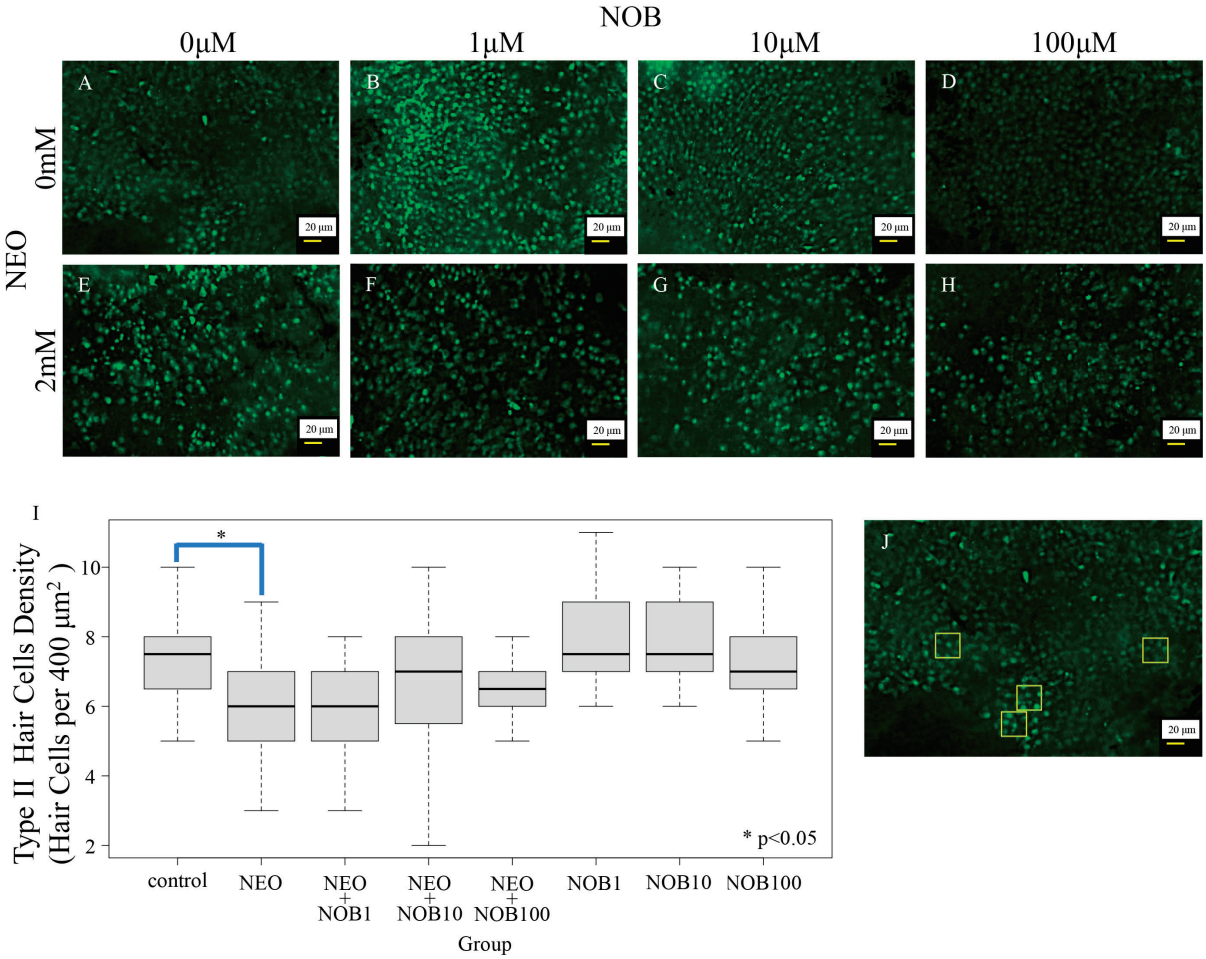

Fig. 2 Microscopic images and statistical analysis of immunostaining using a monoclonal antibody against calmodulin.
Nobiletin (NOB) does not cause hair cell damage but prevents it from neomycin (NEO). The hair cells were treated with the control group (A: no NOB and NEO), NOB group (B:1 μmol, C:10 μmol, D:100 μmol of NOB), NEO group (E:2 mM of NEO), and NEO+NOB group (F:1 μM, G:10 μM, H:100 μM of NOB + 2 mM NEO) and cultured for 24 h. Panel I presents the results of the statistical analysis (n=24 regions from 6 utricles for each group). These cells were labeled with anti-calmodulin (green) and the extrastriolar regions were evaluated. The labeled hair cells were counted in randomly selected regions from each utricle (squares with 20 μm on each side in each utricle) (J). Exposure to NEO caused a decrease in the extrastriolar regions of hair cells, whereas exposure to NOB did not. In addition, no significant decrease in the number of hair cells was observed in the NEO+NOB group at any NOB concentration. Scale bar, 20 μm. Asterisks indicate statistical significance (Kruskal-Wallis test *p<0.05 and **p<0.01).

NOB protects hair cells from NEO-induced death

To evaluate the protective effect of NOB against NEO-induced hair cell death, cells were incubated for 24 h with moderate doses of NEO (2 mM) and various doses of NOB (1, 10, and 100 μM NOB). The utricles were fixed and prepared for hair cell counting using double calmodulin and calbindin labels (Figs. 1 and 2). In the extrastriolar regions, exposure to NEO caused a decrease in the number of hair cells (Fig 2E), whereas exposure to NOB did not (Fig. 2B, 2C, and 2D). In addition, no significant decrease in the number of hair cells was observed in the NEO + NOB group at any NOB concentration tested (Fig. 2F, 2G, and 2H). In the striolar regions, compared to the control group, the NEO group showed a significant decrease in the number of hair cells (p-value was 0.0003). In contrast, no significant decrease in the number of hair cells was observed in the NEO + NOB group (Fig. 1G and 1H). Among the NOB concentrations of 1-, 10-, and 100 μM, those that were effective in inhibiting NEO-induced hair cell death were 10 and 100 μM, respectively. There was no significant difference in the number of hair cells between the NOB concentrations of 10- and 100 μM in the NEO+NOB group. The statistical analysis results using the Kruskal-Wallis test and the Steel-Dwass method are shown in Figs. 1I and 2I. The Kruskal-Wallis test returned a $p<0.01$ for both the extrastriolar and striolar regions.

NOB suppresses the production of 4-HNE

To detect free radical production, immunohistochemistry was performed to determine the amount of 4-HNE, a free radical metabolite, produced using an antibody against 4-HNE. The representative results are shown in Fig 3. There was no increase in 4-HNE levels in the group cultured with 100 μM NOB (Fig 3B). Hair cells containing 4-HNE appeared in the utricles cultured with 2 mM NEO (Fig 3C), but there was a decrease in 4-HNE the utricles cultured with NEO and NOB (Fig 3D). For each group, the fluorescence intensity of 4-HNE was measured and calculated relative to the control group. Statistical analyses using these relative values are shown in Fig 4E. The results indicate that NOB inhibited the formation of free radicals in the utricles exposed to NEO. The fluorescence intensity derived from 4-HNE was significantly stronger in utricles incubated with NEO than in those incubated without NEO (p-value was 0.0002). NOB presence suppressed the fluorescence intensity.

NEO and NOB do not induce the production of 3-NT in hair cells

To detect the production of RNS, immunohistochemistry was performed to investigate the amount of 3-NT produced using an antibody against 3-NT, a marker of protein oxidation. The representative results are shown in Fig 4. There was no increase in 3-NT levels in the groups cultured with 100 μM NOB, 2 mM NEO, or NEO, and NOB (Fig. 4B, 4C, and 4D). For each group, the fluorescence intensity derived from 3-NT was measured and calculated relative to that of the control group. Statistical analyses using these relative values are shown in Fig 4E. The fluorescence intensity derived from 3-NT was not considerably different between the groups compared with the control group. Therefore, we could not confirm RNS production by NEO or NOB.

Discussion

We showed that NOB suppresses NEO-induced hair cell death. OXY adsorption tests were performed to determine the antioxidant capacity of NOB. The results showed that the antioxidant capacity of NOB was 722 μM/mL, which was much higher than the normal value. These results show that NOB had antioxidant capacity. Based on these results, utricle organ culture in a medium containing NOB was performed to evaluate whether it caused damage to hair cells or protected them from oxidative stress induced by NEO. The advantages of using mouse utricle organ culture are as follows: (1) hair cells are derived from mammals with vestibular similarity to humans; (2) hair cell-containing tissues can be cultured stably using a relatively easy technique; and (3) the hair cells can be directly exposed to the drug being evaluated by dissolving it in the culture medium.[24] Therefore, we decided to confirm the protective effect of

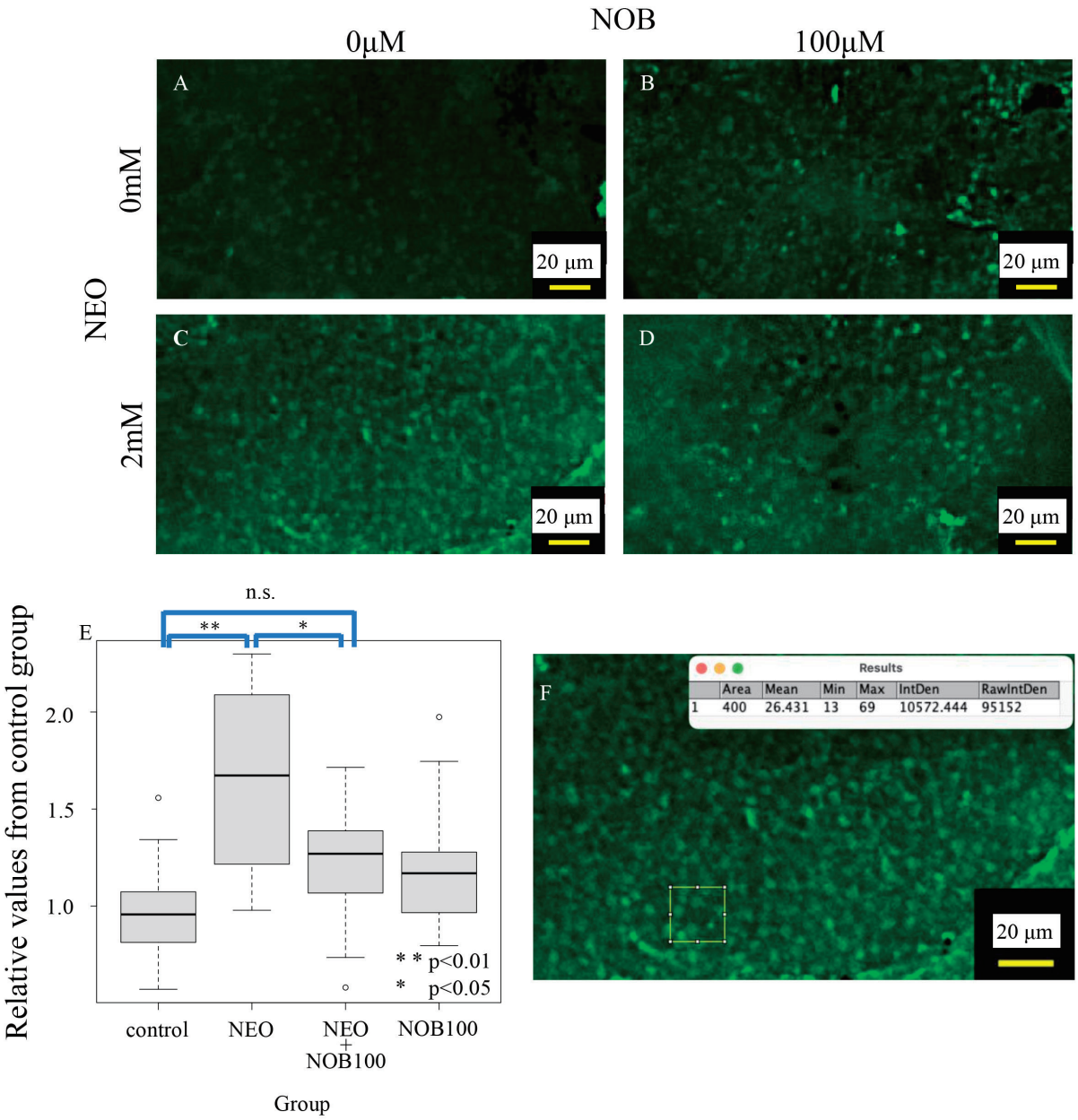

Fig. 3 Fluorescence intensity associated with 4-hydroxy-2-nonenal (4-HNE) production by neomycin (NEO) and nobiletin (NOB) administration and its statistical analysis.
NOB suppresses the production of 4-HNE. The utricles were divided into four groups according to the presence or absence of NOB and NEO: control group (A: no NOB and NEO), NOB group (B: 100 μM NOB), NEO group (C:2 mM NEO), and NEO+NOB group (D: 100 μM NOB + 2 mM NEO). The utricles were then incubated for 12 h. Panel E presents the fluorescence intensity statistics for each group (n=18 regions from 3 utricles for each group). The fluorescence intensity of hair cells was evaluated in each of six randomly selected regions from each utricle (squares with 20 μm on each side in each utricle) (F). The NEO group showed an increase in 4-HNE, whereas the NEO+NOB group showed no increase in 4-HNE. The fluorescence intensity was significantly stronger in utricles incubated with NEO than in those incubated with NEO+NOB. Scale bar, 20 μm. Asterisks indicate statistical significance (Kruskal-Wallis test *p<0.05 and **p<0.01).

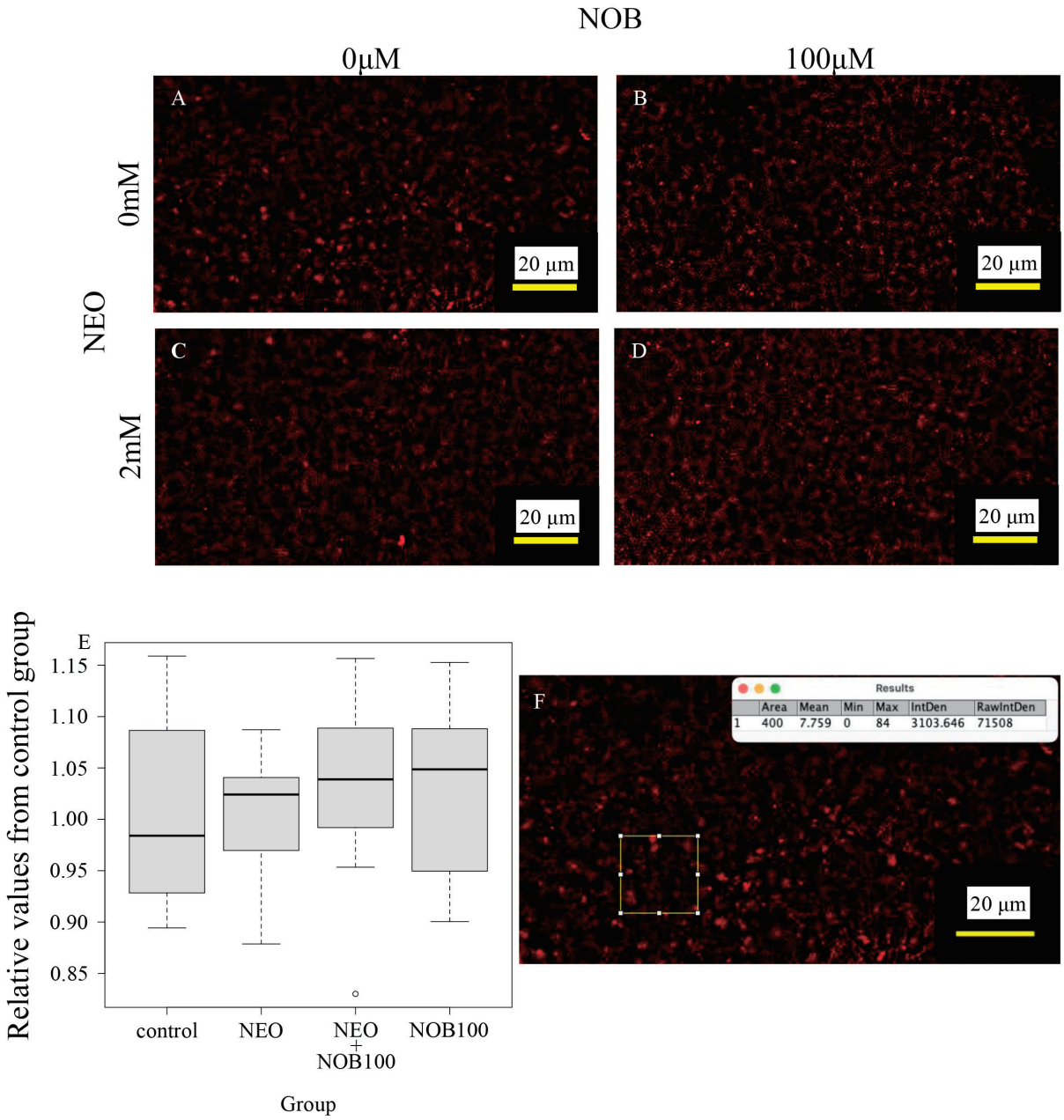

Fig. 4 Fluorescence intensity associated with 3-nitrotyrosine (3-NT) production by neomycin (NEO) and nobiletin (NOB) administration and its statistical analysis.
RNS was not produced by NEO or NOB. The utricles were divided into four groups according to the presence or absence of NOB and NEO: A) control group (no NOB and NEO), B) NOB group (100 μM NOB), C) NEO group (2 mM NEO), and D) NEO+NOB group (100 μM NOB + 2 mM NEO). The utricles were then incubated for 12 h. In each group, the fluorescence intensity derived from 3-NT was measured and calculated relative to the control group. E) Statistical analysis using these relative values (n=18 regions from 3 utricles for each group). The fluorescence intensity of hair cells was evaluated in each of six randomly selected regions from each utricle (squares with 20 μm on each side in each utricle). F) The fluorescence intensity derived from 3-NT was not significantly different in either group compared to the control group. Scale bar, 20 μm (Kruskal-Wallis test).

NOB on hair cells by dissolving NOB in the culture medium and exposing the hair cells to it. NOB did not disrupt hair cells at the concentrations studied. However, there was a downward trend in type I hair cell density when the NOB concentration increased from 1 to 100 μM (without NEO), although the difference was not significant. Among the NOB groups without NEO, the NOB100 group had a wider error bar; however, the distribution of hair cell density was comparable to that in the control group. In contrast, when the utricles were cultured in NOB with NEO, NOB showed a protective effect on hair cells against aminoglycoside-induced hair cell death. In the striolar region, no obvious protective effects were observed with 1 μM NOB, whereas protective effects were observed with 10 and 100 μM NOB. This suggests that, although the protective effect on type I hair cells was concentration-dependent, high concentrations of NOB damaged type I hair cells. Therefore, it is important to expose type I hair cells to NOB at optimal concentrations. However, as no significant difference was observed between 10 and 100 μM NOB, the optimal concentration has not yet been identified. In the extrastriolar region, there were no significant differences between the groups cultured with NEO and NOB and those cultured only with NEO. However, there was a significant decrease in the number of hair cells in the NEO group compared to the control group (p-value was 0.016), whereas there was no substantial difference between the groups cultured with NEO and NOB. This suggests a trend toward a protective effect of NOB on type II hair cells. These results suggest that the optimum concentration of NOB was higher than 100 μM. Another reason for the difference in the degree of protective effect of NOB on hair cells in type I and type II hair cells may be the difference in the sensitivity of each cell to NEO. Type I hair cells are more susceptible to damage by ototoxic agents, such as aminoglycoside antibiotics, than type II hair cells. This is because type I hair cells preferentially take up the drug after administration compared with type II hair cells.[26] In our study, type I hair cells were more strongly damaged by NEO than type II hair cells, which may have caused differences in the protective effects of NOB on striolar and extrastriolar hair cells.

To elucidate the mechanism underlying the antioxidant action of NOB, immunohistochemistry was performed using antibodies against 4-HNE, a free radical metabolite. 4-HNE is formed from n-6 unsaturated fatty acids such as linoleic and arachidonic acids. 4-HNE is an end-product of lipid peroxidation reactions. Owing to the high reactivity of 4-HNE with proteins and nucleic acids and its strong cytotoxicity, it is considered one of the main causative agents of tissue damage associated with oxidative stress.[27,28] Studies have found increased levels of 4-HNE in hair cells exposed to aminoglycoside antimicrobials, suggesting that the elimination of 4-HNE protects hair cells from aminoglycoside-induced death.[15,17] In this study, utricles exposed to aminoglycoside antimicrobials showed a strong fluorescence response derived from 4-HNE, whereas the group cultured in 100 μM NOB showed no increase in 4-HNE. Additionally, a decrease in 4-HNE was observed in the utricles cultured with NEO and NOB. These results indicate that NOB did not promote the production of ROS but inhibited it in the utricles exposed to NEO. 3-NT is produced by the reaction between superoxide and nitric oxide (NO) to form peroxynitrite, which nitroses tyrosine residues in proteins, resulting in oxidative stress.[29,30] Therefore, immunohistochemistry was performed using an anti- 3-NT antibody, a protein nitrosylation modification, to evaluate the production of RNS. In this study, the fluorescence intensity derived from 3-NT was not substantially different between the groups compared with the control group. Therefore, we could not confirm NO production by NEO or NOB. ROS production is an important mediator of aminoglycoside-induced damage in hair cells.[31] Similarly, in the present study, ROS were produced when hair cells were exposed to NEO. In contrast, the administration of NOB and NEO inhibited ROS production. Collectively, the protection of hair cells from aminoglycoside-induced death may result from the inhibition of ROS production by NOB.

These data indicate that the antioxidant effects NOB were useful for hair cell protection

and increased the possibility of developing therapies for prevention or recovery from hair cell death. In contrast, utricular organ culture in a medium containing NOB is different from the physiological route of NOB administration. Therefore, in vivo experiments are essential to validate the results of this study. Thus, this can be the limitation of this study. Clinical trials have already begun to evaluate the efficacy of NOB on the skin (UMIN000031265: Pre-examination: A confirmatory test for efficacy to skin of the rich nobiletin extract from Shikuwasa.). *In vivo* evaluation of the method and duration of administration, dosage, and inner ear migration may lead to clinical applications of hair cell protection.

Conclusions

The results of our study support the hypothesis that NOB suppresses NEO-induced hair cell death. The principle of hair cell protection from aminoglycoside-induced hair cell death suggests that NOB inhibits the formation of ROS in the utricles exposed to NEO. We also found that the protective effect of NOB on hair cells is concentration-dependent. Future *in vivo* experiments on the efficacy of NOB in the inner ear should evaluate the ability of NOB to migrate to the inner ear, and whether there are any systemic side effects.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Tsuprun, V., Keskin, N., Schleiss, M.R., Schachern, P. and Cureoglu, S.: Cytomegalovirus-induced pathology in human temporal bones with congenital and acquired infection. *Am. J. Otolaryngol.*, **40**: 102270, 2019.
2. Hızlı, Ö., Hızlı, P., Kaya, S., Monsanto Rda, C., Paparella, M.M. and Cureoglu, S.: Histopathologic ear findings of syphilis: a temporal bone study. *Eur. Arch. Otorhinolaryngol.*, **273**: 2443-2449, 2016.
3. Sasaki, A., Matsubara, A., Tabuchi, K., Hara, A., Namba, A., Yamamoto, Y. and Shinkawa, H.: Immunoelectron microscopic analysis of neurotoxic effect of glutamate in the vestibular end organs during ischemia. *Acta. Otolaryngol.*, **132**: 686-692, 2012.
4. Mizutari, K., Fujioka, M., Nakagawa, S., Fujii, M., Ogawa, K. and Matsunaga, T.: Balance dysfunction resulting from acute inner ear energy failure is caused primarily by vestibular hair cell damage. *J. Neurosci. Res.*, **88**: 1262-1272, 2010.
5. Duckert, L.G. and Rubel, E.W.: Morphological correlates of functional recovery in the chicken inner ear after gentamycin treatment. *J. Comp. Neurol.*, **331**: 75-96, 1993.
6. Baker, T.G., Roy, S., Brandon, C.S., Kramarenko, I.K., Francis, S.P., Taleb, M., Marshall, K.M., Schwendener, R., Lee, F.S. and Cunningham, L.L.: Heat shock protein-mediated protection against Cisplatin-induced hair cell death. *J. Assoc. Res. Otolaryngol.*, **16**: 67-80, 2015.
7. Callejo, A., Durochat, A., Bressieux, S., Saleur, A., Chabbert, C., Domènech Juan, I., Llorens, J. and Gaboyard-Niay, S.: Dose-dependent cochlear and vestibular toxicity of trans-tympanic cisplatin in the rat. *Neurotoxicology.*, **60**: 1-9, 2017.
8. Cunningham, L.L., Cheng, A.G. and Rubel, E.W.: Caspase activation in hair cells of the mouse utricle exposed to neomycin. *J. Neurosci.*, **22**: 8532-8540, 2002.
9. Lang, H. and Liu, C.: Apoptosis and hair cell degeneration in the vestibular sensory epithelia of the guinea pig following a gentamicin insult. *Hear. Res.*, **111**: 177-184, 1997.
10. Matsui, J.I., Ogilvie, J.M. and Warchol, M.E.: Inhibition of caspases prevents ototoxic and ongoing hair cell death. *J. Neurosci.*, **22**: 1218-1227, 2002.
11. Henderson, D., Bielefeld, E.C., Harris, K.C. and Hu, B.H.: The role of oxidative

stress in noise-induced hearing loss. *Ear Hear.*, **27**: 1-19, 2006.
12. Rybak, L.P., Whitworth, C.A., Mukherjea, D. and Ramkumar, V.: Mechanisms of cisplatin-induced ototoxicity and prevention. *Hear. Res.*, **226**: 157-167, 2007.
13. Takemoto, T., Sugahara, K., Okuda, T., Shimogori, H. and Yamashita, H.: The clinical free radical scavenger, edaravone, protects cochlear hair cells from acoustic trauma. *Eur. J. Pharmacol.*, **487**: 113-116, 2004.
14. Arai, T., Sugahara, K., Mikuriya, T., Miyauchi, Y., Hirose, Y., Hashimoto, M., Shimogori, H. and Yamashita, H.: The free radical scavenger edaravone protects hair cells against aminoglycoside toxicity. *Bull. Yamaguchi Med. School*, **55**: 15-20, 2008.
15. Sugahara, K., Hirose, Y., Mikuriya, T., Hashimoto, M., Kanagawa, E., Hara, H., Shimogori, H. and Yamashita, H.: Coenzyme Q10 protects hair cells against aminoglycoside. *PLoS One*, **9**: e108280, 2014.
16. Hirose, Y., Sugahara, K., Kanagawa, E., Takemoto, Y., Hashimoto, M. and Yamashita, H.: Quercetin protects against hair cell loss in the zebrafish lateral line and guinea pig cochlea. *Hear. Res.*, **342**: 80-85, 2016.
17. Kobayashi, Y., Sugahara, K., Takemoto, Y., Tsuda, J., Hirose, Y., Hashimoto, M. and Yamashita, H.: Protective effect of astaxanthin nanoemulsion on mammalian inner ear hair cells. *PeerJ*, **11**: e15562, 2023.
18. Zou, Z., Xi, W., Hu, Y., Nie, C. and Zhou, Z.: Antioxidant activity of Citrus fruits. *Food Chem.*, **196**: 885-896, 2016.
19. Wang, Y., Liu, X.J., Chen, J.B., Cao, J.P., Li, X. and Sun, C.D.: Citrus flavonoids and their antioxidant evaluation. *Crit. Rev. Food Sci. Nutr.*, **62**: 3833-3854, 2022.
20. Kawaii, S., Tomono, Y., Katase, E., Ogawa, K. and Yano, M.: Quantitation of flavonoid constituents in citrus fruits. *J. Agric. Food Chem.*, **47**: 3565-3571, 1999.
21. Murakami, A., Nakamura, Y., Torikai, K., Tanaka, T., Koshiba, T., Koshimizu, K., Kuwahara, S., Takahashi, Y., Ogawa, K., Yano, M., Tokuda, H., Nishino, H., Mimaki, Y., Sashida, Y., Kitanaka, S. and Ohigashi, H.: Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res.*, **60**: 5059-5066, 2000.
22. Nakajima, A., Aoyama, Y., Nguyen, T.T.L., Shin, E.J., Kim, H.C., Yamada, S., Nakai, T., Nagai, T., Yokosuka, A., Mimaki, Y., Ohizumi, Y. and Yamada, K.: Nobiletin, a citrus flavonoid, ameliorates cognitive impairment, oxidative burden, and hyperphosphorylation of tau in senescence-accelerated mouse. *Behav. Brain Res.*, **250**: 351-360, 2013.
23. Trotti, R., Carratelli, M., Barbieri, M., Micieli, G., Bosone, D., Rondanelli, M. and Bo, P.: Oxidative stress and a thrombophilic condition in alcoholics without severe liver disease. *Haematologica*, **86**: 85-91, 2001.
24. Cunningham, L.L.: The adult mouse utricle as an in vitro preparation for studies of ototoxic-drug-induced sensory hair cell death. *Brain Res.*, **1091**: 277-281, 2006.
25. Desai, S.S., Zeh, C. and Lysakowski, A.: Comparative morphology of rodent vestibular periphery. I. Saccular and utricular maculae. *J. Neurophysiol.*, **93**: 251-266, 2005.
26. Lopez, I., Honrubia, V., Lee, S.C., Schoeman, G. and Beykirch, K.: Quantification of the process of hair cell loss and recovery in the chinchilla crista ampullaris after gentamicin treatment. *Int. J. Dev. Neurosci.*, **15**: 447-461, 1997.
27. Chen, Q., Cai, Y., Zhu, X., Wang, J., Gao, F., Yang, M., Mao, L., Zhang, Z. and Sun, B.: Edaravone dexborneol treatment attenuates neuronal apoptosis and improves neurological function by suppressing 4-HNE-associated oxidative stress after subarachnoid hemorrhage. *Front. Pharmacol.*, **13**: 848529, 2022.
28. Valentovic, M.A., Ball, J.G., Brown, J.M., Terneus, M.V., McQuade, E., Van Meter, S., Hedrick, H.M., Roy, A.A. and Williams, T.: Resveratrol attenuates cisplatin renal cortical cytotoxicity by modifying oxidative stress. *Toxicol. In Vitro*, **28**: 248-257, 2014.

29. Bandookwala, M. and Sengupta, P.: 3-Nitrotyrosine: a versatile oxidative stress biomarker for major neurodegenerative diseases. *Int. J. Neurosci.*, **130**: 1047-1062, 2020.
30. Jomova, K., Vondrakova, D., Lawson, M. and Valko, M.: Metals, oxidative stress and neurodegenerative disorders. *Mol. Cell. Biochem.*, **345**: 91-104, 2010.
31. Hirose, K., Hockenbery, D.M. and Rubel, E.W.: Reactive oxygen species in chick hair cells after gentamicin exposure in vitro. *Hear. Res.*, **104**: 1-14, 1997.