Ceramide/sphingomyelin cycle involvement in gentamicin-induced cochlear hair cell death

doi: 10.1007/s00204-014-1259-x

Ceramide/sphingomyelin cycle involvement in gentamicin-induced cochlear hair cell death

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Abstract Ceramide, a sphingolipid metabolite, regulates diverse cellular processes including apoptosis, cell senescence, the cell cycle, and cellular differentiation. Exogenously administered ceramide reportedly increased cochlear hair cell death due to gentamicin-induced ototoxicity. Ceramide is mainly generated via a ceramide/sphingomyelin cycle by sphingomyelinase and sphingomyelin synthase or via de novo synthesis by serine palmitoyltransferase and ceramide synthase. This study was designed to investigate the possible involvement of neutral sphingomyelinase, sphingomyelin synthase, or serine

palmitoyltransferase in hair cell death due to gentamicin.

The basal turns of the organ of Corti of Sprague–Dawley rats were dissected on postnatal days 3 to 5. Cochlear cultures were exposed to media containing 35 μM gentamicin for 48 hours to assess the effects of GW4869 (a neutral sphingomyelinase inhibitor), 2-hydroxyoleic acid (a sphingomyelin synthase activator), and myriocin (a serine palmitoyltransferase inhibitor). Hair cell loss was significantly decreased in the presence of GW4869 or 2-hydroxyoleic acid. Myriocin had no significant effects against gentamicin-induced hair cell loss. In addition, neutral sphingomyelinase was activated by gentamicin exposure. The present findings strongly suggest that the ceramide/sphingomyelin cycle plays an important role in the protection of hair cells against gentamicin-induced ototoxicity.

Key words ceramide/sphingomyelin cycle; de novo ceramide biosynthesis; gentamicin

Introduction

Ceramide plays a central role in sphingolipid biosynthesis and catabolism and is composed of sphingosine and a fatty acid. As a bioactive lipid, ceramide has been implicated in a variety of physiological functions including apoptosis and, cell growth arrest, differentiation, senescence, migration, and adhesion (Hannun and Obeid 2008). Roles for ceramide as a cell death-inducing factor have also been suggested in a number of pathological conditions including cancer, neurodegeneration, diabetes, microbial pathogenesis, obesity, and inflammation (Wu et al. 2007; Zeidan and Hannun 2007). Moreover, ceramide involvement has been suggested in cochear hair cell loss induced by gentamicin through apoptosis (Nishimura et al. 2010).

Two main ceramide generation pathways are known: via hydrolysis of sphingomyelin at the plasma membrane or via de novo biosynthesis in cells involving the action of the enzymes serine palmitoyltransferase (SPT) and ceramide synthase (Pewzner-Jung et al. 2006). Because ceramide shows toxicity against the cochlea, we supposed that generation of ceramide through the above-mentioned synthesis pathways may affect hair cell damage.

Sphingomyelin is hydrolyzed to ceramide by 3 types of sphingomyelinases (acid, neutral, and alkaline). Of these, magnesium-dependent neutral sphingomyelinase (nSMase) has been identified as a prime candidate for ceramide synthesis. Although 3 nSMases (nSMase1, nSMase2, and nSMase3) exist

(Hofmann et al. 2000; Krut et al. 2006; Tomiuk et al. 1998), nSMase2 is responsible for most of the nSMase activity in many organs, and thus, is the principal generator of ceramide (Aubin et al. 2005; Stoffel et al. 2005). Overexpression of nSMase2 in hepatocytes reportedly resulted in a 40% decrease in SM levels and a concomitant 60% increase in ceramide levels (Karakashian et al. 2004; Marchesini et al. 2003). Although ceramide augments the apoptotic death of outer hair cells (Nishimura et al. 2010), whether nSMase is involved in ceramide generation in gentamicin-induced ototoxicity has not been revealed.

Sphingomyelin synthase (SMS) can also potentially regulate ceramide levels. SMS reconverts ceramide produced by nSMase to sphingomyelin. Namely, catalysis by nSMase and SMS forms a ceramide/sphingomyelin cycle. However, the role of this cycle has not yet been reported.

In the de novo ceramide biosynthesis pathway, SPT is the first and rate-limiting enzyme. Ceramide generation is derived from the de novo biosynthesis pathway by coordinated activation of SPT and ceramide synthase, an endpoint catalyst of ceramide production.

Considering a possible role of ceramide in gentamicin-induced ototoxicity, we hypothesized that hair cell loss could be prevented by modifying the ceramide biosynthesis pathways. In this study, we examined the functions of nSMase, SMS, and SPT in gentamicin-induced ototoxicity.

Materials and Methods

Organ of Corti collection

The basal turns of the organ of Corti were dissected from Sprague-Dawley rats on postnatal days 3 to 5 and cultured according to the methods of Van de Water and Ruben (1974) and Sobkowicz et al. (1993). All animal procedures were carried out according to the guidelines of the Laboratory Animal Research Center of the University of Tsukuba.

Culture techniques

Cochlear explants were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 25 mM HEPES, and 30 U/mL penicillin and cultured in an incubator at 37°C with 5% CO2 and 95% humidity. Cochlear cultures were maintained in the above-described medium overnight (8 - 12 hours) and then exposed to a medium containing 35 μM gentamicin for 48 hours to assess the effects of sphingolipid metabolites (Nakamagoe et al. 2010; Nishimura et al. 2010; Tabuchi et al. 2007).

Pharmacological treatments

GW4869 (an nSMase inhibitor), 2-hydroxyoleic acid (2OHOA, an SMS activator), and myriocin (an SPT inhibitor) were purchased from Sigma Japan (Tokyo, Japan). The tested concentrations of GW4869, 2OHOA, and myriocin were 1 to 50 μM, 10nM to 1 mM and 1 pM to 50 μM, respectively. The tested concentrations were determined based on the previous experiments on the neuronal cells, red blood cells, vascular smooth muscle cells, fibroblasts, glioma cells, lung adenocarcinoma cells and liver cells (Babenko and Kharchenko 2012; Cubi et al. 2013; Gbotosho et al. 2013; Liao et al. 2013; Martin et al. 2013; Qin et al. 2012). The highest concentration of myriocin was determined at 50 μM because of its solubility. GW4869 was routinely stored at -80°C as a 1.5 mM stock suspension in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA). Immediately before use, the suspension was solubilized by addition of 5% methane sulfonic acid (MSA; Sigma). The suspension was mixed and warmed at 37°C until clear (Luberto et al. 2002). 2OHOA was dissolved in DMSO at a stock concentration of 25 mg/mL and stored at -20°C. Myriocin was dissolved in methanol at a stock concentration of 2 mg/mL and stored at -20°C.

Cytochemistry

After 48-hour culture, the explants were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes and then permeabilized with 5% TritonX-100 (Sigma) in PBS with 10% FBS for 10 minutes (Nishimura et al. 2010; Tabuchi et al. 2007; Tabuchi et al. 2010). The specimens were stained with phalloidin with a conjugated Alexa Fluor phalloidin probe (1:100; Molecular Probes, Carlsbad, CA, USA) at room temperature for 1 hour. Phalloidin is a specific marker for cellular F-actin and labels the stereociliary arrays and cuticular plates of hair cells (Nakamagoe et al. 2010; Tabuchi et al. 2007).

Hair cells were characterized as missing if no stereocilia and/or no cuticular plate were observed by phalloidin staining. Quantitative results were obtained by evaluating 30 outer hair cells associated with 10 inner hair cells in a given microscopic field (Nakamagoe et al. 2010; Tabuchi et al. 2007). The average of 3 separate counts was used to represent each culture.

Immunostaining of hair cells by anti-nSMase2 antibody

Rabbit polyclonal anti-nSMase2 (H-195) (sc-67305) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and donkey anti-rabbit FITC (711-095-152), from Sigma.

The hair cells were removed from the culture media, rinsed twice in PBS, and fixed in 4%

paraformaldehyde for 20 minutes, and then double-immunostained. After removal of the fixative, the cells were incubated in 0.1% Triton X-100 and 10% FBS at room temperature for 15 minutes.

The primary anti-nSMase2 antibody was diluted 1:100 in PBS and incubated with cells overnight at 4°C. After 6 rinses in PBS for 5 minutes each time at room temperature, cells were incubated with FITC-conjugated secondary antibody (diluted 1:100 in PBS) for 45 minutes in a dark chamber and then rinsed extensively in PBS at room temperature. The specimens were stained with phalloidin with a conjugated Alexa Fluor phalloidin probe (1:100) at room temperature for 1 hour. One drop of SlowFade Gold Antifade reagent (Invitrogen, Carlsbad, CA, USA) was added, and the slide was sealed with nail polish. The immunofluorescence reaction was followed and documented with an Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

nSMase assay

nSMase activity of the basal turn of the organ of Corti cells during gentamicin exposure was measured using an nSMase assay kit (K-1800; Echelon, Salt Lake City, UT, USA) as described in the manufacturer's protocol. Briefly, organ of Corti cells were harvested and washed with PBS. After the cells had been homogenized, 60 μg total protein was used to examine the nSMase activity. One hundred-microliter samples were mixed with 100 μL reaction buffer and 5 mM dithiothreitol (Thermo

Scientific, Rockford, IL, USA) to block any acid sphingomyelinase activity. The samples were incubated at 37°C for 4 hours. The enzymatic activity was calculated from the graph's slope of the absorbance of light at 595 nm against the concentration of sphingomyelinase and standardized by milligrams of protein (mU/mg, representing the specific enzymatic activity).

Data analyses and statistics

All data were expressed as the means ± SEMs. Statistical analysis was performed using unpaired *t*-tests or 1-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests, as required (StatView 5.0). Probability (*p*) values below 0.05 were considered significant.

Results

Expression of nSMase2 in cochlear hair cells

Immunostaining of nSMase2 in organ of Corti cells revealed the presence of nSMase2 in the inner and outer hair cells (Fig. 1).

GM activated nSMase in organ of Corti cells

Changes in nSMase activity during gentamicin exposure were examined using the organ of Corti explants. Twenty minutes after the initiation of gentamicin exposure, enzymatic activity of the hair cell extracts significantly increased (n = 6 samples of protein, $p < 0.001$), to nearly 3-fold those of the non-gentamicin controls. Thereafter, nSMase activity gradually declined, but at 48 hours, the level of nSMase activity was significantly (n = 6, $p < 0.001$) higher (344%) than that of the non-gentamicin controls (1-way ANOVA and Bonferroni post-hoc test; Fig. 2).

Control pharmacological study

Before the gentamicin experiments, the effects on hair cells of 1 μM to 50 μM GW4869, 10 nM to 100 μM 2OHOA, and 1 pM to 50 μM myriocin, and of 0.01% MSA, 0.25% DMSO, and 1% methanol as solvents were examined. In the control explants maintained in the media containing these agents for 48 hours without exposure to gentamicin, almost all hair cells, in both the inner and the outer rows, were present. Namely, hair cell loss was not statistically significant when explants were cultured for 48 hours in the media containing each agent without gentamicin (data not shown). Furthermore, we also confirmed that the hair cell loss did not differ significantly between the explants exposed to media containing 35 μM

gentamicin with 0.01% MSA, 0.25% DMSO, or 1% methanol for 48 hours and those exposed to media containing 35 μM gentamicin alone (data not shown).

Inhibition of nSMase protected hair cells against gentamicin-induced ototoxicity

The effects of GW4869, an nSMase inhibitor, on gentamicin-induced hair cell damage were examined. GW4869 significantly decreased outer hair cell loss induced by gentamicin at 35 μM (n = 12 explants, p = 0.03), 40 μM (n = 15, $p < 0.001$), 45 μM (n = 13, $p < 0.001$) and 50 μM (n = 16, $p < 0.001$) when compared with the control group exposed to gentamicin alone (1-way ANOVA and Bonferroni post-hoc test; Fig. 3).

Activation of SMS protected hair cells against gentamicin-induced ototoxicity

The effects of 2OHOA, an SMS activator, on gentamicin-induced hair cell damage were examined. 2OHOA significantly decreased outer hair cell loss induced by gentamicin at 10 μM (n = 11 explants, $p < 0.001$) and 100 μM (n = 15, $p < 0.001$) when compared with the control group exposed to GM alone (1-way ANOVA and Bonferroni post-hoc test; Fig. 4).

Inhibition of SPT had no effect on gentamicin-induced hair cell loss

The effects of myriocin, an SPT inhibitor, on gentamicin-induced hair cell damage were examined. Myriocin had no significant effect on the degree of hair cell survival at 1 pM to 50 μM when compared with gentamicin alone (1-way ANOVA; Fig. 5)

Discussion

Ceramide has attracted great attention because of its possible roles in cochlear hair cell apoptosis (Nishimura et al. 2010). Better understanding of the ceramide formation system might offer relevant insights into cochlear hair cell death.

Our immunofluorescence study detected existence of nSMase2 in hair cells. Importantly, nSMase2 is responsible for the main function of nSMase (Aubin et al. 2005; Karakashian et al. 2004; Marchesini et al. 2003; Stoffel et al. 2005). Additionally, gentamicin exposure increased the enzymatic activity of nSMase. GW4869, a specific inhibitor of nSMase, decreased gentamicin-induced hair cell death. On the basis of these findings, ceramide generated by nSMase through the hydrolysis of sphingomyelin may be involved in gentamicin-induced ototoxicity.

In addition, activation of SMS by 2OHOA protected hair cells against gentamicin. Some

researchers speculated on the existence of a ceramide/sphingomyelin cycle. In this cycle, nSMase hydrolyzes sphingomyelin to ceramide and SMS synthesizes sphingomyelin from ceramide. Thus, ceramide levels are balanced by the activities of these 2 enzymes. Our results suggested that such a ceramide/sphingomyelin cycle exists in the organ of Corti and that the balance in this cycle is important for determination of hair cell fate in gentamicin ototoxicity.

We have here documented, for the first time, the immunolocalization and enzymatic activity of nSMase in the cochlea. On the basis of the kinetics, the increase in enzymatic nSMase activity was divided into 2 phases. In the early phase, the total cellular nSMase activity increased by 292% at 20 minutes of gentamicin exposure. Possibly, the nSMase activity rapidly increased as an initial response against gentamicin exposure. Thereafter, the nSMase activity gradually declined. In the late phase, at 48 hours after gentamicin exposure, the level of nSMase activity was 344% higher than that in the non-gentamicin-exposed controls.

In contrast to the results obtained with GW4869 and 2OHOA, myriocin, an SPT inhibitor, known as an inhibitor of the first enzyme in the de novo biosynthesis pathway, did not prevent hair cell death due to gentamicin-induced ototoxicity. This result indicated that the de novo generation of ceramide might not be involved in gentamicin-induced ototoxicity. However, several researchers reported that generation of ceramide via de novo biosynthesis occurred much more slowly than via the sphingomyelin hydrosis pathway (Bose et al. 1995; De Luca et al. 2005; Mullen et al. 2011). Because our study

examined results over a time course of 48 hours, they may indicate that the de novo biosynthesis pathway is activated after a longer period.

In conclusion, the present findings strongly suggest the involvement of a ceramide/sphingomyelin cycle in gentamicin-induced cochlear ototoxicity. Gentamicin-induced ototoxicity may be ameliorated by modifying ceramide synthesis through this cycle.

Acknowledgements This work was supported by a Grant-in-aid for Scientific Research (C) 20591969 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Conflict of interest The authors declare that they have no conflict of interest.

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Figure Captions

Fig. 1 Localization of nSMase2 in cochlear explants (a). nSMase 2 stained with anti-nSMase 2 antibody (indicated in green) (b). Hair cells stained with phalloidin (indicated in red) (c). Merged images of nSMase2 and hair cells. The outer and inner hair cells stained with nSMase2. O: outer hair cell. I: inner hair cell

Fig. 2 Characterization of nSMase enzymatic activity (a). Enzymatic assays of cochlear hair cell extracts significantly increased at 20 minutes (292%) and at 48 hours (344%) after exposure to 35 μM gentamicin (1-way ANOVA and Bonferroni post-hoc test: *p<0.05 versus the non-gentamicin control group at 0 hour). The enzymatic activity expressed as mU/mg protein

Fig. 3 Effects of GW4869 on gentamicin-induced hair cell loss (a). Quantitative analysis of outer hair cell loss. GW4869 protected the cochlea at a concentration of 35 to 50 μM (1-way ANOVA and Bonferroni post-hoc test: *p<0.05 versus the gentamicin control group)

(b-k). Representative photographs of each group (b). Solvent alone (c). Gentamicin control (35 μM gentamicin alone) (d). 35 μM gentamicin plus 1 μM GW4869 (e). 35 μM gentamicin plus 10 μM GW4869 (f). 35 μM gentamicin plus 20 μM GW4869 (g). 35 μM gentamicin plus 30 μM GW4869 (h).

35 µM gentamicin plus 35 µM GW4869 (i). 35 µM gentamicin plus 40 µM GW4869 (j). 35 µM gentamicin plus 45 µM GW4869 (k). 35 µM gentamicin plus 50 µM GW4869

Fig. 4 Effects of 2OHOA on gentamicin-induced outer hair cell loss (a). Quantitative analysis of outer hair cell loss. 2OHOA protected the cochlea at a concentration of 10 nM to 100 µM (1-way ANOVA and Bonferroni post-hoc test: *p<0.05 versus the gentamicin control group)

(b-h). Representative photographs of each group (b). Solvent alone (c). Gentamicin control (35 µM gentamicin alone) (d). 35 µM gentamicin plus 10 nM 2OHOA (e). 35 µM gentamicin plus 100 nM 2OHOA (f). 35 µM gentamicin plus 1 µM 2OHOA (g). 35 µM gentamicin plus 10 µM 2OHOA (h). 35 µM gentamicin plus 100 µM 2OHOA

Fig. 5 Effects of myriocin on gentamicin-induced outer hair cell loss (a). Quantitative analysis of outer hair cell loss. Myriocin had no effect on gentamicin-induced outer hair cell loss at a concentration of 1 pM to 50 µM

(b-l). Representative photographs of each group (b). Solvent alone (c). Gentamicin control (35 µM gentamicin alone) (d). 35 µM gentamicin plus 1 pM myriocin (e). 35 µM gentamicin plus 10 pM myriocin (f). 35 µM gentamicin plus 100 pM myriocin (g). 35 µM gentamicin plus 1 nM myriocin (h). 35 µM gentamicin plus 10 nM myriocin (i). 35 µM gentamicin plus 100 nM myriocin (j). 35 µM gentamicin

plus 1 µM myriocin (k). 35 µM gentamicin plus 10 µM myriocin (l). 35 µM gentamicin plus 50 µM myriocin

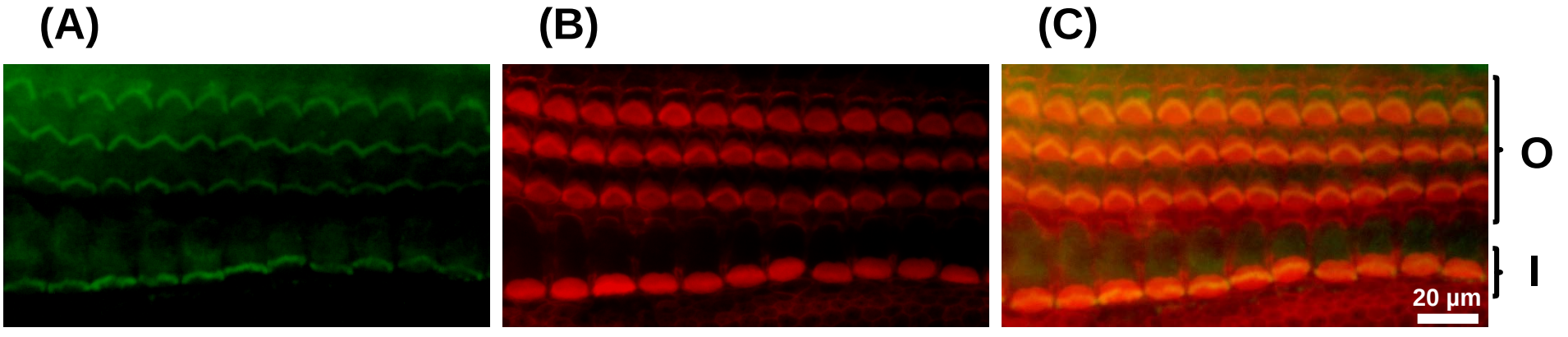


Fig. 1

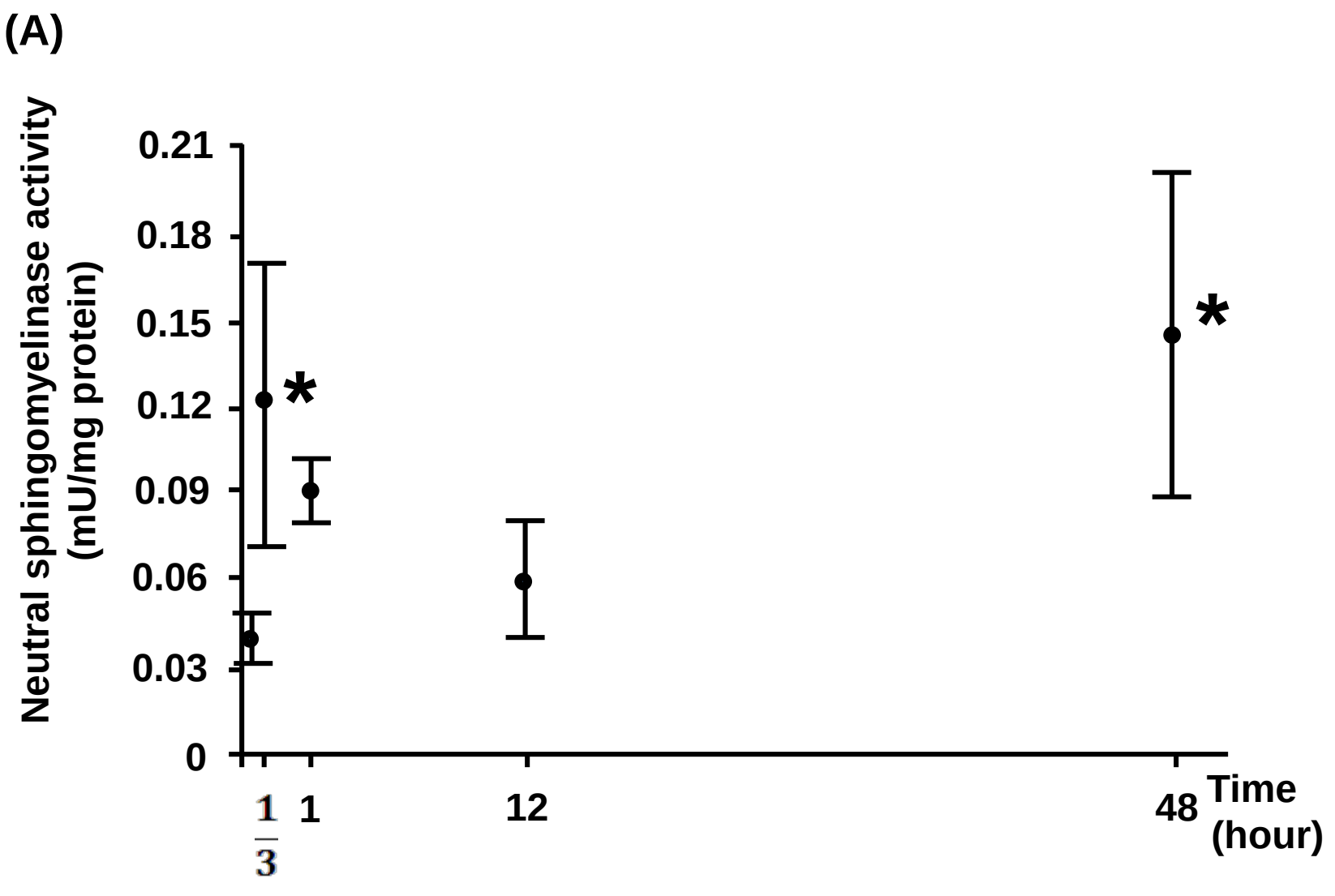
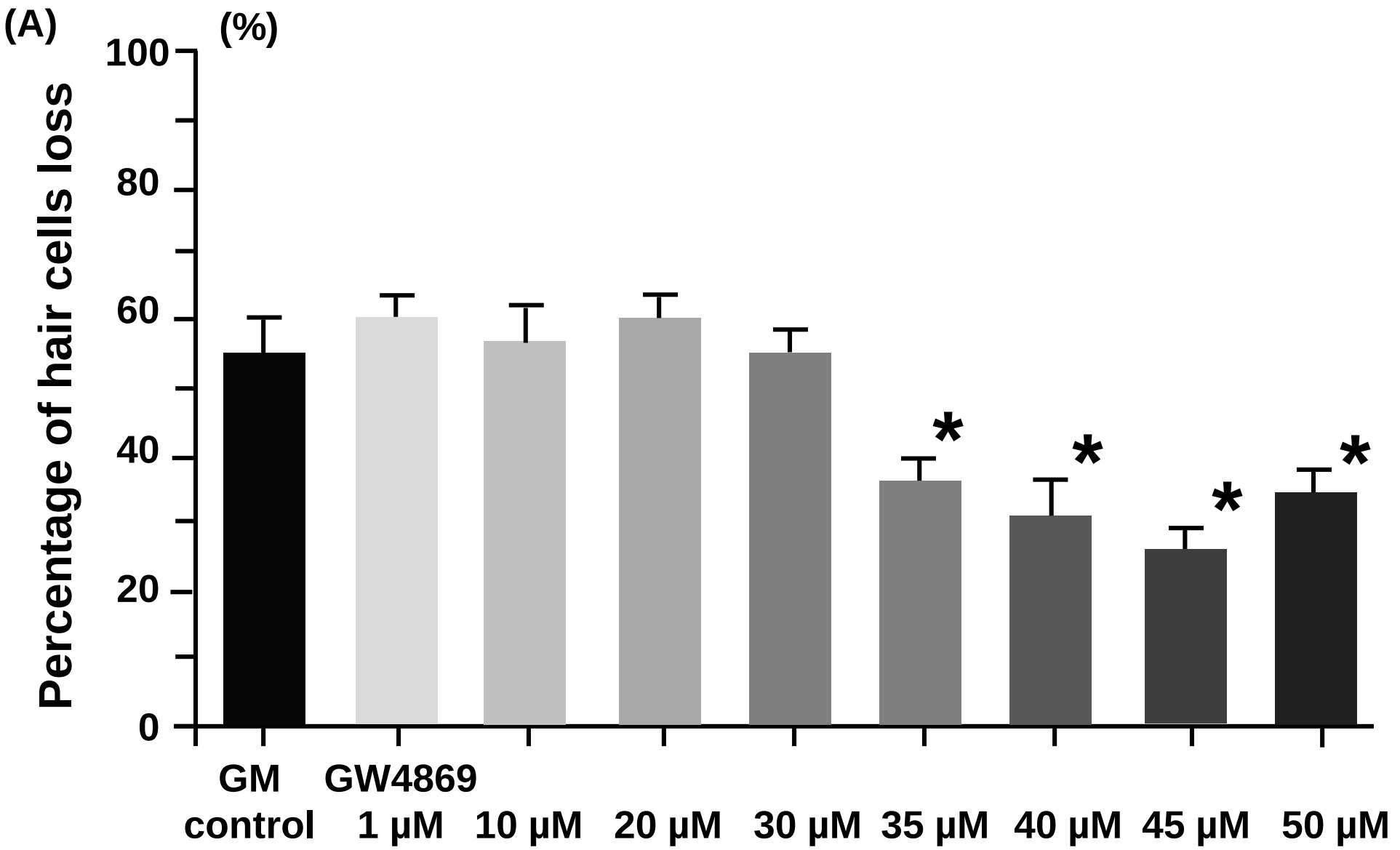

(A)
Neutral sphingomyelinase activity (mU/mg protein)
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0
*
*
1/3
1
12
48
Time (hour)

Fig. 2


(A)
(%)
Percentage of hair cells loss
100
80
60
40
20
0
*
*
*
*
GM control
GW4869 1 μM
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20 μM
30 μM
35 μM
40 μM
45 μM
50 μM

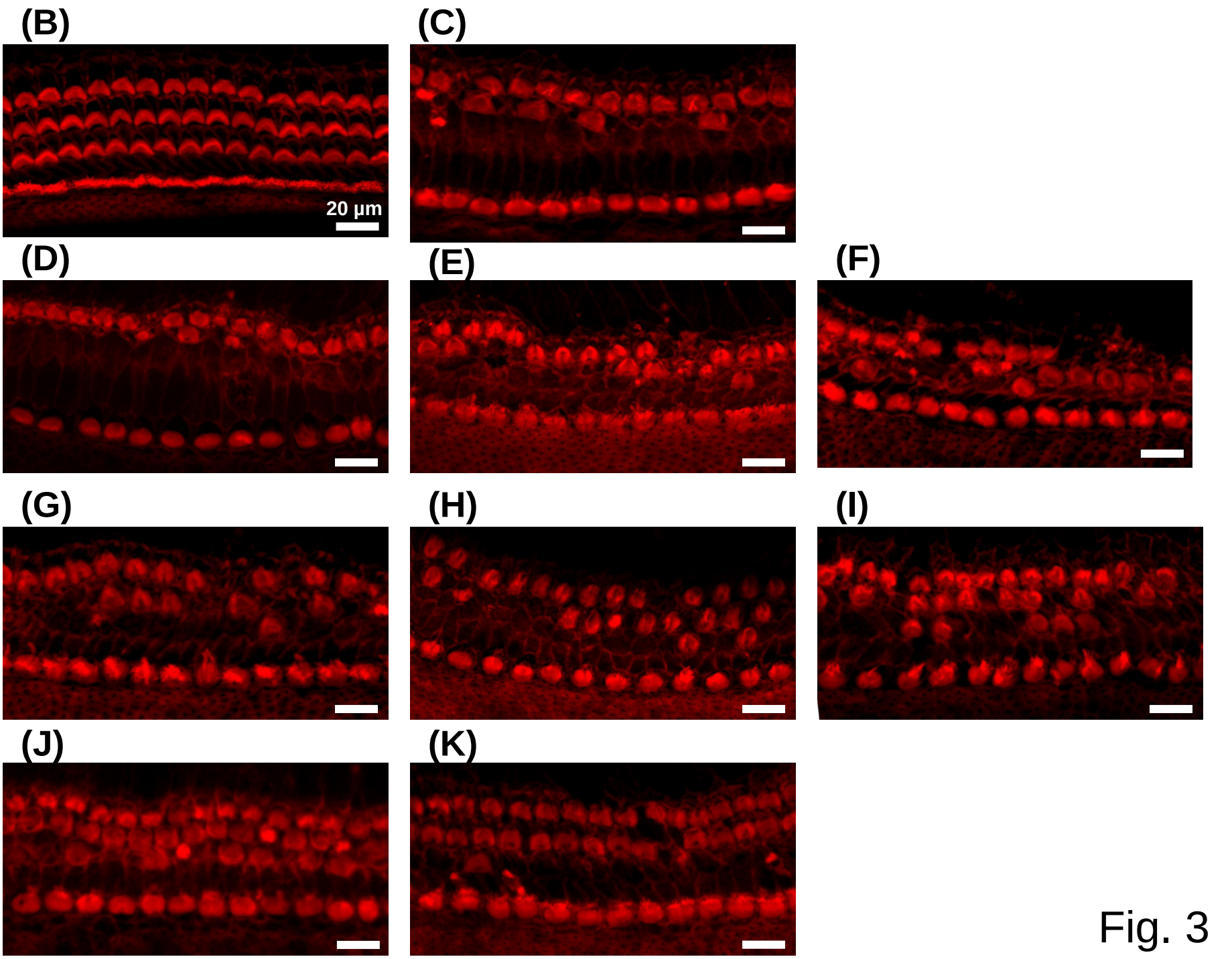
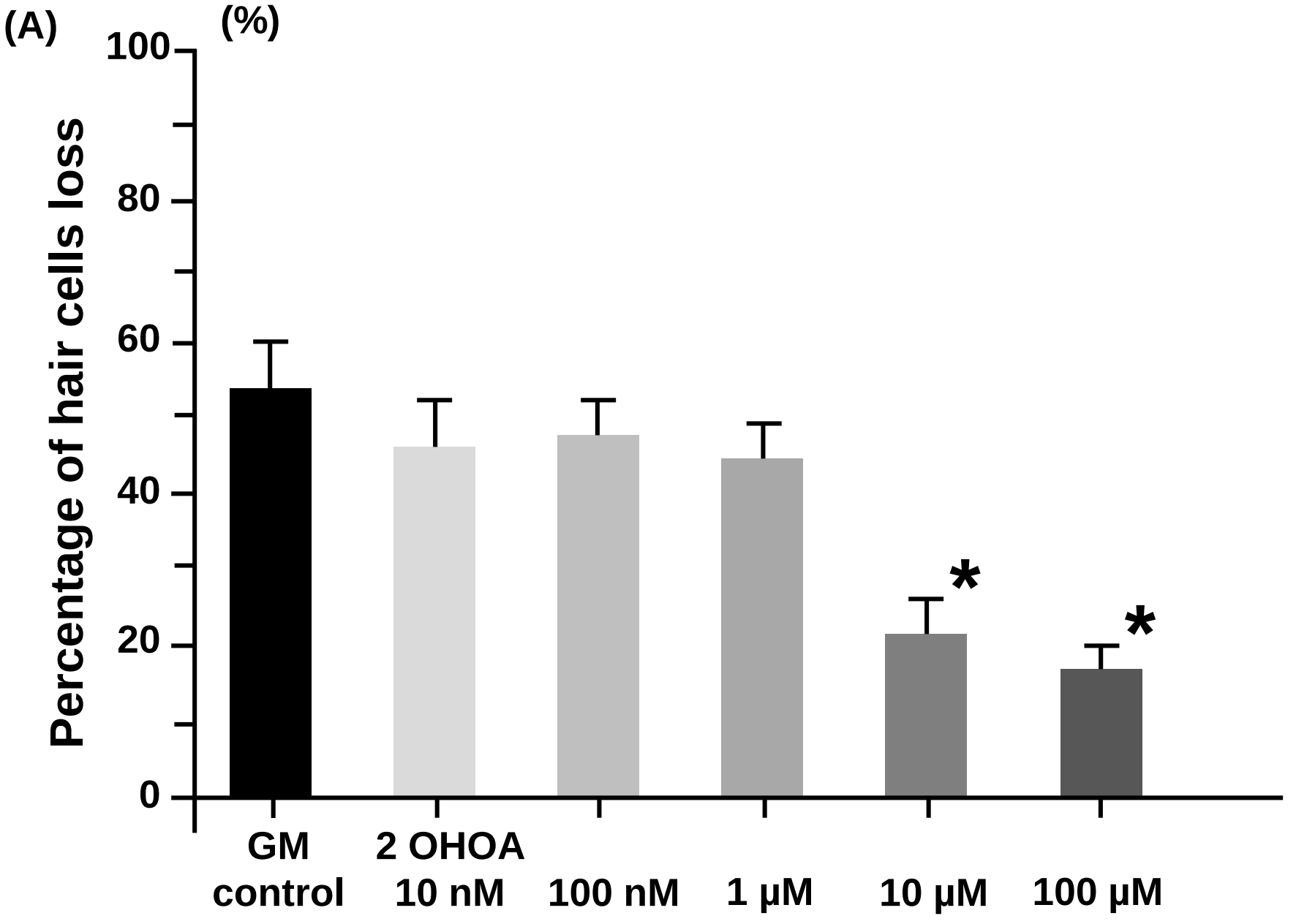

(B)
(C)
20 μm
(D)
(E)
(F)
(G)
(H)
(I)
(J)
(K)

Fig. 3


(A)
(%)
Percentage of hair cells loss
100
80
60
40
20
0
*
*
GM control
2 OHOA
10 nM
100 nM
1 μM
10 μM
100 μM

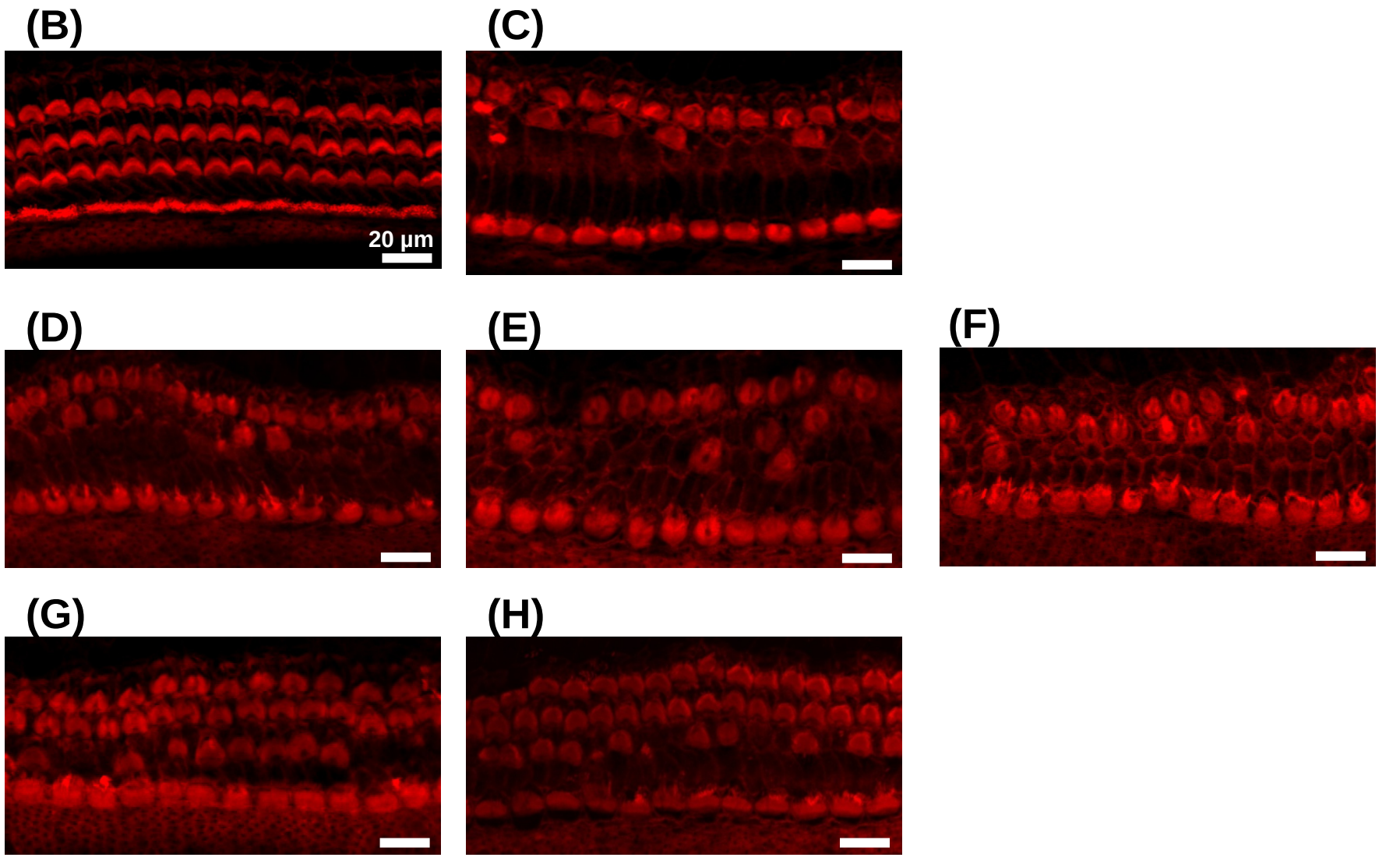

(B)
20 μm
(C)
(D)
(E)
(F)
(G)
(H)

Fig. 4

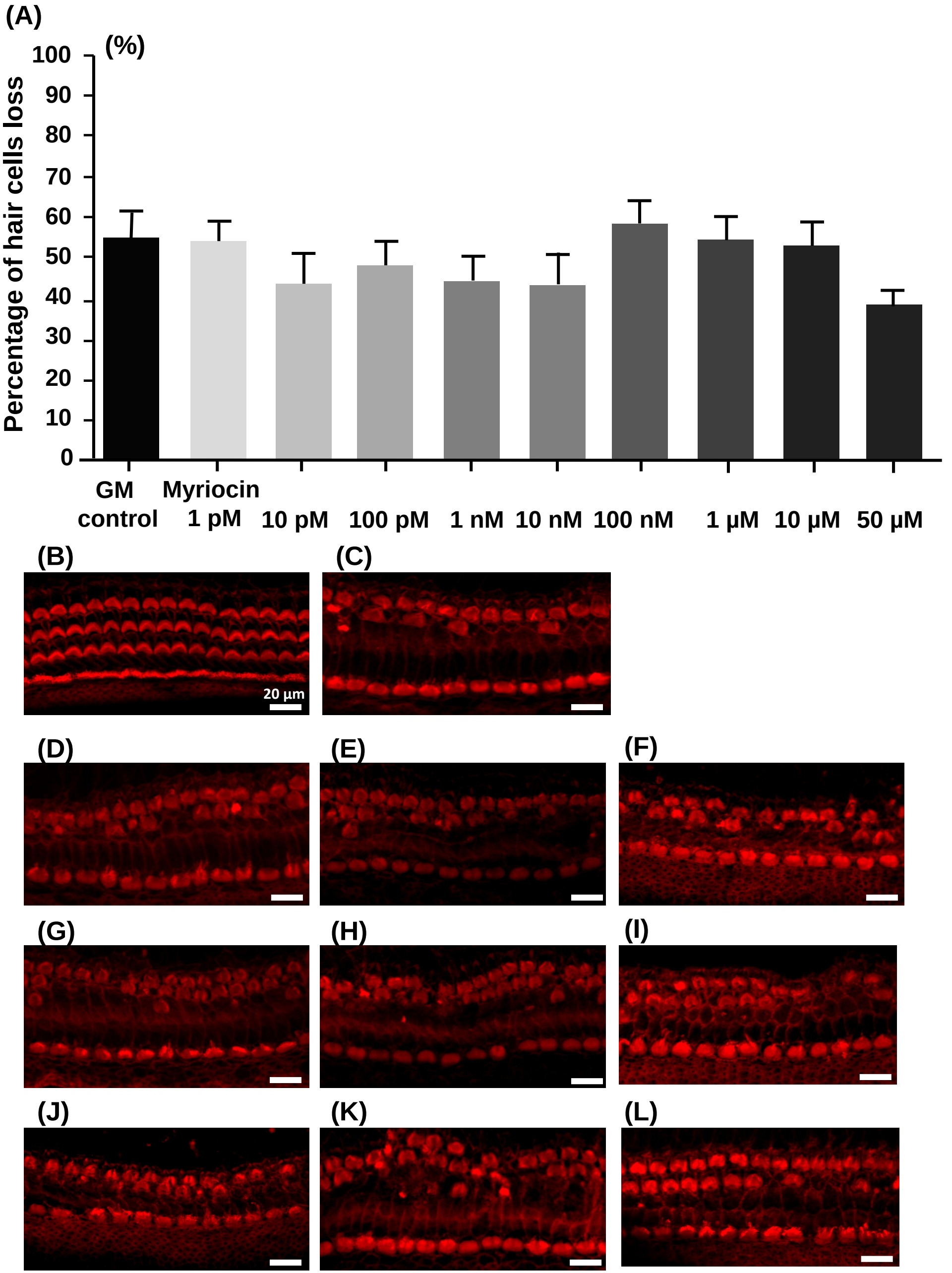

(A)
(%)
Percentage of hair cells loss
100
90
80
70
60
50
40
30
20
10
0
GM control
Myriocin
1 pM
10 pM
100 pM
1 nM
10 nM
100 nM
1 µM
10 µM
50 µM
(B)
(C)
20 µm
(D)
(E)
(F)
(G)
(H)
(I)
(J)
(K)
(L)

Fig. 5