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ORIGINAL ARTICLE

# Glucose Protects Cochlear Hair Cells Against Oxidative Stress and Attenuates Noise-Induced Hearing Loss in Mice

**Hao Xiong[1,2] · Lan Lai[1,2] · Yongyi Ye[1,2] · Yiqing Zheng[1,2]**

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**Abstract** Oxidative stress is the key determinant in the pathogenesis of noise-induced hearing loss (NIHL). Given that cellular defense against oxidative stress is an energy-consuming process, the aim of the present study was to investigate whether increasing energy availability by glucose supplementation protects cochlear hair cells against oxidative stress and attenuates NIHL. Our results revealed that glucose supplementation reduced the noise-induced formation of reactive oxygen species (ROS) and consequently attenuated noise-induced loss of outer hair cells, inner hair cell synaptic ribbons, and NIHL in CBA/J mice. In cochlear explants, glucose supplementation increased the levels of ATP and NADPH, as well as attenuating $H_2O_2$-induced ROS production and cytotoxicity. Moreover, pharmacological inhibition of glucose transporter type 1 activity abolished the protective effects of glucose against oxidative stress in HEI-OC1 cells. These findings suggest that energy availability is crucial for oxidative stress resistance and glucose supplementation offers a simple and effective approach for the protection of cochlear hair cells against oxidative stress and NIHL.

**Keywords** Oxidative stress · Glucose · Cochlea · Hair cell · Hearing loss

## Introduction

Noise-induced hearing loss (NIHL), which is caused by excessive exposure to noise due to occupational or recreational activities, is one of the leading causes of sensorineural hearing loss around the world [1]. Typical features of NIHL include hearing threshold elevation and permanent loss of cochlear hair cells in the inner ear [2–4]. Despite considerable efforts, the comprehensive mechanisms underlying the noise-induced loss of hair cells and NIHL are not fully elucidated. Currently, there are no Food and Drug Administration (USA)-approved pharmacologic agents for the prevention or amelioration of NIHL [5]. Evidence accumulating from animal models shows that oxidative stress is a key determinant in the pathogenesis of NIHL [2, 5–7]. Oxidative stress is attributed to an imbalance between the production and elimination of reactive oxygen species (ROS). Exposure to traumatic noise leads to the accumulation of excessive ROS within the cochlea, which triggers ischemia, inflammation, and apoptosis, leading to cochlear hair cell dysfunction and eventual death [8–11]. Moreover, the causal correlation between oxidative stress and NIHL is also evidenced by the fact that antioxidants can prevent the noise-induced loss of hair cells and NIHL [4, 6, 12].

The cellular response to oxidative stress is an energy-consuming process [13–15]. The basic and direct energy source of living cells is ATP, which is produced through several pathways: glycolysis, glucose oxidation, lactate oxidation, and fatty-acid oxidation. Notably, the mammalian brain relies on glucose as its predominant source of

Hao Xiong and Lan Lai have contributed equally to this work.

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✉ Yiqing Zheng
zhengyiq@mail.sysu.edu.cn

[1] Department of Otolaryngology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China

[2] Institute of Hearing and Speech-Language Science, Sun Yat-sen University, Guangzhou 510120, China

ATP production [16]. Decreased ATP levels following traumatic noise exposure has been reported in cochlear tissue and is correlated with the permanent loss of cochlear hair cells [17–19]. Intriguingly, a recent study has revealed that increasing glucose availability by a high-sugar diet improves the survival of flies challenged with oxidative stress [20]. In the present study, we tested the hypothesis that glucose supplementation protects cochlear hair cells against oxidative stress and attenuates NIHL.

## Materials and Methods

### Animals and Glucose Supplementation

CBA/J mice of both sexes at 8 weeks of age were purchased from Vital River Lab Animal Technology Co., Ltd (Beijing, China). At 9 weeks, auditory brainstem responses (ABRs) were recorded as a baseline. At 10 weeks, mice weighing 20–23 g were exposed to noise. To assess the protective effects of glucose, each mouse received a single intraperitoneal injection of 4.5 g/kg or 9.0 g/kg glucose (Sigma-Aldrich, St.Louis, MO, USA) 30 min before noise exposure. Control mice received the same intraperitoneal volume of saline. Animal research protocols were approved by the Animal Care and Use Committee, Sun Yat-sen University.

### Auditory Brainstem Response Measurement

ABRs were recorded at baseline and 14 days after noise exposure as described previously [21]. In brief, mice were anesthetized with an intraperitoneal injection of a ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture. ABRs were recorded at 8, 16, and 32 kHz in the right ear of each mouse using Tucker-Davis Technologies System III (Alachua, FL, USA). Up to 1024 responses were obtained and averaged. The threshold was determined as the lowest stimulus intensity at which a positive wave was evident in the evoked response trace. The waveforms were saved and analyzed *post hoc* to measure the amplitude of ABR wave I.

### Noise Exposure

CBA/J mice in separate cages were exposed to a broadband noise (2 to 20 kHz) at 115 dB sound pressure level (SPL) for 2 h as previously described [12]. In brief, unrestrained mice were placed within a sound chamber equipped with a loudspeaker (HG220-1, New Retone, Guangzhou, Guangdong, China) driven by a power amplifier (P9500S, Yamaha, Ginza, Tokyo, Japan). Sound levels within the chamber were monitored with a sound level meter during noise exposure. Control mice were kept within the same chamber in silence (the loudspeaker remained off) for 2 h.

### Cochlear Tissue Preparation

For counts of hair cells and synaptic ribbons of inner hair cells (IHCs), the mice were deeply anesthetized and decapitated following final ABR recordings. For immunolabeling of outer hair cells (OHCs), the mice were decapitated 1 h after noise exposure. Cochleae were removed and fixed in 4% paraformaldehyde at 4°C overnight. Then, they were decalcified in 4% sodium EDTA for 4–5 days followed by incubation in 30% sucrose overnight. Afterwards, they were directly processed for immunolabeling and subsequent assessments. For immunoblotting, cochlear tissue was obtained 1 h after noise exposure, dissected, and stored at −80°C.

### Hair Cell Counts in Cochlear Surface Preparations

Following decalcification with EDTA, the cochleae were permeabilized in 0.3% Triton X in PBS for 30 min, then stained with Alexa Fluor 488-conjugated phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) at 1:200 for 30 min. After washing with PBS, each cochlea was dissected into apical, middle, and basal segments. Then all segments were mounted on slides in 50% glycerol. The segments were observed and photographed using an Olympus BX63 microscope. Hair cells were manually counted along the entire length of the cochlear coil. Percentages of hair cell loss were calculated and plotted to construct a cytocochleogram, as described previously [22].

### Immunohistochemistry of Cochlear Surface Preparations

Immunohistochemistry was performed as described in our previous study [23]. In brief, the specimens were incubated with polyclonal rabbit anti-myosin (Santa Cruz, Delaware, CA, USA) at 1:200, polyclonal rabbit anti-4-hydroxynonenal (4HNE, Abcam, Cambridge, UK) at 1:200, monoclonal mouse anti-3-nitrotyrosine (3NT, Abcam, Cambridge, UK) at 1:50, and monoclonal rabbit anti-C-terminal binding protein 2 (CtBP2, BD Biosciences, Franklin, NJ, USA) at 1:200. Following 3 washes with PBS, the specimens were stained with Alexa Flour 594-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) at 1:200. The cytoskeleton was stained with Alexa Fluor 488-conjugated phalloidin at 1:200 and nuclei were counterstained with 4',6-diamidino-2-phenylindole (10 mg/mL, Sigma-Aldrich, St.Louis, MO, USA). Cochlear samples were observed under a Zeiss LSM 710 confocal microscope.

### Quantification of Immunofluorescent Signals from OHCs of Cochlear Surface Preparations

Procedures of quantification of the immunofluorescent signals in OHCs from surface preparations were as described in our previous studies [12, 22]. Briefly, the signals were obtained from original confocal images. All images were captured at identical settings. All surface preparations were processed in parallel using identical solutions. Samples were counterstained with Alexa Fluor 488-conjugated phalloidin to identify comparable regions of OHCs. The immunofluorescent signals were measured at cochlear locations equivalent to the 22 kHz–32 kHz region. Relative fluorescence intensity was calculated by normalizing the intensity of fluorescence from noise-exposed OHCs to that from controls.

### Quantification of Immunolabeled Synaptic Ribbons of IHCs from Cochlear Surface Preparations

Qualitative assessment of synaptic ribbons in IHCs by CtBP2 immunostaining from cochlear surface preparations was performed from original confocal images, as described in our previous study [22]. All z-stack images in each 0.12-mm segment were captured with identical exposure conditions and settings. The number of CtBP2 puncta was counted using Image J (National Institutes of Health, USA).

### Western Blotting Analysis of Whole Cochlear Tissue Homogenates

Western blotting analysis was performed as previously described [23]. In brief, cochlear tissue was homogenized and sonicated in radio-immunoprecipitation assay lysis buffer (Thermo plus, Waltham, MA, USA) on ice, centrifuged at 12,000×*g*, and the supernatant collected. Protein concentrations were measured with a protein assay dye reagent (Bio-Rad, Hercules, CA, USA). The protein samples (50 μg) were subjected to SDS-PAGE and transferred onto a PVDF membrane (Millipore, Boston, MA, USA). After blocking with 5% non-fat dry milk in PBS/0.1% Tween 20, the membranes were incubated overnight with rabbit anti-4HNE (Abcam, Cambridge, UK) at 1:1000, mouse anti-3NT (Abcam, Cambridge, UK) at 1:1000, or anti-GAPDH (Cell Signaling, Boston, MA, USA) at 1:10,000. Following 3 washes, the membranes were incubated with an appropriate secondary antibody (1:10,000) for 1 h. Afterwards, the immuno-reactive bands were visualized using ECL (Millipore, Boston, MA, USA). Band intensities were quantified by densitometry using ImageJ. GADPH was used as an internal control for loading.

### Culture of Mouse Cochlear Explants and Treatment with $H_2O_2$ and Glucose

Primary cochlear explants from CBA/J mice were dissected at postnatal day 3. The whole cochlea was extracted quickly in Hank's balanced salt solution. The epithelium of the organ of Corti was separated after removal of the lateral wall. The explants were incubated in modified Eagle's medium, 1% serum-free supplement (Invitrogen, Carlsbad, CA, USA), and 1% bovine serum albumin under 5% $CO_2$ at 37°C. $H_2O_2$ was used to induce oxidative stress. To evaluate the dose-dependent cytotoxicity of $H_2O_2$, cultured cochlear explants were treated with $H_2O_2$ at 0, 0.1, 0.5, 1, and 2 mmol/L for 5 h followed by incubation in culture medium alone for the next 48 h. To test the protective effects of glucose, the cochlear explants were incubated with fresh medium containing $H_2O_2$ at the indicated concentrations along with glucose at 0, 5, 25, and 50 μmol/L for 5 h followed by 48-h recovery.

### Evaluation of Cytotoxicity in Cochlear Explants

To evaluate the cytotoxicity of $H_2O_2$ and the protective effects of glucose, cochlear explants were immunolabeled with polyclonal rabbit anti-myosin VIIa (Santa Cruz, Delaware, CA, USA) at 1:200 and counterstained with Alexa Fluor 596-conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) at 1:200 to label hair cells. Because hair cells at the apical turn are resistant to $H_2O_2$ cytotoxicity, hair cell counts were performed only at the basal turn [24].

### Mitochondrial ROS Detection in Cochlear Explants

Mitochondrial ROS levels were assessed using MitoSox (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Briefly, cochlear explants exposed to $H_2O_2$ with or without glucose were incubated with MitoSox (5 μmol/L) at 37°C for 30 min. After washing with PBS, the explants were incubated with polyclonal rabbit anti-myosin VIIa (Santa Cruz, Delaware, CA, USA) at 1:200 and counterstained with Alexa Fluor 488-conjugated phalloidin at 1:200. Images were captured using an Olympus BX63 microscope.

### $NADPH/NADP^+$ Measurement in Cochlear Explants

The $NADPH/NADP^+$ ratio was determined with an NADP/NADPH Assay kit (Abcam, Cambridge, UK). Five cochlear explants from identically-treated mice were homogenized with NADP/NADPH extraction buffer. Extracts were clarified by centrifugation and the supernatant was concentrated with Amicon Ultra-0.5 mL 30 kD

centrifugal filters (Millipore, Boston, MA, USA). Finally, the filtrate was used for the assays according to the manufacturer's instructions.

### Measurement of ATP Levels in Cochlear Explants

Intracellular ATP levels from cochlear explants under various conditions were measured using the ATP bioluminescence assay Kit HS II (Roche, Basel, Switzerland) following the manufacturer's instructions. Three cochlear explants from identically-treated mice were pooled for one sample and three replicates were conducted for each condition.

### Culture of HEI-OC1 Cells and $H_2O_2$ Exposure

HEI-OC1 cells, which were kindly provided by Dr. Federico Kalinec at UCLA Health, were cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) at 33°C in an atmosphere of 10% $CO_2$. To evaluate the dose and time effects of $H_2O_2$, HEI-OC1 cells were incubated with fresh medium containing $H_2O_2$ at 0, 0.2, 0.4, and 0.8 mmol/L for 1, 3, and 5 h. Afterwards, HEI-OC1 cells were incubated with the culture medium again and maintained for 24 or 48 h.

### Treatment of HEI-OC1 Cells with Glucose and WZB117

HEI-OC1 cells were treated with glucose at various concentrations for 24 h, and then incubated with $H_2O_2$ at the indicated concentrations for 5 h. Afterwards, the cells were incubated in medium alone and maintained for 24 h. WZB117, a glucose transporter type 1 (GLUT1) inhibitor (Selleck, Houston, TX, USA), was used to block glucose uptake. WZB117 at 20 μmol/L was administrated to HEI-OC1 cells alone or in combination with glucose 24 h before $H_2O_2$ treatment.

### Cell Viability Assay

The procedures for cell viability assay were as described in our previous study [25]. HEI-OC1 cells were treated with various agents and viability was evaluated with Cell Counting Kit-8 (APExBIO, Houston, TX, USA). The absorbance of samples at 450 nm was measured with a microplate reader (Molecular Devices, San Jose, CA, USA).

### Statistical Analysis

All data are presented as the mean ± SD and were analyzed using SPSS 21. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Fisher's *post hoc* test, Student's *t*-test, and one-sample *t*-tests. Differences were considered significant at values of $P < 0.05$.

## Results

### Glucose Protects Against Noise-induced Loss of OHCs and Auditory Threshold Shifts in CBA/J Mice

We first investigated whether glucose supplementation exerts protective effects against noise-induced cochlear hair-cell loss and NIHL. CBA/J mice tolerated glucose supplementation well at both concentrations. The blood glucose level increased to 33 mmol/L 15 min after intraperitoneal injection of 9.0 g/kg glucose, and remained at a high level for the next 3 h. Then the concentration declined to normal within 2 h (Fig. S1). In contrast, although intraperitoneal injection of 4.5 g/kg of glucose also raised the blood level to a high of 30 mmol/L within 15 min, it dropped rapidly to baseline within the subsequent 1 h (Fig. S1).

Then, we tested these two concentrations of glucose for the prevention of NIHL. Our results showed that supplementation with glucose at 4.5 g/kg protected against neither NIHL nor noise-induced loss of hair cells (Fig. S2). Therefore, we used 9.0 g/kg glucose for the rest of the experiments. Noise exposure caused permanent ABR threshold shifts of 35, 38, and 43 dB at 8, 16, and 32 kHz, respectively (Fig. 1A, B). As expected, glucose supplementation remarkably attenuated these shifts by 17 dB at 8 kHz ($t(23) = 7.137$, $P < 0.001$), 15 dB at 16 kHz ($t(23) = 3.974$, $P = 0.001$), and 13 dB at 32 kHz ($t(23) = 5.27$, $P < 0.001$; Fig. 1A, B). In addition, although noise exposure caused a base-to-apex gradient of OHC loss, glucose supplementation significantly reduced the OHC loss at 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mm from the apex compared with controls ($F(1,16) = 277.23$, $P < 0.001$; Fig. 1C, D). However, noise-induced loss of IHCs at the basal turn was not attenuated by glucose supplementation (Fig. 1C). Collectively, these results indicate that glucose supplementation attenuates noise-induced loss of OHCs and NIHL.

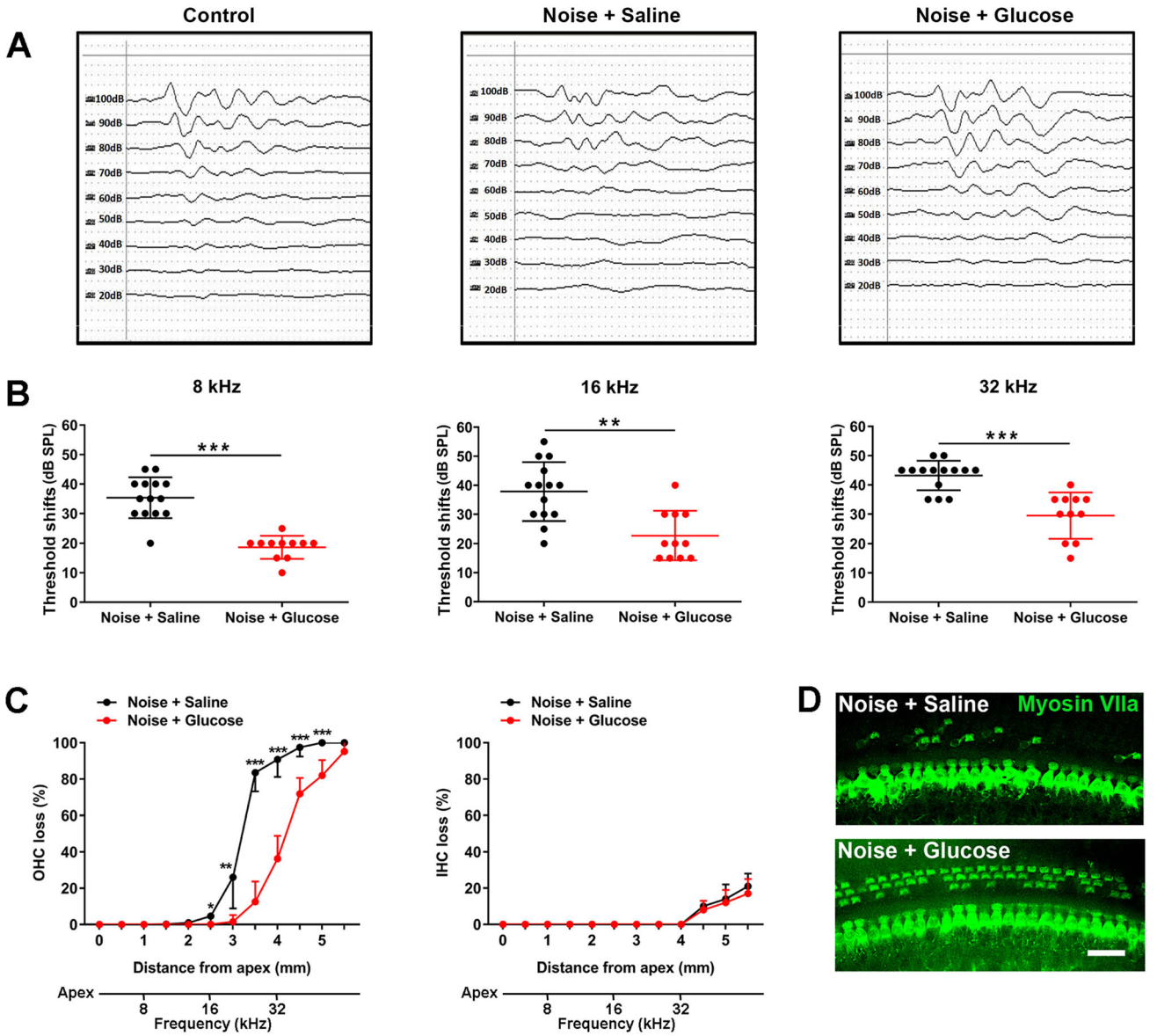


**Fig. 1** Glucose attenuates noise-induced hearing loss and outer hair cell loss in CBA/J mice. **A** Representative ABR waveforms in mice exposed to noise with or without glucose supplementation and controls. **B** Threshold shifts of ABR recorded in mice exposed to noise with or without glucose supplementation and controls. Data are presented as individual points and mean ± SD. $n$ = 11 in noise + glucose group and $n$ = 14 in noise + saline group. **C** Counts of outer hair cells and inner hair cells in mice exposed to noise with or without glucose supplementation and controls (mean ± SD; $n$ = 12 per condition; OHC, outer hair cell; IHC, inner hair cell). **D** Representative images of the upper-basal turn of cochlear surface preparations from mice exposed to noise with or without glucose supplementation and controls (scale bar, 10 μm). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

## Glucose Prevents Noise-induced Loss of IHC Synaptic Ribbons and Reduction of ABR Wave I Amplitude

Because noise-induced loss of IHC synaptic ribbons has been well documented, we next evaluated whether glucose supplementation can reduce this loss 14 days after noise exposure by counting the number of presynaptic ribbons immunolabeled with CtBP2. Noise exposure caused substantial ribbon loss in the 5-, 8-, 16-, 22-, and 32-kHz regions compared with controls (Fig. 2A, B). Again, glucose supplementation significantly reduced the noise-induced loss of IHC synaptic ribbons at 8- ($t(8) = -4.7$, $P$ = 0.004), 16- ($t(8) = -2.294$, $P$ = 0.003), 22- ($t(8) = -3.68$, $P$ = 0.005), and 32-kHz ($t(8) = -6.57$, $P$ = 0.008) regions (Fig. 2A, B).

We then measured the ABR wave I amplitudes at 8 kHz. These amplitudes reflect the activity of the auditory nerve, and infer function of synapses. Noise exposure remarkably decreased the ABR wave I amplitude from 50 to 80 dB

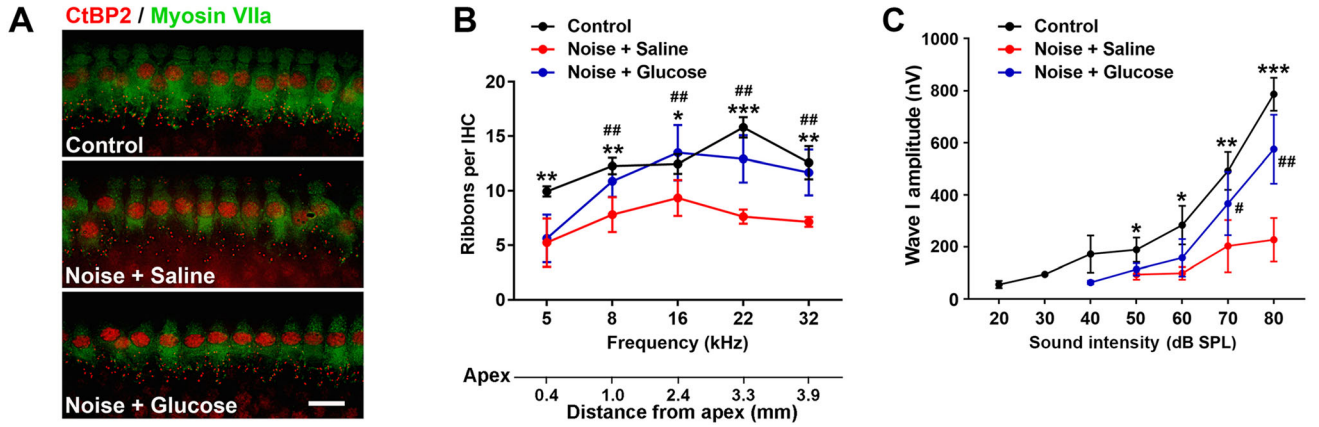


**Fig. 2** Glucose attenuates noise-induced loss of inner hair cell synaptic ribbons and ABR wave I amplitude reduction in CBA/J mice. **A** Representative images of immunolabeled CtBP2 in inner hair cells at the upper-basal turn in mice exposed to noise with or without glucose supplementation and controls (scale bar, 10 μm). **B** Quantification of synaptic ribbons in inner hair cells corresponding to various frequency regions (mean ± SD; $n = 5$ per condition). **C** ABR wave I amplitudes in mice exposed to noise with or without glucose supplementation and controls ($n = 5$ per condition). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (control *vs* noise + saline); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (noise + saline *vs* noise + glucose).

SPL in comparison with non-exposed controls, whereas glucose supplementation significantly mitigated this noise-induced decrease (80 dB, $t(8) = -8.12$, $P = 0.009$; 70 dB, $t(8) = -12.7$, $P = 0.024$; Fig. 2C). These results indicated that glucose prevents the noise-induced loss of IHC synaptic ribbons and the reduction in ABR wave I amplitude.

### Glucose Attenuates Noise-induced Oxidative Stress in OHCs of CBA/J Mice

Since oxidative stress is the key determinant in the pathogenesis of NIHL, we then explored whether glucose protects against NIHL through reducing oxidative stress. Expression of 4HNE and 3NT, markers of oxidative stress, was measured to monitor oxidative stress in cochlear tissue. The immunolabeling of 4HNE ($t(6) = -4.3$, $P = 0.013$) and 3NT ($t(6) = -6.102$, $P = 0.004$) in OHCs was increased 1 h after noise exposure, consistent with previous studies [5, 6]. Glucose supplementation significantly attenuated the noise-induced elevation of 4-HNE ($t(6) = 3.104$, $P = 0.036$) and 3-NT ($t(6) = 4.628$, $P = 0.01$), indicating that glucose can reduce noise-induced oxidative stress in the cochlea (Fig. 3A–D). Notably, the levels of 4HNE and 3NT expression were unchanged in whole cochlea homogenates (Fig. 3E–G).

### Glucose Mitigates the Noise-induced Decrease of SOD1 Expression in OHCs of CBA/J Mice

Energy is crucial for the rapid synthesis of enzymes protective against ROS during oxidative stress [20], therefore we next asked whether glucose supplementation enhances the capacity of antioxidant defense systems during noise exposure in the cochlea. SOD1 (Cu/Zn superoxide dismutase) is one of the key antioxidant enzymes and has been shown to provide protection from NIHL and drug- and age-related hearing loss [26–28]. Therefore, we explored the effects of glucose on SOD1 expression in the cochlea. Our data showed that SOD1 expression in OHCs was significantly decreased 1 h after noise exposure ($t(6) = 6.543$, $P = 0.001$). Conversely, glucose supplementation mitigated the noise-induced decrease of SOD1 expression ($t(6) = -4.007$, $P = 0.007$; Fig. 4A, B), which implies that glucose supplementation improves cochlear antioxidant capacity during noise exposure. However, SOD1 expression was unchanged in whole cochlear tissue homogenates (Fig. 4C, D).

### Glucose Protects Against Oxidative Stress-induced Hair Cell Death in Cochlear Explants

To uncover the molecular events underlying the glucose-conferred protection against noise exposure, we conducted *in vitro* oxidative stress experiments in cochlear explants. $H_2O_2$ was selected as an oxidizing agent to induce oxidative stress due to its wide use in multiple cell types [24, 29, 30]. First, we determined that $H_2O_2$ caused a dose-dependent loss of OHCs at the basal turn ($F(1,14) = 115.643$, $P < 0.001$), while only a slight loss of IHCs was observed upon high-dose $H_2O_2$ treatment $F(1,14) = 20.936$, $P < 0.001$; Fig. 5A). We then chose $H_2O_2$ at 0.5 mmol/L as its half-maximal effective concentration for the rest of the experiments in cochlear explants. Next, we tested the protective effects of glucose at multiple concentrations. Our results showed that supplementation with glucose at 25

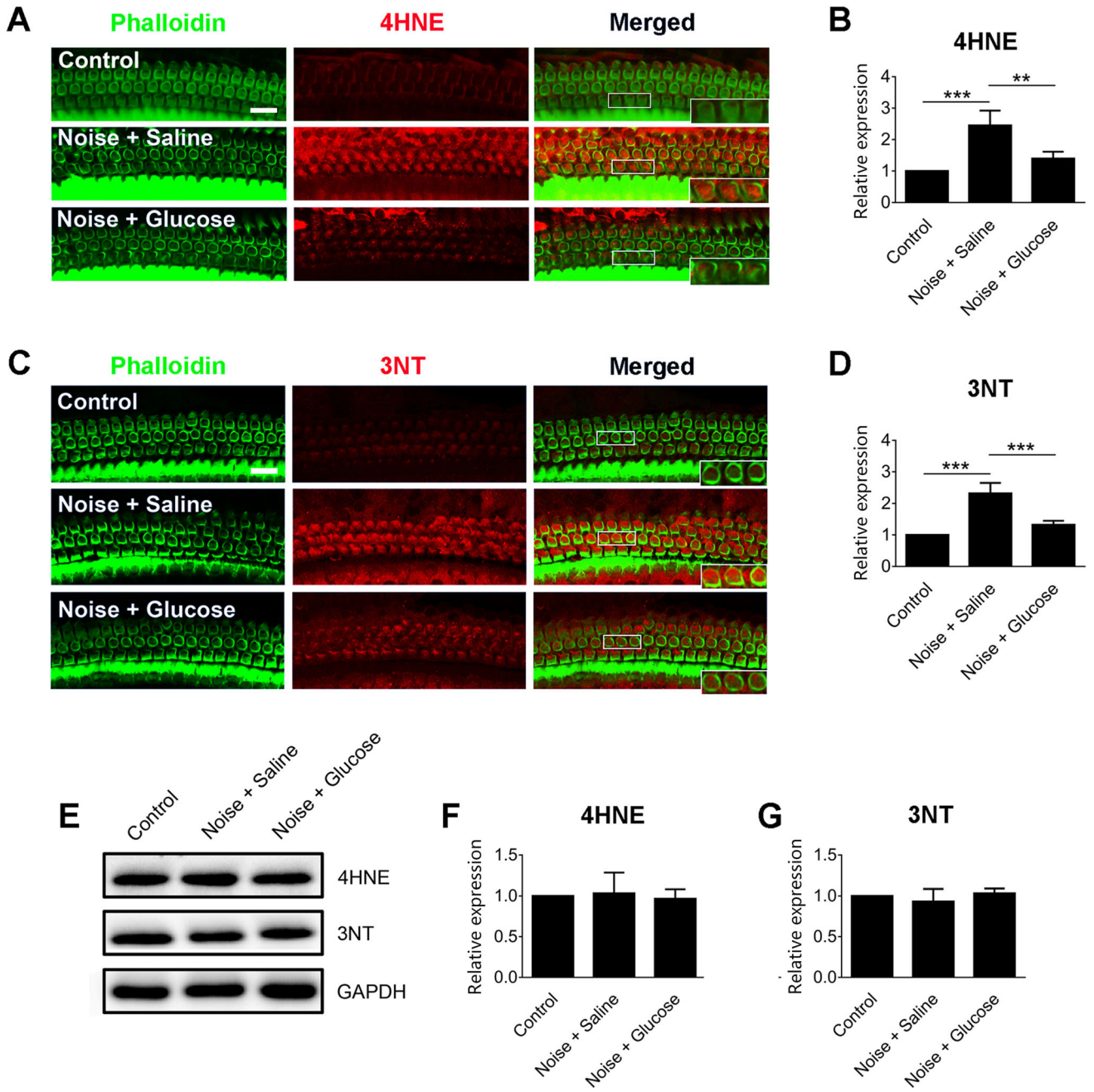


**Fig. 3** Glucose reduces the noise-induced increase of 4HNE and 3NT expression in outer hair cells in CBA/J mice. **A** Immunolabeled 4HNE in outer hair cells at the upper-basal turn in CBA/J mice exposed to noise with or without glucose supplementation and controls (insets, enlarged views of 4HNE immunolabeling). **B** Quantification of 4HNE expression in outer hair cells (means ± SD; $n = 4$ per condition). **C** Immunolabeled 3NT expression in outer hair cells at the upper-basal turn in CBA/J mice exposed to noise with or without glucose supplementation and controls (insets, enlarged views of 3NT immunolabeling; scale bar, 10 μm). **D** Quantification of 3NT expression in outer hair cells (mean ± SD; $n = 4$ per condition). **E–G** Western blots and densitometric analysis of 4HNE and 3NT in whole cochlear tissue homogenates of CBA/J mice exposed to noise with or without glucose supplementation and controls ($n = 4$ per condition). $^{**}P < 0.01$, $^{***}P < 0.001$.

μmol/L significantly attenuated the $H_2O_2$-induced loss of OHCs ($F(1,15) = 52.439$, $P < 0.001$; Fig. 5B, C), while other concentrations of glucose displayed weak protection against $H_2O_2$ treatment. Taken together, these results show that glucose prevents oxidative stress-induced hair cell death in cochlear explants.

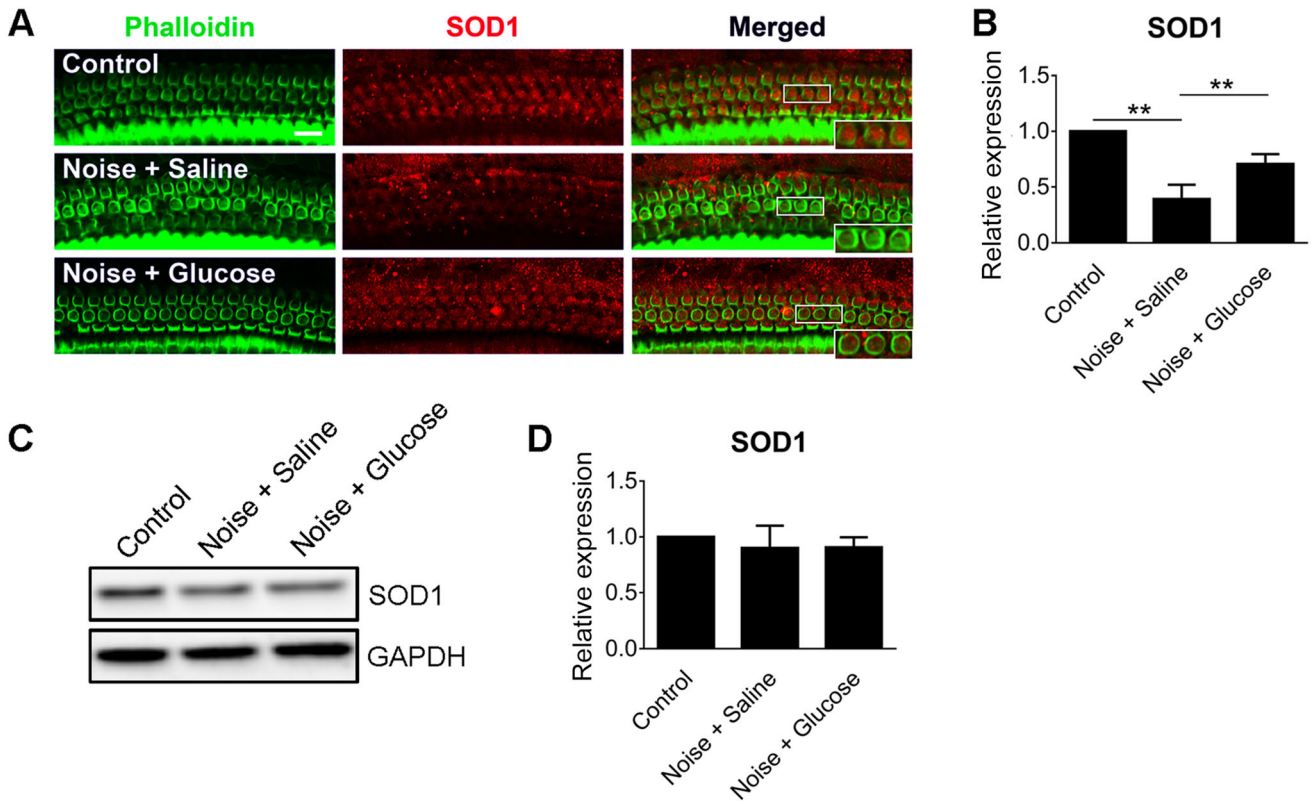


**Fig. 4** Glucose attenuates the noise-induced decrease of SOD1 expression in outer hair cells in CBA/J mice. **A** Immunolabeled SOD1 in outer hair cells at the upper-basal turn in CBA/J mice exposed to noise with or without glucose supplementation and controls (insets, enlarged views of SOD1 immunolabeling; scale bar, 10 μm). **B** Quantification of SOD1 expression in outer hair cells (mean ± SD; $n$ = 4 per condition). **C, D** Western blots and densitometric analysis of SOD1 in whole cochlear tissue homogenates of mice exposed to noise with or without glucose supplementation and controls ($n$ = 4 per condition). $^{**}P < 0.01$.

### Glucose Alleviates $H_2O_2$-induced Oxidative Stress and Attenuates the Decreased Levels of ATP and NADPH in Cochlear Explants

Next, we asked whether glucose protects hair cells by reducing $H_2O_2$-induced oxidative stress in cochlear explants. Based on the above results, glucose at 25 μmol/L was used. MitoSOX Red was used to assess mitochondrial ROS generation. Treatment with $H_2O_2$ significant increased the MitoSOX Red fluorescence intensity in OHCs compared with saline controls ($t(4) = -8.51$, $P = 0.001$; Fig. 5D, E). Conversely, glucose supplementation remarkably weakened the MitoSOX Red fluorescence intensity in $H_2O_2$-treated explants ($t(4) = 4.111$, $P = 0.015$; Fig.5D, E), which further confirms the protective effect of glucose against oxidative stress.

Because glucose is the major energy source for ATP production, we then probed whether glucose supplementation increases ATP levels under oxidative stress. We found that the ATP levels were decreased by 50% in cochlear explants after $H_2O_2$ treatment compared with controls ($t(6) = 14.437$, $P < 0.001$; Fig. 5F). Intriguingly, glucose supplementation significantly increased the ATP levels compared with controls and also attenuated the $H_2O_2$-induced ATP decline ($t(6) = -4.629$, $P = 0.004$; Fig. 5F). Moreover, we found that the NADPH/$NADP^+$ ratio after $H_2O_2$ treatment was decreased ($t(6) = 14.1$, $P < 0.001$), and this decrease was partially reversed by glucose supplementation ($t(6) = -2.05$, $P = 0.045$; Fig. 5G). Because NAPDH is generated primarily during glucose metabolism and can increase glutathione levels and activate multiple antioxidant enzymes [31], our finding implies that glucose appears to reduce oxidative damage, at least partly, through increasing the cellular energy supply and enhancing cellular antioxidant defense.

### Inhibition of Glucose Uptake Abolishes the Protective Action of Glucose Against Oxidative Stress in HEI-OC1 Cells

GLUT1 is the major glucose transporter across multiple cell types and regulates glucose metabolism by controlling cellular glucose uptake [32]. To explore whether inhibition of glucose uptake can weaken the protective action of glucose against oxidative stress, we conducted GLUT1 inhibition experiments in HEI-OC1 cells. HEI-OC1 is an

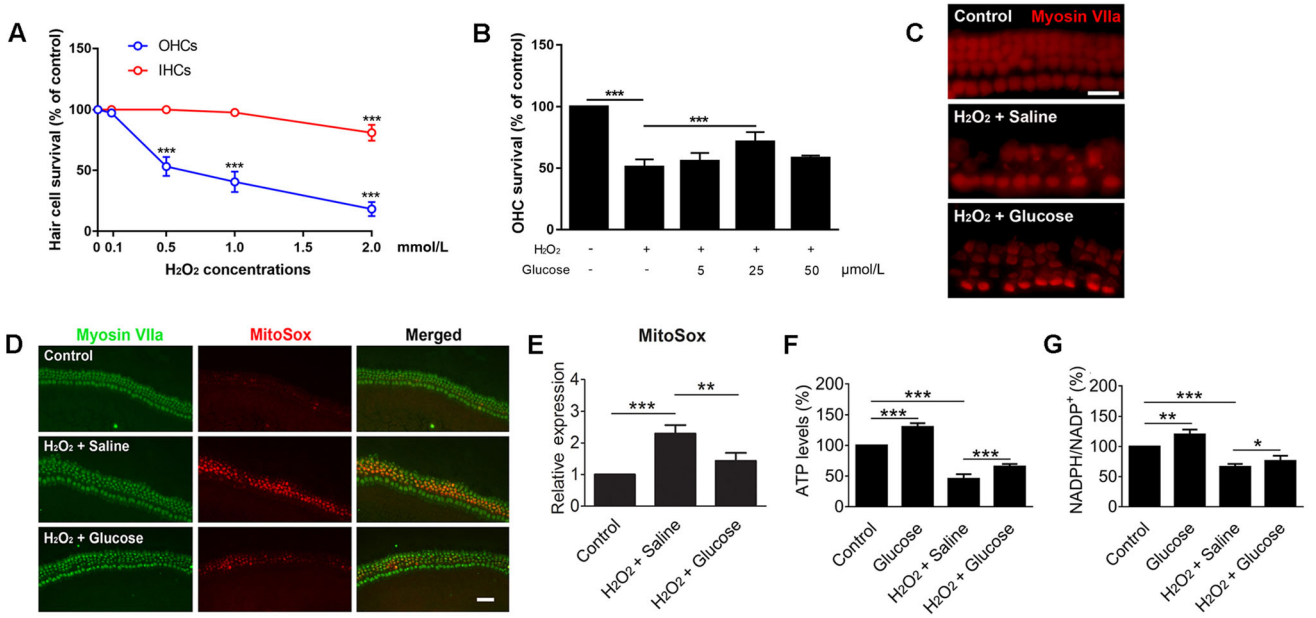


**Fig. 5** Glucose attenuates $H_2O_2$-induced oxidative stress, reduces the $H_2O_2$-induced decreases of ATP and NADPH levels and prevents $H_2O_2$-induced hair cell loss in cochlear explants. **A** Dose-dependent cytotoxic effects of $H_2O_2$ on hair cells in cochlear explants ($n$ = 3; ***$P$ < 0.001 *vs* controls). **B** Cytoprotective effects of glucose at various concentrations on outer hair cells under $H_2O_2$ exposure in cochlear explants (mean ± SD; $n$ = 4 per condition). **C** Immunolabeling for myosin VIIa at the upper-basal turn in cochlear explants for hair cell counts (scale bar, 10 μm). **D** Representative images of immunolabeling for MitoSox Red at the upper-basal turn in cochlear explants (scale bar, 20 μm). **E** Quantification of MitoSox Red immunolabeling reveals significant differences among treatments (mean ± SD; $n$ = 3 per condition). **F** ATP levels in cochlear explants under various conditions (mean ± SD; $n$ = 4 per group). **G** NADPH/$NADP^+$ ratio in cochlear explants under various conditions (mean ± SD; $n$ = 4 per condition). OHCs, outer hair cells; IHCs, inner hair cells; *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001.

inner ear cell line that is widely used in hair cell pathology research [23, 25]. We first determined the HEI-OC1 cell death induced by $H_2O_2$ at various concentrations for different durations. Our results showed that $H_2O_2$ exposure caused significant dose- and time-dependent death of HEI-OC1 cells (Fig. S3). We then chose $H_2O_2$ at 0.8 mmol/L, and a 5-h treatment time followed by 48 h of recovery for subsequent experiments.

We first tested the protective effects of glucose against $H_2O_2$-induced cell death. HEI-OC1 cells were exposed to $H_2O_2$ in combination with glucose at various concentrations from 1 to 100 μmol/L. Intriguingly, glucose exerted a significantly protective action only at 10 and 25 μmol/L ($F(1,29) = 19.207$, $P < 0.001$; Fig. 6A). Then, the GLUT1 inhibitor WZB117 was administered to block glucose uptake. As expected, WZB117 completely abolished the protective effects of glucose (25 μmol/L) against $H_2O_2$-induced death ($t(8) = 6.803$, $P < 0.001$; Fig. 6B). Thus, we conclude that glucose ameliorates $H_2O_2$-induced toxicity in HEI-OC1 cells by increasing glucose metabolism.

## Discussion

Continuous delivery of glucose from blood ensures that the highest energy demand of neurons in the brain is met [16, 33]. Glucose metabolism provides the energy necessary for maintaining physiological brain function through the generation of ATP. A growing body of evidence indicates that impaired glucose metabolism in the brain accounts for several neurological disease, such as neuroglycopenia, epilepsy, Alzheimer's disease, and Parkinson's disease [34–37]. Notably, hearing impairment is common in type 2 diabetes, which is a prevalent metabolic disorder characterized by a decline of pancreatic β-cell function, inability to utilize insulin, impaired glucose tolerance, and hyperglycemia [38]. The hyperglycemic conditions of diabetes are thought to impair cochlear function and cause hearing loss through microangiopathy, advanced glycation end-products, and excessive ROS production [38, 39].

Although persistently high glucose levels have a deleterious impact, a recent study has revealed that short-term glucose supplementation is crucial in tolerance to acute oxidative stress [20]. A recent report showed that the histone methytransferase G9a protects flies against oxidative stress induced by paraquat through inhibiting the over-

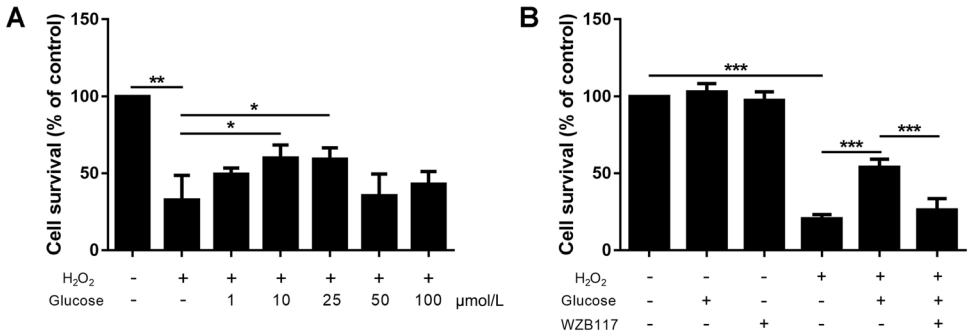


**Fig. 6** GLUT1 inhibition with WZB117 eliminates the protective effects of glucose on oxidative stress in HEI-OC1 cells. **A** Protective effects of glucose at various concentrations against $H_2O_2$-induced cell death ($n = 4$ per condition). **B** Treatment with WZB117 abolishes the protective effects of glucose (25 μmol/L). $n = 4$ per group; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

activation of stress response genes and consequently preventing unnecessary energy consumption during oxidative stress. Moreover, the importance of energy availability during oxidative stress is evidenced by the finding that a high-sugar diet rescues the survival of deficiency of G9a mutants under oxidative stress. Intriguingly, a high-sugar diet is also reported to improve locomotor and lifespan defects in a fly model of amyotrophic lateral sclerosis [40].

Inspired by the findings of this previous work, we explored the possibility that glucose supplementation mitigates NIHL in mice. Here, we found for the first time that administration of glucose shortly before noise exposure significantly attenuated the noise-induced loss of OHCs and IHC synaptic ribbons, as well as NIHL. To be noted, functional deficits measured by ABRs do not correspond to the extent and localization of OHC loss because the ABR threshold does not precisely reflect OHC damage. Actually, although noise exposure always induces a base-to-apex gradient of OHC loss, hearing preservation can be present at both low and high frequencies as measured by ABR, which was evidenced in the present study and previous reports [12, 23, 25]. This discrepancy might be in large part due to the fact that OHCs at the base are more sensitive to noise than those at the apex.

In addition, the levels of oxidative stress markers (4HNE and 3NT) in OHCs increased after noise exposure, while they decreased with glucose treatment. Moreover, glucose supplementation appeared to enhance antioxidant defense (as indicated by an increased SOD1 level) in OHCs during noise exposure. However, changes in these proteins were not found in whole cochlear tissue homogenates. We speculate that the difference in the expression levels of these proteins between the immunolabeled OHCs and immunoblots using whole cochlear tissue homogenates might be due to the fact that multiple cell types (such as spiral ganglion neurons, stria vascularis, and supporting cells) were included in the homogenates, which diluted the changes of protein expression occurring only in OHCs.

The protective effect of glucose was also found in *in vitro* oxidative stress experiments using $H_2O_2$ in cochlear explants. $H_2O_2$-induced oxidative stress and cytotoxicity was significantly reduced in combination with treatment with glucose. Mechanistically, administration of glucose prevented the decrease of ATP and NADPH levels in cochlear explants treated with $H_2O_2$. The cochlea is an energy-demanding organ and depends heavily on efficient ATP production [41, 42]. The levels of ATP in the cochlea are reported to decline dramatically following traumatic noise exposure [17–19]. Consequences of this decrease of ATP are likely to include loss of ion gradients and decreased protein synthesis, which result in cellular dysfunction and eventually in death [43]. On the other hand, NADPH is the electron donor for glutathione generation and thus is crucial for maintaining the antioxidant defense system in various cell types [31]. A recent study has revealed that decreased levels of NADPH cause loss of OHCs and spiral ganglion cells by abnormal ROS accumulation [44]. Last, our findings showed that the protective action of glucose was abolished by the inhibition of GLUT1 in HEI-OC1 cells, further indicating that glucose-conferred protection against oxidative stress is mediated through a direct increase of glucose metabolism, which results in increasing ATP production and enhancing of antioxidant defense.

The mechanisms underlying energy consumption during cellular responses to noise exposure remain incompletely understood. Recently, Ji and colleagues investigated auditory metabolomics during traumatic noise exposure [45] and identified 40 metabolites in the main pathways of central carbon metabolism that are differentially affected by traumatic noise. However, energy-related metabolites were not involved. In addition, noise-induced expression changes in the key enzymes that modulate storage and the utilization of energy still remain elusive. Future studies are needed to further elucidate the characteristics of noise-induced changes in energy metabolism, especially in

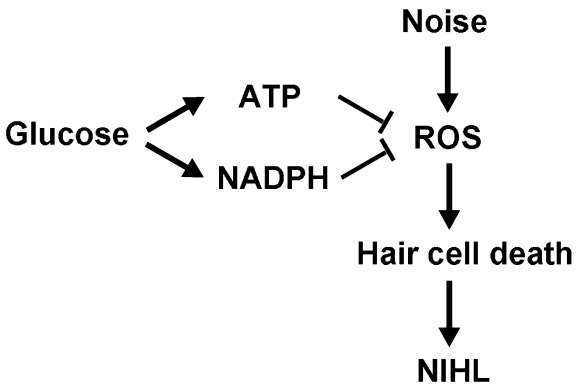


**Fig. 7** Diagram of proposed mechanisms underlying the protective role of glucose against NIHL (ROS, reactive oxygen species; NIHL, noise-induced hearing loss).

glucose metabolism. For example, mice deficient in a rate-limiting enzyme in glycolysis could be generated to test the idea that dysfunctional glucose metabolism underlies the susceptibility to NIHL. Expanding on the present work, it might also be interesting to test whether the simple provision of glucose may promote cochlear hair cell survival and hearing conservation against various hearing loss-relevant insults, such as exposure to ototoxic drugs.

We proposes the immediate provision of accessible energy with glucose as a simple and effective way to prevent noise-induced loss of cochlear hair cells and NIHL. The underlying mechanisms are likely to involve increasing glucose metabolism, resulting in the elevation of ATP levels, which in turn enhance antioxidant defense and consequently reduce oxidative stress (Fig. 7).

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**Conflict of interest** The authors claim that there are no conflicts of interest.

## References

1. Cunningham LL, Tucci DL. Restoring synaptic connections in the inner ear after noise damage. N Engl J Med 2015, 372: 181–182.
2. Henderson D, Bielefeld EC, Harris KC, Hu BH. The role of oxidative stress in noise-induced hearing loss. Ear Hear 2006, 27: 1–19.
3. Brigande JV, Heller S. Quo vadis, hair cell regeneration? Nat Neurosci 2009, 12: 679–685.
4. Fetoni AR, De Bartolo P, Eramo SL, Rolesi R, Paciello F, Bergamini C, *et al.* Noise-induced hearing loss (NIHL) as a target of oxidative stress-mediated damage: cochlear and cortical responses after an increase in antioxidant defense. J Neurosci 2013, 33: 4011–4023.
5. Sha SH, Schacht J. Emerging therapeutic interventions against noise-induced hearing loss. Expert Opin Investig Drugs 2017, 26: 85–96.
6. Yuan H, Wang X, Hill K, Chen J, Lemasters J, Yang SM, *et al.* Autophagy attenuates noise-induced hearing loss by reducing oxidative stress. Antioxid Redox Signal 2015, 22: 1308–1324.
7. Fetoni AR, Paciello F, Rolesi R, Paludetti G, Troiani D. Targeting dysregulation of redox homeostasis in noise-induced hearing loss: Oxidative stress and ROS signaling. Free Radic Biol Med 2019, 135: 46–59.
8. Thorne PR, Nuttall AL, Scheibe F, Miller JM. Sound-induced artifact in cochlear blood flow measurements using the laser Doppler flowmeter. Hear Res 1987, 31: 229–234.
9. Yamane H, Nakai Y, Takayama M, Iguchi H, Nakagawa T, Kojima A. Appearance of free radicals in the guinea pig inner ear after noise-induced acoustic trauma. Eur Arch Otorhinolaryngol 1995, 252: 504–508.
10. Ohlemiller KK, Wright JS, Dugan LL. Early elevation of cochlear reactive oxygen species following noise exposure. Audiol Neurootol 1999, 4: 229–236.
11. Zhang G, Zheng H, Pyykko I, Zou J. The TLR-4/NF-kappaB signaling pathway activation in cochlear inflammation of rats with noise-induced hearing loss. Hear Res 2019, 379: 59–68.
12. Xiong H, Ou Y, Xu Y, Huang Q, Pang J, Lai L, *et al.* Resveratrol promotes recovery of hearing following intense noise exposure by enhancing cochlear SIRT1 activity. Audiol Neurootol 2017, 22: 303–310.
13. Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 2002, 192: 1–15.
14. Yu XJ, Zhao YN, Hou YK, Li HB, Xia WJ, Gao HL, *et al.* Chronic intracerebroventricular infusion of metformin inhibits salt-sensitive hypertension via attenuation of oxidative stress and neurohormonal excitation in rat paraventricular nucleus. Neurosci Bull 2019, 35: 57–66.
15. Liu AH, Chu M, Wang YP. Up-regulation of Trem2 inhibits hippocampal neuronal apoptosis and alleviates oxidative stress in epilepsy via the PI3K/Akt pathway in mice. Neurosci Bull 2019, 35: 471–485.
16. Mergenthaler P, Lindauer U, Dienel GA, Meisel A. Sugar for the brain: the role of glucose in physiological and pathological brain function. Trends Neurosci 2013, 36: 587–597.
17. Chen FQ, Zheng HW, Hill K, Sha SH. Traumatic noise activates Rho-family GTPases through transient cellular energy depletion. J Neurosci 2012, 32: 12421–12430.
18. Munoz DJ, Kendrick IS, Rassam M, Thorne PR. Vesicular storage of adenosine triphosphate in the guinea-pig cochlear lateral wall and concentrations of ATP in the endolymph during sound exposure and hypoxia. Acta Otolaryngol 2001, 121: 10–15.
19. Vlajkovic SM, Housley GD, Munoz DJ, Robson SC, Sevigny J, Wang CJ, *et al.* Noise exposure induces up-regulation of ecto-nucleoside triphosphate diphosphohydrolases 1 and 2 in rat cochlea. Neuroscience 2004, 126: 763–773.
20. Riahi H, Brekelmans C, Foriel S, Merkling SH, Lyons TA, Itskov PM, *et al.* The histone methyltransferase G9a regulates tolerance to oxidative stress-induced energy consumption. PLoS Biol 2019, 17: e2006146.
21. Xiong H, Pang J, Yang H, Dai M, Liu Y, Ou Y, *et al.* Activation of miR-34a/SIRT1/p53 signaling contributes to cochlear hair cell apoptosis: implications for age-related hearing loss. Neurobiol Aging 2015, 36: 1692–1701.
22. Xiong H, Long H, Pan S, Lai R, Wang X, Zhu Y, *et al.* Inhibition of histone methyltransferase G9a attenuates noise-induced cochlear synaptopathy and hearing loss. J Assoc Res Otolaryngol 2019, 20: 217–232.

23. Xiong H, Chen S, Lai L, Yang H, Xu Y, Pang J, *et al.* Modulation of miR-34a/SIRT1 signaling protects cochlear hair cells against oxidative stress and delays age-related hearing loss through coordinated regulation of mitophagy and mitochondrial biogenesis. Neurobiol Aging 2019, 79: 30–42.
24. Benkafadar N, Francois F, Affortit C, Casas F, Ceccato JC, Menardo J, *et al.* ROS-induced activation of DNA damage responses drives senescence-like state in postmitotic cochlear cells: implication for hearing preservation. Mol Neurobiol 2019, 56: 5950–5969.
25. Wu F, Xiong H, Sha S. Noise-induced loss of sensory hair cells is mediated by ROS/AMPKalpha pathway. Redox Biol 2020, 29: 101406.
26. Sha SH, Zajic G, Epstein CJ, Schacht J. Overexpression of copper/zinc-superoxide dismutase protects from kanamycin-induced hearing loss. Audiol Neurootol 2001, 6: 117–123.
27. Keithley EM, Canto C, Zheng QY, Wang X, Fischel-Ghodsian N, Johnson KR. Cu/Zn superoxide dismutase and age-related hearing loss. Hear Res 2005, 209: 76–85.
28. Ohlemiller KK, McFadden SL, Ding DL, Flood DG, Reaume AG, Hoffman EK, *et al.* Targeted deletion of the cytosolic Cu/Zn-superoxide dismutase gene (Sod1) increases susceptibility to noise-induced hearing loss. Audiol Neurootol 1999, 4: 237–246.
29. Ren Y, Li Y, Yan J, Ma M, Zhou D, Xue Z, *et al.* Adiponectin modulates oxidative stress-induced mitophagy and protects C2C12 myoblasts against apoptosis. Sci Rep 2017, 7: 3209.
30. Choi HI, Kim HJ, Park JS, Kim IJ, Bae EH, Ma SK, *et al.* PGC-1alpha attenuates hydrogen peroxide-induced apoptotic cell death by upregulating Nrf-2 via GSK3beta inactivation mediated by activated p38 in HK-2 Cells. Sci Rep 2017, 7: 4319.
31. Fan J, Ye J, Kamphorst JJ, Shlomi T, Thompson CB, Rabinowitz JD. Quantitative flux analysis reveals folate-dependent NADPH production. Nature 2014, 510: 298–302.
32. Macintyre AN, Gerriets VA, Nichols AG, Michalek RD, Rudolph MC, Deoliveira D, *et al.* The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function. Cell Metab 2014, 20: 61–72.
33. Howarth C, Gleeson P, Attwell D. Updated energy budgets for neural computation in the neocortex and cerebellum. J Cereb Blood Flow Metab 2012, 32: 1222–1232.
34. Pascual JM, Wang D, Hinton V, Engelstad K, Saxena CM, Van Heertum RL, *et al.* Brain glucose supply and the syndrome of infantile neuroglycopenia. Arch Neurol 2007, 64: 507–513.
35. Garriga-Canut M, Schoenike B, Qazi R, Bergendahl K, Daley TJ, Pfender RM, *et al.* 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. Nat Neurosci 2006, 9: 1382–1387.
36. Kapogiannis D, Mattson MP. Disrupted energy metabolism and neuronal circuit dysfunction in cognitive impairment and Alzheimer's disease. Lancet Neurol 2011, 10: 187–198.
37. Borghammer P, Hansen SB, Eggers C, Chakravarty M, Vang K, Aanerud J, *et al.* Glucose metabolism in small subcortical structures in Parkinson's disease. Acta Neurol Scand 2012, 125: 303–310.
38. Frisina ST, Mapes F, Kim S, Frisina DR, Frisina RD. Characterization of hearing loss in aged type II diabetics. Hear Res 2006, 211: 103–113.
39. Gupta S, Eavey RD, Wang M, Curhan SG, Curhan GC. Type 2 diabetes and the risk of incident hearing loss. Diabetologia 2019, 62: 281–285.
40. Manzo E, Lorenzini I, Barrameda D, O'Conner AG, Barrows JM, Starr A, *et al.* Glycolysis upregulation is neuroprotective as a compensatory mechanism in ALS. Elife 2019, 8. https://doi.org/10.7554/elife.45114.
41. Fettiplace R, Hackney CM. The sensory and motor roles of auditory hair cells. Nat Rev Neurosci 2006, 7: 19–29.
42. LeMasurier M, Gillespie PG. Hair-cell mechanotransduction and cochlear amplification. Neuron 2005, 48: 403–415.
43. Butterfield DA, Halliwell B. Oxidative stress, dysfunctional glucose metabolism and Alzheimer disease. Nat Rev Neurosci 2019, 20: 148–160.
44. Kim YR, Baek JI, Kim SH, Kim MA, Lee B, Ryu N, *et al.* Therapeutic potential of the mitochondria-targeted antioxidant MitoQ in mitochondrial-ROS induced sensorineural hearing loss caused by Idh2 deficiency. Redox Biol 2019, 20: 544–555.
45. Ji L, Lee HJ, Wan G, Wang GP, Zhang L, Sajjakulnukit P, *et al.* Auditory metabolomics, an approach to identify acute molecular effects of noise trauma. Sci Rep 2019, 9: 9273.