



Microplastic polyethylene induced inner ear dysfunction in murine model

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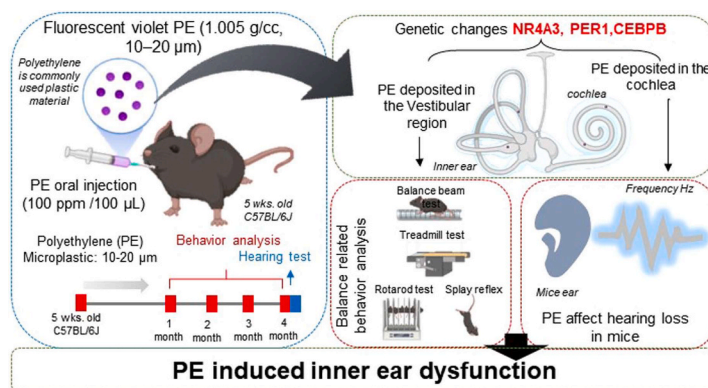
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HIGHLIGHTS

- Microplastic polyethylene induced hearing loss and balance defects in a murine model.
- Upregulated gene expression of PER1, NR4A3 and CEBPB with polyethylene exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

While the hazardous effects of microplastics (MPs) are increasingly reported, it remains uncertain if MPs induce inner ear dysfunction. Nonetheless, prevalence of inner ear dysfunction was observed across all age groups. In this study, we investigated whether MP polyethylene affect inner ear function in a murine model. To detect hearing loss and balance defect after polyethylene (PE) exposure, we evaluated hearing threshold levels, assessed cerebral glucose metabolism, conducted transcriptome analysis, and performed behavioral studies. C57BL/6 J mice (5-week-old) were grouped into control (n = 10) and PE-fed groups (n = 10). Mice were orally administered 100 ppm/100 μL (equivalent to 10 μg) of PE every day for 4 months. We identified the accumulation of PE in the cochlea and vestibular region. The fragmented PE in inner ear was $3.00 \pm 0.38 \mu\text{m}$ in size; the administered PE concentration was $1.14 \pm 1.06 \text{ mg/g}$. Fourier transform infrared spectrometry confirmed that the properties of the MP were identical with those of PE fed to the mice. Transcriptomic analysis showed up-regulation of PER1,

Abbreviations: MP, microplastic; PE, polyethylene; FDG, ¹⁸F- fluorodeoxyglucose.

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NR4A3 and CEBPB at the PE exposed inner ear tissue and it was confirmed using qRT-PCR, western blotting, and immunofluorescence staining. We observed abnormalities in balance related behavior assessment in the PE group. Exposure to PE increased the hearing thresholds and decreased glucose metabolism in the bilateral lateral entorhinal cortex, right primary auditory cortex, and right secondary auditory cortex. We can conclude that PE exposure induced inner ear dysfunction such as hearing loss and balance disorder.

1. Introduction

Microplastics (MPs) with a diameter of < 5 mm are recognized as a new environmental threat; they pose a human health risk and MP contamination of food can no longer be ignored [1–3]. The hazardous effects of MP are increasingly reported. MP is a ubiquitous global contaminant identified from seawater, sediment, public drinking water, feeding bottles and marine organisms [4–9]. The potential global exposure of infants up to 12 months of age in 48 regions range from 14, 600–4,550,000 particles per capita per day [4]. A median concentration of 20 MP (50 to 500 μm in size) per 10 g of human stool was identified in eight volunteers residing in Japan, Russia, the Netherlands, the United Kingdom, Italy, Poland, Finland, and Austria, representing different geographic regions and dietary patterns [10]. Based on a human health risk assessment of MP, MPs detected in seafood and the chemical monomers contained in MP could have a serious pollutants [11].

The inner ear is a minute, complex, fluid-filled structure surrounded by a bony labyrinth and located deep in the temporal bone. The cochlea corresponds to the acoustic end organ, and the vestibular end organs consist of the three semicircular canals with their ampullary tissue, the saccule and the utricle. Inner ear problem was common in any age group, even in young adults and adolescents [12]. The prevalence of inner ear problem such as hearing loss and balance defects increased rapidly with age [12]. Hearing loss was the most common sensory impairment in humans. In 2019, 19.3 % of the world population had hearing loss, and 5.1 % of the population showed over moderate hearing loss (over 35 dB HL) [13]. The lifetime prevalence of vestibular vertigo was 7.8 % [14].

Hearing loss can have a significant influence on an individual's ability to enter the workforce. Hearing-impaired individuals have disadvantages in job seeking or self-selecting out of employment [15]. People with hearing loss typically experience a higher unemployment rate, lower earning potential, earlier retirement, greater absenteeism, reduced productivity, and greater stress related to the hearing impairment [15,16]. There is evidence that hearing loss, and its impact on education, communication, and financial success, can affect overall quality of life [15,17–19]. It is an important health problem in the elderly, and 40 % of the population aged > 65 years have a hearing loss great enough to impair communication [20]. Between 1988–1994 and 2005–2006, there was a significant increase in the prevalence of any hearing loss among adolescents in the United States, rising from 14.9 % to 19.5 % [21]. Ototraumatic agents, such as noise, chemicals, and heavy metals, are pervasive pollutants, mostly produced by human activity, and are critical factors in inducing acquired of inner ear problem such as hearing loss and balance defects [22,23]. Developmental cadmium exposure disrupted zebrafish vestibular calcium channels, interfering with otolith formation and inner ear function [24].

The estimate of concentration with the actual exposure of the MP to human is limited due to the variability of the size, shape, and type of MP. Ingestion of MP is though the primary route of exposure to human besides inhalation and dermal penetration [25]. This finding suggests the potential translocation of MP from the environment into systemic circulation [25]. Subsequently, these circulating MP are transported to distant tissues including the inner ear. In our previous study, we showed MP transited to brain, which was confirmed positron emission tomography (PET) with radioisotopes ^{64}Cu [26,27]. We also identified that MP could cause autism spectrum disorder in a murine model [28]. However, no research has conclusively established the presence of MP in the inner

ear. It remains unknown whether inner ear problems, including hearing loss and balance disorders, are caused by MP. In this study, we investigated whether MP polyethylene (PE) was deposited in the inner ear and later induced inner ear problems such as hearing loss and balance disorders using a murine model. To find evidence of inner ear problems following PE exposure, we evaluated the hearing threshold levels, molecular imaging, transcriptome analysis, and behavioral studies using murine model.

2. Material and methods

2.1. Animal model and PE feeding

C57BL/6 J mice (Shizuoka Laboratory Center, Japan) were used in the experiments. Mice were maintained in temperature-controlled clean racks with a 12 h light/dark cycle. The animals went through a period of adaptation for a week before the experiments. Mice were grouped into phosphate-buffered saline (PBS) control ($n = 10$) and PE groups ($n = 10$). PE (1.005 g/cc, 10–20 μm , 0.1 g; Cosphereic LLC, Santa Barbara, USA) were reconstituted in Tween-80 (Sigma, USA) to make a 10 % weight/volume stock solution. The stock solution was homogenized using a Q-Sonica homogenizer (Newtown, USA). A total of 100 ppm / 100 μL (10 μg) of PE was orally administered to mice every day for 4 months. We used age-matched control mice that were orally administered with 100 μL of PBS. A sterile single-use animal feeding needle (size: 20 $\times$ 1–1/2", Cadence Science, Japan) was used. All animal experiments were performed according to the institutional guidelines of the Korea Institute of Radiology and Medical Sciences (KIRAMS). All protocols were approved by the Institutional Animal Care and Use Committee (IACUC number: KIRAMS 2022–0079).

2.2. PE concentration

To determine the concentration of PE in the cochlea, mice were euthanized using CO_2 . HCl (2 ml, 30 % v/v). The concentrations of PE were determined using a fluorescent spectrophotometer (i3X, Molecular Devices, USA; excitation = 420 nm, emission = 520 nm). A standard curve was generated using serial dilutions of fluorescent PE suspensions from 500 to 0.977 ppm.

2.3. Hearing evaluation

Each mouse underwent auditory brainstem response (ABR) testing before study. Before checking ABR, the mice were anesthetized with isoflurane (Hana pham Co., Republic of Korea), and the ABR tests were performed in a sound-attenuating chamber. The ABR thresholds at clicks and tone-burst sounds were detected using subdermal needle electrodes (SmartEP, Intelligent Hearing System; Miami, FL, USA).

2.4. Regional glucose metabolism evaluated using ^{18}F -FDG PET

PET is a powerful tool for the in vivo monitoring of biological effects. In our previous studies, analysis using ^{18}F -fluorodeoxyglucose (FDG) PET indicated decreased cerebral glucose metabolism in the auditory cortex during hearing loss [29,30]. Siemens Inveon PET was used in this study. ^{18}F -FDG PET scanning was performed to assess cerebral glucose metabolism. Before PET scanning, the mice were fasted for at least 8 h, after which they were anesthetized with 2 % isoflurane in 100 % oxygen

(Forane solution; Choong Wae Pharma, Seoul, Republic of Korea). After injection of ^{18}F -FDG (18.5 MBq /100 g body weight), 40 min of emission PET data were acquired. Emission list-mode data were sorted into 3D sinograms and reconstructed using 3D reprojection algorithms. The image matrix was $512 \times 512 \times 159$, the pixel size was $0.046 \text{ mm} \times 0.046 \text{ mm}$, and the slice thickness was 0.796 mm . The regional differences in cerebral glucose metabolism between the groups were analyzed using voxel-wise statistical analysis using SPM 8 (<http://www.fil.ion.ucl.ac.uk/spm>). For SPM analysis, the brain region of interest was extracted, and a study-specific mouse brain template was constructed [29]. Individual PET data were spatially normalized using affine and non-linear transformations onto a study-specific mouse brain template and then overlaid onto a realigned TPM mouse brain template. The voxel size of spatially normalized images was $0.3 \text{ mm} \times 0.3 \text{ mm} \times 0.3 \text{ mm}$. A 3 mm-gaussian smoothing kernel was applied to enhance the signal-to-noise ratio. Count normalization was performed. Two-sample *t*-tests were used to identify regional differences between groups with a threshold of $p < 0.005$ (uncorrected).

2.5. Tissue preparation

After checking the auditory brainstem response, the mice were deeply anesthetized and transcardially perfused with phosphate-buffered saline (PBS, pH7.2) and then with 4 % paraformaldehyde (PFA) in PBS. The temporal bones containing the inner ear were immediately dissected. The oval and round windows were punched, the inner ear was gently perfused with 4 % PFA in PBS and post-fixed for 24 h at 4°C . After fixation, the samples were incubated in 10 % ethylene diamine tetra acetic acid (EDTA, Sigma-Aldrich) (pH 7.2–7.4) in PBS at 4°C for decalcification. To determine if the sample is adequately decalcified, the inner ear was gently pressed with the forceps. A spongy tissue indicates that the decalcification is complete. The decalcified samples were washed in PBS.

2.5.1. Cochlear surface preparation

Cochlear whole mounts were prepared to examine the morphology of the organ of Corti in the cochlea. The fixed and decalcified cochleae were micro-dissected and cut below the basal turn to separate them from the vestibular organs. The spiral ligament, lateral wall, and Reissner's membrane were carefully cut to ensure that the organ of Corti was visible. Several cuts were made to reduce the thickness of the spiral ganglion axons and to make the turn as flat as possible. The dissected pieces were stained with 4'-6'-amino-2-phenylindole (DAPI 1:5000; Invitrogen, USA). They were mounted on silane-coated micro slides and the images were acquired with a confocal microscope (LSM900, Carl Zeiss). Cochlear whole mounts (two mice per group) were prepared to examine the morphology of the outer hair cells of the cochlea. We compared the amount of PE in the area of apex, middle and base in the cochlear.

2.5.2. Immunofluorescence (IF) analysis

For IF, each mouse was transcardially perfused with PBS and then with 4 % PFA. Inner ear specimens were extracted from the mice. The harvested inner ear was post-fixed in 4 % PFA for 24 h at 4°C and decalcified in 10 % EDTA. The samples were immersed in 10 %, 20 %, and 30 % sucrose solutions at 4°C until they became submerged and then embedded in an OCT compound. The frozen tissues were sectioned at a thickness of $10 \mu\text{m}$, at approximately the mid-modiolar plane using a cryostat microtome. The sections were blocked using 10 % normal donkey serum (Abcam, USA) in 0.3 % Triton X-100 for 1 h at room temperature. Tissue sections were incubated for 1 h at RT with Period circadian protein homolog 1 (PER1) (PER1 1:100; Finetest, USA), nuclear receptor subfamily 4 group A member 3 (NR4A3) (NUR77 1:100; Abcam, UK), or CCAAT Enhancer Binding Protein Beta (CEBPB) (C/EBP beta 1:100; Mybiosource, USA) as the primary antibody (Table 1). The sections were then treated with the Alexa Fluor 488-conjugated donkey

Table 1

List of antibody details.

Antibody	Source	Cat. No.
anti-PER1 antibody	FineTest, USA	FNab06311
NOR1 Polyclonal Antibody	Bioss, USA	BS-11903R
Phospho-C/EBP-beta (Thr235/188) antibody	Mybiosource, USA	MBS9600973
Recombinant Anti-CEBP Beta antibody [E299]	Abcam, UK	ab32358
Anti-NUR77 antibody	Abcam, UK	ab153914
Anti β -actin	Abcam, UK	ab6276
Alexa Fluor 488 donkey anti-rabbit IgG	Invitrogen, USA	A21206

anti-rabbit (1:500; Invitrogen, USA) as the secondary antibody (Table 1) and were mounted using DAPI mounting solution (GBI Labs, WA, USA). Visualization of the slides was performed using a laser scanning confocal microscope (LSM900, Carl Zeiss, Germany).

2.5.3. PE concentration in the inner ear

To identify the concentration of PE in the inner ear after feeding PE for 1 week, the mice ($n = 5$) were euthanized using CO_2 . HCl (2 ml, 30 % v/v) was used to treat 0.1 g at room temperature overnight. The concentrations of MPs in different tissues were determined using a

spectrophotometer (i3X, Molecular device, USA; excitation = 420 nm, emission = 520 nm). A standard curve was generated using serial dilutions of the fluorescent PE suspensions from 500 to 0.977 ppm.

2.5.4. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) images ($\times 195$ and $\times 500$) were obtained using an EVO10 microscope (Zeiss, Germany).

2.5.5. Fourier transform infrared spectrometer (FT-IR)

A Fourier transform infrared spectrometer (FT-IR, Alpha II, Bruker, MA, USA) was used to determine whether the PE detected in the inner ear had the same properties as the PE fed to mice. To confirm the FT-IR peak of PE (after feeding), the results were compared with those of PE (before feeding) without conjugation with a fluorophore. We extracted the inner ear from PE-fed mice and cultured them in 36 % HCl solution 48 h with gentle shaking to collect PE from them. The pH of the solution was adjusted to between 3 and 7 by titration with 10 % NaOH (ACS reagent, Sigma Aldrich, USA). After titration, the solution was filtered through a $149 \mu\text{m}$ PP filter (pore size: $149 \mu\text{m}$, Spectrum™, USA) to remove any undissolved contaminants. Any remaining organic matter was then lysed by incubating the solution in 12 % H_2O_2 solution (DAEJUNG, Republic of Korea) at 60°C for 24 h. The solution was then filtered through a $0.22 \mu\text{m}$ vacuum filter (Corning, USA), and the substances that did not pass through were collected. The collected substances were washed three times with pure ethanol (ACS reagent, Sigma Aldrich, USA) by centrifugal separation.

FT-IR spectroscopy was performed. Typical PE peaks at 2910 cm^{-1} and 1463 cm^{-1} were observed in all the samples.

2.5.6. PE accumulation in the inner ear

To identify whether PE accumulated into the ear after PE feeding, mice were perfused with 4 % PFA in PBS, and the inner ears were dissected with 4 % PFA in PBS. The ears were lyophilized overnight, and the ear samples were treated with $\text{HNO}_3 + \text{H}_2\text{O}_2$ at $80\text{--}90^\circ\text{C}$ overnight. Non-lysed tissue fragments were removed after neutralization with NaOH and ddH_2O . The sample was filtered and attached to a 0.45 nitrocellulose membrane (Millipore, USA) with a vacuum pump. The sample was examined using a laser scanning confocal LSM-880 microscope (Carl Zeiss, Germany) with a $40\times$ objective lens and fluorescent filter (ex = 488 nm, em = 526 nm). The size measurement of the deposited particles was carried out using line tool of Zeiss software (ZEN ver 2.3, Carl Zeiss NTS Ltd, Germany). The scale bar was set initially, and measurements were performed for each detected particle using the line

tool.

2.5.7. Tissue clearing

To make the inner ears transparent, mice were transcardially perfused with PBS and then fixed with 4 % PFA in PBS. Inner ears were dissected from each mouse and incubated in 4 % PFA for 24 h at 4 °C. The fixed samples were rinsed with PBS. The clearing of the inner ear requires the decalcification of the bone as calcified bone is a strong light scatterer. Therefore, the samples were incubated in 10 % EDTA (pH 7.2–7.4) until the tissues were soft. Subsequent transparency processing was performed using SmartBatch+ equipment following the Lifecanvas technologies protocol guidelines (Ver. 5.0.6). Transparency images were obtained from upright confocal microscope (C2 +, Nikon, USA) and lightsheet7 (Carl Zeiss, Germany).

2.6. Behaviors assessment of balance

Behavior study for evaluating the balance, coordination, and motor stability in mice (5-week-old, control; $n = 10$, PE fed; $n = 10$) were conducted following the behavioral test. A PE dose of 100 ppm/ 100 μ L was fed to the mice orally for up to 3 or 4 months, or a dose of 1000 ppm/100 μ L (100 μ g) was fed to the mice orally for 1 month, for confirming an acute response (Fig S1).

2.6.1. Treadmill test

The accelerated treadmill test was conducted to test the motor function and stability at 1, 2, 3 months exposure each [31]. The test was performed by training the mice to briefly explore the treadmill and to experience at least one shock from the grid while running on the treadmill (Jeungdo Bio & Plant Co.Ltd, Seoul, Republic of Korea) at a constant speed of 10 m /min for 5 min. The shock intensity and frequency were fixed at 200 msec/pulse and 3 Hz. The test was conducted on the following day with an acceleration at every 2 min, from 12–46 m / min until the mice became unstable enough to run. The criterion for instability was defined in terms of the inability of the mice to run on the treadmill, as spending time on the shock grid for 5 continuous seconds. The run time, distance travelled, and the max speed tolerated for each mouse were recorded in both groups.

2.6.2. Rotarod test

Rotarod test was conducted for the assessment of balance and motor coordination at 1, 2, 3 months exposure each [32]. Mice were placed on the rotating rod of the rotarod (Jeungdo Bio & Plant Co.Ltd, Seoul, Republic of Korea) at a constant speed of 10 rpm for 5 min. The test was conducted with acceleration increasing every minute from 10–50 rpm; the cumulative number of falls of the mice was recorded. An average of three readings was recorded.

2.6.3. Grip strength test

For the grip strength test, the mice were assessed for forelimb and total paw strength in maintaining balance using their muscle strength and ability to grasp the grid while being suspended from their tail [33–35]. The mice from each group were placed on the grid (10 cm $\times$ 10 cm) by holding their tail and were allowed to grasp the grid with their forelimb. They were then slightly pushed backward until the mice lost its grip. The grip meter (Jeungdo Bio & Plant Co.Ltd, Seoul, Republic of Korea) was used to record the peak kilogram-force (Kgf). Similarly, total force was assessed by allowing the four paws to grasp the grid; the mice were pulled backward via the tail and the peak Kgf was recorded. The average of three consecutive measurements for each mouse were recorded.

2.6.4. Balance beam test

Fine motor coordination and balance, which involves the vestibular system, is evaluated using the balance beam assay, as described previously [36,37]. The mice from each group were trained for the beam

walk. The arrangement consists of a 10 mm round beam (Jeungdo Bio & Plant Co.Ltd, Seoul, Republic of Korea) about 50 cm height from the ground attached to two poles. The beam was designed with stage at one pole for the mice to start from and with a dark box with food from the nesting material at the other pole to attract the mice to the finish point. The time taken to cross the beam and the number of paw slips were recorded. The average of the three consecutive walks for each mice were calculated.

2.6.5. Splay reflex

Splay reflex is a neurophysiological response or proprioception that involves the extension of the limb in response to external stimuli [38]. The splay reflex is a protective mechanism that helps mice maintain balance and stability. We used modified splay reflex scores to highlight the disease onset. The mice were hanged by their tail, and the degree of hindlimb splay was assessed for neurological scoring on a scale of 0–3. The neurological score of 0 (Normal, 80–90 degrees splay to lateral midline) was assigned for full extended splay of the mice from the lateral midline for 2 s or longer. A score of 1 (early symptoms, 55–45 degrees splay to lateral midline) was assigned for the abnormal splay of the hindlimb upon suspending the mice from its tail; either the hindlimbs fully or partially collapse during suspension, trembling during tail suspension, or retraction/clasping. A neurological score of 2 (Onset of abnormal stability, 25–15 degrees splay to lateral midline) was assigned for the abnormal splay of the hindlimb upon suspending the mice from its tail; the hindlimbs fully or partially collapse during suspension, trembling during tail suspension, or exhibiting retraction/clasping. A neurological score of 3 (Abnormal stability, 10–0 degrees splay to lateral midline) was assigned for the abnormal splay of the hindlimb upon suspending the mice from its tail; the hindlimbs fully or partially collapse during suspension, trembling during tail suspension, or exhibiting retraction/clasping. The readings are an average of three consecutive takes for each mouse.

2.7. RNA extraction

For tissue collection, mice were decapitated under deep anesthesia, and both inner ears were dissected from the temporal bone in RNAlater solution (Invitrogen, USA); they were rapidly frozen on dry ice and stored at -80°C . Total RNA from the tissues was extracted using the RNeasy Mini Kit (Qiagen, Germany), following the manufacturer's instructions. The quantity and quality of the total RNA were determined using UV/Vis spectrophotometer (NanoDrop 2000, ThermoFisher Scientific, USA). All RNA samples showed suitable A260/A280 and 28 s/18 s ratios; they were stored at -80°C .

2.7.1. Transcriptome analysis

RNA-sequencing (RNA-Seq) was performed to compare the transcriptomes of the control ($n = 4$) and PE-exposed groups ($n = 4$). Analysis of transcriptome RNA sequencing was performed using the previously prepared RNA. Library preparation involves fragmentation of nucleic acids, adapter ligation, and amplification. Library quantification and size distribution analysis are conducted to ensure optimal quality. Subsequently, cluster generation is performed, wherein library fragments are immobilized onto a flow cell surface and subjected to bridge amplification to generate clusters of identical DNA fragments. NGS was performed using an Illumina NovaSeq 6000 system (Macrogen, Republic of Korea). Base calling converts raw signals into nucleotide sequences, producing FASTQ files containing sequencing reads and quality scores. Data analysis involves quality control checks, alignment to a reference transcriptome, and differential gene expression analysis for RNA-seq data. All top-ranked gene sets were manually curated to confirm their accurate functional and pathway categorizations. The annotated data were filtered for the log2 fold change difference of 2 for up and down regulated gene (FDR adjusted $P < 0.05$).

2.7.2. Real-time quantitative polymerase chain reaction

After the transcriptome analyses, representative genes were selected for qPCR. Total RNA samples (Control: n = 4, PE: n = 3) were diluted in RNase-free dH₂O (ThermoFisher Scientific, USA). Complementary DNA was synthesized using the SuperScript™ IV cDNA Synthesis Kit (Invitrogen, USA), according to the manufacturer's specifications. qPCR was performed on an ABI qPCR system (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Applied Biosystems, USA), following the manufacturer's instructions. The reaction conditions were: 10 min at 95 °C; 50 cycles of 95 °C for 15 s and 60 °C for 60 s. The primer (BIONEER, Republic of Korea) sequences are shown in Table 2. The mRNA concentrations were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as the endogenous control (Schmittgen and Livak, 2008). Relative quantity (RQ) values for mRNA expression were calculated using the equation $RQ = 2^{-\Delta\Delta C_t}$ analysis method.

2.8. Western blotting

Cell extracts were obtained using 1 × extraction buffer (Abcam, UK) containing extraction enhancer buffer (Abcam, UK). The ear tissues from the mice were first cut into small pieces and then lysed through homogenization and sonication in 1 × extraction buffer (Abcam, UK) containing 1 × extraction enhancer buffer (Abcam, UK). Final protein concentrations were determined using a BCA assay (ThermoFisher Scientific, USA), according to the manufacturer's instructions. SDS polyacrylamide gels (10 %) were loaded with 50 µg total protein per lane. Pre-stained protein molecular weight markers (ThermoFisher Scientific, USA) were used as standards. The electrophoresed samples were transferred to PVDF membranes. After transferring using the iBlot2 dry blotting system (ThermoFisher Scientific, USA), the membrane was blocked with 5 % blotting-grade blocker (BIORAD, USA) in 1 × TBST (ThermoFisher Scientific, USA) for 1 h at room temperature (RT). The membrane was then incubated with diluted primary antibodies (Table 1) overnight at 4 °C, rinsed and washed thrice, and incubated with the appropriate HRP-conjugated secondary antibody (Table 1) for 1 h at RT. The membrane was washed and rinsed as previously described. The expressed proteins were detected using the pierce ECL western blotting substrate (Thermo Fisher Scientific, USA), and membranes were visualized using the Amersham Imager 600 (GE Healthcare Life Sciences, USA) documentation system.

2.9. Statistical analysis

All statistical analyses were performed using PRISM software (version 5.0, GraphPad Software; San Diego, CA, USA). Data are expressed as mean ± SD. All data were normally distributed. A two-tailed Student's *t*-test was used. The * denotes *P* < 0.05, ** denotes *P* < 0.01, *** denotes *P* < 0.005. For the presentation of SPM results, the *T* value was determined using two-tailed Student's *t*-tests and overlaid on the TPM mouse brain template. *P*-values < 0.005 were considered significant in the SPM analysis.

Table 2
List of primer details.

Gene	Source	Forward (5' → 3')	Revers (5' → 3')	Product length
PER1	BIONEER, Rep. of Korea	AGATCAACTGCCTGGACAGC	CTGCCCTCTGCTTGTCATCA	132
NR4A3	BIONEER, Rep. of Korea	GTGTCTCAGTGTCTGGGATGG	TTTGGAAGGCAGACGACCTC	79
CEBPB	BIONEER, Rep. of Korea	GACAAGCTGAGCGACGAGTA	TGCTTGAAACAAGTTCGCAG	197
GAPDH	BIONEER, Rep. of Korea	CAAGAAGGTGGTGAAGCAGG	AGGTGGAAGAGTGGGAGTTG	110

3. Results

3.1. PE is deposited in the inner ear

We identified the evidence of MP accumulation in the inner ear (Fig. 1A-C). When 100 ppm/100 µL of PE was fed orally for 4 months, PE accumulated in the spiral ganglion area of the cochlea (Fig. 1A, B) and vestibular region (Fig. 1C). PE were observed at ampulla of semicircular canal as reconstructed 3D image using confocal microscopy (Supplementary video 1). We found 34 PE particles across 11 cochleae. Specifically, there were 26 ea (2.6 PE particles per cochlea) in the basal turn, 2 ea (0.2 PE particles per cochlea) in the mid-turn, 3 ea (0.3 PE particles per cochlea) in the apical turn and 3 ea (0.3 PE particles per cochlea) in the modiolus. Interestingly, the highest amount of PE was found in the basal turn, presumably due to extravasation of PE from arteries in the basal turn area, as blood is supplied to the basal turn first in the artery in cochlea. The original size of the PE, measured using confocal microscopy, was 20.32 ± 3.81 µm. MP can be fragmented after digestion [39]. The concentration of PE was 1.14 ± 1.06 mg/g in the inner ear (Fig. 1D). SEM images (×195 and ×500) of PE were shown in Fig. 1E. FT-IR demonstrated that the PE detected from the inner ear had the same properties as the PE fed to the mouse. Typical PE peaks, at 2910 cm⁻¹ and 1463 cm⁻¹, were observed in the lysed inner ear (Fig. 1F). The representative PE images from confocal microscopy were shown in Fig. 1G. The size of PE was 20.17 ± 2.74 µm before feeding and 6.37 ± 3.28 µm in inner ear (Fig. 1G), indicating that the PE fragments had transitioned to the cochlea after digestion. Fig. 1H shows pre- and post-images of the entire cochlea and vestibular regions following tissue clearing. Tissue clearing and three-dimensional imaging clearly showed the deposited PE in the whole cochlea and vestibular regions, determined by light sheet microscopy (Fig. 1H, I and supplementary video 2).

3.2. Decreased sound pressure level in PE-fed mice

Hearing loss in PE-fed mice was evaluated. The mean threshold of the PE group using click sound increased significantly compared to that in the control group in the right ear (54.0 dB sound pressure level for PE group and 31.7 dB sound pressure level for control group, ** *P* < 0.01, Fig. 2A). In the control group, the mean thresholds using 8 kHz and 16 kHz tone burst stimuli were 28.3 dB in the right ear and 40 dB in the left ear. In the PE group, the mean thresholds at 8 kHz and 16 kHz were 63 dB and 64 dB in the right ear and 62 dB and 65 dB in the left ear. Tone burst stimuli using 8 kHz and 16 kHz showed significant differences in both ears between the two groups (***) *P* < 0.005, Fig. 2A).

3.3. Decreased motor function and balance stability in PE-fed mice

Behavior tests were employed for assessing balance stability (Fig. 3A). Mice fed PE (100 ppm /100 µL) continuously showed a decrease in the auditory and motor function. Accelerated treadmill test was employed to measure the activity and posture stability. PE-fed group (fed for two and three months) showed a significant decrease in the maximum speed from 45.5 ± 0.8 m in the control to 41.2 ± 5.8 m in PE-fed group. The distance covered during treadmill sessions reduced from 275.5 ± 108 m in the control to 159.3 ± 67 m in the PE-fed group. The stable running time on the treadmill decreased from 515.7 ± 121 s in the control to 322.1 ± 90 s in the PE-fed group at 3 months PE

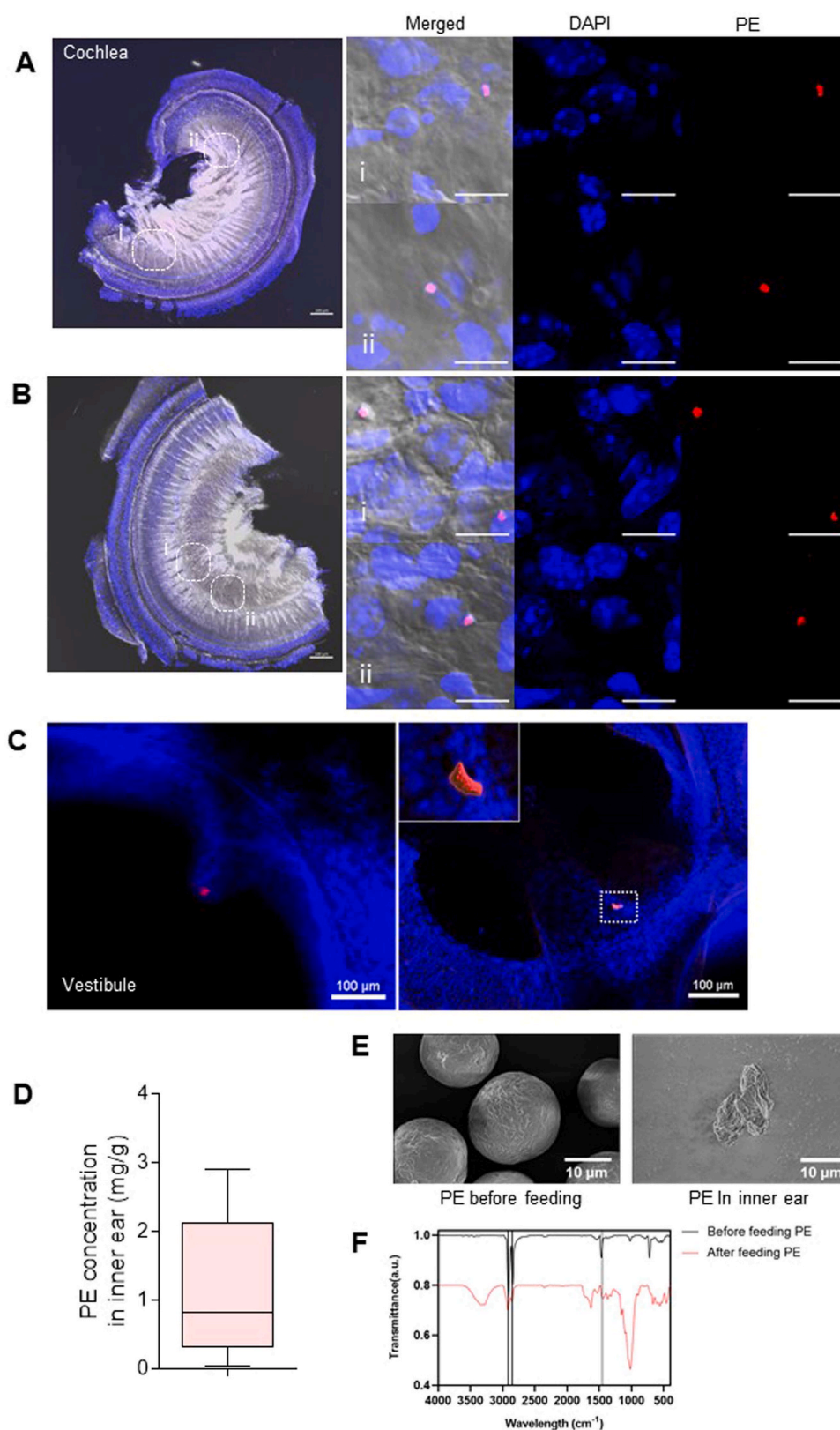


Fig. 1. Confocal microscopy of cochlear whole mount preparation. Cell nuclei are stained in blue (DAPI). (A) Apical turn ($\times 50$) i and ii: spiral ganglion area. Plastic materials are observed as a red dot ($\times 800$). (B) Mid-basal turn ($\times 50$). i and ii: spiral ganglion area. Plastic materials are observed as red dots ($\times 800$) (scale bar = $20\ \mu\text{m}$). (C) PE in the vestibular region. (D) PE concentration in the inner ear. (E) SEM images ($\times 195$ and $\times 500$) of PE. (F) FT-IR results of PE. PE in inner ear was compared with that in the control and the PE reference. (G) Representative images of PE before feeding (scale bar = $20\ \mu\text{m}$) and PE deposited in the inner ear (scale bar = $10\ \mu\text{m}$), along with the distribution of size. (H) pre- and post- images of whole cochlea after tissue clearing. (I) PE in vestibular organ observed in 3D image of inner ear using light sheet microscopy and anatomical structures. Yellow arrow indicated PE particle (red) in the inner ear. A two-tailed Student's *t*-test was used. The * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.005$.

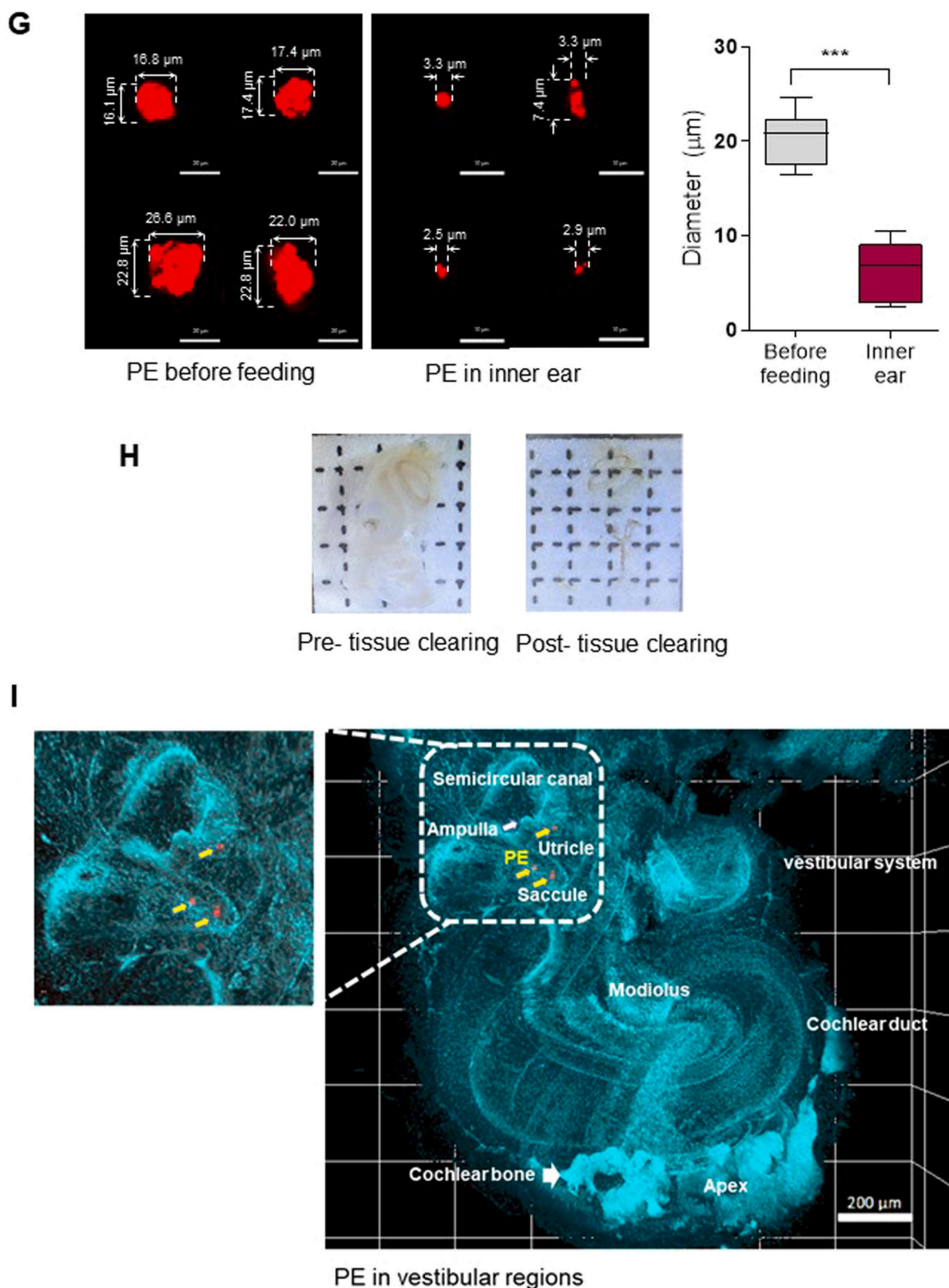


Fig. 1. (continued).

exposure, indicating a compromised ability for sustaining high-speed locomotion (Fig. 3B). The latency to fall (sec) for the PE-fed mice, for maintaining its balance on the rotarod, reduced significantly to 0.5-fold in the 1-month exposed mice and consecutively to 0.7-fold in mice exposed for 2 and 3 months (Fig. 3C). The grip strength, a risk factor for

maintaining the balance, among the PE-fed mice weakened significantly in the forelimb by 0.7-fold, ($** P < 0.01$), 0.6-fold ($*** P < 0.005$), and 0.8-fold (N.S) for 1-, 2-, and 3-months PE-exposed mice, respectively. Similarly, the overall grip of the mice was lowered significantly by 0.7-fold, 0.8-fold, and 0.7-fold in the 1, 2, and 3 months, respectively

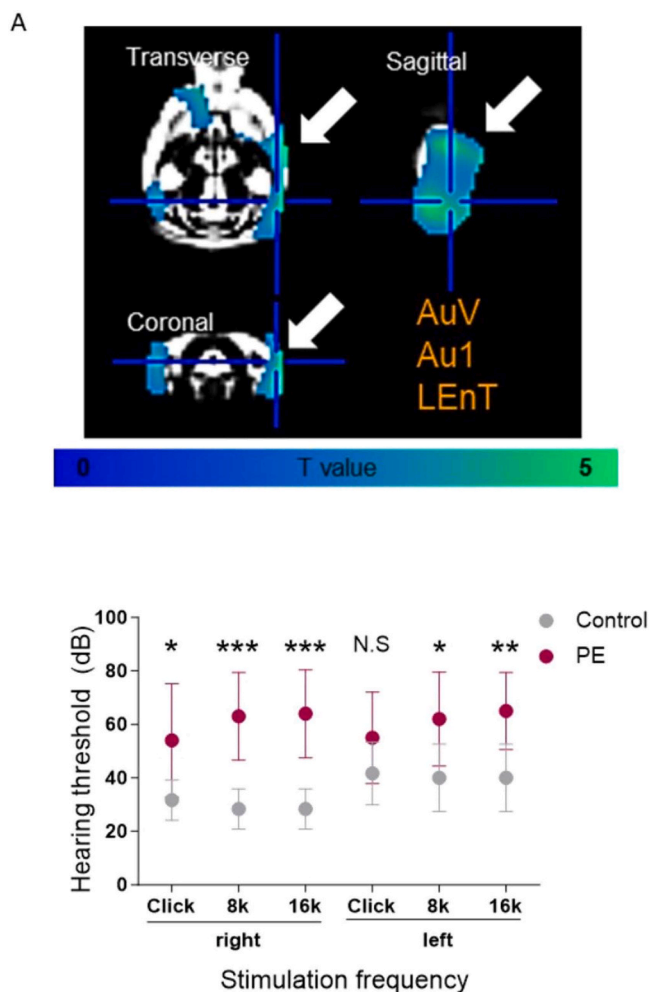


Fig. 2. Decreased glucose metabolism in the auditory cortex of PE-fed mice with hearing loss. Exposure to PE induced hearing loss and decreased glucose metabolism. (A) Decreased cerebral glucose metabolism in the bilateral lateral entorhinal cortex, right primary auditory cortex, and right secondary auditory cortex. A two-tailed Student's *t*-test was used. (B) Hearing thresholds difference between two groups (sound pressure level). The * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.005$.

(Fig. 3D). Similarly, in the balance beam test, which reflects the involvement of the vestibular system, the mice exposed to PE for 4 months showed a significantly lowered stability. The no of slips while trying to maintain balance on the 10 mm beam significantly increased by 2.4-fold, showing that PE induces a lack of stability in maintaining the balance (Fig. 3E). No change was observed in the splay reflex (Fig. 3F). Upon exposure to PE at 1000 ppm/100 μ L, significant changes in the motor and balance stability were observed. (Fig. 1S A-F).

3.4. Decreased glucose metabolism in the auditory cortex of PE-fed mice

The metabolic activity of the auditory cortex of PE-fed mice was evaluated. Fig. 2B shows the results of regional glucose metabolism as determined using SPM analysis [29]. Glucose metabolism was decreased in the bilateral lateral entorhinal cortex (LEnT), right primary auditory cortex (Au1), and right secondary auditory cortex (AuV); these results are consistent with the clinical PET findings of deafness [40].

3.5. Disruption of gene expression in the cochlea in response to PE exposure

Transcriptome analysis was performed to elucidate the changes in

gene expression in response to PE exposure for 12 weeks, compared to that of the control mice. The volcano plot shows the comparison of the average log2 fold-change and p-value between the PE and control groups (Fig. 4A). The heat map shows the 238 genes that satisfy the hierarchical clustering using z score for normalized value (log2 based) with cut off fold change of 2 and raw p-value (Fig. 4B, $*P < 0.05$). Here, 131 genes were upregulated including the apoptotic gene, PER1 and the inflammatory genes, NR4A3 and CEBPB ($\log_2$ FC > 2, $*P < 0.05$); 73 genes were downregulated satisfying $\log_2$ fold change < -2 with $*P < 0.05$ (Fig. 4C). The DAVID GO analysis shows the molecular function related to terms like immune response, phagocytosis and external side of plasma membrane, and defense response to bacterium (Fig. 4D). The gene network analysis is presented in Fig. 4E. The RNA seq DEGs for the genes of interest are shown in Fig. 4F.

We verified the transcriptomic data by conducting quantitative polymerase chain reaction (qRT-PCR) for PER1, NR4A3, and CEBPB. The transcriptomic data revealed a significant fold change of 2.3 for PER1, 3.5 for NR4A3, and 2.5 for CEBPB ($**P < 0.01$), in comparison to the control; this was consistent with the qRT-PCR results. The qRT-PCR results showed a significant fold change of 2.6, 3.6, and 2.7 ($**P < 0.01$) for PER1, NR4A3, and CEBPB, respectively, in response to PE exposure (Fig. 5A). The immunofluorescence (Fig. 5B) and western blotting images (Fig. 5C) confirm the upregulation of the proteins, PER1, NR4A3 and CEBPB following PE exposure. Fig. 5D shows the quantification of the western blot.

4. Discussion

While the hazardous effects of MPs are increasingly reported, and inner ear dysfunction is widespread across all age groups, it remains uncertain if MPs induce inner ear dysfunction. In this study, we showed direct evidence of inner ear problem such as hearing loss and balance defect after exposure to MP. To assess hearing loss and balance defects after PE exposure, we evaluated hearing threshold levels, assessed cerebral glucose metabolism via 18 F-FDG PET, conducted transcriptome analysis, and performed behavioral studies. We identified PE accumulation in the cochlea and vestibular region. According to the report [25], the content of MP ingestion per human was estimated to be 52,000 MP annually from food and water leading to the high necessity of the risk of side effects of MP on human health. Notably, researchers at Columbia University were the pioneers in quantifying and verifying the existence of nano-sized particles in bottled water crafted from polyethylene terephthalate (PET) using stimulated Raman scattering microscopy [41]. They found that, on average, about 240,000 detectable plastic particles are present per liter, which is 10 to 100 times higher than previous estimates based primarily on larger sizes. Based on the results of this paper, advancements in measurement technology may suggest that the concentration of MP accumulating in our bodies could be much higher than previously thought.

We have prepared 100 ppm (0.1 mg/ml) and 1000 ppm (1 mg/ml) to be delivered in 100 μ L. The mice received 100 ppm / 100 μ L (equivalent to 10 μ g) for 3–4 months (data in main text) and 1000 ppm / 100 μ L (equivalent to 100 μ g) for a month (supported as supplemental file). The density of PE was 1.005 g/cc; therefore, our dose level was 0.09 mg/gm per day. According to the dose conversion [42] and age relationship between mice and humans [43] the corresponding human dose is 1.27 mg/kg. For a person weighing 60 kg, the corresponding amount will be 76.65 mg. Regarding dose concentration in relation to actual concentrations reported in humans, according to the result of quantification of MP pollution in human blood [44], the maximum concentration of PET analyzed in a blood sample was 2.4 μ g/ml, for PS this was 4.8 μ g/ml, for PE this was 7.1 μ g/ml. Detected MP concentration such as PET, PS, and PE was 14.3 μ g/ml in blood. The blood volume in the body is approximately 8 % of body weight for males. For example, a male weighing 60 kg would have approximately 4800 ml of blood in their body, then the amount of MP present in the blood would be

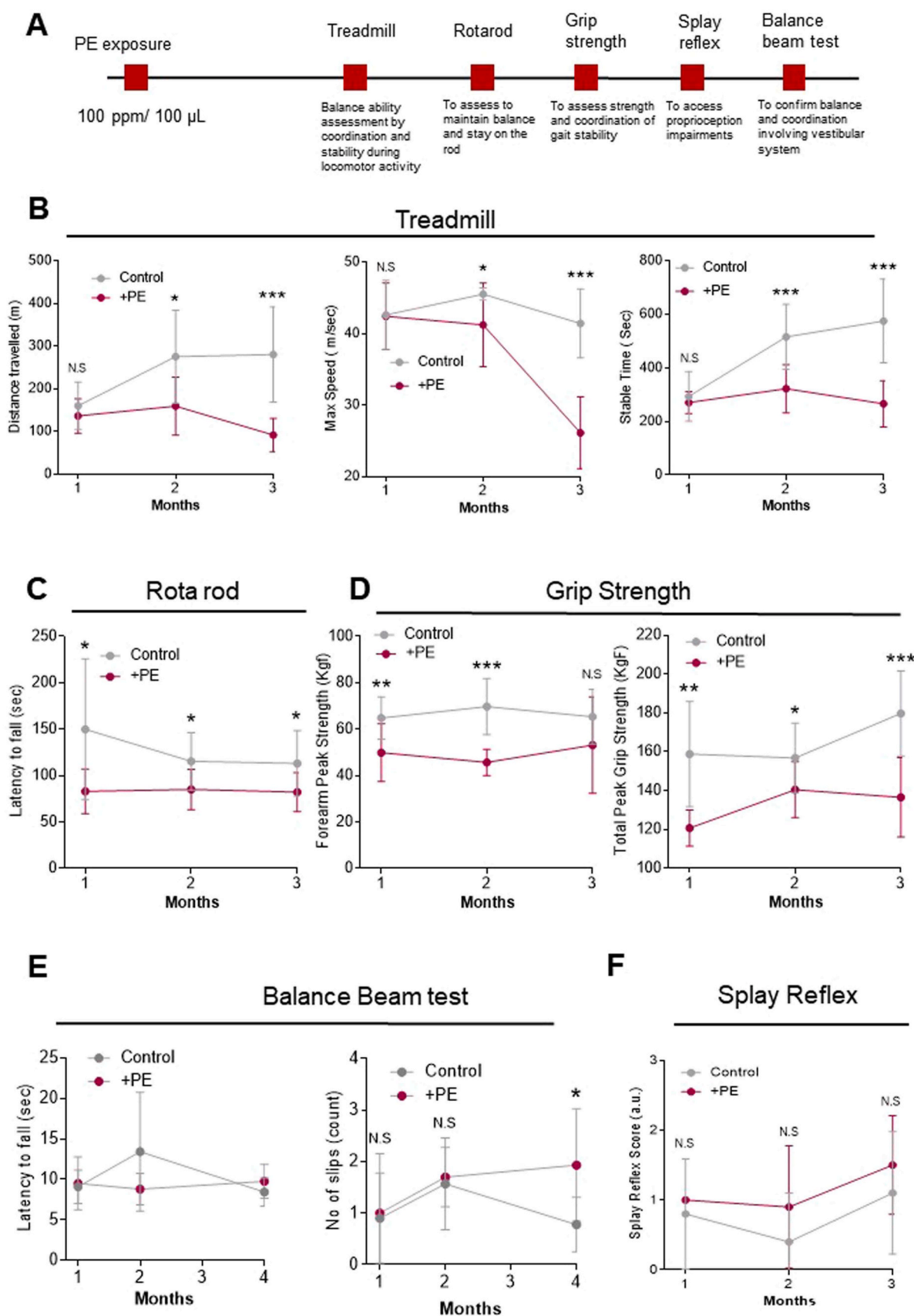


Fig. 3. PE can impair balance system. (A) Behavior assessment criteria for evaluating balance (B) treadmill test, (C) rotarod, (D) grip strength test, (E) balance beam test, (F) splay reflex for control and PE-exposed mice. A two-tailed Student's *t*-test was used. The * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.005$.

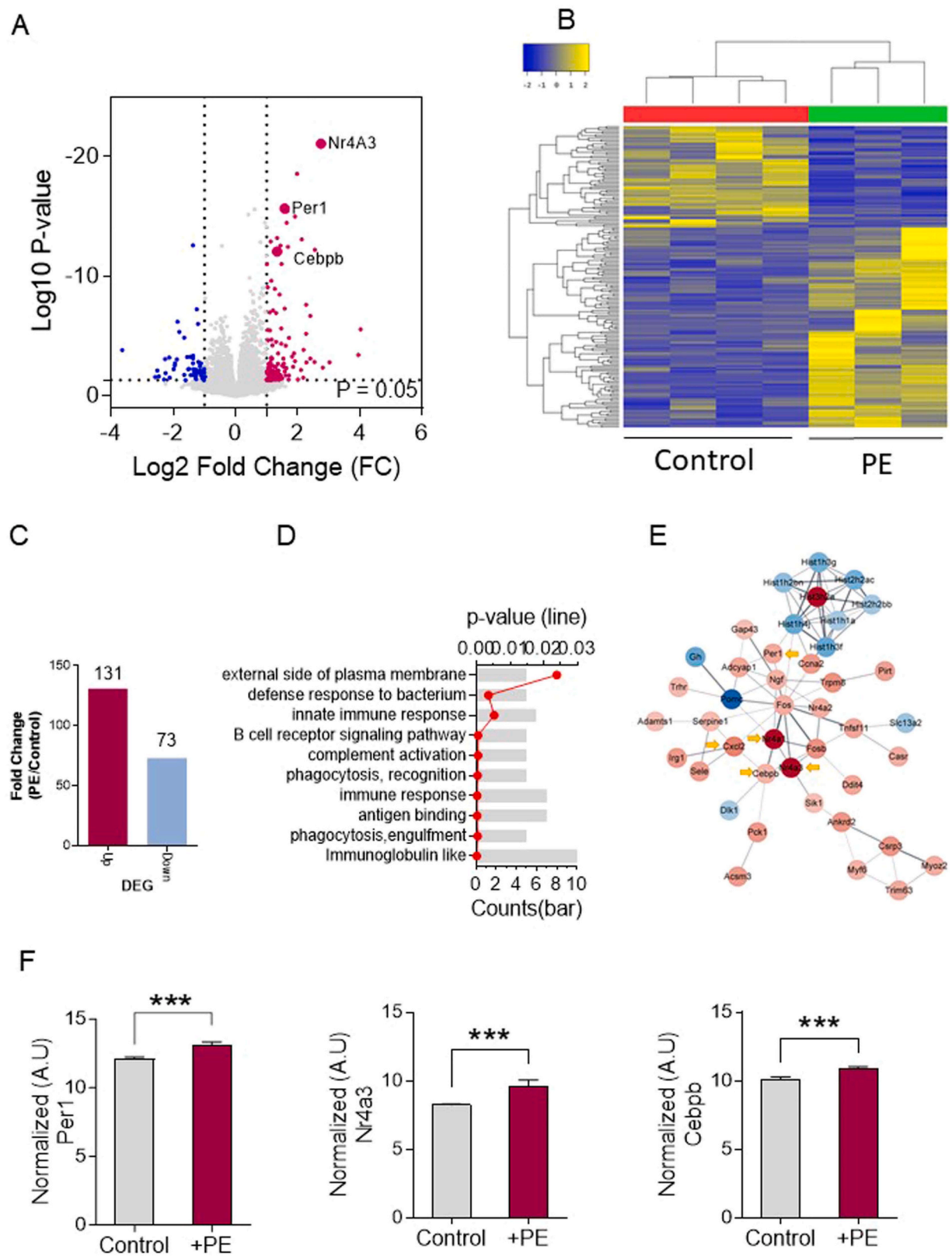


Fig. 4. Differential gene expression in response to PE exposure. Exposure to PE leads to changes in genetic expression, resulting in differential gene expression patterns. (A) A volcano plot was generated to depict the differential gene expression (DEG) resulting from PE exposure. In the plot, genes with a p-value below 0.05 were considered statistically significant. The fold change of gene expression was assessed, and a cutoff point of 2 (upregulated) and - 2 (downregulated) on the fold change scale was applied. Upregulated genes were color-coded in orange, while downregulated genes were color-coded in blue. (B) Heat map of DEG for control and PE exposed group. (C) Number of up and downregulated genes in response to PE exposure. (D) DAVID GO analysis for the top DEG. (E) Cytoscape network analysis. (F) RNA seq data for the gene of interest.

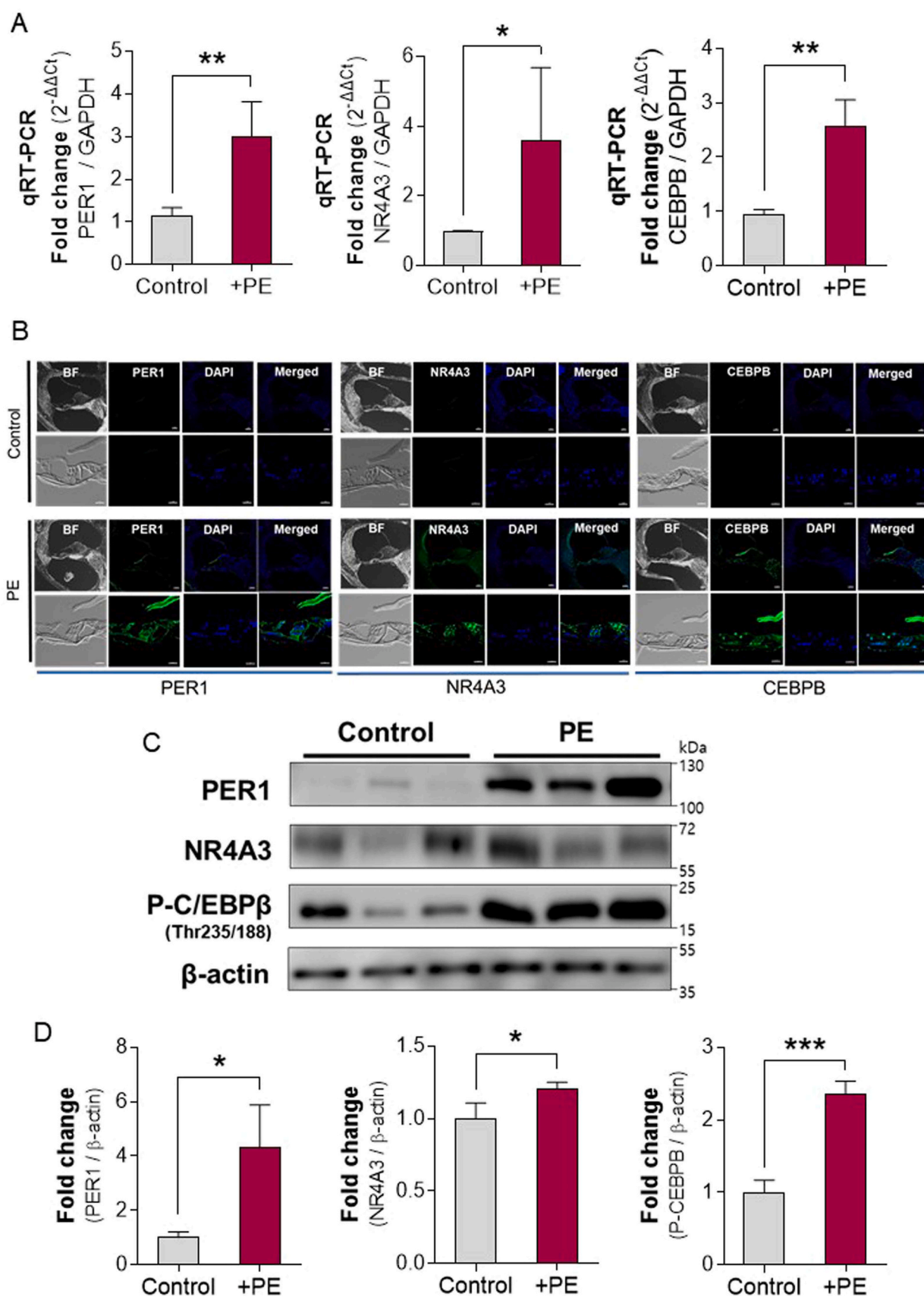


Fig. 5. PE upregulates inflammatory markers at messenger RNA level. PE exposure has the potential to upregulate apoptotic and inflammatory mRNA. (A) qRT-PCR (B) immunofluorescence images for antigen marker (C) western blot results (D) quantification of the western blot bands and transcriptomic changes in PER1, NR4A3 and CEBPB. A two-tailed Student's *t*-test was used. The * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.005$.

68.64 mg. According to the Fig. 4 in Ref [26], it can be observed that 0.236 % of the total administered MP moves into the blood one hour after oral administration. At the 24-hour, 0.068 % of the total administered MP was present in the blood. Based on this data, it can be inferred that 0.04 mg of PE would be present in the blood, assuming a total amount of 68.64 mg. Directly comparing the two data sets is challenging, but it suggests that the dosage we administered is not unrealistically high compared to the real-world environments.

It is important to know how much PE actually enters the inner ear. We estimated the percentage of PE entering the inner ear. The concentration of PE was 1.14 ± 1.06 mg/g in the inner ear (Fig. 1D). The weight of inner ear was 25.46 ± 7.75 mg. Then actual weight of PE deposited in inner ear was 29 μ g. To identify the concentration of PE in the inner ear, we feed 100 ppm / 100 μ L (equivalent to 10 μ g) of PE for 1 week. Then amount of PE exposure was 70 μ g within whole body. To calculate delivered dose of 70 μ g to inner ear, we can use radioisotope PET tracing techniques. However, unfortunately, we have no data of biodistribution of ^{64}Cu labeled PE. Therefore, we can estimate the biodistribution data of ^{64}Cu labeled polystyrene [26] and the PE concentration in the brain [28]. The Fig. 4 in ref [26] shows the biodistribution data from the study, indicating that 0.016 % of polystyrene reaches the brain 48 h after administration. The Fig. 1F in ref [28] shows the concentration of PE in the brain was 1.43 mg/g. Therefore, given that the total administered amount of PE reaching the brain is 0.016 %, with a PE brain concentration of 1.43 mg/g, we can estimate the percentage of the total administered PE reaching the inner ear using the inner ear concentration of 1.14 mg/g. This estimation suggests that 0.012 % of the administered PE reaches the inner ear. Therefore, $70 \mu\text{g} \times 0.012 \% = 0.0084 \mu\text{g}$ is the amount that reaches the inner ear over a week, indicating that 0.0012 μ g of PE reaches the inner ear per day. Therefore, in this study, 0.108 μ g of PE was exposed for 3 months, and 0.144 μ g of PE was exposed for 4 months, respectively.

PE exposure deteriorate hearing ability and decreased glucose metabolism in specific brain regions, leading to changes in gene expression levels and abnormalities in balance-related behavior assessment in the PE group. Consequently, PE exposure induces hearing loss and affects balance, suggesting that environmental MP pollution could contribute to inner ear problems like balance and hearing loss.

Fig. 1 shows that fragmented PE was deposited in the inner ear. Fragmented PE in the cochlea was confirmed using FT-IR. PE was deposited in the spiral ganglion area (Fig. 1A,B) and vestibular regions (Fig. 1C), resulting in hearing and balance loss. The route of the PE from oral administration to the inner ear tissues would involve the bloodstream. Fig. 4 in the reference [26] represents that the ^{64}Cu -labeled polystyrene could distribute into the bloodstream after 1 h. The orally administered PE passes through the stomach and is absorbed into the body through the intestinal mucosa. It is known that PE causes damage to the intestinal mucosa and is absorbed into the body through the damaged area. Then, absorbed PE was known to circulate throughout the body. Once the PE particles have reached the inner ear along the bloodstream, they are expected to reach each turn of the cochlea through the following pathway: Vertebral artery - Basilar artery - Anterior inferior cerebellar artery (AICA) - Labyrinthine artery - Common cochlear artery - Main cochlear artery. PE supplied to the vestibule would be delivered through the following pathway: via the anterior inferior cerebellar artery and anterior vestibular artery to supply blood to most of the anterior semicircular canal and utricle, and via the common cochlear artery and posterior vestibular artery to supply blood to the horizontal/posterior semicircular canal and saccule. When we count the number of PE in areas of apex, middle and base in the cochlear, the highest amount of PE was found in the basal turn, presumably due to extravasation of PE from arteries in the basal turn area, as blood is supplied to the basal turn first in the artery in cochlea. External substances often affect the basal turn more readily, as the vascular supply predominantly reaches this area first. In cases of external auditory stimuli (such as noise-induced hearing loss) or conditions like otitis

media, damage often occurs more prominently in the basal turn. Toxic substances also tend to cause damage starting from the high-frequency (basal turn) region. The apical turn, responsible for low frequencies, is implicated in conditions like Meniere's disease, but these are less common than cases where high frequencies deteriorate first. However, in this present study, our aim was to investigate the impact of PE in the inner ear. We plan to explore the frequency-specific effects of MP in future research.

In this study, we found that upregulated PER1 (Figs. 4, 5), which was reported molecular marker for hearing loss in Meniere's Disease [45]. Upregulated PER1 expression was observed in mice exposed to environment pollutant such as carbon tetrachloride (CCl_4) [46], particulate exposure [47] and toxins [46]. PER1 is a gene involved in the regulation of circadian rhythms, and are crucial for maintaining proper physiological function [48]. Circadian rhythms play a role in regulating the expression of genes involved in maintaining the health and function of the inner ear [48]. Exposure to PE upregulated the inflammatory markers, NR4A3 and CEBPB (Figs. 4,5). The chemical pollutant like polychlorinated biphenyls exposure revealed an increase in NR4A3 activity [49]. Studies have shown the involvement of upregulated NR4A3 in noise mediated cochlear damage [50]. NR4A3 is expressed in the inner ear, where it likely plays a role in the development and maintenance of sensory cells and neurons involved in hearing and balance [51]. According to the gene ontology data from the PubMed Gene database (<https://www.ncbi.nlm.nih.gov/gene/18124>), NR4A3 is associated with inner ear dysfunction and behavioral abnormalities. CEBPB was shown to be elevated in face mask degraded products which are composed of the plastic polymer fiber [52]. Inflammation play significant roles in the development of hearing loss [53]. The changes observed in the expression of inflammatory markers suggest a potential role in the development of hearing loss associated with PE exposure. Inflammatory processes involving the release of pro-inflammatory cytokines and the infiltration of immune cells can contribute to hearing loss by causing damage to the delicate structures of the inner ear; this can effect balance system [53]. We evaluated the balance performance of PE-fed and control mice using the treadmill test where PE fed mice show decreased stability in motor coordination. The PE-fed mice could not maintain the balance to stay on the rotarod. Similarly, the grip strength test, to assess the risk of not maintaining balance [54], showed significant weakness both in the forelimb and hindlimb of the PE-fed mice. We did not observe any significant differences in the splay reflex; however, the PE-fed mice showed a lowered degree of the splay reflex compared to those in the control. To confirm the balance disorder involving vestibular system, we subjected the animals to the balance beam test, which evaluates the ability of mice to maintain their posture and balance. We observed increased foot slips along with the hearing loss over the course of 4 months of PE exposure (Fig. 3). Similar results were observed in the group exposed to a higher dose of 1000 ppm for one month PE exposure (Fig. S1).

Recently, the relationship between heavy metal exposure and inner ear problems were reported. Blood lead levels greater $\geq 2 \mu\text{g/dL}$ was associated with increased odds of high-frequency hearing loss in US adolescents compared to those with lead levels $< 1 \mu\text{g/dL}$ [23]. Environmental ototraumatic agents contribute to the development of hearing loss. Styrene exposure is linked to hearing loss in rodent model [55]. Heavy metals caused a poor balance system in humans [56–58]. The odds of developing balance disorder, which increases the risk of falls, hospital visits, and even mortality, was associated with air pollution [59]. According to the report on the correlation between heavy metal exposure inner ear problems [23], researchers identified the concentration of heavy metals and found a correlation between the concentration of heavy metals and the odds of high-frequency hearing loss. In this study, we reported that MP can cause hearing loss using a mouse model because human auditory organs are tissues that are nearly impossible to biopsy. Temporal bone banks collect postmortem temporal bones for histological study, those collected bones are not targeted for

specific conditions. However, if ear tissue were obtained while alive, it could result in complete hearing loss. Since detecting MP in human ear tissue was challenging, we identified the presence of PE in a mouse model and revealed a correlation between inner ear problems and the presence of PE. If we measure the concentration of MP in the blood (similar to ref [23]) and find a correlation between MP concentration and increased hearing loss, we could demonstrate the association between hearing loss and MP in humans.

Another assumption could be that hearing loss increases with the rise in MP pollution over time, and the increasing prevalence of hearing loss in recent years may provide evidence to support this hypothesis. However, when estimating data on MP pollution levels along the Korean coast and studying the trends of hearing loss in Korea and the United States over the years, it is difficult to find a correlation between MP environmental pollution and hearing loss. According to the report of spatial distribution and historical trend of MP pollution in sediments from enclosed bays of South Korea [60], the chronology of MP pollution in the sediment of Masan Bay and Jinhae Bay identifies an increasing trend of MP pollution from the 1970s to the late 2010s. The study found that the concentration of MP in sediment has been increasing over time. The average annual growth rate of MP has increased dramatically since the 2000s, with Masan Bay tripling from 5 % to 15 % and Jinhae Bay nearly doubling from 4 % to 10 %. However, the trend of hearing loss prevalence showed a gradual decrease from 2010 to 2015 in Korea [61]. According to the NIH reports (<https://www.nidcd.nih.gov/health/statistics/quick-statistics-hearing>), among adults ages 20–69, the overall annual prevalence of hearing loss dropped slightly from 16 % (28.0 million) in the 1999–2004 period to 14 % (27.7 million) in the 2011–2012 period. However, since socioeconomic position can also influence hearing loss [62], it might mitigate the impact of environmental pollution. Therefore, it may not be easy to find a simple correlation between MP pollution and hearing loss. This is because various factors such as economic status are involved in influencing hearing loss. Nevertheless, despite this, when considering the correlation between blood concentration of heavy metals and hearing loss, it can be speculated that there might be a sufficient correlation between MP concentration in blood and hearing loss.

Our results of the PE exposure in mice for upregulated PER1, NR4A3 and CEBPB genes were consistent with the previous studies with exposure to the environmental pollutants. Studies investigating the acute toxic effects of PE have revealed concerning findings regarding their impact on both aquatic organisms and human cells. When exposed to PE, adult zebrafish exhibited notable behavioral changes, indicating potential neurological or physiological disruptions [63]. Concurrently, in human peripheral blood lymphocytes, PE induced genotoxic and cytotoxic effects, raising concerns about DNA damage and cell death [64]. Furthermore, the presence of PE was associated with erythrocytic mutagenicity, suggesting a potential for genetic mutations in red blood cells [65]. These findings collectively emphasize the diverse and deleterious effects of PE. Our research was the first to investigate the impact of PE on the inner ear, revealing its induction of hearing loss and balance disorder. The increased hearing threshold levels and decreased cerebral glucose metabolism are consistent with hearing loss in murine model (Fig. 2). Exposure of mice to PE for 4 months induced hearing loss, which was identified in the context of auditory brainstem response testing and PET. As hearing loss could result from aging, we compared the hearing threshold levels and cerebral glucose metabolism with age-matched mice (21 weeks aged; 4 months PE exposure to 5 weeks aged mice) to avoid the confounding aging effect. Therefore, it is unlikely that the hearing loss observed after PE exposure resulted from aging; it resulted from the hazardous effects of PE.

In this study, five-week-old mice were exposed to PE for 4 months, which corresponds to 30 human years considering the age relationship between mice and humans [43]. As individuals age, they experience deterioration in hearing, cognition, and balance abilities [66]. Hence, our findings indicate that prolonged, chronic exposure to PE may

potentially lead to inner ear problem, including hearing and balance disorder in older individuals. For further investigation, it is essential to examine the correlation between the level of microplastic exposure and inner ear issues through actual human studies.

5. Conclusion

We demonstrated that PE induced inner ear dysfunction, based on the evidence of accumulated PE in the inner ear, elevated hearing thresholds, behavioral studies, transcriptome analysis, and the evidence of PET. These preclinical findings may serve as an impetus for additional epidemiological studies investigating the impact of MP exposure on inner ear problem.

CRediT authorship contribution statement

This study investigated the impact of polyethylene (PE) exposure on inner ear function in a murine model, focusing on hearing loss and balance defects. Based on these findings, the importance of environmental influence is highlighted. Polyethylene, a common plastic material, was found to accumulate in the inner ear regions of exposed mice, leading to observed abnormalities in balance-related behavior and increased hearing thresholds. This study underscores the negative impact of environmental exposure to PE on inner ear function, emphasizing the need for awareness regarding the consequences of plastic pollution on both biodiversity and human health. It suggests the necessity for sustainable environmental policies and consumer behavior changes, particularly addressing appropriate measures in plastic usage and disposal.

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CRediT authorship contribution statement

Jin Su Kim: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Min-Hyun Park:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ho Sun Lee:** Methodology, Formal analysis, Data curation. **Javeria Zaheer:** Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Jonghoon Choi:** Formal analysis, Data curation. **Hyeonggi Kim:** Data curation. **Jaehee Jang:** Formal analysis, Data curation. **Seungyou Kim:** Methodology, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.135193.

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