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# Reduced mitophagy in the cochlea of aged C57BL/6J mice

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의학과

오정현

# Reduced mitophagy in the cochlea of aged C57BL/6J mice

노화성 난청 마우스에서 와우내
마이토파지의 변화

2020년 8월 28일

조 선 대 학 교 대 학 원
의 학 과
오 정 현

# Reduced mitophagy in the cochclea of aged C57BL/6J mice

지도교수 : 조 성 일

이 논문을 의학박사 학위신청 논문으로 제출함.

2020년 5월

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오 정 현

# 오정현의 박사학위논문을 인준함.

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# ABSTRACT

## Reduced mitophagy in the cochlea of aged C57BL/6J mice

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An increase in mitochondrial damage has been associated with a decline in the ability to mitigate damage through mitophagy in age-related pathologies. The present study aimed to investigate the changes in mitophagy in a mouse model with age-related hearing loss. C57BL/6J mice were divided into two groups: young (1 month) and aged (12 months). Hearing tests were conducted by measuring auditory brainstem response (ABR). Mitochondrial DNA copy number, the level of mitochondrial DNA damage, mitochondrial biogenesis, and mitophagy-related genes and proteins were investigated using real-time PCR and western blot analyses. Mitophagosome and lysosome coexpression in the cochlea was investigated through immunofluorescence imaging analysis. Major players of mitophagy, *Parkin* and *BNIP3*, were also investigated through immunohistochemical staining in the cochlea. Hearing thresholds were observed to have increased in the aged group. The mitochondrial DNA copy number, *PGC-1α*, and *PGC-1β* significantly decreased in the cochlea of mice in the aged group. mRNA levels of *PINK1*, *Parkin*, *MUL1*, *Atg5*, *Atg12*, *Atg13*, *NIX*, and *BNIP3* significantly decreased in the cochlea of the mice in the aged group. The level of mitochondrial DNA damage significantly increased in the cochlea of mice in the aged group. Protein levels of *PINK1*, *Parkin*, *BNIP3*, *COX4*, LC3B, and all OXPHOS subunits significantly decreased in the cochlea of the mice in the aged group. Immunofluorescence imaging

analysis of mitophagosomes and lysosomes revealed decreased colocalization in the cochlea of mice in the aged group. Immunohistochemical imaging analysis of *Parkin* and *BNIP3* revealed their decreased expression in aged cochlea. Our results indicate that reduced mitophagy with aging might be attributed to the cellular changes that occur in aged cochlea in the development of age-related hearing loss.

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**Keywords:** Mitochondria; Cochlea; Age-related hearing loss; Mitophagy.

# 국문초록

## 노화성 난청 마우스에서 와우내 마이토파지의 변화

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미토콘드리아 손상의 증가는 노화 병리에 있어 마이토파지를 통해 손상을 완화시키는 능력의 감소와 관련이 있다. 본 연구는 노화성 난청의 마우스 모델에서 마이토파지의 변화를 조사하는 것을 목표로 하였다. C57BL/6J 마우스를 젊은 연령(1 개월) 및 고령(12 개월)의 두 그룹으로 나누었다. 청력검사는 청성뇌간반응(ABR)을 통해 시행하였다. 미토콘드리아 DNA 복제 횟수, 미토콘드리아 DNA 손상 수준, 미토콘드리아 생성, 마이토파지 관련 유전자 및 단백질을 실시간 PCR 및 웨스턴 블롯 분석을 통해 확인하였다. 와우에서 마이토파고솜과 리소좀의 공동발현과 마이토파지의 주요 관련 인자인 *Parkin*과 *BNIP3*는 면역조직 화학염색을 통해 확인하였다. 청력 역치는 고령군에서 높은 것으로 나타났으며 와우에서 미토콘드리아 DNA 복제 횟수, *PGC-1α* 및 *PGC-1β*는 젊은 연령군에 비해 유의하게 감소하였다. *PINK1, Parkin, MUL1, Atg5, Atg12, Atg13, NIX* 및 *BNIP3*의 mRNA 발현량은 고령군의 와우에서 유의하게 감소하였으며 미토콘드리아 DNA 손상의 정도는 고령군에서 유의하게 증가함을 확인하였다. *PINK1, Parkin, BNIP3, COX4,* LC3B 및 모든 OXPHOS 서브 유닛의 단백질 수준은 고령군에서 현저하게 감소하였다. 마이토파고솜와 리소좀의 공동발현은 면역형광 검사상 고령군의 와우에서 감소함을 보였고 *Parkin* 및 *BNIP3*의 발현은 면역조직화학 염색상 고령군에서 감소하였다. 결과적으로 노화에 따른 마이토파지의 감소는 노화성 난청 발생시 노화된 와우에서 발생하는 세포적 변화에 기인함을 보여준다.

핵심어: 미토콘드리아; 와우; 노화성 난청; 마이토파지

# 1. Introduction

Among the elderly, age-related hearing loss is common. Aging is widely associated with the process of oxidative damage caused by reactive oxygen species (ROS) (Beckman et al., 1998). This results in mitochondrial dysfunction and contributes to cochlear cell degeneration and hearing loss (Cheng et al., 2005; Someya et al., 2010). Age-related degeneration in the cochlea is most frequently associated with damage to the organ of Corti and spiral ganglion neurons, which have sensory hair cells and sensory neurons (Gates et al., 2005). Mitochondria play an important role in redox homeostasis and supplying energy in the form of adenosine triphosphate (ATP). The accumulation of defective mitochondria and the declining function of mitochondria result in the decline of cellular function during the aging process (Linnane et al., 1989). Autophagy involves the degradation of damaged and dysfunctional cellular components through the formation of the autophagolysosome (Mejías-Peña et al., 2017). Appropriate induction of autophagy is required to extend lifespan and maintain metabolic balance in stressful environments; however, there is a reduced occurrence of autophagy in dysfunctional aged tissues and organs (Cuervo et al., 2014). The beneficial roles of autophagy in the inner ear have been reported, and the studies have noted that upregulation of autophagy contributes to alleviating damage to the inner ear and hearing loss (Ye et al., 2019). Autophagy including mitophagy protects the hair cells by suppressing ROS accumulation and apoptosis (He et al., 2017). The induction of mitophagy in the auditory cell line minimized cytotoxicity after carbonyl cyanide m-chlorophenyl hydrazone (CCCP) exposure (Setz et al., 2018). Additionally, basal autophagy is important in the maintenance of hair cells and hearing acuity (Fujimoto et al., 2017). Selective autophagy can be classified depending on its selective organelle. Mitophagy is a form of mitochondrial autophagy, and it is important in regulating the turnover of damaged mitochondria and maintenance of healthy mitochondria (Fivenson et al., 2017). Reductions in the turnover of damaged

mitochondria via mitophagy are associated with various age-related pathologies (Palikaras et al., 2015). Compromised mitophagy may be a cause of cell degeneration due to mitochondrial dysfunction in age-related hearing loss. The present study aimed to investigate the changes in mitophagy in age-related hearing loss using a mouse model.

# 2. Materials and Methods

## 2.1. Mice and animal care

C57BL/6J mice, a mouse model of age-related hearing loss, were provided by the Animal Facility of Aging Science, KBSI Gwangju Center (Gwangju, South Korea). The animals were maintained and approved by the Chosun University Institutional Animal Care and Use Committee (approval no. CIACUC2016-A0035). All experiments were performed in strict accordance with the relevant guidelines and regulations approved by the committee. The mice were randomly divided into two groups (n = 5, 2 males and 3 females per group): young (1 month) and aged (12 months). Following hearing tests, mice were euthanized with cervical dislocation under 5% isoflurane anesthesia, and then their cochleae were removed.

## 2.2. Auditory brainstem response measurement

Hearing tests were conducted in a soundproof chamber. Before testing, mice (n = 5 per group) were anesthetized by intraperitoneal injection of ketamine (50 mg/mL, Yuhan, Seoul, Korea) and xylazine (23.32 mg/mL, Bayer Korea, Seoul, Korea) at a dose of 0.1 mL/20 g body weight. Body temperature was maintained at 37–38 ℃ by placing the anesthetized mice on a heating pad and monitoring with a rectal probe throughout the recording. The auditory brainstem response (ABR) was recorded from the scalp of the mice using the computerized Intelligent Hearing System (IHS, FL, USA) with the Smart-EP software. Waveforms from 256 stimuli (rate: 21.1/s) were examined and averaged using frequency-specific tone-burst stimuli (8, 16, and 32 kHz). ABR waveforms were recorded from 10 to 80 dB sound pressure level (SPL) intervals down from the maximum amplitude. The threshold was defined as the lowest stimulus level at which response peaks for waves were visible in the evoked trace.

## 2.3. Real-time PCR analysis

The cochleae were carefully removed from the anesthetized mice (n = 5 per

group). For the estimation of mitochondrial DNA (mtDNA) copies in mouse cochlea, genomic DNA was isolated from cochlea with the Accuprep Genomic DNA extraction Kit (Bioneer, Daejeon, Korea). Total DNA (40 ng) was used for PCR. The control region of mouse mtDNA was amplified using the primer pair D1 (5′-CCCAAGCATATAAGCTAGTAC-3′) and D2 (5′-ATATAAGTCATATTTTGGGAACTAC-3′) (Fuke et al., 2011). For the determination of the amount of nuclear DNA, the apoB gene was used as a reference: forward, 5′-CGTGGGCTCCAGCATTCTA-3′ and reverse, 5′-TCACCAGTCATTTCTGCCTTTG-3′ (Fuke et al., 2011). The thermal cycling protocol used was 45 cycles of 95 ℃ for 5 s and 60 ℃ for 34 s after an initial denaturation step at 95 ℃ for 30 s. PCR was performed with SYBR *Premix Ex Taq*$^{TM}$II (Tli RNaseH Plus) (TaKaRa Bio, Shiga, Japan).

For the analysis of mtDNA damage, total DNA (20 ng) was used for PCR. A long fragment of mtDNA (10.1 kb, *Mus musculus*) was amplified using the primer 5′-GCCAGCCTGACCCATAGCCATAATAT-3′ and 5′-GAGAGATTTTATGGGTGTAATGCGG-3′. A short fragment of mtDNA (117 bp, *Mus musculus*) was amplified with the primers 5′-CCCAGCTACTACCATCATTCAAGT-3′ and 5′-GATGGTTTGGGAGATTGGTTGATGT-3′ (Santos et al., 2006). The conditions for long-fragment amplification were 95 °C for 15 s and 60 °C for 720 s for 45 cycles after an initial denaturation at 95 °C for 30 s. The conditions for short-fragment amplification were 95 °C for 15 s and 60 °C for 90 s for 45 cycles after an initial denaturation at 95 °C for 30 s. PCR was performed with SYBR *Premix Ex Taq*$^{TM}$II. The short fragment of mtDNA was used as the reference gene.

For the analysis of mitochondrial biogenesis, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*) and peroxisome proliferator-activated receptor gamma coactivator 1-beta (*PGC-1β*) expression levels in mouse cochleae were evaluated. Primers used for real-time PCR were as follows: *PGC-1α* forward, 5′-AGTTTTTGGTGAAATTGAGGAAT-3′; reverse, 5′-TCATACTTGCTCTTGGTGGAAGC-3′ (Meirhaeghe et al., 2003), *PGC-1β* forward, 5′-TGCGGAGACACAGATGAAGA-3′; reverse, 5′-GGCTTGTATGGAGGTGTGGT-

3′ (Gali Ramamoorthy et al., 2015), and housekeeping internal control gene, *GAPDH* forward, 5′-GTATTGGGCGCCTGGTCACC-3′; reverse, 5′-CGCTCCTGGAAGATGGTGATGG-3′(Jang et al., 2013). *GAPDH* was used as the reference gene.

To confirm the mRNA expression levels of mitophagy-related genes, total RNA was purified from the cochlea with Hybrid-RTM (GeneAll, Seoul, Korea) according to the manufacturer's protocol. cDNA was synthesized using 1 μg of RNA with the M-MLV cDNA Synthesis Kit (Enzynomics, Daejeon, Korea). Real-time PCR analysis was performed using the SYBR *Premix Ex Taq* kit (TaKaRa Bio, Shiga, Japan). Primers used for real-time PCR were as follows: phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (*PINK1*) forward, 5′-GCTTGCCAATCCCTTCTATG-3′ ; reverse, 5′-CTCTCGCTGGAGCAGTGAC-3′ (Zhou et al., 2007), *Parkin* forward, 5′-AAACCGGATGAGTGGTGAGT-3′; reverse, 5′-AGCTACCGACGTGTCCTTGT-3′(Bouman et al., 2011), activating molecule in Beclin1-regulated autophagy (*AMBRA1*) forward, 5′-CTACTGGGACCAGCTAAGTGAAA-3′; reverse, 5′-ACGTGGCTCTGCTGGTTC-3′ (Cianfanelli et al., 2015), mitochondria lubiquitin ligase 1 (*MUL1*) forward, 5′-GTCATCGAAGGAGCTGTGC-3′ ; reverse, 5′-GTAGTTCGGTTCCACACCATC-3′(Yun et al., 2014), nip3-like protein X (*NIX*) forward, 5′-GCAGGGACTAGCTCTCAG-3′ ; reverse, 5′-TGCTCAGTCGCTTTCCAATA-3′ (Lu et al., 2012), Bcl2/adenovirus E1B 19 kDa-interacting protein 3 (*BNIP3*) forward, 5′-GCTCCCAGACACCACAAGAT-3′ ; reverse, 5′-TGAGAGTAGCTGTGCGCTTC-3′, Bcl2/adenovirus E1B 19 kDa-interacting protein 3-like (*BNIP3L*) forward, 5′-CCTCGTCTTCCATCCACAAT-3′ ; reverse, 5′-GTCCCTGCTGGTATGCATCT-3′ (Ha et al., 2007), autophagy-related protein 3 (Atg3) forward, 5′-CCATTGAAAACCATCCTCATCTC-3′ ; reverse, 5′-GCCTTCTGCAACTGTCTCAATAATT-3′, autophagy-related protein 5 (Atg5) forward, 5′-GGACAGCTGCACACACTTGG-3′ ; reverse, 5′-TGGCTCTATCCCGTGAATCAT-3′, autophagy-related protein 7 (Atg7) forward, 5′-GGCCTTTGAGGAATTTTTTGG-3′ ; reverse, 5′-ACGTCTCTAGCTCCCTGCATG-3′, autophagy-related protein 12 (Atg12) forward, 5′-TGAATCAGTCCTTTGCCCCT-3′; reverse, 5′-CATGCCTGGGATTTGCAGT-3′ (Kang et al., 2012), and

autophagy-related protein 13 (Atg13) forward, 5′-TGGCGGAAGATTTGGACTCC-3′ ; reverse, 5′-GGGTTTCCACAAAGGCATCG-3′ (Zhang et al., 2017). *GAPDH* was used as the reference gene.

The fold change in gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Cts for the gene of interest (GOI) in both the young group (1 month) and the aged group (12 months) samples were adjusted in relation to anormalizer (reference gene) Ct from the same two samples. Relative mtDNA copies, mtDNA damage, *PGC-1α* and *PGC-1β*, and mitophagy-related gene expression of the aged group were calculated by comparing to basal activity of the young group to obtain relative fold expression.

## 2.4. Western blot analysis

The cochleae were carefully removed from the anesthetized mice (n = 3 per group) and homogenized by a homogenizer (Omni international, GA, USA) in 300 μL lysis buffer (20 mM HEPES pH 7.4; 2 mM EGTA; 50 mM glycerol phosphate; 1 % Triton X-100; 10 % glycerol; 1 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml leupeptin; 10 μg/ml aprotinin; 1 mM Na3VO4; and 5 mM NaF). The homogenized samples were sonicated by a sonicater (Sonics & Materials Inc., CT, USA) two times for 10 s each and centrifuged at 13,000 rpm for 15 min to gain the soluble extract proteins. The extracts were separated by 10−12 % SDS-PAGE and transferred to PVDF membranes (Millipore Corp, MA, USA). The membranes were blocked in TBS-T (20 mM Tris-HCl, 137 mM NaCl, pH 7.5, and 0.1 % Tween-20) with 5 % skim milk at room temperature for 1 h. After washing with TBS-T, the membranes were incubated with primary antibodies overnight at 4 °C and then washed with TBS-T. The primary antibodies used were *PINK1* (1:1000, ThermoFisher Scientific, MA, USA), *Parkin* (1:1000, ThermoFisher Scientific), *BNIP3* (1:1000, ThermoFisher Scientific), cytochrome c oxidase subunit 4 (*COX4)* (1:1000, Cell signaling Technology, MA, USA), LC3B (1:1000, Cell signaling Technology),

oxidative phosphorylation (OXPHOS) Rodent WB Antibody Cocktail (6 μg/mL, Thermo Fisher Scientific), and β-actin (1:4000, Santa Cruz Biotechnology, CA, USA). The membranes were washed three times with TBS-T for 10 min and then incubated with secondary antibodies for 2 h. The secondary antibodies used were anti-mouse in sheep (1:4000, Jackson ImmunoResearch, PA, USA) and anti-rabbit in donkey (1:4000, Jackson ImmunoResearch). The protein bands were visualized using a western blot detection system (MILLIPORE, MA, USA) and developed by an image analyzer (LAS-3000 imaging system, Fujifilm, Tokyo, Japan).

## 2.5. Immunostaining

The cochleae were carefully removed from the anesthetized mice (n = 5 per group), fixed in 4 % formaldehyde 37 % solution (VWR International Co., PA, USA) overnight at 4 ℃, and decalcified for 7 days in 0.12 M EDTA (Sigma, MO, USA) in PBS. The decalcified cochleae were then embedded in paraffin wax and sliced into 4-μm-thick serial paramodiolar sections. The sections were mounted on glass slides, and representative sections were used for experiments. The sections of two groups (1 month, 12 months) were labelled simultaneously with the same solutions.

For immunofluorescence, after blocking in 0.5% BSA solution, the slides were incubated with primary antibodies against translocase of the outer membrane 20 (TOM20) (1:200; Santa Cruz Biotechnology, CA, USA), LC3B (1:200; Cell Signaling Technology, MA, USA), and lysosomal-associated membrane protein 1 (LAMP1) (1:200, Santa Cruz Biotechnology) overnight at 4 ℃. Then, the slides were incubated with the FITC-conjugated secondary antibodies: chicken anti-rabbit Alexa Fluor 488 and chicken anti-mouse Alexa Fluor 594 (1:200, Invitrogen, CA, USA), and nuclei were counterstained with DAPI (GBI Labs, WA, USA). Immunofluorescence was detected by confocal microscopy (Carl Zeiss, Oberkochen, Germany) with Zeiss microscope image software ZEN (Carl

Zeiss). The colocalization coefficients were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

For immunohistochemistry, six sections for each animal were deparaffinized, rehydrated, and then rinsed three times in 0.1 M PBS. After incubation in 0.01 M sodium citrate buffer for the microwave antigen retrieval step, the slides were treated with 0.3 % hydrogen peroxide for 20 min to block endogenous peroxidase activity and then rinsed with PBS. They were then blocked with normal horse serum in 0.5 % BSA solution for 30 min at room temperature and incubated with primary antibodies against *Parkin* and *BNIP3* (1:100, ThermoFisher Scientific, MA, USA) overnight at 4 ℃. The next day, the sections were rinsed with PBS and incubated with biotinylated anti-rabbit IgG. The immunoreactivity of cells in the sections was visualized using an avidin-biotin-peroxidase kit (Vector Laboratories, CA, USA). The chromogen used was 3, 3′-diamino-benzidine. Counterstaining was performed using thionin for 30 s. The sections were mounted with Polymount mounting medium (Polysciences, IL, USA). *Parkin* and *BNIP3* immunoreactivities were determined by scoring for staining intensity (1, none; 2, weak; 3, moderate; 4, strong) and percent positive cells (1, <5%; 2, 6–25%; 3, 26–50%; 4, 51–75%; 5, >76%), and then expressed as the product of both scores.

## 2.6. Statistical analysis

The results were statistically analyzed using SPSS 24.0 software (SPSS Inc., IL, USA). The Mann-Whitney U test was used to analyze the data. A $p$ value < 0.05 was considered statistically significant.

# 3. Results

## 3.1. Assessment of ABR thresholds in C57BL/6J mice

C57BL/6J mice are an animal model for age-related hearing loss. To confirm hearing loss in the aged group, hearing thresholds were evaluated by measuring ABR. The hearing thresholds of 8, 16, and 32 kHz in the young group (1 month) were 34 ± 2.5 dB SPL, 22 ± 2.0 dB SPL, and 26 ± 2.5 dB SPL, respectively. The hearing thresholds in the aged group (12 months) significantly increased to 64 ± 2.5 dB SPL, 62 ± 2.0 dB SPL, and 68 ± 5.8 dB SPL, respectively (n = 5, $p < 0.01$, Fig. 1).

## 3.2. Aging results in increased mitochondrial damage and decreased mitochondrial biogenesis in the cochlea of C57BL/6J mice

To examine the change in mitochondria in the aged group, the mitochondrial DNA copy number and the level of mitochondrial DNA damage were investigated relative to those in the young group. The mitochondrial DNA copy number in the cochlea of the aged group significantly decreased to 0.51 ± 0.01 (n = 5, $p < 0.05$, Fig. 2A). The level of mitochondrial DNA damage in the cochlea of the aged group significantly increased to 1.53 ± 0.08 (n = 5, $p < 0.01$, Fig. 2B). *PGC-1α* and *PGC-1β* mRNA expression levels in the aged group were also investigated relative to those in the young group to evaluate mitochondrial biogenesis. Relative mRNA ratios of *PGC-1α* and *PGC-1β* in the cochleae of the aged group significantly decreased to 0.58 ± 0.12 and 0.15 ± 0.03, respectively (n = 5, $p < 0.05$, Fig. 2C). These results indicated that mitochondrial damage increased and mitochondrial biogenesis decreased in aged cochlea.

## 3.3. Aging results in decreased levels of mitophagy-related genes and proteins in the cochlea of C57BL/6J mice

To examine the change in mitophagy in the aged cochlea, representative genes associated with mitophagy pathways were investigated relative to those in the

young group. The mRNA levels of *PINK1*, *Parkin*, *MUL1*, *Atg5*, *Atg12*, *Atg13*, *NIX*, and *BNIP3* significantly decreased to 0.54 ± 0.13, 0.43 ± 0.12, 0.54 ± 0.06, 0.6 ± 0.03, 0.74 ± 0.08, 0.43 ± 0.05, 0.6 ± 0.04, and 0.34 ± 0.03, respectively, in the cochleae of the aged group (n = 5, $p < 0.05$, Fig. 2D). The mRNA levels of *AMBRA1*, *Atg3*, *Atg7*, and *BNIP3L* showed as slight, but not significant, decrease in the cochleae of the aged group. Representative proteins associated with mitophagy pathways were investigated in the young group. A mitochondrial marker, *COX4*, and a reliable marker of autophagy, LC3B, were also investigated in the young group. The protein levels of *PINK1*, *Parkin*, *BNIP3*, *COX4*, and LC3B were significantly decreased in the cochleae of the aged group (n = 3, $p < 0.05$, Fig. 2E and F). The level of mitochondrial function was investigated using western blotting for the protein levels of the mitochondrial OXPHOS complex to examine the change in mitochondrial function in the aged cochlea. All OXPHOS subunits (complex I–V) significantly decreased in the cochleae of the aged group (n = 3, $p < 0.05$, Fig. 2E and G). These results indicated that the expression of mitophagy-related genes and proteins and mitochondrial function decreased in the aged cochlea.

### 3.4. Aging results in decreased levels of mitophagolysosomes and mitophagy-related proteins in the organ of Corti and spiral ganglions of C57BL/6J mice

To examine the change in mitophagosomes and mitophagolysosomes in the aged cochlea, immunofluorescence analysis was performed. Mitophagosomes were investigated by observing colocalization of a marker of autophagy, LC3B, with a major mitochondrial receptor, TOM20. Immunofluorescence analysis of LC3B and TOM20 showed that their coexpression was significantly decreased in the organ of Corti and spiral ganglion in 12-month-old mice compared to that in 1-month-old mice (n = 5, $p < 0.01$, Fig. 3A). The fusion process of mitophagosomes with lysosomes was investigated by observing colocalization of

a marker of lysosome, LAMP1, with TOM20. Immunofluorescence analysis of LAMP1 and TOM20 showed that their coexpression was significantly decreased in the organ of Corti and spiral ganglion in 12-month-old mice compared to that in 1-month-old mice (n = 5, $p < 0.01$, Fig. 3B).

To evaluate the major pathways of mitophagy, the expression levels of *Parkin* and *BNIP3* were investigated through immunohistochemical staining. Immunohistochemistry (IHC) scores of *Parkin* in the organ of Corti and spiral ganglion (apical turn/basal turn) were 13.0 ± 1.34/12.2 ± 1.11 and 8.6 ± 1.29/11 ± 1.38, respectively, in the young group; however, the scores significantly decreased to 6.6 ± 0.87/4.2 ± 0.8 and 5.2 ± 0.49/1.2 ± 0.49, respectively, in the aged group. IHC scores of *BNIP3* in the organ of Corti and spiral ganglion (apical turn/basal turn) were 15.4 ± 2.18/13.8 ± 1.91 and 5.0 ± 1.14/7.4 ± 1.03, respectively, in the young group; however, the scores significantly decreased to 11.0 ± 1.67/7.8 ± 1.36 and 3.6 ± 0.98/2.6 ± 0.4, respectively, in the aged group (n = 5, $p < 0.05$, Fig. 4). In the aged group, the IHC score of Parkin in the basal turn of the spiral ganglion showed a significant decrease compared to the score in the apical turn. The IHC scores of *Parkin* in the basal turn of the organ of Corti and *BNIP3* in the basal turn of the cochlea showed a slight but not significant decrease compared to the scores in the apical turn. These results indicate that the expression of *Parkin* and *BNIP3* decreased in aged cochlea, as observed through immunostaining analysis.

## 4. Discussion

Mitochondria play a key role in maintaining the balance of cellular processes; however, aging affects mitochondrial homeostasis (Palikaras et al., 2012). mtDNA is sensitive to oxidative damage, which increases in the cochlea with aging. Mutations in mtDNA and mitochondrial dysfunction in the cochlea are associated with age-related hearing loss (Markaryan et al., 2008) as damaged mitochondria generate less ATP and produce more ROS, which can trigger cell death (Rugarli et al., 2012). The constant turnover of dysfunctional mitochondria is important for maintaining healthy mitochondria with aging. Mitophagy and mitochondrial biogenesis are two key processes in mitochondrial turnover, and increasing mitophagy and mitochondrial biogenesis can affect the turnover and activity of mitochondria (Chen et al., 2014).

Mitophagy, a type of selective autophagic process for mitochondria, is the key mechanism in the clearance of damaged mitochondria (Ding et al., 2012). Down-regulation of mitophagy with aging was shown in the present study. This hinders the elimination of damaged mitochondria and results in the accumulation of dysfunctional mitochondria. Inadequate clearance of damaged mitochondria results primarily from reduced mitophagy rather than the reduction in mitochondrial biogenesis upon aging because mitophagy plays an essential role in the clearance of functionally impaired mitochondria and the maintenance of a healthy mitochondrial population (Markaki et al., 2018).

In the present study, significant losses in mitochondrial biogenesis along with mitophagy in the cochlea with aging were revealed. *PGC-1α* and *PGC-1β* are key regulators in modulating the expression of genes involved in mitochondrial biogenesis (Scarpulla et al., 2011). Mitochondrial biogenesis coordinates the regulation between gene expression, transcription of mtDNA, and protein import (Carter et al., 2015). *PGC-1α* controls the mitochondrial genome copy number (Gouspillou et al., 2014). Decreased expression of *PGC-1α* and *PGC-1β* can result in overall loss of mtDNA.

The most widely studied pathways in mitophagy are the *PINK1*/*Parkin* (Pickrell et al., 2015) and *BNIP3/NIX* pathways (Ney, 2015). *PINK1* and Parkin-dependent mitophagy is activated by the decrease in mitochondrial membrane potential due to mitochondrial damage and leads to the degradation of mitochondria (Lazarou et al., 2015). *BNIP3* and *NIX* are involved in the regulation of mitophagy and induce the programmed clearance of dysfunctional mitochondria through direct binding with LC3 (Ney, 2015; Novak, 2012). *NIX* also induces mitophagy by decreasing mitochondrial membrane potential in a *PINK1/Parkin*-independent manner (Song et al., 2016). *MUL1* can cleave mitochondria in a *PINK1*-dependent manner (Rojansky et al., 2016). *AMBRA1* induces autophagy in a *Parkin*-independent manner via direct binding with LC3 (Strappazzon et al., 2015). The present study showed the down-regulation of these pathways in the cochlea of 12-month-old mice.

The general machinery for autophagy involves Atg proteins that form multi-molecule complexes and regulate autophagosome formation (Mizushima et al., 2011). *Atg13* is a member of the core complex for initiating autophagy (Mizushima, 2010). *Atg4*, *Atg7*, and *Atg3* convert LC3-I to LC3-II (Kabeya et al., 2000). The *Atg12-Atg5-Atg16* complex also facilitates LC3 lipidation (Hanada et al., 2007). *Atg7* also involves a delayed clearance of mitochondria (Zhang et al., 2009). Our results showed a general decrease in Atg mRNA levels in the cochlea of 12-month-old mice. TOM20 is the mitochondrial import receptor and is distributed throughout the whole cochlea (Balaker et al., 2013). In the present study, a decrease in LC3B, LAMP1, and TOM20 coexpression was shown in the aged cochlea, indicating a decrease in mitophagy.

Xiong et al. (2019) reported upregulation of mitophagy during aging, which is in contrast to the results of the present study. Animal mitophagy can be investigated using demonstration of colocalization analyses of autophagosomes and mitochondria (Klionsky et al., 2016). While Xiong et al. investigated the colocalization in an auditory cell line only, we investigated the colocalization in

the cochlea and identified the next step of mitophagy: the fusion process of mitophagosomes with lysosomes. Our results showed a decrease in the colocalization of autophagosomes and mitochondria and decrease in the fusion process of mitophagosomes with lysosomes in the aged cochlea.

To date, impaired mitochondrial homeostasis by reduced mitophagy has been reported in other age-related diseases, including Parkinson's disease, Alzheimer's disease, and Huntington's disease (Chen et al., 2009; Burté et al., 2015; Martinez-Vicente et al., 2010). Inducing mitophagy can effectively prevent the increase in ROS from damaged mitochondria and the accumulation of dysfunctional mitochondria, thus mitigating cell death during aging (Zhang et al., 2008; Ryu et al., 2016).

In conclusion, the present study revealed a decrease in mitophagy in aged cochlea, which can aggravate cellular damage and increase hearing loss. Reduced mitophagy with aging might be a major cause of cellular damage in aged cochlea, resulting in age-related hearing loss.

## 5. Conclusion

The maintenance of mitochondrial function and mitophagy is essential to the cells, because mitochondria are involved in both adaptive metabolism and survival in response to aging. Mitophagy decrease with age in the cochlear and auditory cortex of C57BL/6J mice. Although the studies analyzed do not exhibit a general consensus, it seems that aging impairs mitochondrial biogenesis and dynamics and decreases the mitophagic function of the organism in cochlear and auditory cortex. So, further research is necessary to determine the mechanisms of interaction between mitochondrial functions, aging, and hearing loss, as well as to analyze possible factors that are supposed to influence these processes.

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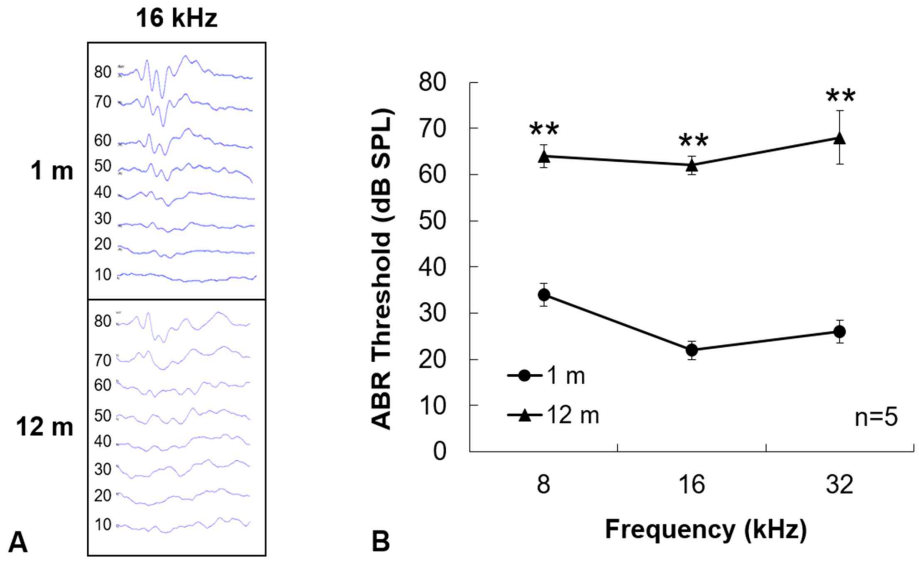


**Fig. 1. The hearing thresholds of 1- and 12-month-old mice.** (A) Representative waveforms of ABR measurement using 16 kHz tone-burst stimuli. The lowest stimuli levels that produced response peaks in the young and the aged groups were 20 and 70 dB SPL, respectively. (B) The hearing thresholds of 8, 16, and 32 kHz in the aged group were significantly increased compared with those of the young group. The data are shown as the means ± S.E.M. of five mice per group (1 m: 1 month, 12 m: 12 months; $^{**}p < 0.01$).

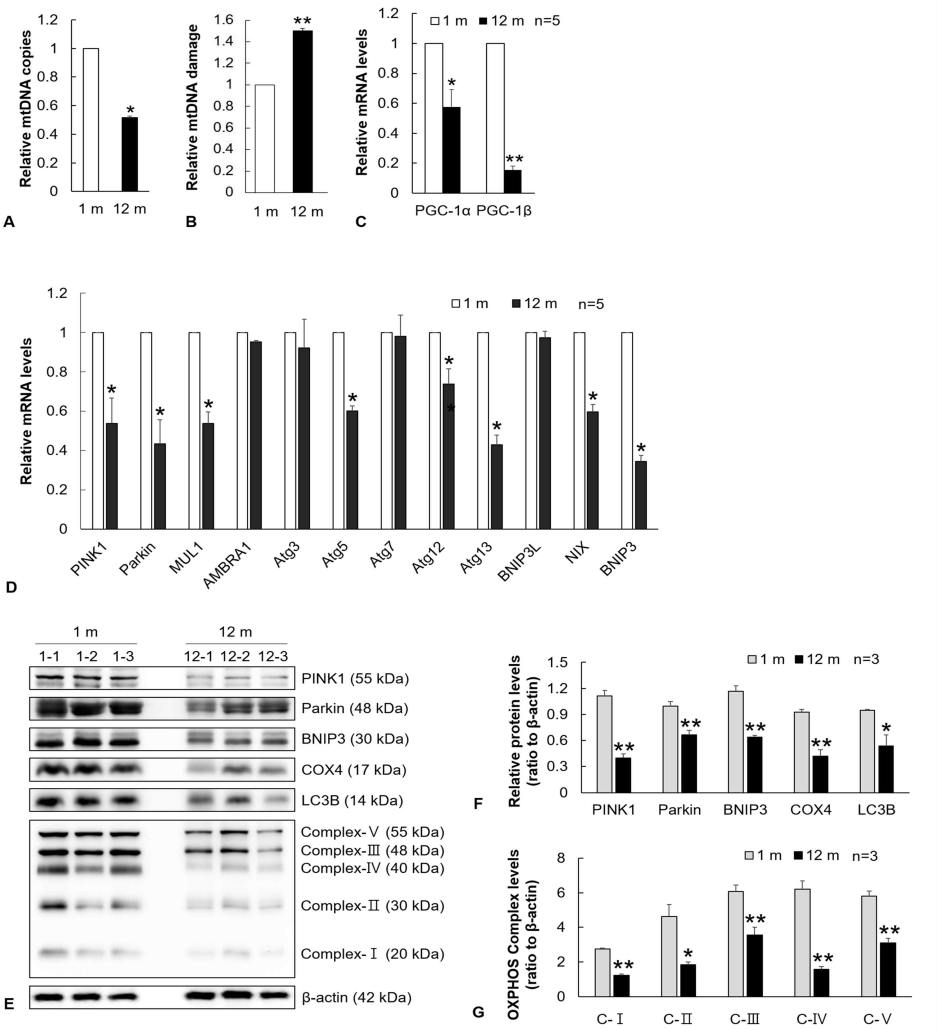


**Fig. 2. The changes in mitochondrial DNA copy number, the level of mitochondrial DNA damage, mitochondrial biogenesis, and the expression of mitophagy-related genes and proteins in the aged cochlea.** (A) Relative mitochondrial DNA copy number in the cochleae of mice in the aged group was significantly decreased compared with that in the cochleae in the young group (n = 5 per group). (B) The level of relative mitochondrial DNA damage in the cochleae of mice in the aged group was significantly increased compared to that in the cochleae in the young group (n = 5 per group). (C) Relative mRNA ratios

of *PGC-1α* and *PGC-1β*, which are key regulators of mitochondrial biogenesis, in the cochleae of mice in the aged group significantly decreased compared with those of mice in the young group (n = 5 per group). (D) Relative mRNA levels of *PINK1*, *Parkin*, *MUL1*, *Atg5*, *Atg12*, *Atg13*, *NIX*, and *BNIP3* significantly decreased in the cochleae of mice in the aged group (n = 5 per group). (E) Western blot analysis of *PINK1*, *Parkin*, *BNIP3*, *COX4*, LC3B, and OXPHOS complex. (F,G) Relative protein levels of *PINK1*, *Parkin*, *BNIP3*, *COX4*, LC3B, and OXPHOS complex significantly decreased in the cochleae of mice in the aged group (n = 3 per group). The data are shown as the means ± S.E.M. of three to five mice in each group (1 m: 1 month, 12 m: 12 months; $*p < 0.05$, $**p < 0.01$).

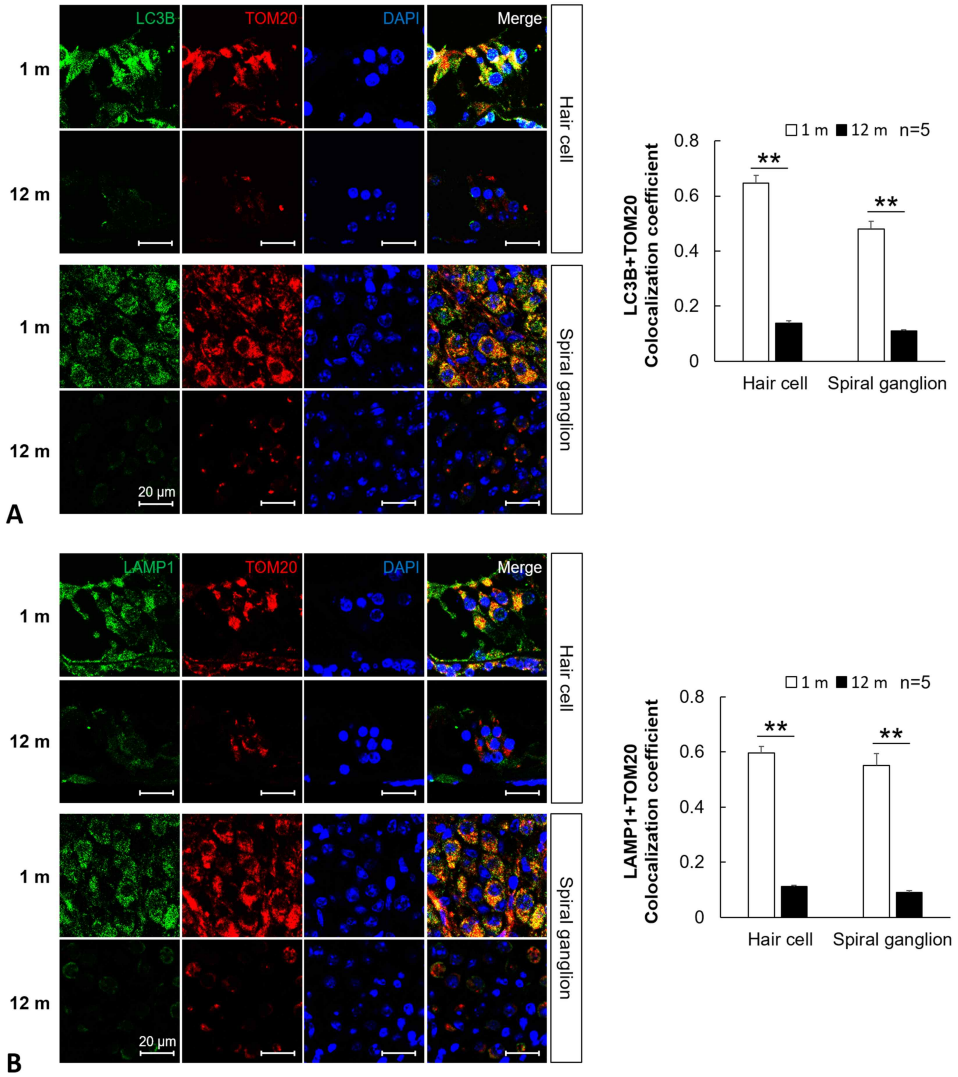


**Fig. 3. Reduced mitophagy in the aged organ of Corti and spiral ganglion.** (A) Colocalization analysis of autophagosomes and mitochondria. Immunofluorescence of LC3B (green) and TOM20 (red) showed that their colocalization (yellow, the overlap color) was significantly decreased in the hair cell and spiral ganglion of 12-month-old mice compared with that in the hair cell and spiral ganglion of 1-month-old mice. (B) Colocalization analysis of lysosomes and mitophagosomes. Immunofluorescence of LAMP1 (green) and TOM20 (red) showed that their

colocalization (yellow, the overlap color) significantly decreased in the hair cell and spiral ganglion of 12-month-old mice compared with that in the hair cell and spiral ganglion of 1-month-old mice. The data are shown as the means ± S.E.M of five mice in each group (1 m: 1 month, 12 m: 12 months; $^{**}p < 0.01$).

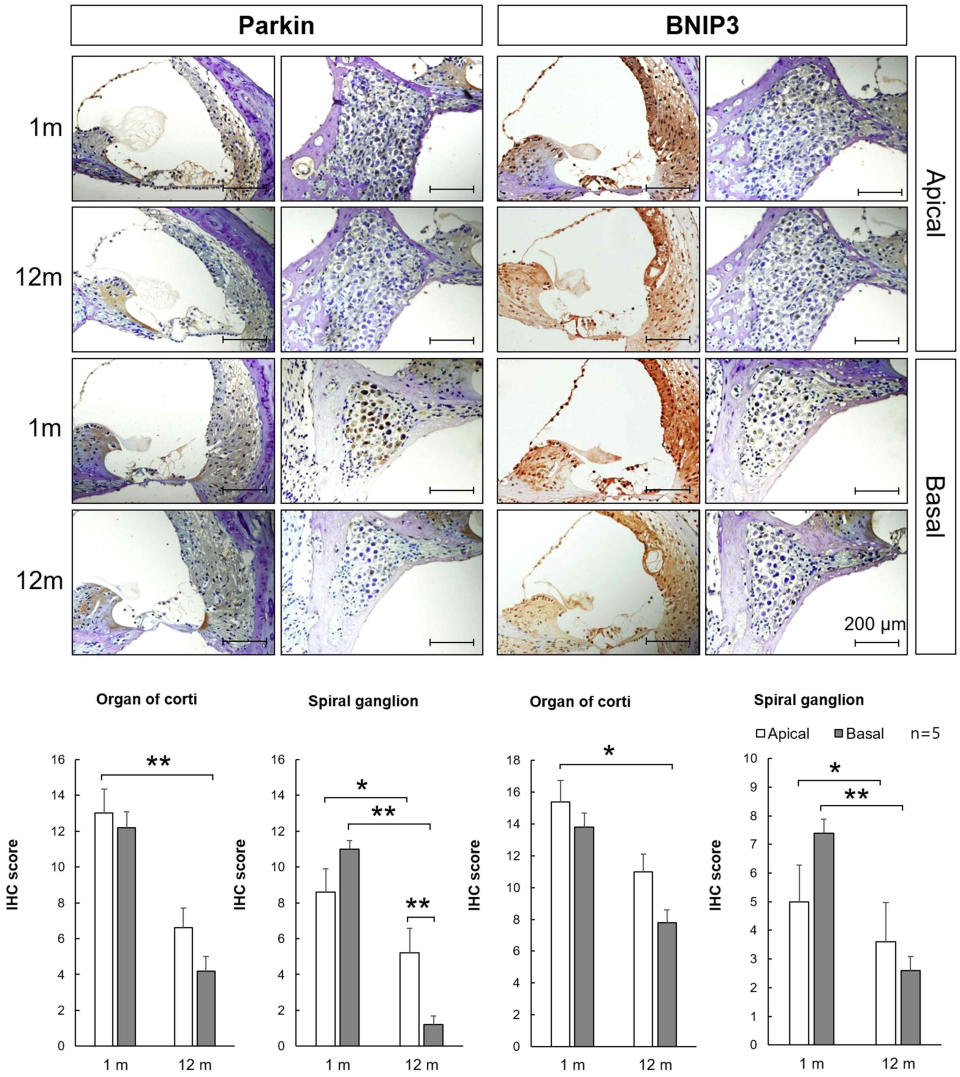


**Fig. 4. Decrease in *Parkin* and *BNIP3* in the aged cochlea.** Immunohistochemistry (IHC) scores of *Parkin* and *BNIP3* in the organ of Corti and spiral ganglion of mice in the aged group significantly decreased in both the apical turn and the basal turn. IHC score of *Parkin* in the basal spiral ganglion of mice in the aged group significantly decreased compared with that in the apical spiral ganglion of mice in the aged group. The data are shown as the means ± S.E.M. of five mice in each group (1 m :1 month, 12 m: 12 months; *$p < 0.05$, **$p < 0.01$).