Increased mitochondrial DNA copy number protects hair cells and HEI-OC1 cells against drug-induced apoptosis

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Abstract. Several factors trigger apoptosis in cochlear hair cells. Previous studies have shown that mitochondria play key roles in apoptosis, but the role of mitochondrial deoxyribonucleic acid (mtDNA) copy number in the pathogenesis of hair cell apoptosis remains largely unknown. We used mouse cochlear hair cells and House ear Institute-organ of Corti 1 (HEI-OC1) cells to explore the relationship between mtDNA copy number and cell apoptosis. We found that the mtDNA copy number of hair cells was reduced relative to mitochondrial mass and hypothesized that increasing it might have a protective effect. We then increased the mtDNA copy number of the hair and HEI-OC1 cells by transfecting them with an adenovirus-associated virus (AAV) vector containing mitochondrial transcription factor A (TFAM). We found that the apoptosis rates decreased upon inducing apoptosis with neomycin or cisplatin (DDP). To elucidate the mechanisms, we analyzed the mitochondrial-membrane permeability and mitochondrial function of HEI-OC1 cells. Our results suggested that the increase in mtDNA copy number could protect hair cells and HEI-OC1 cells against drug-induced apoptosis by stabilizing the permeability of the mitochondrial membrane and mitochondrial function.

Introduction

Disabling hearing loss affects more than 5% of the world's population (466 million individuals). It is estimated that by 2050, more than 900 million people will be affected by disabling hearing loss (http://www.who.int/en/news-room/fact-sheets/detail/deafness-and-hearing-loss). Sensorineural hearing loss (SNHL) is a common disease worldwide that accounts for most hearing loss. Currently, there are no effective treatment options available. Factors that may lead to SNHL include aging, exposure to loud noise, heredity, and some medications (1). All of these factors may result in the apoptosis or loss of cochlear hair cells. Because hair cells cannot regenerate once they are lost (2,3), how to protect them against apoptosis, either by inhibiting processes that lead to damage or by enhancing their survival, is obviously an important research goal.

The mitochondrion plays an important role in cell apoptosis; it is also the main site of intracellular oxidative phosphorylation and adenosine triphosphate (ATP) synthesis (4). Mitochondria have their own nucleic acid, mitochondrial deoxyribonucleic acid (mtDNA). As reported (5,6), a variation in the mtDNA copy number can affect cell apoptosis. Transgenic mice with mtDNA deletions cannot survive the embryonic period, and a large number of apoptotic cells have been found in such embryos (5). Our previous research also found that decreased mtDNA copy number sensitizes cancer cells to chemotherapeutic drugs (6). However, its effect on hair cells has not been elucidated. Increased mtDNA copy number may enhance the survival of hair cells and protect them against apoptosis. In the present study, the mtDNA copy number of mouse cochlear...
hair cells was assessed and it was found that normal cochlear hair cells were relatively lacking in mtDNA. Then the mtDNA copy number of hair cells and House Ear Institute-Organ of Corti 1 (HEI-OC1) cells was increased by mitochondrial transcription factor A (TFAM) transfection to ascertain whether it had a protective effect. Finally, the mechanisms by which increased mtDNA copy number protects against apoptosis were explored.

Materials and methods

Cochlear basal membrane and HEI-OC1 cell culture. A total of 50 newborn C57BL/6j mice (P1-P3), each weighing ~1.5-2.5 grams, were used in this experiment, and their gender selection was random. International Council for Laboratory Animal Science (ICLAS) Ethical Guideline (https://iclas.org/guidelines-for-researchers) and the ethical requirements of experimental animals in Fudan University (http://labanimal.fudan.edu.cn/95/6f/c5213a38255/page.htm) were followed. Our laboratory animals were cared for by a full-time breeder; our experiment was approved by the Animal Care and Use Committee of the Affiliated Eye and ENT Hospital, Fudan University (no. 201526). Mouse pups were decapitated and dissections were performed under a light microscope in a Petri dish containing ice-cold phosphate-buffered saline (PBS). The cochleae were removed from the skulls and opened with forceps. The basal membranes were detached and transferred into pre-prepared culture dishes containing Dulbecco’s modified Eagle’s medium-Ham’s F-12 (DMEM/F12; Thermo Fisher Scientific, Inc.) supplemented with N2 and B27 (Thermo Fisher Scientific, Inc.), and cultured at 37°C with 5% CO₂.

Measurement of mitochondrial mass and immunofluorescence. Mitochondrial mass was used to determine mitochondrial mass, and MitoSoX red was utilized to mark the apoptotic cells.

TFAM forward, ATGGGTTTCTCCGAAGCAT and reverse, CAGATGAAACCCACCTCGGTA; COI forward, ACTCT ACCACCATCATTTTCC and reverse, GGCCTAGTTTCC GGCTAGGG; COII forward, CTTGGTCTACAAGACGCC AC and reverse, CTATTGCAGAAGCCTCGG; 15SrRNA forward, AATGAGATGCACACCCG and reverse, GGG TGATGGCCAGATGCTTT; ATP6 forward, AAGCTCCT TGCCCCACTC and reverse. GGAACCGGACTCTAT GC; GAPDH forward, TGGCACTTTACAGCAACTC and reverse, CTTGGTCTAGTGCTCCTGCT; ND1 forward, ACC ATTTGCAGGCGCTAA and reverse, TGAAATTTTTT GGCTACGG; ACTB forward, TCCAGTTGTAACATG CCA and reverse, TGTTCCCTTCCAGGGTTGT. qPCR conditions consisted of an initial denaturing step of 30 sec at 95°C followed by 35-40 cycles of 5 sec of denaturation at 95°C, 20 sec of annealing at 60°C and 20 sec of extension at 72°C. The results were calculated using the comparative-cycle threshold (ΔΔCq) method (8).

Fluorescence in situ hybridization (FISH). The probe was prepared using the FISH Tag DNA Kit (Thermo Fisher Scientific, Inc.), and the spectrum of the intended probe was from 278 to 1435 bases (NCBI Reference Sequence: NC_005089.1) in mouse mtDNA.

The cochlear basal membranes or HEI-OC1 cells were cultured, collected at proper time points on slides, and then fixed with 4% polyoxymethylene and permeabilized with 0.5% Triton X-100. Then, the FISH procedure was conducted as previously described (6).

Measurement of mitochondrial mass and immunofluorescence. Mitotracker Green FM (Thermo Fisher Scientific, Inc.) was used to determine mitochondrial mass, and MitoSOX Red (Thermo Fisher Scientific, Inc.) was used to determine reactive oxygen species (ROS) levels. Cochlear basal membranes were grown in culture medium for a certain period of time, the culture medium was removed and the samples were washed with PBS. We then added a pre-warmed (37°C) solution containing Mitotracker Green FM or MitoSOX Red and the cells were incubated at 37°C for 20 min. After staining, the samples were washed in PBS and imaged (living-cell imaging) under a Leica SP8 confocal microscope (magnification, x40; Leica Biosystems). To label hair cells, anti-myosin VIIA rabbit polyclonal antibody (ab3481, Abcam) was added and the cells were incubated for 8 h (4°C) at a dilution of 1:200 after cochlear basal membranes had been fixed with 4% polyoxymethylene and permeabilized with 0.5% Triton X-100. We washed the samples 3 times with PBS and incubated them for 2 h with fluorescence-conjugated goat anti-rabbit immunoglobulin G.
(IgG) secondary antibody (ab150077, Abcam) at room temperature (RT).

Since GFP expression was extremely low on the second day after TFAM transfection and signals could not be detected after FISHAM treatment, we used anti-GFP (ab290, Abcam) to enhance the signals. The immunofluorescence procedure was the same as mentioned above.

**Flow cytometry (FCM).** To measure mitochondrial permeability, we conducted FCM using a MitoProbe Transition Pore Assay kit (M34153, Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocols. For each sample, we prepared 3 aliquots: 1 contained calcein-acetoxymethyl (calcein AM) only; 1 contained calcein AM and CoCl2; and 1 contained calcein AM, CoCl2 and ionomycin. The samples were incubated at 37°C in the dark for 15 min. Detailed information can be found in the manufacturer’s protocols.

To determine mitochondrial membrane potential (MMP) and analyze reactive oxygen species (ROS), the HEI-OC1 cells were cultured, trypsinized and collected; and then resuspended in a pre-warmed (37°C) solution containing MitoTracker Red CMXRos or MitoSOX Red (Thermo Fisher Scientific, Inc.) for 10 min. The HEI-OC1 cells were then washed with PBS; and they were analyzed by FCM. For apoptosis analysis, a TUNEL assay kit was utilized. First, the cells were collected and fixed with paraformaldehyde, and then centrifuged for 5 min at 300 x g and then the supernatant was discarded. next, the cells were washed in PBS, pelleted by centrifugation and then resuspended at a concentration of 1x10^6 cells/ml. We added a dead cell apoptosis Kit with fluorescein a M, cocl2 and ionomycin. The samples were incubated at 37°C in the dark for 15 min. Detailed information can be found in the manufacturer’s protocols.

Results

**Relative deficiency of mtDNA copy number and high levels of ROS in hair cells.** Mitochondria are the powerhouse of cells. As auditory receptors and mechano-electrical transducers, hair cells need a large number of mitochondria, which we confirmed in this experiment. After culturing mouse cochlear basal membranes in vitro for 24 h, we measured their mitochondrial mass using MitoTracker Green, for which the location in mitochondria is not affected by MMP. We observed that the fluorescence of hair cells was extremely intense compared with that of supporting cells, indicating that the mitochondrial mass of hair cells was significantly higher (Fig. 1A and D; t=14.024; n=5; P=0.000000649). We also performed FISH on mtDNA in cochlear basal membranes after culturing them for 24 h in vitro. In this experiment, we found that the mtDNA copy number of hair cells was also higher than that of supporting cells (Fig. 1B and E; t=4.083; n=6; P=0.00274), but the difference in fluorescence intensity between hair cells and supporting cells was smaller than the difference in mitochondrial mass between both types of cells. This demonstrated that the copy number per mitochondrion was relatively deficient in hair cells compared with that of supporting cells (Fig. 1G; t=9.366; n=5; P=0.0000138), which may have influenced normal mitochondrial function. Finally, we measured the ROS level using MitoSOX Red and observed that it was higher in hair cells than that in supporting cells (Fig. 1C and F; t=4.6422; n=5; P=0.00166), which may have been caused in part by relative mtDNA copy number deficiency.

**Protective effect of increased mitochondrial DNA copy number on hair and HEI-OC1 cells.** TFAM is an important gene that regulates the copy number of mitochondrial DNA, and its overexpression has no effect on mitochondrial mass (9). We used AAV-TFAM GFP to increase hair cell mtDNA copy number in this experiment (see our ‘Materials and methods’ section for detailed information). After culturing mouse cochlear basal membranes in vitro for 24 h, AAV-TFAM was added into the medium, and FISH was performed on mtDNA and qPCR on TFAM RNA. It was found that the mtDNA copy number was significantly increased in some hair cells at the second day after transfection (Fig. 2A, B and E; t=4.44; n=9; P=0.000412). The result was confirmed in our qPCR experiment (Fig. 2F; t=5.052; n=3; P=0.00722). We also found that mRNA levels of TFAM were significantly increased (Fig. 3A; t=13.698, 19.14, 17.638; n=3, 3, 3; P=0.000165, 0.0000439, 0.0000607). At the fifth day after transfection, it was found that the GFP signal remained in some hair cells, but the mtDNA copy number of transfected hair cells was no longer higher than the non-transfected cells (Fig. 3B-D). The results indicated that the increase in mtDNA copy number occurred only early on after TFAM transfection. As time progressed, TFAM overexpression may have blocked mtDNA replication and offset its effect of increasing mtDNA copy number.

In the apoptosis induction experiment, we added neomycin into the medium at a final concentration of 1 mM on the second day after TFAM transfection. Twelve hours later, we collected the basal membranes and compared hair cell loss or apoptosis rates between the TFAM transfection and control groups using a TUNEL kit. It was demonstrated that the basal membranes of the TFAM transfection group had an obviously lower apoptosis rate than did those of the control group (Fig. 2C, D and G; t=3.638; n=3; P=0.022).

To verify the previous results on hair cells, we repeated the experiment on HEI-OC1 cells. After transfecting them with TFAM, we collected the cells at different time points. First, we...
analyzed TFAM RNA expression levels by qPCR and found that the expression had significantly increased, peaking at the second day (Fig. 4A; \( t = 21.516; n = 3; P = 0.0000276 \)). Then, we analyzed the change in mtDNA copy number in HEI-OC1 cells by qPCR. The mtDNA copy number was also increased and peaked at the third day, lagging just behind the change in RNA levels (Fig. 4B; \( r = 6.556; n = 3; P = 0.0028 \)). However, the mtDNA copy number was obviously decreased after the fifth day but had no statistically significant difference from the control (Fig. 4B). The results were consistent with those we found in hair cells. In the apoptosis induction experiment, the HEI-OC1 cells were treated with 0.07 mM DDP for 24 h, and then the cells were collected and labeled with TUNEL or Annexin V/PI. The apoptosis rates were analyzed by FCM and the results revealed that the rates of the TFAM transfection group were lower (Fig. 4C and D). After quantification of the result, it was found that the difference was statistically significant (Fig. 4E and F; \( t = 4.797; 16.067; n = 4, 3; P = 0.00301, 0.0000878 \)).

**Increased mtDNA copy number exerts a protective effect by decreasing mitochondrial membrane permeability.** In this experiment, the protection mechanisms of mtDNA copy number in HEI-OC1 cells were explored. As previously mentioned, mtDNA copy number peaked at the third day after TFAM transfection, whereupon we induced apoptosis in the HEI-OC1 cells by DDP for 12 h. We then analyzed the mitochondrial permeability of HEI-OC1 cells by FCM using a Mitochondrial Permeability Transition Pore Assay Kit (Fig. 5A) and found that the fluorescence signal was obviously stronger in the TFAM transfection group (Fig. 5B-a). The result were quantified and the difference was found to be statistically significant, indicating that the mitochondrial permeability had decreased (Fig. 5B-b; \( t = 7.772; n = 3; P = 0.00148 \)). The increased...
Molecular Medicine Reports 21: 338-346, 2020

mtdna copy number may have blocked the mitochondrial-permeability transition pore (mPTP) when apoptosis was induced in the Hei-oc1 cells. The decrease in mitochondrial permeability may have stabilized mitochondrial function. Then, we analyzed the mitochondrial transmembrane potential of Hei-oc1 cells by FCM using MitoTracker red MXros at the same time point after ddP treatment. Mitochondrial transmembrane potential was higher in the TFAM transfection group (Fig. 5c-a), but the difference was not statistically significant (Fig. 5C‑b). Meanwhile, we analyzed the ROS level of Hei-oc1 cells by FCM using MitoSoX Red. The ROS level was lower in the TFAM transfection group, and the difference was statistically significant (Fig. 5D; t=9.766; n=3; P=0.000616). After TFAM transfection, increased mtdna copy number may have blocked the mPTP, further inhibiting the decline of mitochondrial transmembrane potential and suppressing the production of ROS. Ultimately, its protective effect was exerted when apoptosis was induced in the cells.

**Discussion**

Mitochondria have their own genetic material, mtdna, which encodes 13 proteins, 22 transfer RNAs (tRNAs), and 2 ribosomal RNAs (rRNAs), which are involved in maintaining the mitochondrial function. mtdna copy number varies by cell (10) and by developmental stage (11,12), and depends on the dynamic equilibrium between mtdna synthesis and degradation. As reported, while a decreased mtdna copy number or mtdna depletion may break cellular mitochondrial function and result in many diseases, an increased mtdna copy number has a protective effect (6,13 -15). Given the highly active and vulnerable nature of hair cells, increasing their mtdna copy number is a potential way to enhance their survival.
In the present study, we measured the mitochondrial mass, ROS level, and mtDNA copy number in newborn mouse cochlear hair cells. It was found that the mitochondrial mass of hair cells was significantly higher than that of the supporting cells, and the same was true for their ROS level. These results were anticipated as hair cells require a great deal of energy from mitochondria to sustain their normal function, and the levels of by-product ROS increase during the ATP production process. Then, the mtDNA copy number in hair cells was then assessed and it was higher than that of the supporting cells, but to a smaller degree than was true for mitochondrial mass, meaning that mtDNA copy number was reduced relative to mitochondrial mass in hair cells. This result was not what we had expected. We think the reduced mtDNA copy number may have influenced the normal mitochondrial function to a certain extent, giving rise to a high level of ROS and leading to the fragility of hair cells. Therefore, increased mtDNA copy number may have a protective effect on hair cells.

The regulation of intracellular mtDNA copy number is complicated. The catalytic subunit of DNA polymerase γ and its processivity factor (both encoded by the POLG2 gene), together with the Twinkle helicase (encoded by the TWNK gene), DNA replication helicase/nuclease 2 (encoded by the DNA2 gene), single-stranded DNA binding protein 1 (encoded by the SSBP1 gene), primase and polymerase (DNA-directed; encoded by the PRIMPOL gene), mitochondrial genome maintenance exonuclease 1 (encoded by the MGME1 gene), and the mitochondrial transcription factor A (encoded by the TFAM gene), play key roles in mitochondrial DNA maintenance (13,16,17). Of all the genes, TFAM has been the most researched and may be the best choice for increasing the mtDNA copy number. In addition, human TFAM overexpression can increase a mouse cell mtDNA copy number (9,18). Therefore, we used an AAV vector containing the human TFAM gene to increase murine cell mtDNA copy number in our research. After TFAM transfection, we collected the cochlear basal membranes and analyzed the mtDNA by FISH. We found that the mtDNA copy number was increased obviously in transfected hair cells, although the increase lasted just a few days.

In the apoptosis induction experiment, we used neomycin, which is an aminoglycoside antibiotic and can specifically damage hair cells in the cochlear basal membrane but does not damage the surrounding supporting cells (19-21). We added neomycin and found that the hair cells had an obviously lower apoptosis rate after TFAM transfection. The results implied the protective effect of increased mtDNA copy number against apoptosis.

To further confirm the protective effect of mtDNA copy number, we repeated these experiments using HEI-OCl cells. The results were the same as for hair cells. The mtDNA copy number was increased and remained higher for approximately 1 week after TFAM transfection. During induction of apoptosis, we tried many times but found that the cell line was not sensitive to neomycin. Even at a high concentration of the drug, we found no difference in apoptosis rates between the neomycin-induced and control groups. HEI-OCl cells originate from mouse cochleas, but they are not real hair cells. Therefore, we finally chose cisplatin. We also found that the TFAM-transfected HEI-OCl cells had a lower apoptosis rate, consistent with that in hair cells.

Increased mtDNA copy number may exert its protective effect by enhancing the mitochondrial function of the cell. However, the detailed mechanisms are not clear. When the mtDNA copy number was increased in HEI-OCl cells, we analyzed the transcription of mtDNA genes (ND1, COI, COII, 12SrRNA, ATP6) through PCR, but we found no significant increase (data not shown). Therefore, mitochondrial genes are not activated and involved in the protection against apoptosis.

Mitochondrial DNA is compacted into nucleoids with numerous nucleoid-associated proteins, each nucleoid containing just 1 copy of mtDNA (22-24). As previously reported,
the nucleoids attach to the inner membranes of mitochondria and interact with many mitochondrial membrane-bound proteins, such as ATPase family AAA domain-containing protein (ATAD3) (22,25,26). We therefore assume that increased mtDNA copy number may result in a number of mitochondrial nucleoids attaching to the inner membrane, which, together with their interaction with membrane proteins, may further affect mitochondrial-membrane permeability.

The mitochondrial permeability transition pore (mPTP) is formed in the inner membrane of the mitochondrion, and it plays an important role in the physiological regulation of Ca²⁺ and in ROS homeostasis. The opening of the mPTP initiates the production and release of ROS, which damages mitochondrial and nuclear DNA, proteins, and phospholipids (27-29). In addition, mPTP has been found to be involved in cell death induced by hypoglycemia, ischemia/reperfusion damage, and neurodegenerative and neuromuscular disorders (30,31).
We analyzed the mitochondrial permeability of the HEI-OCl cells by FCM using a mitochondrial-permeability transition pore assay kit and found that permeability was decreased in the TFAM group, which had a higher mtDNA copy number. The results confirmed our hypothesis that the change in the mitochondrial permeability may have influenced the mitochondrial function. We then analyzed the mitochondrial transmembrane potential and the ROS level of the HEI-OCl cells after TFAM transfection, and found that the potential was higher in the TFAM group (though not to a statistically significant degree), whereas the ROS level was lower. Therefore, decreased mitochondrial permeability enhances the mitochondrial function of cells by stabilizing MMP and reducing ROS, exerting a protective effect on the cells.

In summary, we found that the mtDNA copy number of cochlear hair cells was reduced relative to mitochondrial mass, which may contribute to the fragility of these cells. The mtDNA copy number can be increased in vitro by TFAM overexpression and may help protect the cells against apoptosis. Although the detailed mechanisms, which we intend to study in the future, remain unclear, it is apparent that increased mtDNA copy number may interfere with the opening of the mPTP, reduce the production of ROS and ultimately result in lower rates of apoptosis. These findings provide a potential novel strategy for research into the protection of cochlear hair cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HM, DM and HY performed the experiments, collected the data and wrote the manuscript; SS performed the PCR analysis and collected the data. YC and YZ performed the transfection experiments and collected the data. RC performed the flow cytometry analysis and collected the data. HL designed the research and gave final approval of the version to be published. HL and SS directed the research and provided supporting materials.

Ethics approval and consent to participate

Experimental protocols and procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Affiliated Eye and ENT Hospital, Fudan University (Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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