REVIEW ARTICLE

Acupuncture Attenuates Ototoxicity Induced by Gentamicin in Mice

Yang Yu, MSc; Lihua Cao, MSc; Ningning Guan, MSc; Yiping Tang, MSc; Mei Han, BSc

ABSTRACT

Context • Presbycusis is age-related, progressive, and symmetrical hearing loss in both ears. Acupuncture can play a vital role in the diagnosis and treatment of deafness, but its functional mechanism is still not entirely clear.

Objective • The study intended to explore acupuncture's protective effects and mechanism of treatment in addressing ototoxicity induced by gentamicin (GM) in aged mice.

Design • The research team designed an animal study, and a mouse model of ototoxicity induced by GM was established.

Setting • The study took place in Nanchong Central Hospital, Sichuan, China.

Animals • The animals were 48 male, Kunming mice, with sixteen being three months old and 32 being 18 month old.

Intervention • The three-month-old mice were randomly assigned to a control group (n=8) and a GM group (n=8). The 18-month-old mice were randomly divided into four groups with eight mice each: a positive control group; a negative control group, the GM group; and two intervention groups, the acupuncture + GM group and the drug + GM group. The GM groups were intraperitoneally injected with 100 mg/kg daily of GM for 10 consecutive days. The acupuncture + GM group received acupuncture, and the drug + GM group was injected intraperitoneally with Genadol.

Outcome Measures • The effects of GM induction and treatment with acupuncture or a drug on the numbers of auditory cochlear hair cells were evaluated via an auditory test and cell staining. A real-time polymerase chain reaction (PCR) was performed for gene detection. The levels of superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) were measured.

Results • The aged mice were susceptible to GM ototoxicity. After acupuncture, the threshold of the auditory brainstem response and the number of cochlear hair cells increased significantly. Acupuncture inhibited oxidative stress via the nuclear factor erythroid-derived factor 2-related factor 2 (NRF2) signaling pathway in the mice.

Conclusions • The data demonstrated that acupuncture can alleviate GM ototoxicity via the NRF2 signaling pathway, providing important support for acupuncture in treatment of GM ototoxicity. (*Altern Ther Health Med.* 2022;28(2):78-83).

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With a growing standard of living and current medical treatment in China, life expectancy is rising. More attention is needed on older adult's life quality. Hearing loss or deafness can severely worsen that life quality. According to statistics, 72% of the world's population over 65 years old suffer from hearing loss and the incidence of deafness is about 30% in the people over 60 years old.

Presbycusis is age-related, progressive, and symmetrical hearing loss in both ears, and mostly appears as high-frequency, sensorineural hearing loss. So far, no unified clinical standard exists for treatment of presbycusis. Decreased auditory acuity and reduced speech recognition, especially in a noisy environment, are the main clinical features.[1]

The pathogenesis of presbycusis is still unclear. The cochlea, a component of the peripheral auditory system, is responsible for feeling and transmitting sound waves. Abundant mitochondria exist in the cochlea, which is very sensitive to hypoxia.

The blood flow in the cochlea decreases with age, and oxygen and blood may be inadequately provided to it, which has been attributed to the weak metabolic capacity of older adults. Microcirculation in the inner ear is influenced by blood flow of the vertebral basilar artery system, autonomic nerves, and a local regulatory mechanism.[2]

Poor microcirculation can result in generation of excessive oxygen free radicals and reduction in antioxidant substance, disrupting the balance between the activity of the radicals and the substance. Under a hypoxic condition, damage from lipid peroxidation can occur in addition to production of excessive free radicals, leading to cochlear impairment and even loss of hearing.[3]

High oxidative stress levels in older adults have been confirmed to damage cochlear hair cells through excess production of reactive oxygen free radicals, resulting in hearing loss.[4, 5] The aggregation of oxygen free radicals not only damages the cochlear tissue and causes the functional decline of cochlear hair cells, but also acts on the satellite glial cells (SGC) and the stromal vascular (SV) cells of the cochlea to decrease their functionality, thus leading to the occurrence of deafness.[6] Therefore, a promising avenue for treating presbycusis is inhibition of oxidative stress.

Ferino et al found that NRF2 signaling pathway was the crucial signaling pathway in regulating oxidative stress.[7] Sun et al and Shaw et al have confirmed that NRF2 signaling pathway is a pivotal regulatory pathway in oxidative stress.[8,9] Previous studies have shown that NRF2 is a key target for prevention of noise-induced hearing loss by reducing oxidative damage of cochlea.[10]

Considerable evidence has demonstrated that causes of hearing loss include genetic factors, intrauterine infections, chronic ear infections, loud sounds/noises, age-related sensorineural degeneration, and ototoxic medicines.[11,12] Aminoglycosides displaying concentration dependent bactericidal activity rather than bacteriostatic potential are relatively potent antibiotics used against aerobic Gram-negative bacteria including Enterobacteriaceae and Pseudomonas spp., tuberculosis, neonatal sepsis and some other life threatening infection.[13,14] As ototoxic medicines, aminoglycosides, including GM, can be actively absorbed by inner ear cells. GM binding to ribosomal RNA causes mRNA mistranslation, resulting in the generation of superoxide free radicals, which leads to hair cell necrosis.[12,15] The study of Vysakh A et al confirmed that GM induced oxidative stress and nephrotoxicity in Wistar rats.[16] Consider the role of acupuncture in the regulation of oxidative stress,[17] We speculate that acupuncture may alleviate GM induced deafness by inhibiting oxidative stress.

Su et al concluded in their study that gentamicin (GM) ototoxicity is mainly reflected in cochlear hair cell injuries.[18] O'Reilly et al found that GM can accumulate in the inner lymphatics, and hair cells have a great affinity for GM, which often leads to hair cell death in patients at the early stage of GM exposure and even permanent hearing loss.[19] GM induces excessive generation of reactive oxygen species in cochlear tissues, which breaks through the defensive ability of the intracellular antioxidant system, leading to cell apoptosis through mitochondria-dependent endogenous apoptotic pathways.[20,21] Excessive oxygen free radicals can damage mitochondrial DNA, mitochondrial membranes, respiratory chain proteins, and other structures and eventually can lead to cell death. Therefore, the oxidative stress injury in GM ototoxicity is also a crucial aspect on which researchers should focus.

Although certain benefits have been gained through conventional treatments for presbycusis, including improvement of microcirculation, antiviral actions, and anticoagulant treatments, the prognosis is unfavorable.[22] It's urgent to develop an efficient and reasonable treatment, and acupuncture offers a possible alternative. Acupuncture has been confirmed as a promising avenue for salvaging hearing loss. Jiang et al and Zhang et al found that the hearing of deaf patients can be remarkably elevated after acupuncture.[23,24] Su et al found that acupuncture can alleviate endoplasmic reticulum stress and oxidative stress in ovariectomized rats.[25] Xu et al found that acupuncture can boost blood circulation, resulting in a regulatory effect on brain and nerve tissue.[26] Those researchers also found shown that acupuncture functions on a particular part of the body's surface or acupoint through various forms of electric energy transfer, generating certain electromotive force. Subsequently, the body sends the generated information into the related organs to adjust abnormal biological levels, resulting in the regulation of pathological activities in organs.[26] Furthermore, Huang et al found that acupuncture can promote blood circulation and improve metabolism and has a specific regulatory effect on viscera, organs, and blood by acting on certain meridians.[27] Modern medical research has revealed that the circulation of meridians is consistent with the circulatory distribution of nerves, blood vessels, and lymphatics, which are closely associated with the body's stress system.[28]

The current study intended to explore acupuncture's protective effects and mechanism of treatment in addressing ototoxicity induced by gentamicin (GM) in aged mice.

METHODS

Animals

The study used 48 male mice of the Kunming species that were healthy, good mental state, and auricle-reflex sensitive. The mice were purchased from the Laboratory Animal Center of Medicine at Jilin University. Mice were allowed free access to food and water and maintained at a controlled temperature (20°C ± 2°C) and a controlled humidity (40%-70%).

Interventions

Sixteen of the mice were three months old, and eight were randomly assigned to a control group and eight to a GM group, which were intraperitoneally injected with 100 mg/kg daily of GM (Beijing Solarbio Technology Co., Ltd, Beijing, China) for 10 consecutive days.

Thirty-two mice (18-month-old) were randomly divided into four groups with eight mice each, of which one was a positive control group, the control group; one was a negative control group, the GM group; and two were intervention groups, the acupuncture + GM group and the drug + GM group. The positive and negative control groups received no treatments. The negative control group were intraperitoneally injected with 100 mg/kg daily of GM (Solarbio) for 10 consecutive days. The acupuncture + GM group received acupuncture at the Tinggong, Tinghui and Yifeng acupoints, for 10 minutes each days after intraperitoneally injected with 100 mg/kg daily of GM (Solarbio) for 10 consecutive days. The drug + GM group was injected intraperitoneally with 100 mg/kg daily of Genadol and 100 mg/kg daily of GM (Solarbio) for 10 consecutive days. Subsequently, mice underwent 10 days treatment were tested in follow-up experiments.

Outcome Measures

The auditory brainstem response (ABR) threshold was detected at baseline before treatment and on the first day postintervention. The mice were then sacrificed by decapitation after being anesthetized. Subsequently, peripheral blood and cochlea tissues were removed.

ABR test. The mice were intraperitoneally injected with 10% chloral hydrate. The ABR test was conducted after anesthesia using alternating clicks. Band-pass filtering was 100 to 3000 Hz, superposing 999 times. The test's sound intensity decreased by 10 decibel (dB) after each test, from 60 dB. The stable III wave, with good repeatability, was set as the benchmark threshold.

Basement-membrane separation and hair-cell staining. After the mice were sacrificed, the otic vesicle was removed. The cochlear window and vestibular window were opened and filled with 4% paraformaldehyde. Then the otic vesicle was immersed in the stationary solution. After 24 h, the cochlea was decalcified in a 10% ethylenediamine tetraacetic acid (EDTA) solution for one week.

After being washed with phosphate-buffered saline (PBS), the basement membrane was separated. Then the cells were stained with 50 ug/ml of Heochst33342 for 20 mins. After staining the cell nucleus for 20 mins and sealing it with mounting medium, the cells were observed under laser confocal microscopy.

Fluorescence, real-time, quantitative, polymerase chain reaction (PCR) detection. The total RNA was extracted from the cochlear tissue and quantified using a micro-nucleic-acid detector. The reverse transcription into complementary DNA (cDNA) was conducted using a reverse transcription kit from Takara (Dalian, Liaoning,China). The reaction agent was prepared according to the manufacturer's protocol. PCR were performed using FastStart Essential DNA Green Master kit The test was performed three times for each sample. The reaction condition was set as follows: 95°C for 3 mins, 40 cycles at 60°C for 30 s and at 72°C for 30 s, and an extension at 60°C for 5 mins. Calculation of relative gene level was performed via using 2-ΔΔCt. The primers were as follows:

- Nuclear factor erythroid-derived factor 2-related factor 2 (NRF2):
  Forward—5'-TTCCTCTGCTGCTGCCATTAGTCAGTC-3';
  Reverse—5'-GCTCT TCCATTTCCGAGTCACTG-3'
- Heme oxygenase-1 (HO-1):
  Forward—5'-ACAGA AGAGGCTAAGACCG-3';
  Reverse—5'-CAGGCATCTCCTTCCATT-3'
- Nitrite reductase [NAD(P)H] quinone dehydrogenase 1 (NQO1):
  Forward—5'-ATGTATGACAATGGACCCTTCC-3',
  Reverse—5'-TCCCTTGCAGAGTGTCCATGG-3'
- Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH):
  Forward—5'-ATGACATCAAGACGGTGGTG-3',
  Reverse—5'-CATACCAGGTATGAGCTTG-3'

Assessment of malondialdehyde (MDA) level. The MDA level in serum was detected using an MDA assay kit from Beyotime (Shanghai, China), according to the manufacturer's protocol. The cells were collected to extract the total protein, and the bicinchoninic acid (BCA) method was used for the protein's quantification. The standard substance was diluted into 1, 2, 5, 10, 20, and 50 μM to establish the standard curve. Then 200 μl of supernatant was added to the 96-well plates. The sample was detected using a microplate reader at 532 nm. The calculation of MDA concentration was conducted according to the standard curve.

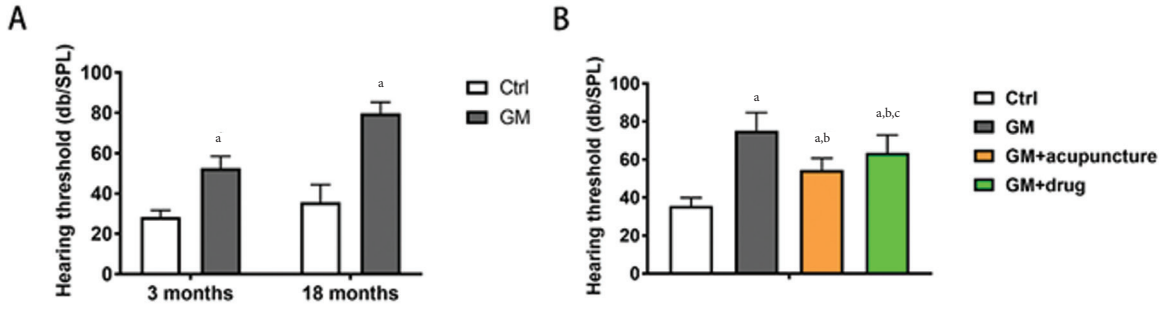
Assessment of superoxide dismutase (SOD) level. The SOD level was measured using an SOD-activity detection kit (Beyotime), according to the manufacturer's protocol. The cells were collected via centrifugation at 600 g for 5 mins and washed with ice-cold PBS. The cells were added into lysis and centrifuged at 12 000 g at 4°C for 5 mins to collect the supernatants. The SOD level was determined via detection of absorbance at 450 nm using a microplate reader.

Assessment of glutathione (GSH) level. The GSH level was assessed using a GSH peroxidase assay kit (Beyotime), according to the manufacturer's protocol. The cells were lysed in cell-lysis buffer, and the total protein was extracted via certification. The quantification of protein was performed using a BCA kit. Then the buffer, sample, and GSH working solution were added to the 96-well plates. The GSH level was detected using a microplate reader.

Statistical Analysis

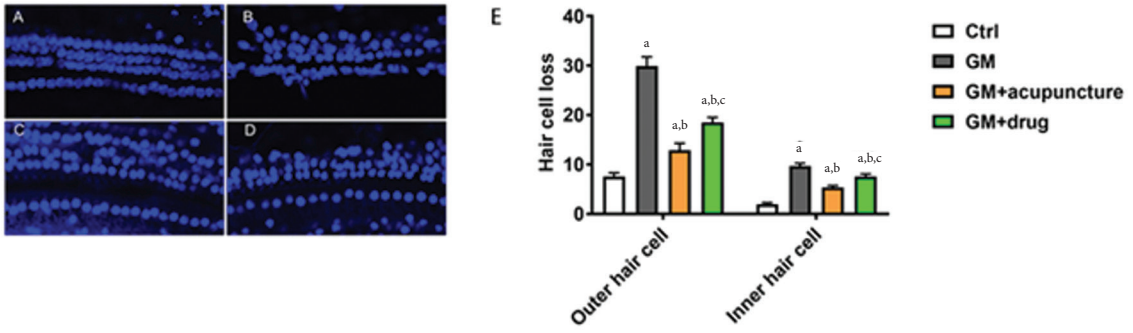
All data were displayed as means ± standard deviations (SDs). Comparisons between two groups were performed using a *t* test, and comparisons among multiple groups were carried out using variance analysis followed by a Tukey test. $P < .05$ was considered to be statistically significant.

Figure 1. The Influence of GM Induction and Acupuncture or Drug Treatment on the Hearing Thresholds of 3-month-old and 18-month-old Mice With Gentamicin (GM) Ototoxicity. Figure 1A shows the hearing thresholds in the control and GM groups after GM induction. Figure 1B shows the hearing thresholds for the 18-month-old mice in the control, GM, GM + acupuncture, and GM + drug groups after GM induction and treatments in the intervention groups.



[a]$P < .05$, statistically significant differences between the positive control groups and the GM negative control groups (Figure 1A), or between the positive control group and the GM negative control group and the two intervention groups (Figure 1B)
[b]$P < .05$, statistically significant differences between the GM group and the GM + acupuncture and the GM + drug groups (Figure 1B)
[c]$P < .05$, statistically significant differences between the GM + acupuncture group and the GM + drug group (Figure 1B)

Figure 2. The Impact of Acupuncture in the Groups on the Hair Cells of 18-month-old Mice With Gentamicin (GM) Ototoxicity. Figures 2A-D show images of the hair-cell staining in the different groups—the control group, the GM group, the BM +acupuncture group, and the GM + drug group, respectively. Figure 2E shows the histogram of hair cell loss in the study groups.



[a]$P < .05$, statistically significant differences between the positive control group and the GM negative control group and the two intervention groups
[b]$P < .05$, statistically significant differences between the GM group and the GM + acupuncture and the GM + drug groups
[c]$P < .05$, statistically significant differences between the GM + acupuncture group and the GM + drug group

RESULTS

Sensitivity to GM Ototoxicity

For the three-month-old mice, the ABR threshold value was 28.4 ± 3.33 db/sound pressure level (SPL) before GM induction, and this value increased to 52.6 ± 5.86 db/SPL after GM induction. For the 18-month-old mice, the ABR threshold value changed from 35.6 ± 4.3 db/SPL before GM induction to 75.2 ± 9.52 db/SPL after GM induction.

After GM induction, the ABR threshold value in the 18-month-old mice increased more than that of the three-month-old mice (Figure 1A).

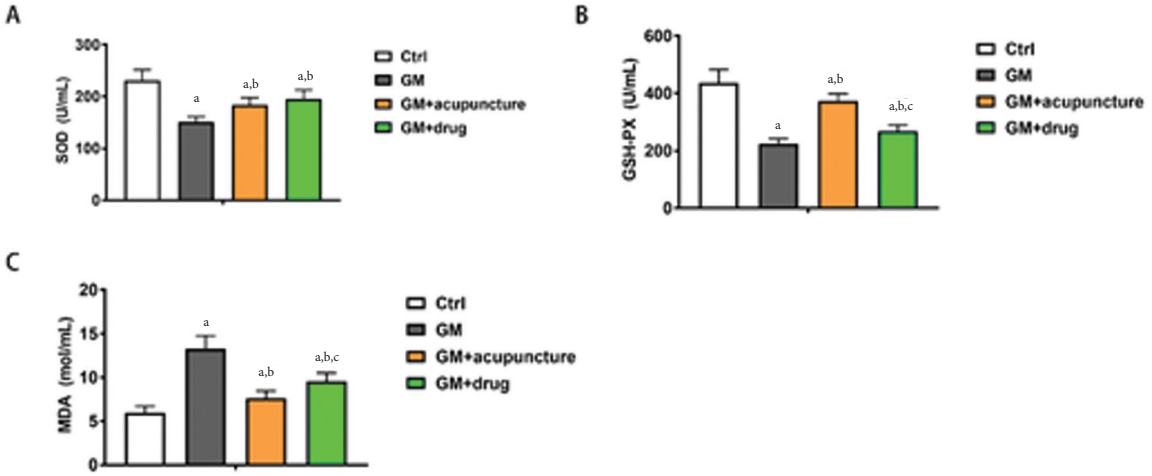
Acupuncture and GM Ototoxicity in Aged Mice

The ABR threshold value for the 18-month-old mice in the GM group increased from 35.6 ± 4.3 db/SPL before GM induction to 75.2 ± 9.52 db/SPL after GM induction, suggesting hearing impairment in the aged mice (Figure 1B). The ABR threshold value for the acupuncture + GM group was 54.34 ± 6.3 db/SPL, which was lower than that of the GM group, with no treatment, and of the drug + GM group, with a threshold of 63.56 ± 9.31db/SPL after drug treatment.

Cochlear Hair Cells and Apoptosis

For the 18-month-old mice, the number of outer cochlear hair cells decreased significantly upon GM induction, and this effect was reduced by acupuncture. Hair-cell survival increased by about 50% in the acupuncture group compared with the GM group (Figure 2A-E).

Figure 3. The Effects of Acupuncture in the Groups on Oxidative Stress in 18-month-old Mice With Gentamicin (GM) Ototoxicity. Figure 3A shows the level of SOD; Figure 3B shows the level of GSH-px; and Figure 3C shows level of MDA.



[a]$P < .05$, statistically significant differences between the positive control group and the GM negative control group and the two intervention groups
[b]$P < .05$, statistically significant differences between the GM group and the GM + acupuncture and the GM + drug groups
[c]$P < .05$, statistically significant differences between the GM + acupuncture group and the GM + drug group

Abbreviations: SOD, superoxide dismutase; GSH-px, glutathione peroxidase; MDA, malondialdehyde.

Acupuncture and Oxidative Stress

For the 18-month-old mice, MDA, SOD, and GSH-PX (peroxidase), as indicators of oxidative stress, were detected. The SOD and GSH-PX levels increased, while the MDA level was lower in the acupuncture + GM group and the positive control group when compared with the GM group (Figure 3A-C).

Suppression of Oxidative Stress and NRF2 Signaling Pathway

The related gene of the NRF2 signaling pathway was detected using PCR. After GM induction, the acupuncture + GM group and the drug + GM group showed significantly elevated levels of NRF2 and HO-1 when compared to that of the GM group (Figure 4), suggesting an activating effect for acupuncture on the NRF2 signaling pathway. In addition, the NRF2 and HO-1 levels were increased more by acupuncture than that by the drug.

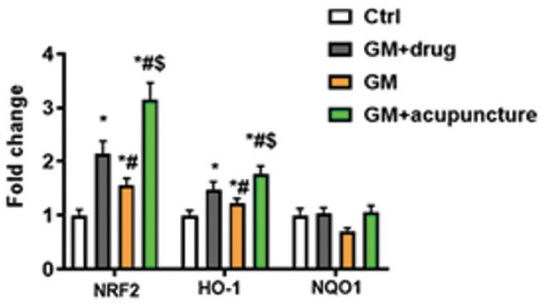
DISCUSSION

The current study's results support the theory that aged mice are more likely to suffer from GM ototoxicity, having established a GM ototoxicity model with mice of different months of age.

Prior studies have indicated that acupuncture can improve hearing loss,[11,12] and the hearing threshold value was elevated significantly by acupuncture in the current study, indicating that it can be an efficient way to reduce GM ototoxicity. It also was superior to the drug treatment, indicating a promising avenue for reducing GM ototoxicity in aged mice.

Consistent with O'Reilly et al's results,[19] the current study found that the hair cell apoptosis was higher in mice

Figure 4. Acupuncture Functions Via Activating the NRF2 Signaling Pathways in the Groups. The figure shows the gene levels of NRF2, HO-1, and NQO1.



[a]$P < .05$, statistically significant differences between the positive control group and the GM negative control group and the two intervention groups
[b]$P < .05$, statistically significant differences between the GM group and the GM + acupuncture and the GM + drug groups
[c]$P < .05$, statistically significant differences between the GM + acupuncture group and the GM + drug group

Abbreviations: GM, gentamicin; NRF2, nuclear factor erythroid factor 2-related factor 2; HO-1, heme oxygenase-1; NQO1, Nitrite reductase [NAD(P)H] quinone dehydrogenase 1

induced with GM that received no treatments, further confirming that the GM ototoxicity model was established successfully.

Consistent with Su et al's study,[25] the current study found that acupuncture down-regulated the serum MDA level and increased the activities of antioxidant enzymes SOD and GSH-Px in the GM ototoxicity model, supporting the conclusion that acupuncture has an anti-oxidative-stress effect and can prevent mice from experiencing an oxidative-stress injury after GM induction.

The current study also found that acupuncture upregulated the expression of NRF2 and its downstream genes HO-1 and NQO1, indicating that acupuncture exerts anti-oxidative stress via the NRF2 signaling pathway.

The current study had some limitations. First, the most efficient acupoint during acupuncture was still not identified. Second, it remains uncertain if acupuncture functions through some other mechanisms. Lastly, the side effects of acupuncture treatment remain unknown.

CONCLUSIONS

The current study found that acupuncture can decrease GM-induced ototoxicity in mice through inhibition of oxidative stress by activating the NRF2 signaling pathway, providing a theoretical basis for acupuncture in treating GM ototoxicity.

AUTHORS' DISCLOSURE STATEMENT

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