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Protective Effects of Acupuncture Against Gentamicin-Induced Ototoxicity in Rats: Possible Role of Neurotrophin-3

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Background: The aim of this study was to investigate the protective effects of acupuncture against gentamicin-induced ototoxicity and explore the possible protective role of neurotrophin-3 (NT-3).

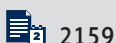
Material/Methods: Twenty-four rats were divided randomly into 4 groups: control group, gentamicin group, neitinggong group, and tinggong group. Rats in the gentamicin, neitinggong, and tinggong groups received intraperitoneal injection of gentamicin (100 mg/kg) for 14 consecutive days. Rats in the neitinggong and tinggong groups further received acupuncture at neitinggong or tinggong acupoints once every 2 days for 20 days. Rats in the control group received intraperitoneal injection of saline. Auditory brainstem response (ABR) was tested in all rats on the day before treatment (day 0), and again on day 14 and day 20 to determine the average threshold value of ABR for each treatment group. The expression of NT-3 in the cochlear nucleus and the inferior colliculus nucleus were detected by immunohistochemical staining.

Results: The average threshold value of ABR was significantly higher in the gentamicin group as compared with that of the control group on day 14 ($P < 0.05$). On day 20, the average threshold values of ABR in the neitinggong and tinggong groups were significantly lower than that of the gentamicin group ($P < 0.05$). No statistically significant differences in NT-3 expression in the cochlear nucleus were observed among the groups ($P > 0.05$). However, the expression of NT-3 in the inferior colliculus nucleus in both the neitinggong and tinggong groups was significantly higher than that of the gentamicin group ($P < 0.01$).

Conclusions: A decrease in NT-3 expression in the inferior colliculus nucleus may contribute to gentamicin-induced ototoxicity in rats. Acupuncture at neitinggong or tinggong acupoints effectively improved hearing, which was attributed partially to the rescue of NT-3 expression in the inferior colliculus nucleus. Therefore, preserving NT-3 expression in the auditory system may be a viable strategy to counteract gentamicin-induced ototoxicity.

MeSH Keywords: **Acupuncture • Cochlear Nucleus • Neurotrophin 3**

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Background

Clinical use of aminoglycoside antibiotics is complicated by ototoxicity, a condition characterized by damage to cochlear hair cells, cochlear spiral ganglion cells, and the auditory center [1,2]. Understanding the molecular events behind the ototoxicity of aminoglycoside antibiotics and developing novel, non-toxic treatments are of great clinical importance. Neurotrophin-3 (NT-3), a member of the neurotrophin family, is widely expressed in the inner ear and auditory center. NT-3 has been reported to play an important role in the structural and functional regulation of the nervous system. Specifically, NT-3 is essential for the development, survival, growth, and differentiation of auditory epithelial cells and neurons; NT-3 also plays an important role in neuronal repair after injury and in the plasticity of remnant neurons. Reduced NT-3 expression in the auditory center has been observed after hearing damage [3]. Additionally, it has also been shown that NT-3 expression can protect the inner ear and mitigate hearing loss through various mechanisms [4,5]. Thus, increasing the local expression of NT-3 in auditory nerve cells may represent a promising approach to protecting against ototoxicity.

Acupuncture, a traditional Chinese therapy, has been found to yield beneficial effects on deafness and to promote hearing recovery [6,7]. However, the mechanism behind these protective effects is not clear. To date, most studies examining this issue have focused on the effects of acupuncture on the inner ear and cochlear spiral ganglion [8–10]. To the best of our knowledge, it has not been reported whether the auditory benefits of acupuncture involve the auditory nuclei. Therefore, in the present study, we aimed to assess the preventive effect of acupuncture on gentamicin-induced hearing impairment by measuring auditory brainstem responses (ABRs) before and after treatment. In addition, we sought to determine whether the underlying protective mechanism may involve NT-3 in the cochlear and inferior colliculus nuclei.

Material and Methods

Animals

A total of 24 healthy Sprague-Dawley rats (250–350 g) with normal reflection of the auricle were provided by the Animal Laboratory of Xi'an Jiaotong University. All rats were housed in an air-conditioned room with a 12-h light/dark cycle and provided free access to standard rodent chow diet and water. After acclimation for 1 week, rats were randomly divided into the following 4 groups: control group, gentamicin group, neitinggong group, and tinggong group. For 14 consecutive days, control group rats received an intraperitoneal injection of saline each morning (2.5 mL/kg), while rats in the other groups

received an intraperitoneal injection of gentamicin (100 mg/kg Tianjin Pharmaceuticals Group Co., Ltd.) each morning. The rats were weighed daily to ensure proper dosing. Starting on the first day of intraperitoneal injection, rats in the neitinggong and tinggong groups also underwent acupuncture at the neitinggong or tinggong acupoints, respectively, once every 2 days for 20 days. The neitinggong and tinggong acupoints are located in the middle ear and adjacent to the antilobium, respectively [11]. For rats in the neitinggong group, filiform needles (0.35×100 mm) were acupunctured around the umbo of the tympanic membrane using an electric auriscope, at a depth of about 2 mm, which was adjacent to the promontory of the tympanum. For rats in the tinggong group, filiform needles (0.35×35 mm) were inserted ~4 mm subcutaneously, then manipulated by the reinforcing-reducing method every 10 min for 30 s each time. For both the neitinggong and tinggong groups, the needles were removed 30 min after application.

Auditory brainstem response (ABR)

All rats were anesthetized by intraperitoneal injection with a 10% (w/v) chloral hydrate solution (0.3 mL per 100 g body mass; Shanghai Mingbo biological technology co., LTD) every afternoon prior to testing the ABR. ABR was determined using brainstem-evoked potentiometer detection (Denmark ICS CHARTR EP). The potentiometer was set to generate a stimulatory clicking sound every 100 μ s (15-ms scanning time, 100–3000-Hz filtering range, and 1024 overlaps). Electrodes were made from 0.35×50 mm platinum acupuncture needles and were inserted subcutaneously at the vertex (active) and ventrolaterally to the right (reference) and left (ground) ears. Recording electrodes were placed at the intersections of the cranial sagittal midline and connected with the external auditory canal, reference electrodes were placed in retroauricular subcutaneous tissue, and grounding electrodes were placed in the nasion. Click stimuli were then presented. ABRs were averaged by the computer and displayed on the screen on a fixed scale. The threshold was obtained by first reducing the sound pressure level (SPL) in 10-dB steps, then fine tuning the level in 5-dB steps up and down to identify the lowest level at which an ABR pattern could be recognized. The minimal SPL intensity able to induce wave III of the ABR waveform was considered to be the ABR threshold.

Immunohistochemistry

After the experiment, rats were anesthetized by a 10% chloral hydrate solution, and then secured on an animal operating table. A perfusion cannula was inserted into the aorta through which 250 mL of saline solution was perfused quickly, followed by 2000 mL of phosphate-buffered saline perfused slowly over 1–2 h. Rats were killed rapidly by decapitation and the bilateral cochlear nucleus and inferior colliculus were removed [12].

Table 1. ABR average threshold values at different time points at day 0, 14 and 20. Values are reported as mean \pm SEM.

Group	Day 0	Day 14	Day 20
Gentamicin	9.50 \pm 5.50	30.00 \pm 6.67*	44.5 \pm 9.27*
Neitinggong	10.00 \pm 5.27	21.05 \pm 10.01*	22.50 \pm 7.55**
Tinggong	8.50 \pm 4.12	22.50 \pm 5.40*	16.00 \pm 8.76**
Control	9.50 \pm 4.38	11.00 \pm 5.16	8.00 \pm 4.22

N=12 for all groups. * $P<0.05$, compared to control group; ** $P<0.05$, compared to gentamicin group.

After paraffin embedding, antigen retrieval, and blocking, sections were incubated overnight at 4°C with a rabbit anti-rat NT3 antibody (Boster Biological Technology, China). After washing with PBS, the sections were incubated with a biotinylated secondary antibody (Boster Biological Technology, China) for 30 min at room temperature. Immunoreactivity was visualized by 3,3-diaminobenzidine (DAB). Sections were washed thoroughly, mounted onto gelatin-coated slides, dehydrated, and cover-slipped before being examined under a microscope operated in the bright field mode. Images (40 \times magnification) from 5 non-overlapping horizons were taken for each slice. The average optical density value was measured by Image-pro Plus (version 6.0).

Data analysis

Results are expressed as means \pm SEM. One-way analysis of variance (ANOVA) was used for statistical analysis (SPSS17.0 software). Differences were considered statistically significant when $P<0.05$.

Results

General condition of rats

There was no significant difference in the general condition of the rats in the control group before versus after the experiments, and their body weights increased steadily. In contrast, rats in the other 3 groups exhibited behaviors characteristic of poor health, such as decreased spontaneous activity, loss of appetite, shaggy hair, and arrested weight gain. These rats also exhibited an absence of the auricle reflex to differing extents. Under the otoendoscope, we observed small tympanic membrane perforations around the handle of malleus in rats from the neitinggong group; these perforations were partially healed, and did not exhibit discharge or infection. The integrity of tympanic membranes in rats from the other experimental groups was not affected.

Average threshold value of ABR in rats

As shown in Table 1, on day 14, the average ABR threshold values in rats from the gentamicin, neitinggong, and tinggong groups were significantly increased compared to those of the control group, indicating that their hearing had been impaired. Compared with the values for the gentamicin group, the average threshold ABR values for the neitinggong and tinggong groups appeared to be slightly, though not significantly, decreased. On day 20, the average threshold ABR value for the gentamicin group was significantly higher than that of the control group. The average threshold ABR values for the neitinggong and tinggong groups were significantly decreased compared with that of the gentamicin group.

Expression of NT-3 in the cochlear nucleus

As shown in Figure 1, NT-3 immunopositivity (yellow) was readily observed in the cytoplasm of cells in the cochlear nucleus area. NT-3 expression in the cochlear nucleus did not differ significantly among the groups (Table 2, $P>0.05$).

Expression of NT-3 in the inferior colliculus

As shown in Figure 2, NT-3 was also observed in the cytoplasm of cells in the inferior colliculus. As shown in Figure 2 and Table 2, the expression of NT-3 in the inferior colliculus was significantly decreased by gentamicin treatment ($P<0.01$), compared with that in the control group. Of note, expression of NT-3 in the inferior colliculus in the neitinggong and tinggong groups was significantly higher than that in the gentamicin group ($P<0.01$), showing a restoration trend toward physiological levels. These results indicate that acupuncture at the neitinggong or tinggong acupoints suppressed the inhibitory effect of gentamicin on NT-3 expression in the inferior colliculus ($P<0.01$).

Discussion

The results of our study support the notion that acupuncture can alleviate gentamicin ototoxicity. Specifically, on day 20 the

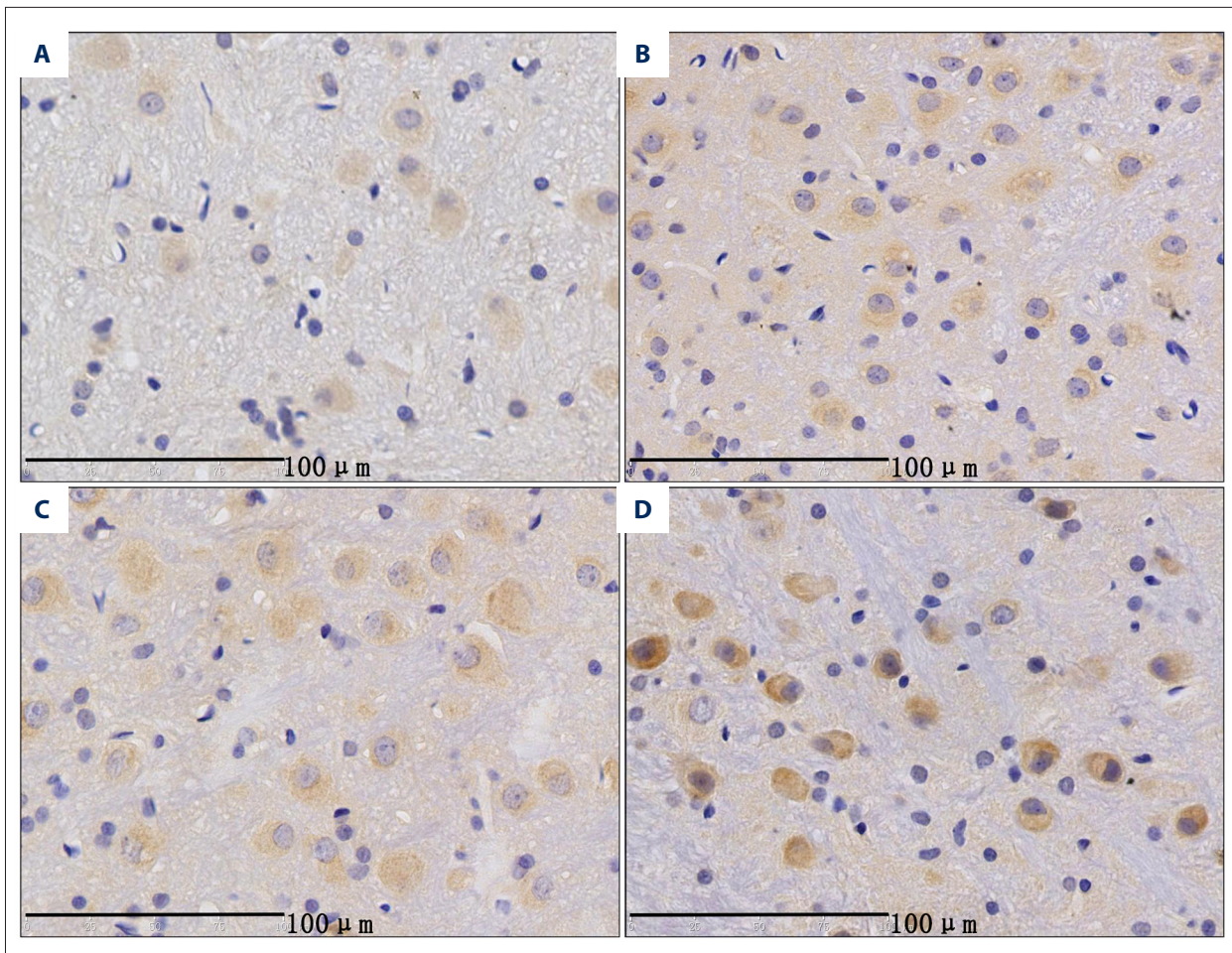


Figure 1. Expression of NT-3 in the cochlear nucleus. (A) Gentamicin group, (B) neitinggong group, (C) tinggong group, and (D) control group. NT-3 immunopositivity (yellow) was distributed in cytoplasm and absent from nuclei. Scale bars, 100 μ m.

Table 2. Average optical density values for NT-3 expression in the cochlear nucleus and inferior colliculus. Optical density values are reported as the mean \pm SEM.

Group	Cochlear nucleus	Inferior colliculus
Gentamicin group	0.017 \pm 0.013	0.016 \pm 0.003*
Neitinggong group	0.016 \pm 0.008	0.030 \pm 0.007
Tinggong group	0.017 \pm 0.008	0.029 \pm 0.008
Control group	0.020 \pm 0.007	0.025 \pm 0.008

N=12 for all groups. * Compared with other groups, $P < 0.01$.

average threshold ABR values of rats treated with neitinggong or tinggong acupuncture were much lower than that of the gentamicin group. These results are consistent with the prior observation of Ma et al. that neitinggong acupuncture attenuated gentamicin-induced ototoxicity [6]. Previous results from clinical trials and animal experiments have demonstrated that acupuncture can improve inner ear microcirculation and the hemorrheology index, release blood vessel contraction of the

inner ear, promote the regeneration and repair of hair cells, and influence brainstem response, all of which may contribute to the attenuation of sensorineural deafness [9,13–16]. Together, these data suggest that acupuncture may serve as a promising treatment of auditory impairment induced by various factors.

Regarding the mechanism by which acupuncture may counter gentamicin-induced ototoxicity, there are several possible

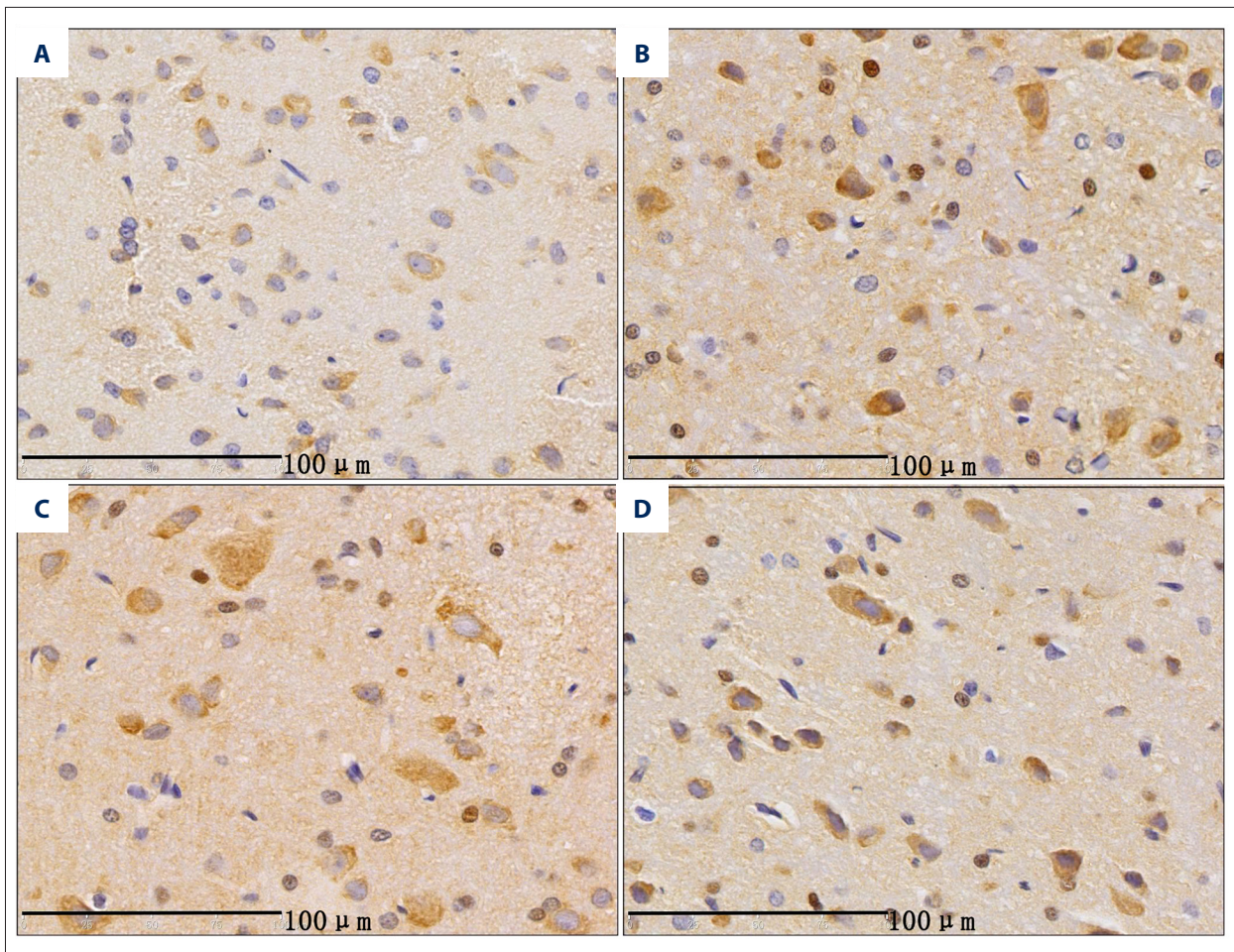


Figure 2. Expression of NT-3 in the inferior colliculus. (A) Gentamicin group, (B) neitinggong group, (C) tinggong group, and (D) control group. NT-3 immunopositivity (yellow or brown) was distributed in the cytoplasm. Scale bars, 100 μ m.

mechanisms to consider. First, previous studies have shown that acupuncture can protect against hair cell damage directly by improving inner ear microcirculation and energy metabolism, stabilizing the mitochondria and lysosomes, scavenging oxygen free radicals, preventing inner ear lesions, increasing afferent signals, reducing the damage to the inferior colliculus nucleus, and inducing neuroplastic changes [7]. Second, acupuncture could stimulate the auditory center directly to produce NT-3, which exerts neurotrophic effects on the inferior colliculus nucleus neurons [13]. Third, acupuncture could produce these effects indirectly, perhaps by improving renal blood flow and glomerular filtration, thereby alleviating renal pathological damage, which in turn could promote the excretion of gentamicin [17].

In this work we found that acupuncture directed at the neitinggong or tinggong acupuncture site increased NT-3 expression selectively in the inferior colliculus nucleus in rats. Recent studies have found that acupuncture can increase the expression of NT-3 in neurons. For example, Chen et al. found that

NT-3 expression in the spared L6 dorsal root ganglion (DRG) in cats that received electro-acupuncture was markedly higher than that of other groups [18]. Wang et al. evaluated the effect of electro-acupuncture in cats subjected to dorsal rhizotomy, and found that NT-3-positive neurons were increased in lamina II on the acupuncture side [19]. Several studies have shown altered NT-3 expression levels in the auditory center after hearing damage. For example, Suneja et al. measured NT-3 levels in adult guinea pig brain stem auditory nuclei after unilateral cochlear ablation, and found that NT-3 levels were depressed by 22–44% on day 3, elevated transiently by 28–124% on day 7, and then returned to normal levels by day 60 [3]. In mice injected with gentamicin for 15 days, Li et al. and Song found that the NT-3 expression in the cochlea, the cochlear nucleus, and the inferior colliculus nucleus was significantly decreased. However, in the mice that received early treatment with herbal compounds for reinforcing kidneys, activating blood, and arousing consciousness (HCRAA), hearing was obviously improved and the expression of NT-3 was elevated [20,21]. In the present study, we found that the

expression of NT-3 in the inferior colliculus nucleus was decreased significantly in the gentamicin group, which may be partially explained by direct transport of gentamicin to the inferior colliculus nucleus through blood circulation where it caused hyopsecretion of NT-3.

In the present study, NT-3 expression in the cochlear nucleus did not differ significantly among the groups. This observation could be due to some NT-3 in the cochlear nucleus coming from the cochlea through axonal transport [22], which may mask reductions in NT-3 expression in the cochlear nucleus. Alternatively, it could be due to expression of NT-3 being related to an increase in synaptic plasticity, given that cochlear injuries may influence NT-3 expression through regulation of synaptic plasticity in auditory nuclei [23].

These results suggest that acupuncture is a simple and effective way to increase NT-3 expression in auditory nuclei, maintain auditory neuron survival, and promote the repair of hearing loss. However, the kinetics of NT-3 expression in auditory brain stem nuclei are not clear. Further studies are needed that focus on the dynamics of NT-3 expression in different parts of the auditory nerve nuclei and under different conditions, such

as acupuncture treatment alone (as a positive group). Such experiments would provide more direct evidence for the efficacy of acupuncture treatment for hearing loss and pave the way for elucidating the mechanism of the protective effects of acupuncture.

Conclusions

In this study, acupuncture at the neitinggong or tinggong acupoints was shown to cure sensorineural deafness caused by gentamicin in rats. Acupuncture therapy increased the expression of NT-3 in the inferior colliculus nucleus, which plays a key role in maintaining the integrity of the hearing pathway and mediates, partially, the benefits of acupuncture on auditory repair. Increasing NT-3 expression in the inferior colliculus nucleus may represent a new strategy for preserving auditory capabilities.

Conflict of interest

The authors declare no conflict of interest.

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