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Effectiveness of intratympanic Epigallocatechin-3-gallate injection on Cisplatin-induced hearing loss in rats

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Abstract

Cisplatin is an imperative drug in the treatment of a wide range of cancers. However, it has various side effects including ototoxicity causing bilateral sensorineural hearing loss. Despite lacking a clearly defined mechanism, Reactive Oxygen Species (ROS) production and damage to Outer Hair Cells (OHCs) have been implicated as possible culprits. The ideal otoprotective drug would target inflammation and oxidative stress without compromising cisplatin's efficacy. This

study aimed to scrutinize whether intratympanic Epigallocatechin-3-Gallate (EGCG) exhibits a protective effect against cisplatin-related ototoxicity. Twenty-four adult male Wistar rats (8–10 weeks, 200–250 g) were randomly divided into four groups (n = 6 per group): Cisplatin, EGCG, Cisplatin+EGCG, and Control. Cisplatin (15 mg/kg) was administered intraperitoneally, and/or EGCG (10 mg/mL, 200 μL) was injected transtympanically into the right ear, depending on group assignment. Control rats received intratympanic distilled water. After 72 h, auditory function was assessed by Auditory Brain Response (ABR), followed by histopathological and Signal Transducer and Activator of Transcription-1 (STAT1) immunohistochemistry evaluation of cochlear sections. All animals underwent baseline otoscopy and ABR to rule out pre-existing hearing impairment. Data were analyzed using repeated measures of Analysis of Variance (ANOVA) and paired t-tests for ABR thresholds, and Kruskal–Wallis/Mann–Whitney tests for histopathological and Immunohistochemistry (IHC) scoring. Statistical significance was defined as $p < 0.05$. EGCG injections failed to prevent hearing threshold increases in the Cisplatin group. According to histopathologic and STAT-1 IHC evaluation in the treatment group, reduced OHC damage, apoptosis, and cochlear hyperemia were observed. Administration of EGCG alleviated apoptosis and prevented OHCs damage in animals. However, it could not prevent hearing loss significantly.

Introduction

Cisplatin (Cis-diamminedichloroplatinum) is an effective antineoplastic drug against various solid tumors, such as sarcomas, carcinomas, lymphomas, etc.[1] However, it has severe side effects like neurotoxicity, nephrotoxicity, myelotoxicity, and ototoxicity, particularly after high-dose administration, making its clinical use challenging.[2] Ototoxicity, especially cochlea damage, is irreversible, with detrimental effects compromising the patient's quality of life.[3] Previous studies have confirmed that cisplatin profoundly degenerates Outer Hair Cells (OHCs) in the hook area, basal and middle turns of the cochlea.[4] Furthermore, cisplatin is capable of inducing injury in the spiral ganglion, organ of Corti, spiral ligament, and stria vascularis.[5] This ototoxicity is bilateral,

cumulative, dose-dependent, and involves high frequencies.[5] Experimental studies have suggested that cisplatin may cause cellular damage by directly affecting DNA, inducing inflammation, oxidative stress, and initiating apoptosis.[6]

The exact mechanisms behind cisplatin-induced ototoxicity are not yet clearly understood; however, recent reports have highlighted the role of increased Reactive Oxygen Species (ROS) in causing damage to OHCs in the cochlea.[7,8] Cisplatin-induced oxidative stress is thought to be due to glutathione depletion, reduced antioxidant enzyme activity, and increased lipid peroxidation.[9] Mitochondrial apoptotic pathways are also involved in this damage.[10] Therefore, the ideal otoprotective drug would target inflammation and oxidative stress without compromising the chemotherapeutic efficacy of cisplatin. The use of antioxidants in treating cisplatin-induced hearing loss initially appeared promising;[11] however, concerns that antioxidants could interfere with cisplatin's chemotherapeutic efficacy have reduced their use in otoprotective procedures.[12] Cisplatin has been shown to activate the Mitogen-Activated Kinase (MAPK) pathway, thereby activating the Signal Transducer and Activator of Transcription-1 (STAT1) and p53.[9] Consequently, the OHCs become inflamed and apoptotic, and hearing loss occurs.[13-15] In several cancers, STAT1 contributes to the development of drug resistance.[16,17] It was thought that inhibiting STAT1 would protect the OHCs while facilitating the killing of cancer cells induced by cisplatin. Many experimental studies have proven the protective effect of antioxidants on cisplatin-induced hearing loss, such as N-Acetyl Cysteine (NAC), Sodium thiosulfate, D-Methionine, and Epigallocatechin-3-Gallate (EGCG).[18-20]

In green tea extract, EGCG is an abundant polyphenol that possesses antioxidant, anti-inflammatory, and anti-tumorigenic properties, as well as being known to inhibit STAT1.[21-23] It has been demonstrated that EGCG has beneficial effects in treating various diseases such as diabetes, cancer, neurodegenerative disorders, cardiovascular diseases, and obesity.[24,25] Studies conducted both *in vitro* and *in vivo* have demonstrated its protective effects on hair cells.[9]

Antioxidant drugs' use against cisplatin-induced ototoxicity is challenging, potentially reducing treatment efficacy by interfering with cisplatin's function. Furthermore, a critical obstacle lies in achieving optimal antioxidant concentrations within the inner ear upon systemic delivery, underscoring the escalating demand for precise localized drug delivery methods.[26] Clinical investigations into the impact of systemic antioxidants on mitigating sensory-neural hearing loss

have yielded a spectrum of outcomes, likely stemming from the inability to reach the intended drug levels within the cochlea following systemic dosing. Moreover, local drug administration for intratympanic applications, specifically targeting the cochlea, has distinct advantages over systemic approaches.[26] These benefits include the ability to establish precise drug concentrations within the perilymph and circumvent the Blood-Labyrinth-Barrier (BLB), as highlighted in existing literature.[27] In this study, we aim to use the intratympanic method to more effectively deliver the drug at a higher concentration to the inner ear. Of note, one of the hypotheses examined in this study was the answer to the question of whether intratympanic injection is superior to intraperitoneal injection or not. Therefore, we administered intratympanic EGCG for the first time and investigated STAT1 as a potential therapeutic target for cisplatin-induced hearing loss. Furthermore, we examined the protective effect of intratympanic EGCG, an antioxidant, against cisplatin-related ototoxicity in a rat model.

Materials and Methods

Drugs and materials

EGCG and STAT 1 antibodies were purchased from Santa Cruz Biotechnology, Dallas, TX, USA. Cisplatin was purchased from Mylan Company, France. Ketamine and Xylazine were purchased from Alfasan/Rompun, Tehran, Iran.

Animal procedures and sample collection

Twenty-four 8-10 weeks adult male Wistar rats (200-250 g) were given free access to commercial food and water and were housed in temperature-controlled rooms with a 12 h light/dark cycle. To rule out otitis media and hearing loss in the rats, each of them underwent an otoscopy and Auditory Brain Response (ABR) before entering the study.

Preliminary trial and cisplatin dose response

The literature shows significant variation in the dosages of cisplatin injections. Therefore, a dose-response study was conducted on 12 Wistar rats to determine the optimal concentration of cisplatin injection that induces a significant hearing loss. The rats were divided into three groups (N=3) and injected intraperitoneally with cisplatin at doses of 8, 11, and 15 mg/kg, respectively.[28,29] A control group was included with 3 rats without injections (not sacrificed following ABR). A significant difference was found in the average hearing threshold after intraperitoneal injection of cisplatin at a dose of 15 mg/kg ($p < 0.001$). Accordingly, this dose is considered the amount of cisplatin that causes hearing loss (graph not shown here).

Study protocol

After anesthesia with a mixture of ketamine/xylazine (80/10 mg/kg), rats underwent otoscopic and ABR examination. The animals were randomly divided into four experimental groups (N=4): i) Cisplatin group (CP): A single-dose injection of cisplatin (15 mg/kg) was administered intraperitoneally (IP); ii) Cisplatin+EGCG group (CP+EGCG): rats first received 15 mg/kg cisplatin IP, and then single dose of EGCG (10 mg/mL in distilled water) IT as treatment (9, 30); in this group, to investigate the systemic effects (systemic toxicity) of cisplatin and EGCG, after administration of cisplatin, EGCG was injected into the right ear; the hearing threshold was taken before and after the injection; iii) EGCG group (EGCG): received 10 mg/mL intratympanic EGCG in distilled water in the right ear (total injection volume: 200 µL); iv) Control group (CO): received intratympanic distilled water in the right ear. Post-treatment ABRs were performed for 72 h following cisplatin administration, after which the animals were decapitated, and the cochleae were isolated for histopathologic and STAT-1 immunohistochemistry evaluation. All injections were performed in the right ear of the animals.

ABR measurements

ABR was recorded in a soundproof booth by the Audiology Lab system (Oto consult, Frankfurt a. M., Germany) under anesthesia conditions. The acoustic stimuli were presented by a calibrated loudspeaker (DT48, Beyer Dynamic, Heilbronn, Germany) via a plastic Cone located in the outer ear canal. The Subdermal needle electrodes were positioned at the vertex (noninverting), under

the left mastoid (inverting), and the right (ground) ears.[31] Clicks were used as auditory stimuli with specific parameters: bandpass filters of 0.3–3.0 kHz and a repetition rate of 21/s. Threshold determination involved varying sound pressure levels from 90 dB to 10 dB Sound Pressure Level (SPL) in 10 dB steps during measurements. The threshold was defined as the lowest intensity that consistently evoked a visually detectable response with waveforms waves II.[32]

Histopathologic study

Isolated adult rat cochleae were perfused with 4% paraformaldehyde and kept overnight at 4°C in formalin 10% for fixation. After 48h fixation, cochleae were decalcified in 0.1 M Ethylenediaminetetraacetic Acid (EDTA) (pH 7.4) with stirring at room temperature for 14 days. Then, the post-fix process was done, and the samples were gradually dehydrated and embedded in paraffin blocks. Paraffin embedding was conducted, and coronal serial sections of 7 μm (30 μm interval) in thickness along the whole length of the cochlea (basal to apical) were prepared using a rotary microtome (Leica RM2235, Leica Biosystems, Chicago, IL, USA). We analyzed six cochlear sections per rat, staining them with Hematoxylin and Eosin (H&E) as well as conducting Immunohistochemistry (IHC), and finally evaluated them under light microscopy.[33] The sections were evaluated for outer hair cell damage and stria vascularis hyperemia. The severity of injury in the histopathological examination was examined as none, mild, moderate, and severe as specified in Table 1.[6] A blinded trial group pathologist developed and applied this scoring scale by determining the minimum and maximum scores appropriate for the specimens, thereby quantifying these two characteristics.

Immunohistochemistry examination

All sections organized for immunochemistry examination were passed through the gradient of xylol and alcohol series. The endogenous peroxidase was deactivated by 10 min exposure to 3% H_2O_2. The tissues were incubated with the primary antibody of STAT-1 (Cat no: 592 Santa Cruz Biotechnology, Dallas, TX, USA) to distinguish the apoptosis according to the manufacturer's instructions. 3-3′ Diaminobenzidine was used as chromogen. Immunopositivity was considered

as none, mild, moderate and severe as demonstrated in Table 1.[6] IHC staining was evaluated by two independent experienced pathologists, who were blinded to the trial data.

Statistical analysis

All data are presented as means ± Standard Error of Mean (SEM). Statistical analyses were performed using GraphPad Prism version 10. Repeated measures of Analysis of Variance (ANOVA) and paired t-tests were applied to assess differences in ABR threshold changes. For the comparison of semi-quantitative histopathological scores, the non-parametric Kruskal–Wallis test was used, followed by the Mann–Whitney U test for pairwise group comparisons. A p-value of less than 0.05 was considered statistically significant.

Results

ABR results

A significant increase in the auditory threshold value in the CP group after 72 hours of cisplatin injection was shown ($p<0.001$) (Figure 1). This suggests that treatment with cisplatin (15 mg/kg - IP) can cause significant hearing loss after 72 hours (Figure 1A). In the CP+EGCG group (intraperitoneal cisplatin and intratympanic EGCG in the right ear), data analysis shows that the auditory threshold value was not significantly different between contralateral ears after 72 hours ($p=0.36$) (Figure 1.B). A significant increase in auditory threshold after 72 hours was observed in both ears compared to the time before injections ($p<0.05$). This indicates that intratympanic injection of EGCG failed to protect against cisplatin-induced hearing loss. In EGCG and Control groups, One-Way ANOVA analysis showed no significant changes in auditory threshold in both Control and EGCG (no difference) groups, after 72 hours in comparison to the primary auditory thresholds (p value=1). This indicates that intratympanic injection of EGCG and distilled water could not change the auditory threshold (Figure 1B).

Histopathological results

A normal histopathological structure was observed in both EGCG and Control groups (Figure 2). However, in the control group, we observed minor inflammatory changes in both the middle ear cavity and cochlea that refer to the injection needle. In group CP, endothelial cells showed degeneration and necrosis along with severe hyperemia in the stria vascularis. There was morphological impairment in OHCs which also decreased in number. Furthermore, severe degeneration was observed in spinal ganglion cells in this group (Figure 2). As a result of EGCG treatment, the cochlear tissues of rats revealed mild hyperemia in stria vascularis, mild reduction in the number of OHCs and mild degeneration in spinal ganglion cells (Figure 2). Histopathological results are summarized in Table 2.

Immunohistochemical results

Negative STAT 1 staining was observed in the cochleas of the rats in the control group (Figures 3, 4). IHC results revealed that cisplatin adversely affects several internal ear areas. In CP group, a severe STAT 1 immunopositivity was observed in endothelial cells in stria vascularis, OHCs and particularly in spinal ganglion cells (Figures 3, 4). Immunochemical examination of the cochleas of rats after treatment with EGCG revealed mild immunopositivity for STAT 1 in OHCs, spinal ganglion cells and endothelial cells of stria vascularis (Figures 3, 4). Immunochemical results are summarized in Table 2.

Discussion

There are wide ranges of malignancies in head and neck regions that can be treated with cisplatin, which is a well-known chemotherapeutic agent.[6] The use of this product has been restricted due to side effects such as nephrotoxicity, ototoxicity, and neurotoxicity.[34] Despite the large amount of research devoted to preventing adverse effects, protocols for treating ototoxicity caused by cisplatin are lacking. This study demonstrates that EGCG is an effective treatment against cisplatin-induced toxicity. We demonstrated that EGCG protects against cell degeneration and can provide positive effects on apoptosis cascade in multiple regions of the cochlea, including OHCs, spiral ganglion, and, stria vascularis. Moreover, *in vivo* results show that EGCG possesses anti-inflammatory and anti-apoptotic properties and alleviated the

expression level of STAT1, which was found to be another mechanism of protection. For the first time, we also administered an intratympanic injection of the EGCG. Our results confirmed that the intratympanic injection route can be preferred over IP injection.

Prior studies have mentioned that the auditory threshold increases in the first 24 to 72 hours after cisplatin injection. García-Berrocal *et al.* have shown that IP injection of single-dose cisplatin in rats made a threshold rise on the second day.[28] Furthermore, in the De Freitas et al study, IP injection of 16 mg/kg cisplatin intensified the auditory threshold in rats on the third day.[35] Thus, to relieve cisplatin's long-term side effects on animals, and to avoid repeated anesthesia's potential mortality, we considered 72 hours as an ABR follow-up checkpoint. The CP group significantly increased its auditory thresholds 72 hours after receiving 15 mg/kg of cisplatin injection which is compatible with the studies mentioned above.[28,35] In the CP+EGCG group, there was no difference between the auditory thresholds of the contralateral ears after 72 hours. Borse *et al.* conducted a study in 2017 in which EGCG was administered orally to rats injected with cisplatin at a dose of 100 mg/kg for four consecutive days.[9] This study resulted in protection against cisplatin-induced hearing loss at all frequencies. Blood-Labyrinth-Barrier (BLB) is a protection against agents of systemic blood circulation for the inner ear, which may also reduce the effect of systemic drugs on the inner ear. As a result of this issue, intratympanic injections are becoming more common for targeting inner ear diseases.[26,36] A lower concentration of EGCG and a single intratympanic injection may explain the inability to protect against threshold rise. It is expected that intratympanic EGCG which passes through the rat's Eustachian tube to the nasopharynx would be absorbed in the gastrointestinal system and act as an oral agent.[37] However, to evaluate whether EGCG could alter cisplatin efficacy in cancers, Borse *et al.* screened various cancer cells, such as University of Michigan Squamous Cell Carcinoma 10B (UMSCC 10B), Human Colorectal Carcinoma 116, Wild Type (HCT116 WT), and Human Ovarian Carcinoma (A8HEYA8) against which cisplatin is used clinically *in vitro*. Interestingly, their results not only showed that EGCG did not reduce the anticancer effects of cisplatin, but also that EGCG by itself significantly killed head and neck tumor cells.[9] As a result of this controversy in results, it may be worthwhile to repeat this study using the sustained-release form of EGCG in future studies in order to avoid the rapid clearance of EGCG in the middle ear.

Our findings indicate that the use of EGCG can improve the structure of the ganglion neurons without impacting the ABR thresholds. Our findings are consistent with a study that showed free EGCG might target processes that support or regenerate ganglion cells without directly influencing neuronal firing patterns that are crucial for a ABR response.[38] Improving ganglion cell health may not lead to changes in ABR results, perhaps because this response depends on the integrity of various components in the auditory pathway, such as hair cells, synapses and neural connections, or because the present study used a single dose and a low dose of EGCG. However, there seems to be a need for further research in this area.

Oxidative stress constitutes the principal factor in the development of cisplatin-induced ototoxicity. Cisplatin's enhancement of ROS production and suppression of antioxidant enzyme systems trigger a range of different pathophysiological events in the cochlea.[39] The cochlea possesses high metabolic activity, making it sensitive to hypoxic events and ischemic reperfusion injury.[6] Furthermore, Cisplatin has been shown to activate MAPK pathway, thereby activating STAT1 and p53.[9] Increased ROS, STAT1 and p53 production results in apoptosis and necrosis through a range of histopathological changes and functional impairment in the cochlea and hearing loss occurs.[39] According to our IHC data, cisplatin injection increased STAT1 expression in spiral ganglion cells and OHCs in the CP group, while after EGCG treatment STAT1 expression was attenuated.

The polyphenol EGCG in green tea extract has antioxidant, anti-inflammatory, and anti-tumorigenic properties, as well as inhibitory properties against STAT1.[21-23] Histopathologic evaluation of the cochlea with H&E staining revealed severe damage to endothelial cells, OHCs, and supporting cells in the CP group. Moreover, the basal membrane and tectorial membrane had severe morphologic changes in this group and there was hyperemia in stria vascularis. Many previous studies have confirmed that CP has a wide range of congestion and engorgement on cochlea structures.[6,40] In contrast, in the EGCG-treatment group, all these anatomical elements were shown to improve. Although EGCG was administered intratympanic for the first time in this study, previous studies have only utilized oral administration.[9]

In the present study we faced some limitations. EGCG was injected intratympanic once and a 72-hour follow-up was conducted. Adding more intratympanic shots and extending the follow-up time may have provided more accurate data, however, would have put the rats at risk of repeated

anesthesia. A Distortion Product Otoacoustic Emission (DPOAE) analysis could have provided valuable additional information on auditory function, particularly regarding the integrity and activity of the OHCs in the cochlea. However, we were unable to perform this assessment in our study because the software available with our auditory testing system did not support DPOAE recording and analysis, representing a technical limitation. A sustained release form of EGCG using biodegradable and biocompatible agents might have reduced drug elimination bias in the middle ear, resulting in a stronger conclusion.

Conclusions

Although cisplatin has serious side effects, it is still used frequently in oncology. To date, no clinical successes have been reported in terms of otoprotection during cisplatin therapy. Our study data demonstrate that intratympanic injection of EGCG protects OHCs against cisplatin-induced ototoxicity and ameliorates cellular apoptosis. Nevertheless, ABR evaluation suggests that it cannot prevent auditory threshold rises. The effectiveness of EGCG needs to be further investigated in more detail through additional clinical trials.

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Table 1. Definition of histopathological and immunochemical scale.

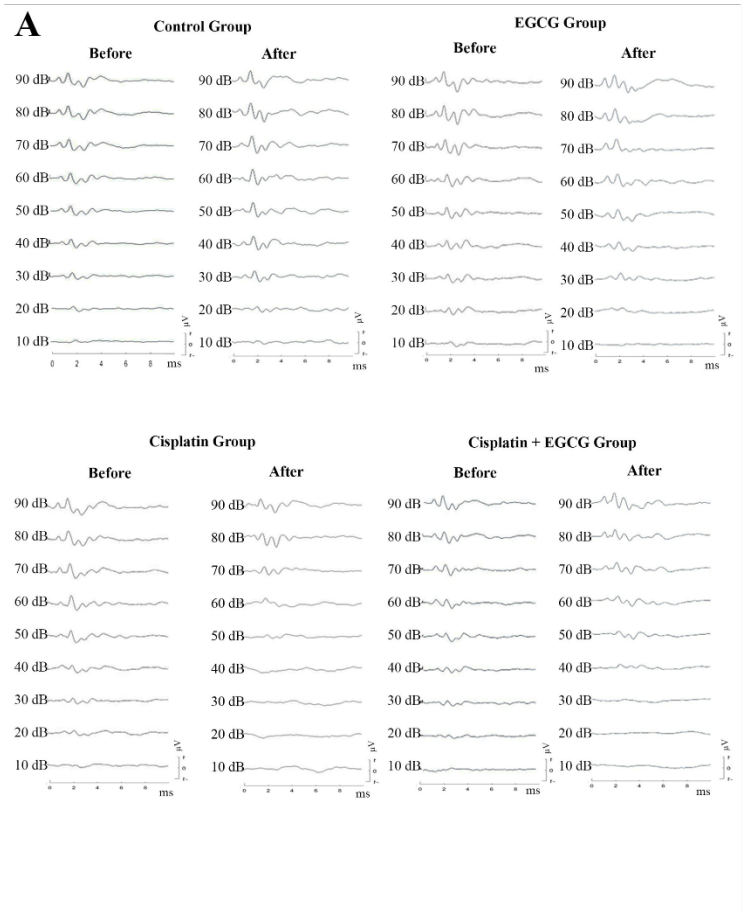
***** Signal Transducer and Activator of Transcription-1

	None (-)	**Mild (+)**	**Moderate (++)**	**Severe (+++)**
Hyperemia in stria vascularis (Diameter of vessel)	< 1 µm	1-2 µm	3-5 µm	> 5 µm
Structural impairment in outer hair cells (Impaired cell number)	0	3-5	6-10	> 10
STAT-1* expression (Number of positive cells)	0	3-5	6-10	> 10

Table 2. Histopathological and immunohistochemical results.

	Control group	**CP* group**	**EGCG** group**	**Treatment group**
Hyperemia in stria vascularis	-	+ +	-	+
Decrease in the number of outer hair cells	-	+ + +	-	+ +
Degeneration in spiral ganglia	-	+ +	-	+
Immunopositivity for STAT-1* **	-	+ + +	-	+ +

*Cisplatin; ** Epigallocatechin-3-Gallate; *** Signal Transducer and Activator of Transcription-1



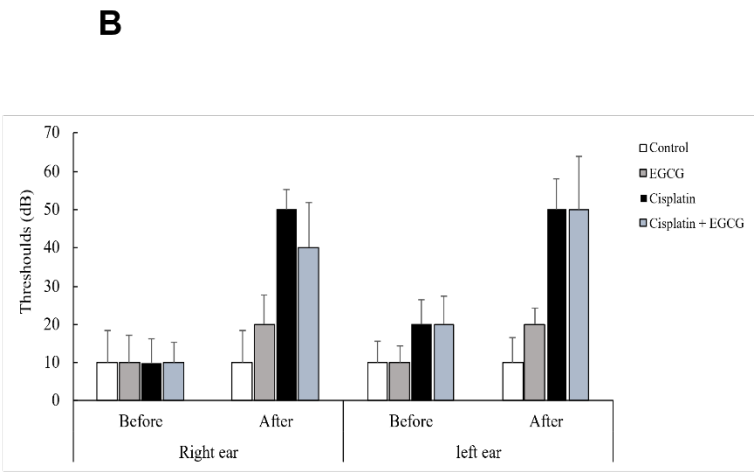


Figure 1. Auditory Brain Response (ABR) thresholds measurements: a) Example of ABR responses to acoustic click stimulation before and after injection of Cisplatin and Epigallocatechin-3-Gallate (EGCG) in rats with normal hearing; b) ABR thresholds change before and after intervention in the right and left ear. The results showed that intratympanic injection of EGCG could not protect against cisplatin-induced hearing loss. (n =6). Results are shown as mean ± Standard Error of Mean (SEM).

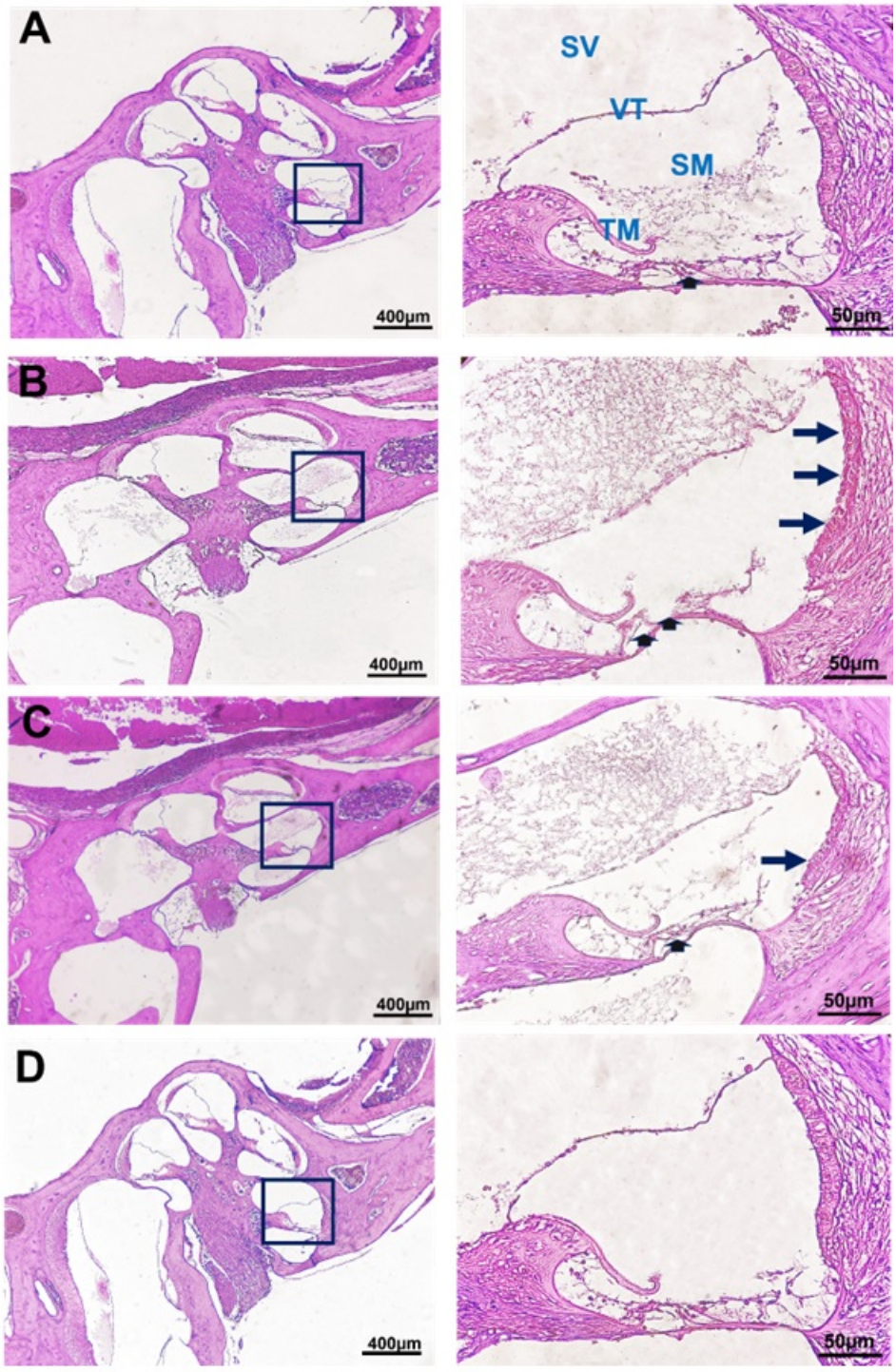

A
SV
VT
SM
TM
400μm
50μm
B
400μm
50μm
C
400μm
50μm
D
400μm
50μm

Figure 2. Histopathologic appearance of the cochlea. Each row represents an experimental group, while the right column presents high-magnification images of the corresponding inserts. A: Control Group: Normal histopathological structure of the cochlea with a few inflammatory changes in both middle ear cavity and cochlea. B: Cisplatin Group: Severe hyperemia in the stria vascularis (arrows), morphological impairment and severe decrease in the number of Outer Hair Cells (OHCs) (arrow heads). C: Cisplatin + Epigallocatechin-3-Gallate (EGCG) Group: Mild hyperemia in the stria vascularis (arrows), mild decrease in the number of OHCs (arrowhead). D: EGCG Group: Normal histopathological structure of the cochlea. SM: Scala Media SV: Scala Vestibuli VT: Vestibular membrane TM: Tectorial Membrane.

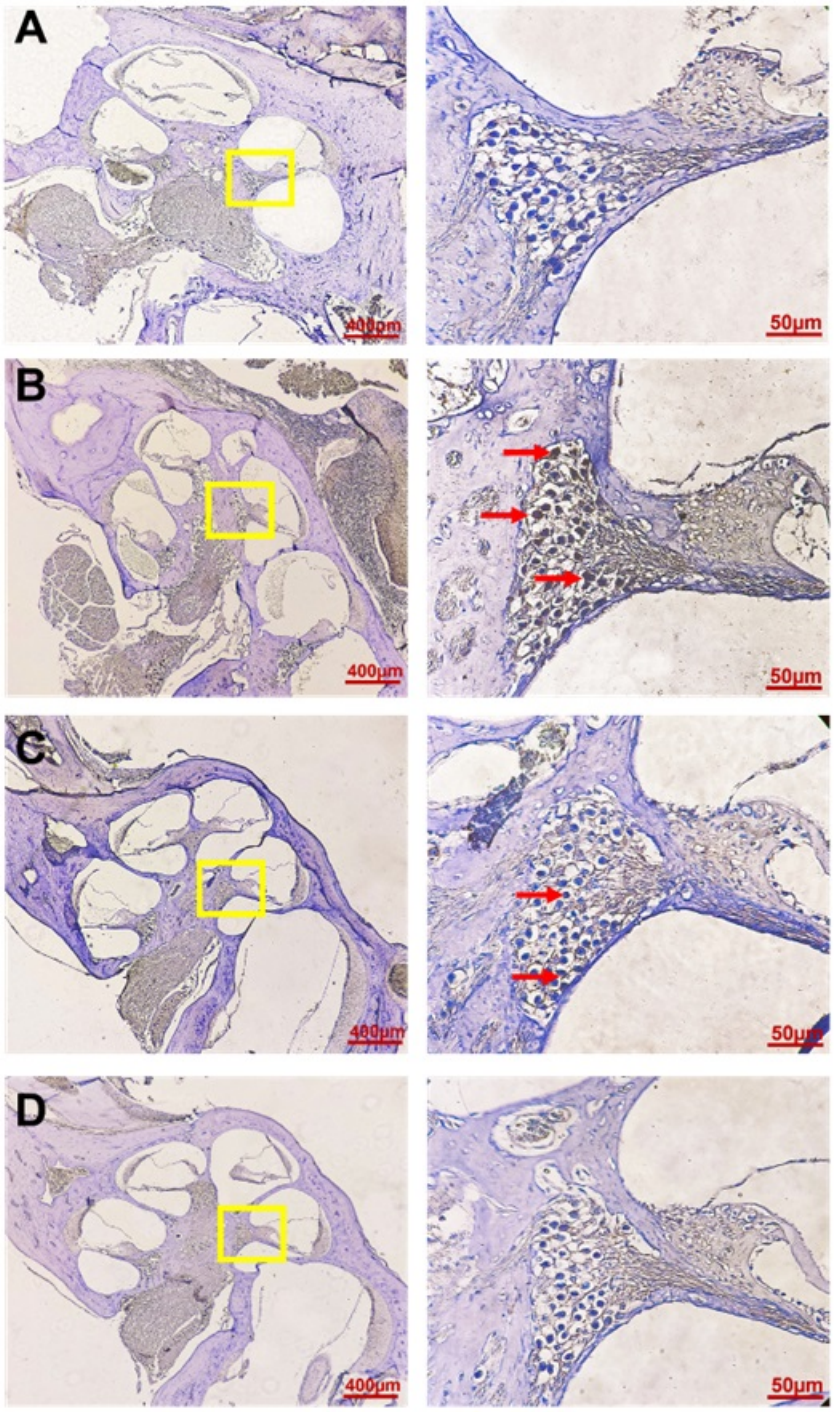


Figure 3. Immunohistochemical staining with severe Signal Transducer and Activator of Transcription-1 (STAT1) in spiral ganglion. Each row represents an experimental group, while the right column presents high-magnification images of the corresponding inserts: A) Control Group: Negative STAT1 expression; B) Cisplatin Group: STAT1 immunopositivity

(Arrowhead); C) Cisplatin + Epigallocatechin-3-Gallate (EGCG) Group: Moderate STAT1 immunopositivity (Arrowhead); D) EGCG Group: Negative STAT1 expression.

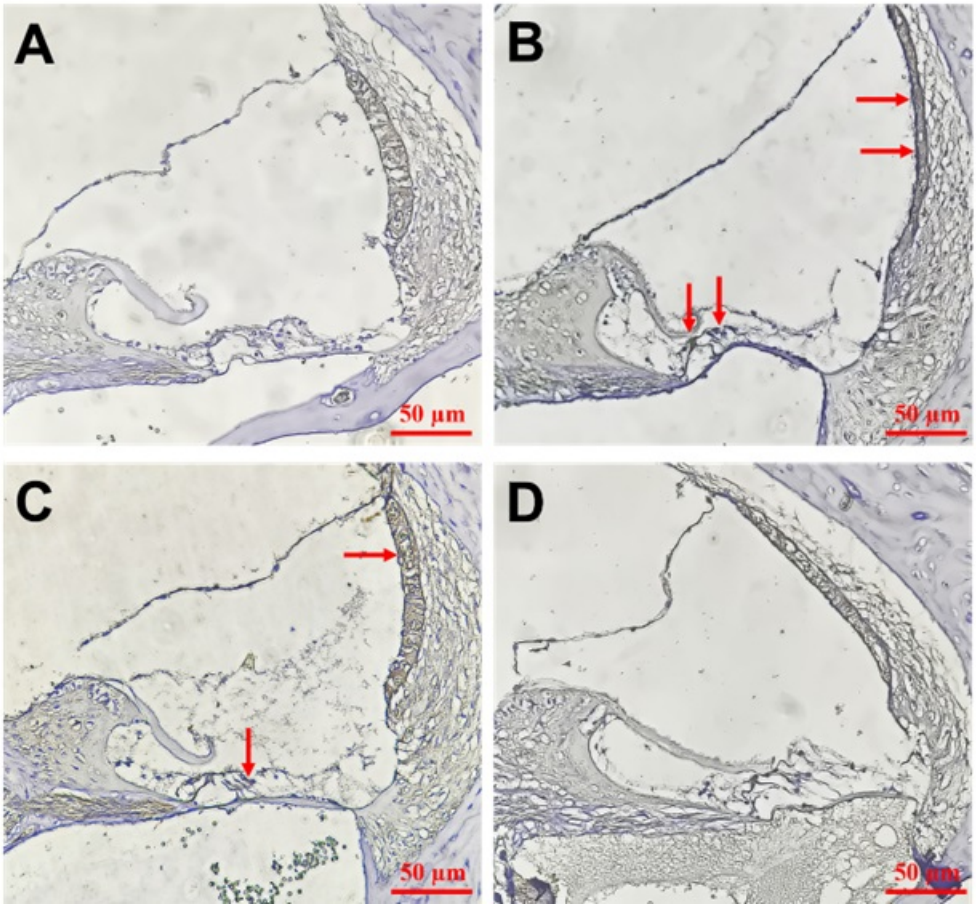


Figure 4. Immunohistochemical staining with severe signal Transducer and Activator of Transcription-1 (STAT1) in Outer Hair Cells (OHCs) and stria vascularis. Experimental groups. Control Group: without STAT1 expression; B) Cisplatin Group: severe STAT1 immunopositivity in wide range of internal ear (arrowhead); C) Cisplatin + Epigallocatechin-3-Gallate (EGCG) Group: Moderate STAT1 immunopositivity (arrowhead); D) EGCG Group: Negative STAT1 expression.