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Curcumin Reduces the Noise-Exposed Cochlear Fibroblasts Apoptosis

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Abstract

Introduction The structural changes underlying permanent noise-induced hearing loss (NIHL) include loss of the sensory hair cells, damage to their stereocilia, and supporting tissues within the cochlear lateral wall.

Objective The objective of this study is to demonstrate curcumin as a safe and effective therapeutic agent in the prevention and treatment for fibroblasts damage within the cochlear supporting tissues and lateral wall through cell death pathway.

Methods   We divided 24 Rattus norvegicus into 4 groups, Group 1: control; Group 2: noise (+); Group 3: noise (+), 50 mg/day curcumin (+); Group 4: noise (+), 100 mg/day curcumin (+). We provided the noise exposure dose at 100 dB SPL for two hours over two weeks and administered the curcumin orally over two weeks. We examined all samples for the expressions of calcineurin, nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), and apoptotic index of cochlear fibroblasts.

Results   We found significant differences for the expressions of calcineurin ($p < 0.05$) in all groups, significant differences for the expressions of NFATc1 ($p < 0.05$) in all groups, except in Groups 1 and 4, and significant differences for the apoptotic index ($p < 0.05$) in all groups.

Conclusion Curcumin proved to be potentially effective in the prevention and treatment for fibroblasts damage within the cochlear supporting tissues and lateral wall regarding the decreased expression of calcineurin, NFATc1, and apoptotic index of cochlear fibroblasts.

Keywords
► noise
► curcumin
► calcineurin
► NFATc1
► apoptosis
► cochlea

Introduction

Exposure to excessive noise is the major avoidable cause of permanent hearing impairment.[1] It is mostly found in the developing and industrial countries with bad hearing conservation.[2] In 2012, there were 360 million persons in the world with disabling hearing loss, ∼5.3% of the world's population, of which 328 million (91%) were adults (183 million men, 145 million women) and 32 (9%) million were children. Sixteen per cent of the disabling hearing loss in the adult population in the world resulted from excessive noise exposure in the workplace, ranging from 7% to 21% in the various subregions.[1,3]

The cochlear spiral ligament is a connective tissue lining the space between stria vascularis and the bony otic capsule. It plays diverse roles in normal hearing and is composed of sub-populations of specialized fibrocytes, which are suggested to play distinct roles in fluid homeostasis, inflammatory responses, predicted by their protein expression profiles. Certain fibrocyte sub-types express ion transport proteins and, thus, are likely to regulate K^+ and Cl^- within the lateral wall perilymph.[4]

Evidence from various cell lines shows that cell death can be stimulated by oxidative stress and excitotoxicity through Ca^{2+} overload. Acoustic overstimulation increases the Ca^{2+} concentration in auditory hair cells. Elevated Ca^{2+} has been implicated in the impairment of hair cell function and may initiate hair cell damage after noise exposure. There are several pathways through which Ca^{2+} may contribute to cell death, involving activation of nitric oxide synthase (NOS), phospholipase A2, proteases, and calcineurin.[5]

For years, studies have emerged based on the use of natural compounds plant-derived as potential therapeutic agents for various diseases in humans.[6] Curcumin, a yellow pigment obtained from the rhizomes of *Curcuma longa* Linnaeus (Family: Zingiberaceae), is a major component of turmeric and has been used as a traditional medicine that possesses therapeutic potential against various diseases. Curcumin is capable of modulating numerous molecular targets involved in each stage of disease development by regulating transcription factors, growth factors, receptors, cytokines, kinases, enzymes, cell survival, metastatic, and apoptotic molecules.[7]

The role of curcumin in the prevention of and treatment of fibroblast damage within the supporting tissues and the cochlear lateral wall through the apoptosis inhibition mechanism contributed by calcineurin in cochlear fibroblasts has never been studied and serves as the focus in this study. The objective of this study is also to demonstrate that higher doses of curcumin (100 mg/day) exert more beneficial effects in inhibiting apoptosis rather than low doses of curcumin (50 mg/day).

Methods

This study is an experimental study with randomized posttest-only control group design using male Wistar strain white *Rattus norvegicus* rats (150 - 250 g, 8 - 12 weeks of age). The dose and frequency of noise exposure was 100 dB SPL and 1 - 10 kHz for 2 hours.

Curcumin used in this study was derived from *Curcuma longa Linnaeus* (Turmeric) with curcumin content levels of $28.1 \pm 1.0\%$ w/w compared with standard, suspended in 0.5% carboxymethyl cellulose. Afterwards, we administered the suspension directly to the stomach of each rat via nasogastric tube, once a day for two weeks. The samples were composed of 24 *Rattus norvegicus* divided into 4 groups. Group 1: the control group; Group 2: noise (+); Group 3: noise (+), 50 mg/day curcumin (+); Group 4: noise (+), 100 mg/day curcumin (+). We provided noise exposure doses of 100 dB SPL for two hours over two weeks.

After two weeks, the rats underwent termination by ether inhalation and necropsy procedure on their temporal bone. All samples underwent standard tissue processing with fixation in buffered formaldehyde, followed by dehydration in graded alcohol solutions. Thereafter, they were embedded in paraffin blocks, serially cut into 4 µm thick sections, and put on glass slides. Representative sections were stained with hematoxylin and eosin (H&E). We performed immunohistochemical staining to examine the expressions of calcineurin and nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) and apoptotic index of cochlear fibroblasts by terminal deoxynucleotidyl transferase (TdT) 2'-deoxyuridine 5'-triphosphate (dUTP) nick-end labeling (TUNEL) Assay.

Immunohistochemistry procedures were performed as follows. We cleared the slide in xylene and rehydrated it through graded series of alcohol solutions. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in absolute methanol. We prevented nonspecific binding of the second layer antibody by incubation with 10% nonimmune serum (0.25% Triton X-100 in phosphate-buffered saline phosphate-buffered saline). Anti-Calcineurin A antibody (abcam ab71149, Abcam plc., Cambridge, USA) and NFATc1 antibody 7A6 (sc-7294, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) served as the first antibodies and were separately applied to each specimen and incubated in a humid chamber. After rinsing with phosphate-buffered saline, we incubated sections with biotinylated secondary antibody. Later, we washed them once more and incubated with a horseradish streptavidin–peroxidase conjugate. Next, we added a substrate–chromogen solution (3–3'-diaminobenzidine tetrahydrochloride). This reaction involved peroxidase catalysis of the substrate and conversion of the chromogen to a brown deposit that marked the antigen. The final steps included counterstaining with H&E and application of coverslips.

The TUNEL assay (The ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore Corporation, Darmstadt, Germany) procedures were described as follows. We cleared the slide in xylene and rehydrated by transferring the slides through a graded ethanol series. We blotted away the excess water carefully and added proteinase K solution to cover sections. Afterwards, it was incubated at room temperature. We inactivated endogenous peroxidases by covering sections with 2% hydrogen peroxide. Later, the slide was washed and the excess water also was blotted away carefully. We added TdT equilibration buffer to cover sections and then removed the buffer. TdT reaction buffer was added to cover sections afterwards. The slide was incubated in a humidified chamber. To conserve reagents, a reduced volume of TdT buffer may be carefully covered with a glass coverslip during the incubation. The reaction was stopped by incubating the slide. Then we rinsed it in phosphate-buffered saline and blocked nonspecific binding by covering tissue sections with 2% Bovine Serum Albumin solution. Later, the slide was incubated in working strength stop/wash buffer. After applying stop solution, the sample was washed and incubated with anti-digoxigenin peroxidase conjugate. We developed the slide with 3–3'-diaminobenzidine tetrahydrochloride substrate, counterstained with methyl green, dehydrated, and cover-slipped.
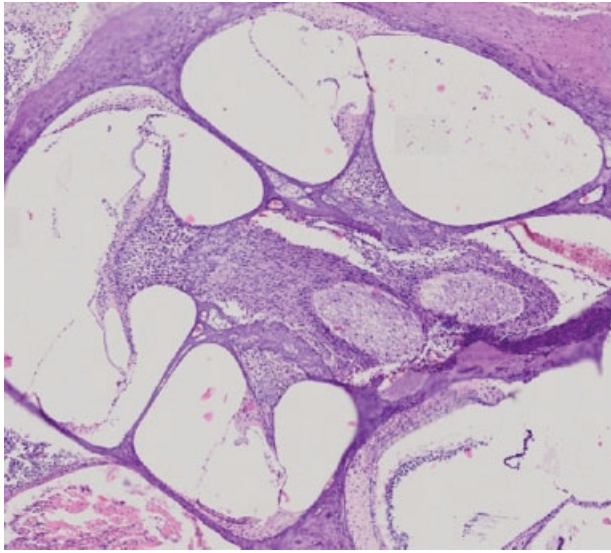
Fig. 1 The cochlear supporting tissues and lateral wall with H&E staining (20x zoom).

Three observers examined the samples in each slide. The fibroblasts within the cochlear supporting tissues and lateral wall, which expressed calcineurin and NFATc1, and apoptotic index in all fields were calculated manually with a hand counter. We calculated the expressions of calcineurin and NFATc1 quantitatively for the average distribution of fibroblasts unit with single nucleus expressing calcineurin and NFATc1 (showing brown-colored cytoplasms) and the occurrence of apoptosis (showing brown-colored nucleus).

We processed the data using the Statistical Package for the Social Sciences (SPSS) one-way analysis of variance (ANOVA) and used a p value of 0.05 as the cut-off for statistical significance.

Results

We performed H&E staining of rat cochlea to get a detailed view of the tissue (►**Fig. 1**).

The expression of calcineurin, after being evaluated with the immunohistochemistry method, showed an increased expression in Group 2 (►**Fig. 2B**), compared with other groups. The curcumin-treated groups showed lower density seen in the brown color, and less calcineurin-expressed fibroblasts than Group 2 (►**Fig. 2C, D**).

Data in ►**Table 1** show significant differences for the expressions of calcineurin ($p < 0.05$) in all groups. A dose of curcumin 100 mg per day showed statistically significant decreases in the expressions of calcineurin rather than a dose of curcumin at 50 mg per day.

The expression of NFATc1 after being evaluated with the immunohistochemistry method showed an increased expression in Group 2 (►**Fig. 3B**) compared with other groups. The curcumin-treated groups showed less NFATc1-expressed fibroblasts than Group 2 (►**Fig. 3C, D**).
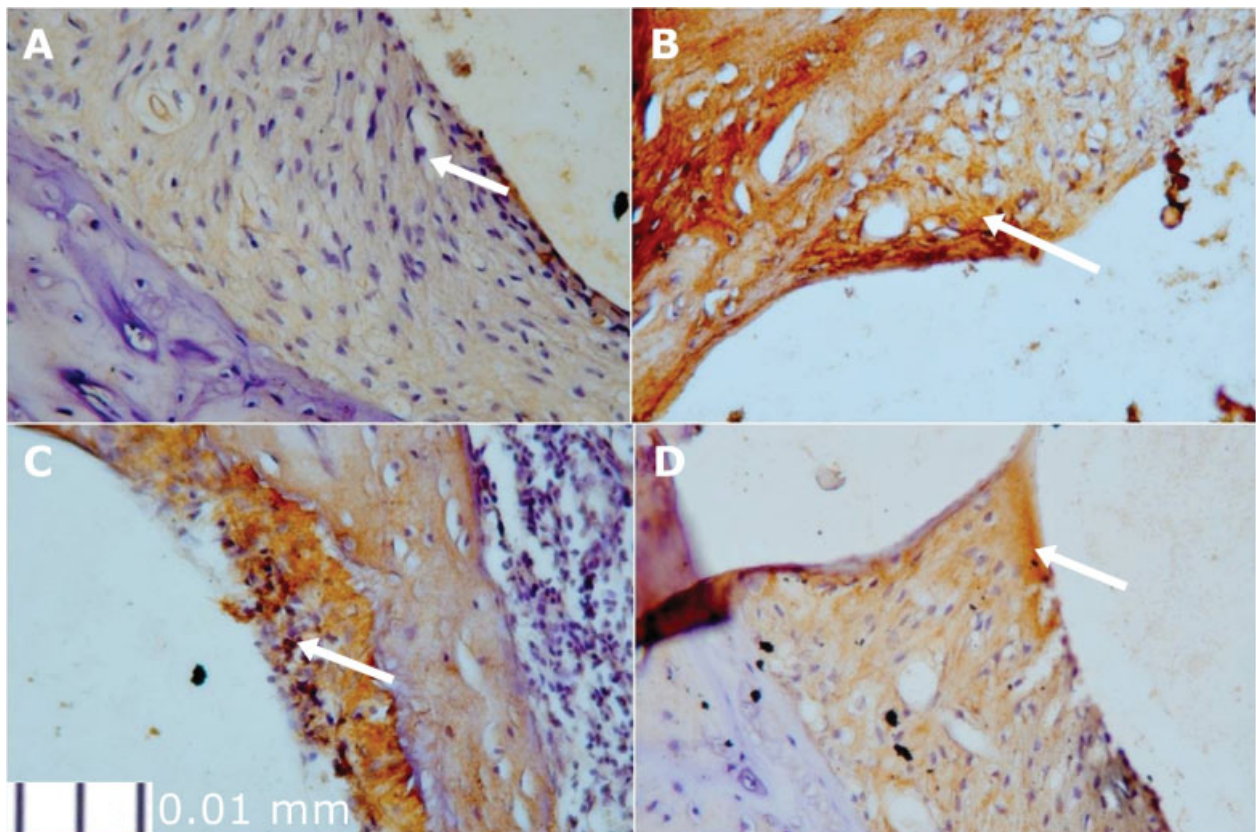

Fig. 2 The expression of calcineurin in each group (1000x zoom): (A) Group 1/control; (B) Group 2; (C) Group 3; (D) Group 4. The white arrow indicates the expression of calcineurin in cochlear fibroblasts marked by the brown color.

Table 1 ANOVA test results in terms of the expressions of calcineurin

Group		Mean difference ± Standard deviation	P value
Group 1	Group 2	22.000 ± 1.392	0.000*
	Group 3	17.500 ± 1.392	0.000*
	Group 4	12.500 ± 1.392	0.000*
Group 2	Group 3	4.500 ± 1.392	0.025*
	Group 4	9.500 ± 1.392	0.000*
Group 3	Group 4	5.000 ± 1.392	0.011*

*Denotes statistically significant.

Data in ►**Table 2** above showed significant differences for the expressions of NFATc1 ($p < 0.05$) in all groups, except in Groups 1 and 4. A dose of curcumin of 100 mg per day showed statistically significant decreases in the expressions of NFATc1, rather than a dose of curcumin 50 mg per day.

The apoptotic index, after being evaluated with TUNEL assay showed an increased apoptotic index in group 2 (►**Fig. 4 B**) compared with other groups. The curcumin-treated groups showed less apoptotic cells-expressed fibroblasts than group 2 (►**Fig. 4C, D**).

Data in ►**Table 3** showed significant differences for the apoptotic index ($p < 0.05$) in all groups. A dose of curcumin 100 mg per day showed statistically significant decreases in the apoptotic index rather than a dose of curcumin 50 mg per day.

Discussion

Acoustic overstimulation induces Ca^{2+} overload and mediated cell death pathways, involving activation of calcineurin.[5,8] In this study, we found that the expression of calcineurin statistically increases in the cochlear fibroblast noise-exposed group (Group 2) when compared with the control group. Calcineurin belongs to the family of Ca^{2+}/calmodulin-dependent protein phosphatases, protein phosphatase 2B. Calcineurin is activated by binding of Ca^{2+}/calmodulin and the only protein phosphatase regulated by a second messenger Ca^{2+}.[9,10]

It has been recently reported that calcineurin is activated in outer hair cells following noise exposure in mice exposed to broadband noise (2 - 20 kHz).[8] In another experimental study in guinea pigs, after intense noise exposure (4 - 10 kHz, 120 dB, for 5 hours), varying degrees of hair cells loss and calcineurin immunoreactivity were detected immunohistochemistrically in outer hair cells and concentrated at the cuticular plate.[5]
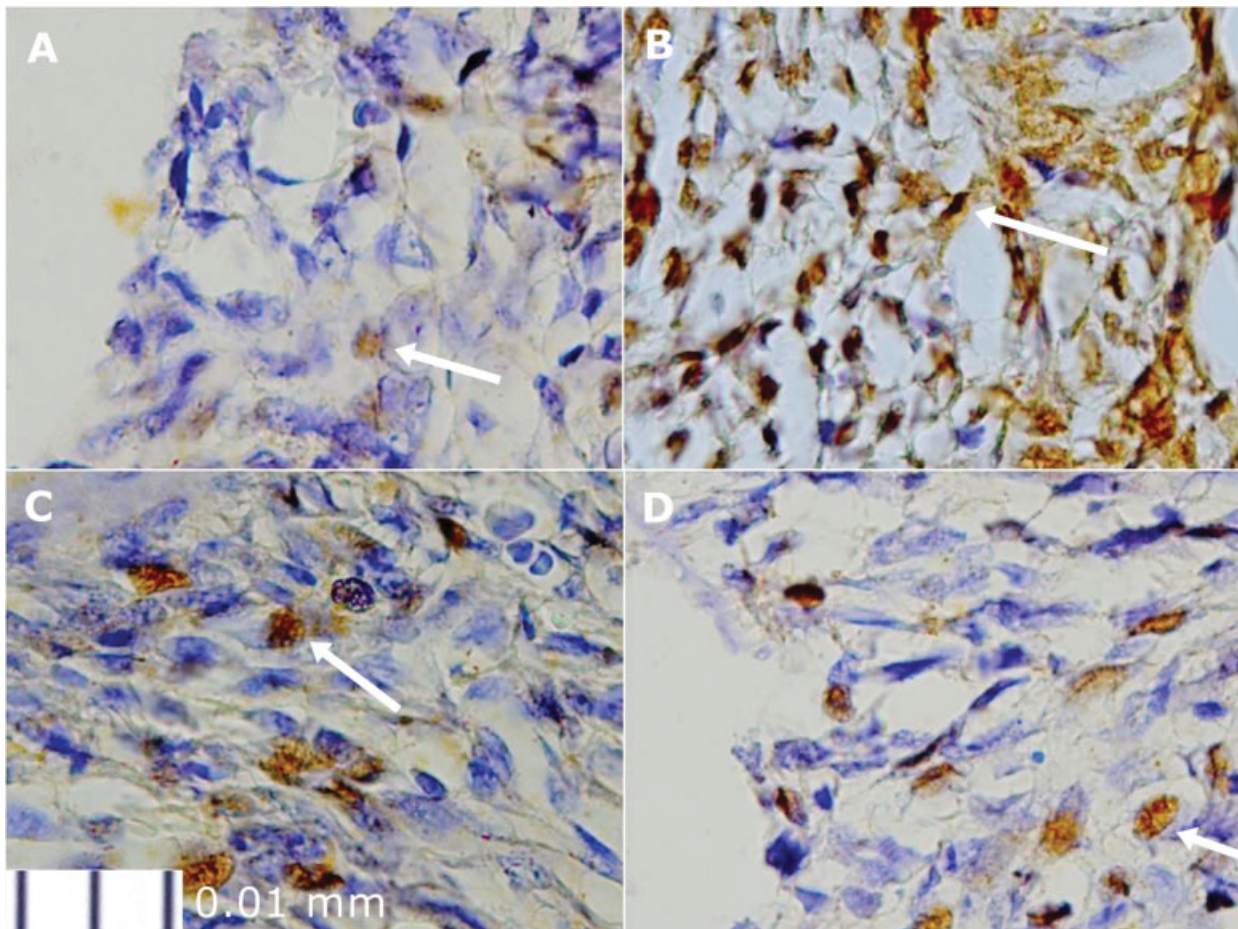

Fig. 3 The expression of NFATc1 in each group (1000x zoom): (A) Group 1/control; (B) Group 2; (C) Group 3; (D) Group 4. The white arrow indicates the expression of NFATc1 in cochlear fibroblasts marked by the brown color.

Table 2 ANOVA test results in terms of expressions of NFATc1

Group		Mean difference ± Standard deviation	P value
Group 1	Group 2	10.833 ± 0.792	0.000*
	Group 3	5.667 ± 0.792	0.000*
	Group 4	1.833 ± 0.792	0.189
Group 2	Group 3	5.167 ± 0.792	0.000*
	Group 4	9.000 ± 0.792	0.000*
Group 3	Group 4	3.833 ± 0.792	0.001*

*Denotes statistically significant.

Table 3 ANOVA test results in terms of the apoptotic index

Group		Mean difference ± Standard deviation	P value
Group 1	Group 2	12.333 ± 1.004	0.000*
	Group 3	6.833 ± 1.004	0.000*
	Group 4	3.667 ± 1.004	0.010*
Group 2	Group 3	5.500 ± 1.004	0.000*
	Group 4	8.667 ± 1.004	0.000*
Group 3	Group 4	3.167 ± 1.004	0.030*

*Denotes statistically significant.

The expression of NFATc1 was found to be statistically higher in the cochlear fibroblast noise-exposed group (Group 2) compared with the control group. Ca^{2+} overload activates calcineurin-dephosphorylated NFATs, leading to their translocation to the nucleus. In addition to this first wave of NFAT activation, in a second step of NFATc1/αA generation, a short isoform of NFATc1 is strongly induced.[11,12]

The apoptotic cochlear fibroblasts were statistically higher in the cochlear fibroblast noise-exposed group (Group 2) compared with the control group. Calcineurin has a dual function and may exert its effects on apoptosis either by the activation of specific transcriptional pathways or by direct dephosphorylation of proteins including Bad (B cell lymphoma 2 antagonist of cell death) and caspase-9 involved in the apoptotic pathway.[10,11]

Calcineurin dephosphorylates Bad, resulting in the disruption of the binding of Bax (B cell lymphoma 2 associated x protein) to Bcl-2 (B cell lymphoma 2) or Bcl-xL (B cell lymphoma 2-extra large) at the outer membrane of mitochondria through intrinsic pathway. Free Bax translocate to mitochondria and activate the transport system to release cytochrome c.[8,13,14]
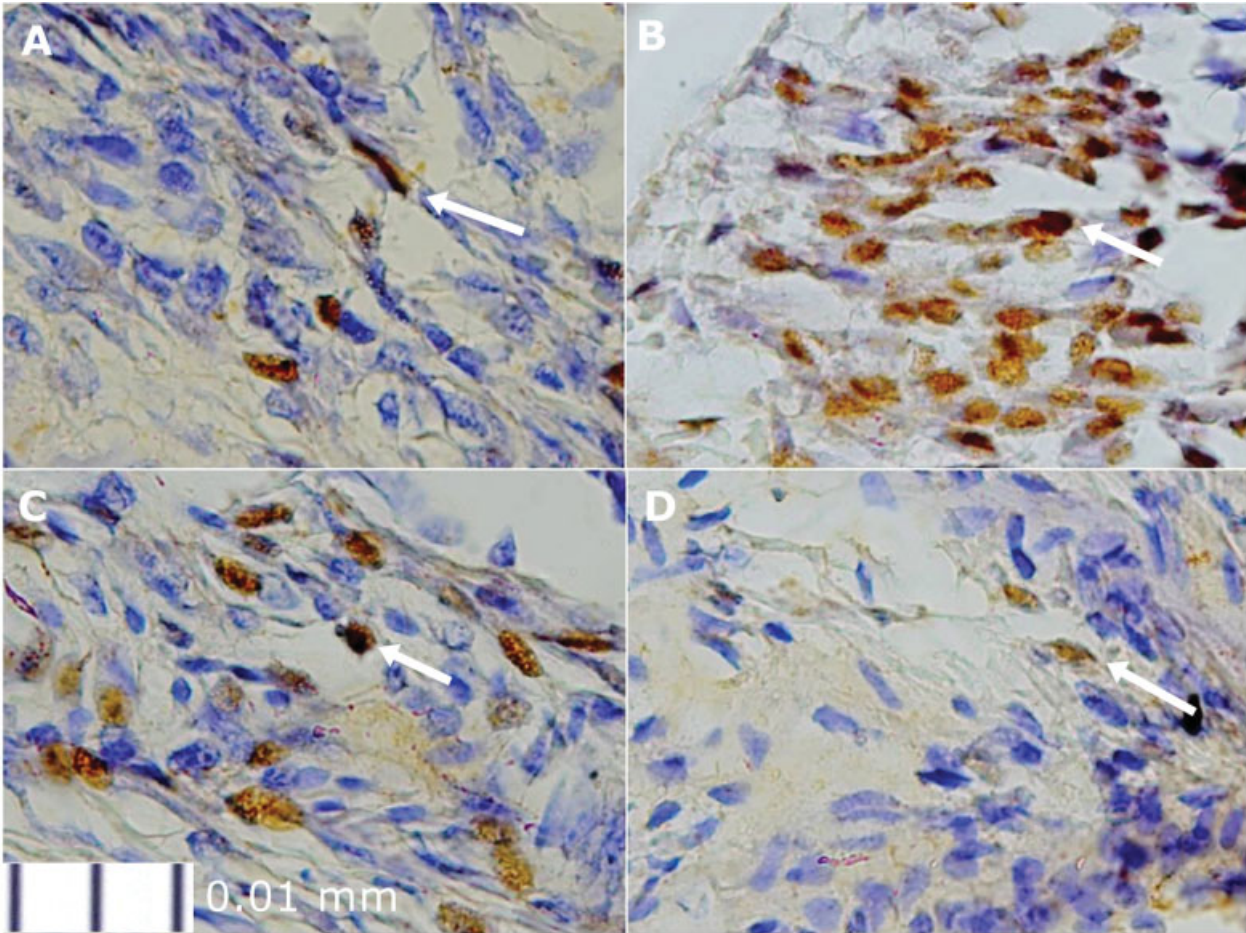

Fig. 4 The apoptotic index in each group (1000x zoom): (A) Group 1/control; (B) Group 2; (C) Group 3; (D) Group 4. The white arrow indicates the apoptotic cochlear fibroblasts marked by the brown color.

In the extrinsic pathway, outside the mitochondria, it has been previously described that expression of the membrane-bound death receptor ligand FasL is mediated by NFAT. When FasL binds to its receptor Fas, the intracellular machinery associated with the death receptor Fas is activated and eventually leads to apoptosis by caspase activation and subsequent DNA cleavage.[2] Caspase-8 directly cleaves caspase-3 as well as cytosolic Bid (BH-3 interacting domain death agonist), its active fragment (tBid) translocates to mitochondria to release cytochrome c.[15]

Cytochrome c interacts with proteins such as Apaf-1 (apoptotic protease activating factor-1), dATP, and procaspase-9 to produce apoptosome and then activates caspase-9. This complex degrades procaspase-3 to caspase-3.[15] In apoptotic cells, activated caspase-3 cleaves inhibitor of caspase activated DNAse (ICAD) to release CAD. CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies.[14]

An experimental study in mice exposed to broadband noise (2 - 20 kHz) has discovered that noise exposure induces activation of mitochondria-mediated cell death pathways in outer hair cells of the cochlea through activation of Bad by calcineurin. The localization of Bad was analyzed by immunohistochemistry. Total Bad was observed both in the sensory cells and in the supporting cells and also in the nerve fibers projecting to the sensory cells. The results of this study strongly support the Bad as a link between Ca^{2+} influx after noise exposure and the death of outer hair cells.[8] In another study, after intense noise exposure (4 - 10 kHz, 120 dB, for 5 hours) in guinea pigs, some calcineurin-immunopositive hair cells demonstrated condensed and swollen nuclei, indicating that calcineurin is related to both apoptosis and necrosis.[5]

This study proved that curcumin was able to decrease the expression of calcineurin and NFATc1 in cochlear fibroblasts, where a dose of curcumin 100 mg per day showed statistically significant decreases in the expression of calcineurin and NFATc1 compared with a dose of curcumin 50 mg per day. This is due to the speculation that curcumin inhibits the regulation and expression of calcineurin, and prevents the dephosphorylation of NFATc1 by calcineurin, thus, reducing its translocation to the nucleus. The expression of NFATc1 in Group 4 was statistically insignificant compared with the control group, indicating that curcumin administration at higher doses is able to prevent NFATc1 activation, thereby its expression was found to be nearly similar to the control group (without noise exposure).

Researchers have recently investigated the therapeutic efficacy of curcumin in attenuation of left ventricular hypertrophy and sought to delineate the associated signaling pathways in blunting the hypertrophic response in nephrectomized rats. Curcumin attenuates cardiac hypertrophy and remodeling through deactivation of multiple hypertrophic signaling pathways. This study reported that cytosolic NFAT was significantly decreased in rats that underwent nephrectomy and was significantly attenuated by curcumin. NFAT in the nucleus was decreased by curcumin with quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis.[16] Another study showed an action of curcumin as an NFAT inhibitor through the Ca^{2+} signaling pathway blocking. This experiment also demonstrates that curcumin inhibits NFAT transcriptional activity by preventing its nuclear translocation from the cytoplasm into the nucleus upon phorbol myristate acetate (PMA)/ionomycin stimulation of Jurkat T-cells with laser scan microscopy (LSM) analysis.[17]

Curcumin significantly promoted nonischemic wound healing in a dose–response fashion compared with controls as judged by increased reepithelialization and granulation tissue formation. Improved wound healing was associated with significant decreases in pro-inflammatory cytokines interleukin (IL)-1 and IL-6 as well as the chemokine IL-8. Curcumin also significantly reduced hypertrophic scarring.[18]

Another study concluded that pre- and coreceiving curcumin can significantly protect the cochlear morphology and functions on paclitaxel-induced ototoxicity in rats using light microscopy and distortion product otoacoustic emissions (DPOAEs) to evaluate histopathological, immunohistochemical, and functional changes in hearing. Curcumin might be considered as a potential dietary supplement from a natural product given to patients undergoing paclitaxel chemotherapy.[19]

Curcumin can also be used as an efficient adjuvant to cisplatin cancer therapy. This treatment strategy in head and neck cancer could mediate cisplatin chemoresistance by modulating therapeutic targets (Signal transducer and activator of transcription 3 and NF-E2 p45-related factor 2) and, at the same time, reduce cisplatin-related ototoxic adverse effects.[20]

Preclinical studies demonstrated that systemic curcumin attenuates ototoxicity and provides molecular evidence for a role of hemeoxigenase (HO-1) as an additional mediator in attenuating cisplatin-induced hearing loss.[21]

A previous study found the effect of curcumin on peroxynitrite (ONOO)-induced damage in rat spiral ganglion neurons. Pretreatment with curcumin abrogated cytochrome c release, blocked activation of caspase-3, and altered the expression of Bcl-2 family triggered by ONOO. Curcumin can attenuate ONOO-induced damage in spiral ganglion neurons by the anti-oxidative activity, as well as protect mitochondria from oxidative stress.[22]

In the present study, we proved that curcumin is able to decrease the apoptotic cochlear fibroblasts, whereby a dose of curcumin 100 mg per day showed significant decreases in the apoptotic index compared with a dose of curcumin 50 mg per day. This is due to the speculation that curcumin inhibits calcineurin activation, therefore its role in Bad dephosphorylation can be prevented. Furthermore, the complex of Bcl-2 or Bcl-XL remains inseparable and both function in preventing cytochrome c release into cytosol is inhibitable and apoptosis becomes an avoidable process. Curcumin also inhibits the dephosphorylation of NFATc1 leading to reduced translocation to the nucleus, therefore, preventing apoptosis by caspase-8 activation.

Conclusion

This study indicates that curcumin is safe and effective as a therapeutic agent in the prevention and treatment of the damage of fibroblasts within the supporting tissues and the cochlear lateral wall through the cell death pathway. Moreover, the study provides more insight into the mechanism of curcumin against apoptosis and shows that curcumin inhibits multiple apoptosis signaling pathways, including calcineurin and NFATc1. The study may serve as a scientific basis in the traditional systems of medicine for the management of NIHL in the future.

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