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Calpain mediated cisplatin-induced ototoxicity in mice

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Research Highlights

(1) Calpain participates in gentamicin-, neomycin- and kanamycin-induced inner ear cell apoptosis. Cisplatin has been shown to be an anticancer drug. However, cisplatin can lead to severe ototoxicity, induce cochlear cell apoptosis, and result in hearing decrease or loss, which limits the application of cisplatin in a clinical setting to a certain degree.
(2) Cisplatin has been shown to activate transient receptor potential V1 and cause Ca^{2+} influx and overload, finally resulting in cochlear hair cell injury and death. Thus, it remains unclear whether calpain expression was detected during cisplatin-induced cochlear hair cell death.
(3) This study established a BALB/c mouse model of cisplatin-induced ototoxicity. Calpain sion in the mouse cochlea was observed and the possible effect of calpain on cisplatin-induced ototoxicity was explored using immunofluorescence staining, image analysis and western blotting, in combination with an auditory brainstem response test.
(4) Results from this study verified that cisplatin upregulated calpain 1 and calpain 2 expression in the mouse cochlea in a dose-dependent manner, and that calpain 2 plays a leading role in this process.

Abstract

Ototoxic drug-induced apoptosis of inner ear cells has been shown to be associated with calpain expression. Cisplatin has severe ototoxicity, and can induce cochlear cell apoptosis. This study assumed that cisplatin activated calpain expression in apoptotic cochlear cells. A mouse model of cisplatin-induced ototoxicity was established by intraperitoneal injection with cisplatin (2.5, 3.5, 4.5, 5.5 mg/kg). Immunofluorescence staining, image analysis and western blotting were used to detect the expression of calpain 1 and calpain 2 in the mouse cochlea. At the same time, the auditory brainstem response was measured to observe the change in hearing. Results revealed that after intraperitoneal injection with cisplatin for 5 days, the auditory brainstem response threshold shifts increased in mice. Calpain 1 and calpain 2 expression significantly increased in outer hair cells, the spiral ganglion and stria vascularis. Calpain 2 protein expression markedly increased with an increased dose of cisplatin. Results suggested that calpain 1 and calpain 2 mediated cisplatin-induced ototoxicity in BALB/c mice. During this process, calpain 2 plays a leading role.

Key Words

neural regeneration; biological factor; cisplatin; mice; cochlea; apoptosis; calpain; auditory tem response; ototoxicity; immunofluorescence staining; image analysis technique; western blotting; grants-supported paper; neuroregeneration

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INTRODUCTION

Cisplatin is a divalent platinum compound that has a strong broad-spectrum anticancer effect and a synergistic effect with many antineoplastic agents. Cisplatin lacks cross-resistance and has been considered as a first-line agent for cancer[1]. However, cisplatin has severe adverse effects including ototoxicity, which can induce damage to the cochlea, resulting in bilateral hearing decrease or loss[2-4]. Thus, clinical application of cisplatin has been limited. Numerous studies have confirmed that the mechanism of cisplatin ototoxicity is associated with cisplatin-induced cochlear cell apoptosis[5-8]. Alam *et al* [5] demonstrated that terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL)-positive staining appeared in outer hair cells, the spiral ganglion and stria vascularis in the basal turn of the gerbil cochlea after injection of cisplatin. In addition, Watanabe *et al* [6] found that TUNEL-positive cells were detected in the cochlear stria vascularis of guinea pigs after administration with 10 mg/kg cisplatin (critical dose of ototoxicity). Moreover, Sun *et al* [9] verified that hearing decreased, and cochlear spiral ganglion cell apoptosis appeared in mice injected with cisplatin, and that caspase-3 was involved in this apoptosis, which further confirmed that apoptosis is a mechanism of cisplatin ototoxicity.

Inner ear cell apoptosis induced by ototoxic drugs such as gentamicin, kanamycin and neomycin are associated with calpain[10-13]. Calpain, a Ca^{2+}-dependent cysteine proteinase, exists in various cells, and is involved in cytoskeleton reconstitution, signal conduction, cell apoptosis and necrosis[14]. So far, at least 15 mammalian calpain family members have been identified; of them, calpain 1 and calpain 2 have been intensively studied[15]. Under physiological conditions, calpain mainly exists in an inactive form and maintains the renewal of the cytoskeleton. Under pathological conditions, a persistent abnormal increase in Ca^{2+} levels activates calpain, which degrades the cytoskeleton, membrane proteins and signal transduction-associated enzymes and transcription factors, finally resulting in cell apoptosis and necrosis.

Numerous studies have confirmed that cisplatin activates transient receptor potential vanilloid 1, induces Ca^{2+} influx and overload, and results in cochlear hair cell injury and death[16-18]. However, calpain expression during cisplatin-induced cochlear hair cell death remains poorly understood. Thus, this study was designed to investigate the effect of cisplatin on cochlear calpain expression and to explore the possible effects of calpain on cisplatin ototoxicity. We aimed to provide evidence for the clinical prevention and treatment of cisplatin ototoxic deafness in a healthy BALB/c mouse model of cisplatin-induced ototoxicity using immuno- fluorescence staining, image analysis, western blot, and auditory brainstem response testing.

RESULTS

Quantitative analysis of experimental animals

A total of 65 BALB/c mice were randomly assigned into five groups: control group, 2.5, 3.5, 4.5, and 5.5 mg/kg cisplatin groups (n = 13; 26 ears). Cisplatin groups received an intraperitoneal injection of cisplatin injection 2.5, 3.5, 4.5 or 5.5 mg/kg separately. The control group received an intraperitoneal injection of an equal volume of saline. At 5 days after administration, no death or infection was detected. All 65 mice were included in the final analysis.

Cisplatin effects on hearing in ototoxic mice

At 5 days following intraperitoneal injection of cisplatin, under various frequencies of stimulation, auditory brainstem response threshold shifts in various-dose cisplatin groups were significantly greater when compared with the control group (P < 0.01). Following cisplatin treatment, auditory brainstem response threshold shifts significantly increased in a noticeable dose-effect relationship (P < 0.01; Table 1).

Table 1 Auditory brainstem response threshold shifts (dB SPL) in mice from each group

Group	Frequency of stimulation (kHz)		
	8	12	24
Control	1.0±2.1	0.6±1.7	0.8±1.9
2.5 mg/kg cisplatin	7.1±5.5[a]	7.9±4.6[a]	10.6±5.9[a]
3.5 mg/kg cisplatin	11.9±5.3[ab]	13.5±4.3[ab]	18.8±6.1[ab]
4.5 mg/kg cisplatin	21.9±8.2[ac]	24.2±8.8[ac]	29.0±10.0[ac]
5.5 mg/kg cisplatin	42.7±6.6[ad]	46.0±7.7[ad]	47.7±9.9[ad]

[a]$P < 0.01$, *vs*. control group; [b]$P < 0.01$, *vs*. 2.5 mg/kg cisplatin group; [c]$P < 0.01$, *vs*. 3.5 mg/kg cisplatin group; [d]$P < 0.01$, *vs*. 4.5 mg/kg cisplatin group. Data are expressed as mean ± SD, n = 26 ears. Intergroup comparison was performed using one-way analysis of variance and least significant difference t-test.

Effect of cisplatin on calpain immunoreactivity in the mouse cochlea

Results of immunofluorescence staining revealed that in the control group, calpain 1 expression (pale green) was mainly in outer hair cells, the spiral ganglion and stria vascularis (Figure 1A). The regions where calpain 1 expression was detectable in the mouse cochlea of the various dose cisplatin groups were similar to that in the control group, but the intensity of the green fluorescence was noticeably stronger when compared with the control group (Figure 1B–E). However, no significant difference in fluorescence intensity of calpain 1 was visible among the different dose cisplatin groups.

Image analysis results demonstrated that calpain 1 immunoreactivity was significantly greater in the various dose cisplatin groups than that of the control group ($P < 0.01$), but no significant difference in calpain 1 immunoreactivity was observed among the various dose cisplatin groups (Table 2).

In the control group, calpain 2 immunoreactivity (pale red) was mainly in outer hair cells, the spiral ganglion and stria vascularis (Figure 2A). The regions where calpain 2 immunoreactivity was detected in the mouse cochlea of various dose cisplatin groups were similar to that in the control group, but the intensity of red fluorescence was noticeably stronger when compared with the control group (Figure 2B–E). Moreover, with increased dose of administration, calpain 2 immunoreactivity noticeably enhanced. Image analysis results demonstrated that calpain 2 immunoreactivity was significantly greater in the various dose cisplatin groups than that of the control group ($P < 0.05$). With increased dose of cisplatin, calpain 2 immunoreactivity was significantly increased in a noticeable dose-effect relationship ($P < 0.01$; Table 3).

Effect of cisplatin on calpain protein expression in the mouse cochlea

At 5 days after administration, calpain 1 and calpain 2 expression was low in the mouse cochlea in the control group. Calpain 1 and calpain 2 expression was significantly higher in the different dose cisplatin groups than those of the control group (Figure 3).

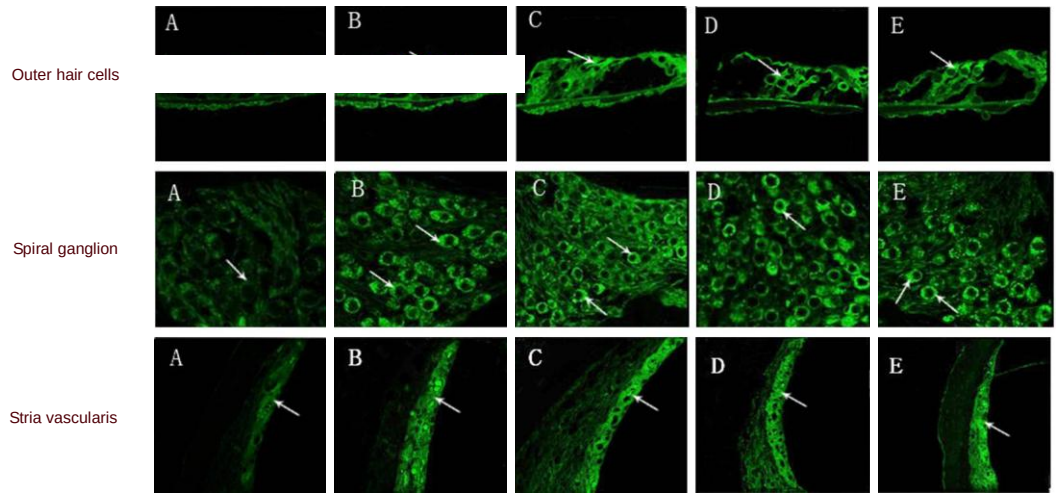

Figure 1 Calpain 1 immunoreactivity in the mouse cochlea (immunofluorescence staining, paraffin section, × 200).

Calpain 1 immunoreactivity appeared green fluorescence (fluoresceine isothiocyanate, arrows), and was mainly observed in outer hair cells, the spiral ganglion and stria vascularis. The regions where calpain 1 immunoreactivity was detectable in the mouse cochlea of 2.5, 3.5, 4.5, 5.5 mg/kg cisplatin groups (B–E) were similar to that of the control group (A), but the intensity of green fluorescence was noticeably stronger when compared with the control group.

Table 2 Calpain 1 immunoreactivity (average absorbance) in the mouse cochlea in each group

Group	Outer hair cells	Spiral ganglion	Stria vascularis
Control	1.70±0.20	3.17±0.16	1.44±0.26
2.5 mg/kg cisplatin	3.56±0.23[a]	5.27±0.21[a]	3.44±0.28[a]
3.5 mg/kg cisplatin	3.57±0.22[a]	5.27±0.37[a]	3.46±0.64[a]
4.5 mg/kg cisplatin	3.58±0.31[a]	5.29±0.42[a]	3.49±0.36[a]
5.5 mg/kg cisplatin	3.60±0.29[a]	5.30±0.27[a]	3.49±0.18[a]

[a]$P < 0.01$, *vs*. control group. Data are expressed as mean ± SD, $n = 8$ ears. Intergroup comparison was performed using one-way analysis of variance and least significant difference *t*-test.

Semi-quantitative analysis results revealed that calpain 1 and calpain 2 expression was significantly higher in the various dose cisplatin groups than those in the control group ($P < 0.01$). No significant difference in calpain 1 protein expression was detected among the different dose cisplatin groups, but calpain 2 protein expression significantly increased with increased dose of cisplatin ($P < 0.01$; Table 4).

DISCUSSION

BALB/c mice are an inbred mouse line, characterized by clear gene properties, strong productivity, and low cost. In addition, it is easy to obtain antibodies, probes and gene mutants. Thus, BALB/c mice are widely used for biomedical studies. A previous study demonstrated that BALB/c mice were vulnerable to AmAn ototoxicity[19], but whether these mice are vulnerable to cisplatin-induced ototoxicity remains poorly understood. Results from this study showed that after intraperitoneal injection of various doses of cisplatin in BALB/c mice for 5 days, auditory brainstem response threshold shifts increased to different degrees, and significantly increased with increased doses of cisplatin in a noticeable dose-effect relationship, which was consistent with a previous study[20]. The above-described results demonstrated that cisplatin has toxic effects on the cochlea of BALB/c mice. Moreover, auditory brainstem response threshold shifts were highest under 24 kHz conditions in the cisplatin groups at 5 days after administration, indicating that cisplatin-induced ototoxicity in BALB/c mice was developed from high frequency to low frequency, which was identical to ototoxicity in rats[21-24]. The damage to lower regions of the cochlea mainly impacted high-frequency hearing, but the damage to upper regions of the cochlea mainly impacted low-frequency hearing. Results demonstrated that the influence of cisplatin on the cochlea of BALB/c mice was from lower region to upper region.

Calpain contains two isomers: calpain 1 and calpain 2. Their activation depends on Ca^{2+}, but the concentration of Ca^{2+} needed is different. Calpain 1 can be activated by micromolar concentrations of Ca^{2+}, thus, it is also called μ-calpain. Calpain 2 requires millimolar concentrations of Ca^{2+}, and is therefore called m-calpain[25]. Under normal conditions, Ca^{2+} in the human body is altered at micromolar concentration levels.

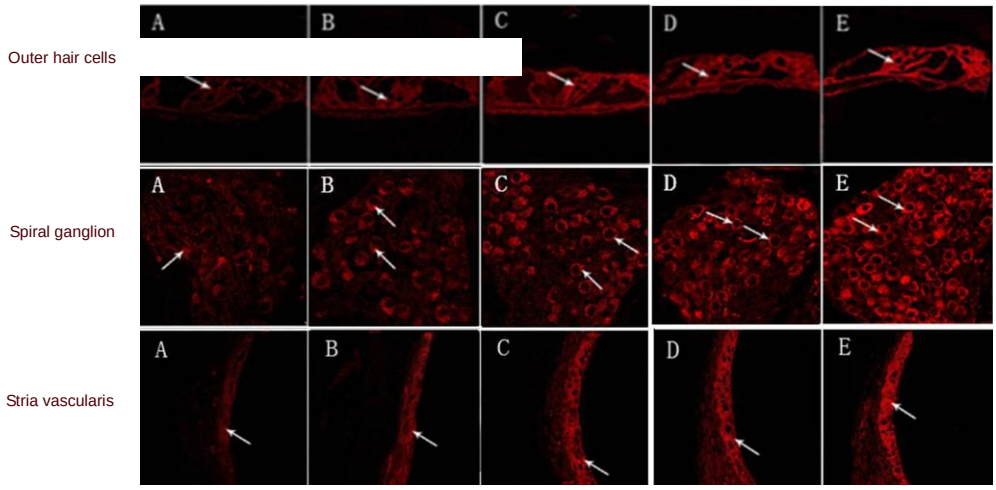

Figure 2 Calpain 2 immunoreactivity in the mouse cochlea (immunofluorescence staining, paraffin section, × 200).

Calpain 2 immunoreactivity appeared red (tetramethyl rhodamin isothiocyanate, arrows), and was mainly observed in outer hair cells, the spiral ganglion and stria vascularis. The regions where calpain 2 immunoreactivity was observed in the mouse cochlea of 2.5, 3.5, 4.5, 5.5 mg/kg cisplatin groups (B–E) were similar to that of the control group (A), but the intensity of red fluorescence was noticeably stronger when compared with the control group.

Table 3 Calpain 2 immunoreactivity (average absorbance) in the mouse cochlea in each group

Group	Outer hair cells	Spiral ganglion	Stria vascularis
Control	1.9±0.3	3.9±0.5	1.6±0.3
2.5 mg/kg cisplatin	2.5±0.9[a]	4.3±0.4[a]	1.9±0.3[a]
3.5 mg/kg cisplatin	3.2±0.5[ab]	5.1±0.2[ab]	2.9±0.5[ab]
4.5 mg/kg cisplatin	4.4±0.4[ac]	7.3±0.5[ac]	4.0±0.5[ac]
5.5 mg/kg cisplatin	5.4±0.4[ad]	8.7±0.7[ad]	5.2±0.5[ad]

[a]$P < 0.05$, *vs*. control group; [b]$P < 0.01$, *vs*. 2.5 mg/kg cisplatin group; [c]$P < 0.01$, *vs*. 3.5 mg/kg cisplatin group; [d]$P < 0.01$, *vs*. 4.5 mg/kg cisplatin group. Data are expressed as mean ± SD, $n = 8$ ears. Intergroup comparison was performed using one-way analysis of variance and least significant difference *t*-test.

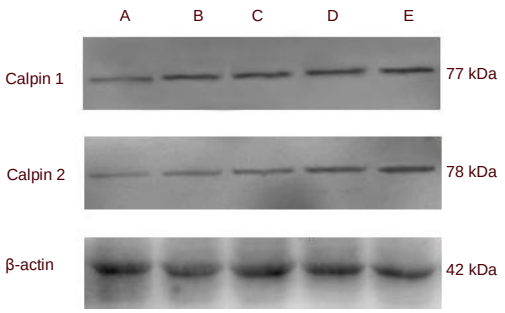

Figure 3 Cisplatin effects on calpain 1 and calpain 2 expression in the cochlea of mice with cisplatin-induced ototoxicity.

Calpain 1 and calpain 2 expression was significantly higher in the 2.5, 3.5, 4.5, 5.5 mg/kg cisplatin groups (B–E) than that of the control group (A).

Table 4 Calpain 1 and calpain 2 protein expression in the mouse cochlea in each group

Group	Calpain 1	Calpain 2
Control	0.38±0.01	0.08±0.01
2.5 mg/kg cisplatin	0.63±0.02[a]	0.18±0.01[a]
3.5 mg/kg cisplatin	0.63±0.02[a]	0.27±0.01[ab]
4.5 mg/kg cisplatin	0.64±0.02[a]	0.48±0.01[ac]
5.5 mg/kg cisplatin	0.64±0.01[a]	0.61±0.02[ad]

Data are expressed as the ratio of absorbance of target protein/β-actin. [a]$P < 0.01$, *vs*. control group; [b]$P < 0.01$, *vs*. 2.5 mg/kg cisplatin group; [c]$P < 0.01$, *vs*. 3.5 mg/kg cisplatin group; [d]$P < 0.01$, *vs*. 4.5 mg/kg cisplatin group. Data are expressed as mean ± SD, $n = 9$ mice; each six cochleas served as a specimen. Intergroup comparison was performed using one-way analysis of variance and least significant difference *t*-test.

When Ca^{2+} concentrations abnormally increase due to various reasons, abundant amounts of calpain are activated, which can degrade cytoskeletal proteins, channel proteins and various enzymes. Thus, protein and enzyme function loss is observed, finally resulting in cell apoptosis and necrosis.

Noise- and ototoxic drug-induced inner cell apoptosis is strongly associated with calpain. The calpain inhibitor BN82270 has been shown to effectively prevent noise-induced cochlear hair cell damage in a dose-dependent manner in the rat[26]. In addition, amikacin increased calpain expression in rat cochlear hair cells and spiral ganglion cells, suggesting that calpain plays a key role in amikacin-induced cochlear cell apoptosis[27]. When intracellular Ca^{2+} concentration increased, calpain inhibitors protected spiral ganglion cells against injury, suggesting that calpain restrained nerve axon growth by degradation[28]. Neomycin induced apoptosis of C57BL/6J mouse cochlear hair cells cultured *in vivo*, indicating calpain participated in neomycin-induced cochlear hair cell apoptosis[13].

Results from the present study demonstrated that calpain 1 and calpain 2 expression in outer hair cells, the spiral ganglion and strial vascularis of the mouse cochlea were obviously increased when compared with the control group at 5 days following intraperitoneal injection of various doses of cisplatin, and that calpain 2 expression significantly increased with an increased dose of cisplatin. Simultaneously, auditory brainstem response threshold shifts gradually increased. Taken together, calpain is also involved in cisplatin-induced injury to mouse cochlear cells. Calpain 1 expression did not increase with an increased dose of cisplatin, which suggests that calpain 1 did not play a leading role in cisplatin-induced mouse cochlear cell apoptosis.

At present, the mechanisms by which activated calpain further causes cell apoptosis remain poorly understood. It is generally thought that there are two pathways: (1) mitochondrial pathway: increased intracellular Ca^{2+} concentrations can activate calpain, which cleave the apoptosis-related protein Bid in the cytoplasm to activated Bid. Bid combined with the transmembrane protein Bak in mitochondria can induce changes in Bak conformation, forming pores. Thus, outer mitochondrial membrane permeability increases, and cytochrome C is released, finally resulting in cell apoptosis. (2) Proteolysis pathway: if calpain hyperactivity can induce degradation of cytoskeletal proteins, such as spectrin and calmodulin kinase, this can result in cell apoptosis. Whether calpain leads to cochlear cell apoptosis by the above-mentioned mechanisms requires further investigation.

In summary, cisplatin increased calpain 1 and calpain 2 expression in the mouse cochlea. With increased dose of cisplatin, calpain 2 expression gradually increased, in-

dicating that calpain 1 and calpain 2 mediated cisplatin-induced ototoxicity, and that calpain 2 plays a leading role in cisplatin-induced ototoxicity.

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

Experiments were performed at the Department of Physiology, Liaoning Medical University, China and Center for Scientific Experiments, Liaoning Medical University, China from July 2011 to March 2012.

Materials

Animals

A total of 65 healthy, adult, BALB/c mice of both genders, weighing 26–30 g, with normal auricle reflex, were supplied by the Experimental Animal Center, Dalian Medical University, China, Animal license No. SCXY(Liao) 2008-0002.

The protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by Ministry of Science and Technology of China[29].

Drugs

Cisplatin injection (specification 30 mg/6 mL, lot No. 100703; Jiangsu Ausen Pharmaceutical Joint-Stock Co., Ltd., Jiangsu Province, China) was diluted in sterile saline, and then intraperitoneally injected into animals in accordance with 0.02 mL/g[30].

Methods

Establishment of cisplatin-induced ototoxicity model

In accordance with a previous study[20], 2.5, 3.5, 4.5, 5.5 mg/kg cisplatin was intraperitoneally injected to the appropriate cisplatin groups, and an equal volume of saline was injected in the control group, once a day, for 5 consecutive days.

Auditory brainstem response measurement for auditory function in mice

The auditory brainstem response test was performed before administration and at 1 day following drug withdrawal in each group in the sound insulation room. The mice were intraperitoneally anesthetized with 1% (v/v) sodium pentobarbital 100 mg/kg. The positive electrode was subcutaneously placed at the center of calvaria, and the negative electrode was placed in the posterior and inferior auricle of the detected side, and the ground electrode was placed in the posterior and inferior auricle of the corresponding side. Using the auditory evoked potential-otoacoustic emission recording system (Intelligent Hearing Systems, Miami, FL, USA), the mice were stimulated with tone burst at frequencies of 8, 12 and 24 kHz, and the auditory brainstem response threshold (audibility threshold) was recorded. Each frequency of stimulation stacked 1 024 times. The intensity of sound stimulus began from 95 dB SPL, and gradually reduced at 5 dB. Auditory threshold was assessed taking I wave appearance (repeated at least twice) as the standard. Auditory brainstem response threshold shifts were calculated according to the difference of auditory threshold before and after administration under the same stimulation frequency[31].

Immunofluorescence staining for calpain 1 and calpain 2 expression in mouse cochlear cells

Four mice were randomly selected from each group, and sacrificed immediately after the final test of auditory brainstem response. The auditory vesicle was obtained. The fenestra cochleae and fenestra vesticuli were opened and slowly perfused with 0.1 mol/L PBS (pH 7.4) containing 4% (w/v) paraformaldehyde. The specimens were fixed in paraformaldehyde at 4°C for 24 hours, washed with PBS, incubated in 4% (v/v) ethylenediamine tetraacetic acid, decalcified at 4°C for 5 days, dehydrated through a graded alcohol series, permeabilized with xylene, embedded in paraffin, and then serially sliced into 5 μm sections.

Sections were conventionally dewaxed, hydrated, immersed in PBS for 5 minutes, followed by retrieval with a microwave for 10 minutes. After cooling in the air at room temperature, the specimens were blocked with 5% (w/v) bovine serum albumin (Sigma, St. Louis, MO, USA) at room temperature for 2 hours, washed with PBS, and treated with rabbit anti-calpain 1 and calpain 2 polyclonal antibody (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China; 1:100) at 4°C overnight. On the next day, sections were rewarmed for 1 hour, washed with PBS, incubated with fluorescein isothiocyanate (FITC; Sigma)- and tetramethyl rhodamine isothiocyanate (TRITC; Sigma)-labeled goat anti-rabbit IgG (1:200) at room temperature in the dark for 1 hour, washed with PBS, mounted with antifade mounting medium, and observed under a laser confocal microscope. PBS was used for negative control sections instead of primary antibody, and the remaining procedures were identical to the above-mentioned methods.

Image analysis

Eight sections were randomly selected from each group, and observed under a microscope (Olympus, Tokyo, Japan). Absorbance values of calpain 1- and calpain 2-positive products in the outer hair cells, spiral ganglion and stria vascularis in each group were measured using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA)[32]. The great absorbance value represented strong protein expression. Final results were expressed as the average absorbance value.

Western blot analysis for calpain 1 and calpain 2 expression in cochlear cells

The remaining nine mice from each group were sacrificed immediately after the final test for auditory brainstem response. The auditory vesicle was obtained. The bilateral cochlea was obtained in 10 mmol/L HEPES-NaOH buffer, and every six cochleae were considered as a specimen. The specimens were lysed in radioimmunoprecipitation assay buffer for 15 minutes, centrifuged at 4°C for 10 minutes at 12 000 r/min. Supernatant was obtained to measure protein content. After pouring glue, 40 μg of protein sample from each group was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel, and transferred to a membrane. The membranes were blocked in 2% (w/v) defatted milk powder at room temperature for 1 hour, washed in Tris-buffered saline with Tween, and then incubated in rabbit anti-calpain 1 and calpain 2 polyclonal antibody and rabbit anti-β-actin polyclonal antibody (Beijing Biosynthesis Biotechnology Co., Ltd.; 1:200) at 4°C overnight. On the next day, the membranes were washed with Tris-buffered saline with Tween and incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:5 000; Sigma) and then on a shaking bed at 4°C for 1 hour. After a wash in Tris-buffered saline with Tween, the enhanced chemiluminescence method was used to show image bands.

The EDAS290 gel image analysis system (Eastman Kodak, Rochester, NY, USA) was utilized to analyze electrophoresis strips. Absorbance values of each strip were read. β-actin served as an internal reference. The ratio of absorbance of calpain 1/β-actin and the ratio of absorbance of calpain-2/β-actin represented calpain-1 and calpain-2 protein expression levels, respectively.

Statistical analysis

Data were expressed as mean ± SD, and analyzed using SPSS 16.0 statistical analysis (SPSS, Chicago, IL, USA). Intergroup comparisons were performed using one-way analysis of variance and least significant difference *t*-test. A value of $P < 0.05$ was considered statistically significant.

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