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Gene transfection mediated by polyethyleneimine-polyethylene glycol nanocarrier prevents cisplatin-induced spiral ganglion cell damage

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Abstract

Polyethyleneimine-polyethylene glycol (PEI-PEG), a novel nanocarrier, has been used for transfection and gene therapy in a variety of cells. In our previous study, we successfully carried out PEI-PEG-mediated gene transfer in spiral ganglion cells. It remains unclear whether PEI-PEG could be used for gene therapy with X-linked inhibitor of apoptosis protein (XIAP) in the inner ear. In the present study, we performed PEI-PEG-mediated *XIAP* gene transfection in the cochlea of Sprague-Dawley rats, *via* scala tympani fenestration, before daily cisplatin injections. Auditory brainstem reflex tests demonstrated the protective effects of *XIAP* gene therapy on auditory function. Immunohistochemical staining revealed XIAP protein expression in the cytoplasm of cells in the spiral ganglion, the organ of Corti and the stria vascularis. Reverse transcription-PCR detected high levels of *XIAP* mRNA expression in the cochlea. The present findings suggest that PEI-PEG nanocarrier-mediated *XIAP* gene transfection results in XIAP expression in the cochlea, prevents damage to cochlear spiral ganglion cells, and protects hearing.

Key Words: *nerve regeneration; polyethyleneimine-polyethylene glycol; spiral ganglion cells; X-linked inhibitor of apoptosis protein; gene therapy; nanocarrier; cisplatin; neural regeneration; ototoxicity; cochlea*

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Introduction

Ototoxicity is a potentially serious side-effect of many drugs commonly used in the clinic, including aminoglycoside antibiotics, chemotherapy drugs and diuretics (Schacht et al., 2012). Indeed, drug-induced hearing loss is the primary cause of deafness in Chinese children, with 30,000 new cases diagnosed each year, of which more than 60% suffering from drug-induced deafness (Zheng et al., 2008). With increases in tumor incidence, hearing impairment due to chemotherapy, such as cisplatin, is increasingly common. Mammalian hair cells and spiral ganglion neurons have poor regeneration capacity, and damage to these cells can cause irreversible hearing loss. Therefore, there is an urgent need to protect spiral ganglion neurons, improve cell regeneration and promote functional reconstruction (Atkinson et al., 2012; Schacht et al., 2012).

Previous studies investigating the protection and repair of functional cells of the inner ear have proposed a number of viable treatments, including the use of various types of neurotrophic polypeptides, such as nerve growth factor, brain-derived neurotrophic factor (Liu et al., 2011), and neurotrophin-3 (Jiang et al., 2007; Atkinson et al., 2012; Rak et al., 2014); oxygen free radical scavenging and anti-apoptotic therapy (Rybak et al., 2000); and stem cell therapy (Nakagawa, 2014). Because of the high molecular weight and short half-life of neurotrophic factors and anti-apoptotic drugs, and the presence of the blood-labyrinth barrier (Boussif et al., 1995; Lin and Trune, 1997), direct administration into the inner ear is challenging. With systemic administration, it is difficult to maintain a therapeutically effective drug concentration in the inner ear. Local injection and fenestration surgery often require repeated treatments, limiting their application. Delivering therapeutic genes into the inner ear would be a better solution, with the potential to maintain lifelong expression of the gene. Anatomical and immunological studies of the inner ear have shown that the cochlea is a suitable target organ for *in vivo* gene therapy (Xia and Yin, 2013; Nakagawa, 2014), with excellent therapeutic prospects, but a suitable gene vector remains to be found. Vectors can be viral or non-viral. Viral vector-mediated gene therapy in the inner ear faces safety issues and immune rejection (Sun and Wu, 2013; Xia and Yin, 2013). However, traditional non-viral vector liposomes also have some disadvantages such as poor expression efficiency and duration (Sun and Wu, 2013). The transfection efficiency of viral vector-mediated genes into spiral ganglion cells is

more than 70% (Husseman and Raphael, 2009; Okada et al., 2012). Transfection efficiency is notably lower in non-viral vectors (approximately 10%) than viral vectors, and is a major restricting factor in the application of non-viral vectors in gene therapy of the inner ear (Pyykkö et al., 2011). Therefore, it is necessary to find a non-viral vector with high efficiency and good safety.

Polyethyleneimine-polyethylene glycol (PEI-PEG), a novel nanocarrier, has been successfully used for gene transfection in a variety of cells. However, the application of PEI-PEG to gene therapy for the inner ear has, to our knowledge, not yet been investigated. In our previous study (Chen and Xu, 2014), the non-viral vectors PEI-PEG and lipofectamine 2000 were transfected into *in vitro* cultured spiral ganglion cells. PEI-PEG transfected spiral ganglion cells with an efficiency of 16.5%, higher than that of lipofectamine 2000 (11.2%). Moreover, the cytotoxicity to spiral ganglion cells was lower with the vector system than with lipofectamine 2000. After transfection, the morphology of spiral ganglion cells was not noticeably abnormal. These results indicate that PEI-PEG performs better than conventional cationic liposomes in several measures, in studies of spiral ganglion cells. X-linked inhibitor of apoptosis protein (XIAP) is frequently used in anti-apoptotic studies (Wang et al., 2011; Wu et al., 2014). In the present study, we sought to determine whether PEI-PEG would mediate *XIAP* gene expression in the inner ear, and if so, in which cell types and in which parts of the cochlea. Furthermore, we investigated whether PEI-PEG-mediated *XIAP* gene expression would protect the cochlea against damage by cisplatin, to assess the potential of PEI-PEG in gene therapy of the inner ear.

Materials and Methods

Preparation of plasmid DNA (pDNA)

Plasmid pIRES2-EGFP/XIAP (constructed in-house) was amplified by *E. coli*, extracted, and purified using a plasmid extraction kit (Qiagen, Dusseldorf, Germany). The content of prepared plasmids was measured at 260 and 280 nm using an ultraviolet spectrometer (Youke, Shanghai, China). Electrophoresis was performed on 1.0% agarose gel at 100 V for 40 minutes. DNA purity was in accordance with the requirements of the experiment, according to the ratio A_{260}/A_{280}. Samples were stored at −20°C.

Preparation of PEI-PEG/XIAP composite

Preliminary *in vitro* tests showed that good transfection efficiency was achieved in spiral ganglion cells when the molar ratio of amino groups in the polymer to DNA phosphate groups was equal to 15. Therefore, polymer to DNA phosphate groups = 15 was used in the present study. pIRES2-EGFP/XIAP plasmid (1 μg) was diluted in 50 μL of Dulbecco's modified Eagle's medium and gently shaken. The corresponding amount of PEI-PEG, 4.6 at polymer to DNA phosphate groups = 15 (constructed in-house; Chen and Xu, 2014), was added to an Eppendorf tube containing 50 μL of Dulbecco's modified Eagle's medium. The composite was obtained after 5 minutes of shaking at room temperature.

Experimental animals

A total of 38 clean-grade male and female Sprague-Dawley rats, aged 2–3 months and weighing 250–300 g, were provided by the Guangdong Medical Experimental Animal Center in China (animal license No. SCXK (Yue) 2013-0002). The rats were housed in a dark, quiet environment at 18–26°C and at 40–70% relative humidity. The protocols were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Guangzhou Medical University, China.

Upon examination of the ears, the external auditory canal of the rats was smooth, the tympanic membrane was normal, and pinna response was normal. All rats underwent auditory brainstem response (ABR) audiometry. Two rats with ABR thresholds above a decibel sound pressure level (dB SPL) of 40 dB were excluded. The remaining rats were randomly assigned to three groups (n = 12 per group): D-Hanks, XIAP, and control. Following 2 weeks of injection in the cochlea scala tympani in the three groups, drilling of the scala tympani was performed. A single dose of D-Hanks' solution was injected into cochleas in D-Hanks group and PEI-PEG/XIAP composite was injected into the XIAP group. Nothing was injected into the control animals. 2 weeks after cochlea injection, 3 mg/kg cisplatin (Sigma-Aldrich, St. Louis, MO, USA) was daily intraperitoneally injected for 6 days.

Drilling of the scala tympani and administration of composite component

In the XIAP group, rats were anesthetized with 1% sodium pentobarbital (45 mg/kg, i.p.), placed on the operating table, and the skin around the left ear was sterilized. The auditory vesicle of the left ear was exposed under a binocular dissecting microscope (Carl Zeiss, Oberkochen, Germany). A hole (0.1–0.2 mm in diameter) was made in the outer wall of the scala tympani in the cochlear substrate, using an electric surgical drill (Stryker, Kalamazoo, MI, USA) with a diamond head. PEI-PEG/XIAP composite (10 μL, 0.01 g/L was slowly injected over 5 minutes using a microsyringe (Hamilton, Bonaduz, Switzerland) to a maximum depth of 0.2 mm. After injection, the needle was maintained in place for a further 2 minutes to prevent liquid outflow. Upon removal of the needle, a small amount of sarcoplasm was used to block the drilled hole, and the wound was sutured. After the surgery, penicillin (200,000 U/d) was injected intramuscularly for 3 consecutive days to prevent infection. The rats in the D-Hanks group underwent the same procedure but 10 μL D-Hanks' solution was injected instead of PEI-PEG/XIAP composite. The rats in the control group underwent anesthesia and drilling of the scala tympani but were not injected.

Detection of ABR thresholds

The rats were anesthetized with 1% sodium pentobarbital (45 mg/kg, i.p.). Reference electrodes (Fude Medical Electronics Co., Ltd., Xi'an, China) were placed in the auditory

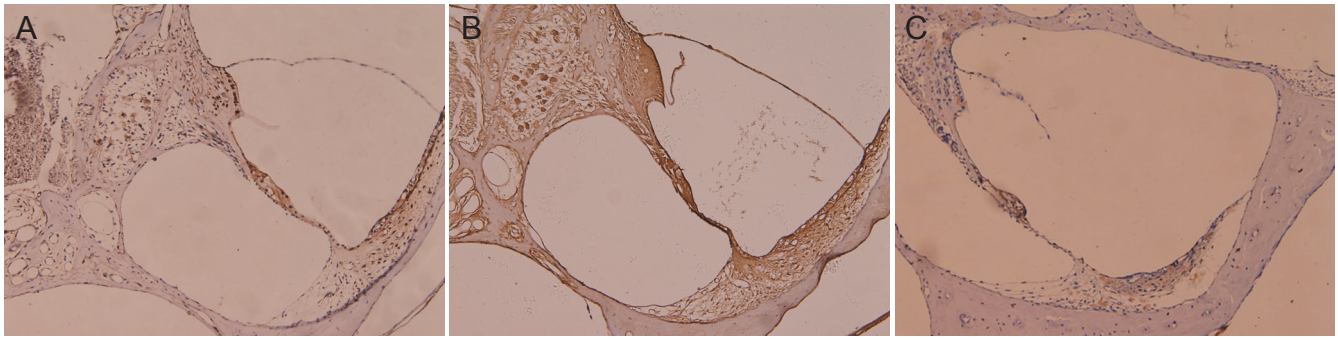

Figure 2 XIAP immunoreactivity in the cochlea after PEI-PEG-mediated *XIAP* gene transfection into the cochlea of rats with cisplatin-induced ototoxicity (immunohistochemical staining, × 100).
XIAP immunoreactivity (brown staining) was visible in the spiral ganglion cells, organ of Corti and stria vascularis of the cochlea in the D-Hanks (A), XIAP (B) and control (C) groups. XIAP immunoreactivity was the strongest in the XIAP group. XIAP: X-linked inhibitor of apoptosis protein; PEI-PEG: polyethyleneimine-polyethylene glycol.

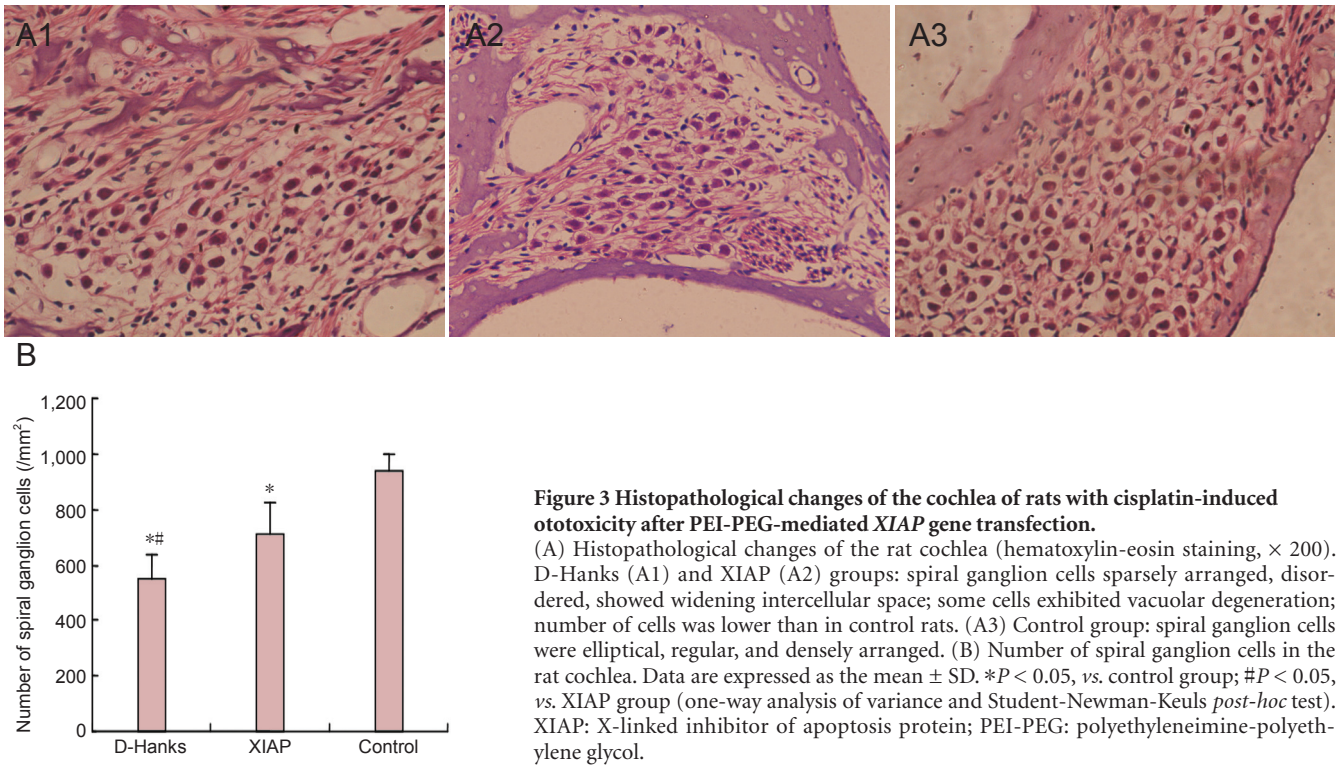

Figure 3 Histopathological changes of the cochlea of rats with cisplatin-induced ototoxicity after PEI-PEG-mediated *XIAP* gene transfection.
(A) Histopathological changes of the rat cochlea (hematoxylin-eosin staining, × 200). D-Hanks (A1) and XIAP (A2) groups: spiral ganglion cells sparsely arranged, disordered, showed widening intercellular space; some cells exhibited vacuolar degeneration; number of cells was lower than in control rats. (A3) Control group: spiral ganglion cells were elliptical, regular, and densely arranged. (B) Number of spiral ganglion cells in the rat cochlea. Data are expressed as the mean ± SD. $*P < 0.05$, *vs.* control group; $\#P < 0.05$, *vs.* XIAP group (one-way analysis of variance and Student-Newman-Keuls *post-hoc* test). XIAP: X-linked inhibitor of apoptosis protein; PEI-PEG: polyethyleneimine-polyethylene glycol.

vesicle behind the ipsilateral (left) ear. Ground poles were placed in the papilla or nasal tip behind the contralateral (right) ear. Recording electrodes were placed in the midpoint of the bilateral external auditory canal on the scalp. Low-intensity white noise (< 20 dB SPL) was applied to the contralateral side. Short sound (click) stimulation was used at a frequency of 4,000 Hz, filter range 100–3,000 Hz, 1,000 times, at a repetition rate of 10 times/second and at an intensity of 5–100 dB SPL. ABR was measured and hearing thresholds were recorded 24 hours before cochlear injection, 24 hours after cochlear injection, and 24 hours after the final injection of cisplatin.

Preparation of cochlear sections

After the final ABR measurement, all rats were intraperitoneally anesthetized with 1% pentobarbital (50 mg/kg). The sternum was cut open to expose the heart, which was perfused with 4°C physiological saline and 4°C fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.4). The rats were rapidly decapitated, and the left auditory vesicle was obtained. The cochlea was dissected in an ice bath, and postfixed in the same fixative at 4°C overnight. Samples were decalcified in 10% sodium ethylenediaminetetraacetic acid solution (pH 7.4) for 14 days, embedded in paraffin, and sliced into 5-μm-thick sections parallel to the cochlear axis.

Hematoxylin-eosin staining for spiral ganglion cell counts in the rat cochlea

Paraffin-embedded sections of rat cochlea were dewaxed with xylene, dehydrated through a graded alcohol series, stained with hematoxylin and eosin for 5 minutes, washed with running water and decolorated with 1% hydrochloric acid in ethanol for a few seconds. 0.5% ammonium hydroxide was then added to stain the hematoxylin. After rinsing under running tap water for 1 hour, samples were placed in distilled water, dehydrated with 70 and 90% alcohol (10 minutes each), and stained with ethanol eosin for 2–3 minutes. Finally, the sections were dehydrated with pure ethanol, permeabilized in xylene, and mounted with neutral resin. From the serial sections proximal to the cochlear axis, one section was obtained from every contiguous five, resulting in a total of six sections from each rat. The density of spiral ganglion cells in Rosenthal's canal was quantified with Image Pro Plus 6.0 image analysis system (MediaCybernetics, Bethesda, MD, USA). Cell density (n/mm^2) was equal to the number of spiral ganglion cells/section area of Rosenthal's canal, and the mean of all sections was calculated.

Immunohistochemical staining for XIAP expression in the rat cochlea

Sections of rat cochlea were treated with 3% H_2O_2 for 20 minutes to inactivate endogenous peroxidase, washed with distilled water, and antigen retrieval was performed using a microwave. Sections were then incubated with goat anti-XIAP polyclonal antibody (1:200; Boster, Wuhan, Hubei Province, China) at 4°C overnight, biotinylated rabbit anti-goat IgG (1:300; Boster) secondary antibody at 37°C for 30 minutes, and streptavidin-biotin complex at 37°C for 30 minutes. After washing, sections were visualized with 3,3′-diaminobenzidine and mounted with neutral resin. PBS, instead of primary antibody, was used as a blank control. Samples were observed under a light microscope (CX31; Olympus, Tokyo, Japan).

Reverse transcription for *XIAP* mRNA expression in the rat cochlea

The rats were sacrificed by overdose of anesthesia, their fur was soaked in 70% ethanol, and they were decapitated. Auditory vesicles were removed in an ice bath to obtain the inner ears. Both ears of each animal were considered a metering unit. Total RNA was extracted. Reverse transcription-PCR was performed using a one-step reverse transcription kit (Qiagen, Dusseldorf, Germany), according to the manufacturer's instructions. β-Actin served as the internal reference. PCR conditions were as follows: reverse transcription at 50°C for 30 minutes; initial activation at 95°C for 15 minutes, 35 cycles of denaturation at 94°C for 50 seconds, annealing at 60°C for 50 seconds, extension at 72°C for 60 seconds; extension at 72°C for 10 minutes. XIAP primer sequences: upstream primer, 5′-AGG AAC CCT GCC ATG TAT TG-3′; downstream primer, 5′-TGT TGT TCC CAA GGG TCT TC-3′. β-Actin primer sequence: upstream primer, 5′-CGC ACC ACT GGC ATT GTC AT-3′; downstream primer, 5′-TIC TCC TTG ATG TCA CGC AC-3′. The product length was 494 bp. PCR products were electrophoresed on a 1.5% agarose gel. A Gel Imaging Analysis System (Tanon2020; Tanon, Shanghai, China) was employed to measure optical density. The expression of XIAP mRNA was calculated as the average optical density ratio of the target band to the internal reference band.

Statistical analysis

Measurement data were expressed as the mean ± SD and analyzed using SPSS 13.0 Statistical Software (SPSS Inc., Chicago, IL, USA). Two-way repeated measures analysis of variance was used to compare ABR thresholds. Intergroup differences of spiral ganglion cell number and *XIAP* mRNA expression were compared by one-way analysis of variance, followed by the Student-Newman-Keuls test. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of PEI-PEG-mediated *XIAP* gene transfection on ABR thresholds in rats with cisplatin-induced ototoxicity

Baseline ABR thresholds were similar between rats from each group ($P > 0.05$), meeting the test requirements. Cochlear injection did not produce a significant difference in ABR thresholds ($P > 0.05$). However, after intraperitoneal injection of cisplatin for 6 days, ABR thresholds were significantly higher in the D-Hanks and XIAP groups than in the same groups after cochlear injection ($P < 0.05$), and also higher than in the control group ($P < 0.05$). However, ABR thresholds were lower in the XIAP group than in the D-Hanks group after the injection of cisplatin ($P < 0.05$), suggesting that *XIAP* gene therapy reduced the damage of cisplatin to hearing (**Figure 1**).

Effect of PEI-PEG-mediated *XIAP* gene transfection on cochlear tissue in rats with cisplatin-induced ototoxicity

Immunohistochemical staining revealed that a weak immunoreactivity of XIAP in the cytoplasm of cells in the spiral ganglion, organ of Corti, and stria vascularis border of the cochlea in the D-Hanks and control groups. XIAP immunoreactivity was strongest in rats in the XIAP group (**Figure 2**).

Hematoxylin-eosin staining revealed the complete structure of the cochlea. Spiral ganglion cells, organ of Corti and stria vascularis were prominent in the three groups. In the control group, spiral ganglion cells were elliptical, regular and densely arranged, with uniform staining in Rosenthal's canal of the cochlea. In the D-Hanks and XIAP groups, spiral ganglion cells were sparsely arranged and disordered, the intercellular spaces were wider than in the control group, cytoplasm was lightly stained, and some cells exhibited vacuolar degeneration. There were fewer spiral ganglion cells in the experimental groups after treatment with cisplatin; spiral ganglion cell number, ranked in descending order, was as follows: control group > XIAP group > D-Hanks group ($P < 0.05$; **Figure 3**).

Effect of PEI-PEG-mediated *XIAP* gene transfection on *XIAP* mRNA expression in cochlear tissue of rats with cisplatin-induced ototoxicity

Reverse transcription-PCR showed that *XIAP* mRNA expression was greater in the XIAP group than in the other two groups ($P < 0.05$; **Figure 4**), with no difference in expression between the D-Hanks and control groups ($P > 0.05$).

Discussion

Cisplatin is widely used as a first-line anti-cancer drug. It has a strong broad-spectrum effect by inhibiting DNA replication in cancer cells. However, it has severe side effects, which include gastrointestinal reactions, bone marrow suppression, and kidney and inner ear damage. Of these, the side effects in the kidneys and the inner ear are the most serious (Dille et al., 2012). A large proportion (50–100%) of patients undergoing chemotherapy with cisplatin experience a degree of hearing loss (Rybak et al., 2007; Dille et al., 2012). Cisplatin damage to hearing has a complex multifactorial mechanism. Previous studies demonstrated that cisplatin ototoxicity was associated with drug-induced oxygen free radical damage, with oxidative stress ultimately resulting in the apoptosis of inner ear hair cells and spiral ganglion cells *via* a series of cascades (Deavall et al., 2012; Pereira et al., 2012; Fu et al., 2013). Human inner ear hair cells and spiral ganglion cells are nonrenewable, so it is important to prevent drug ototoxicity.

Hearing loss induced by noise, aging or ototoxicity is correlated with cochlear hair cell and spiral ganglion cell death. The final pathway is cell apoptosis. Anti-apoptotic therapy is therefore effective in ameliorating the damage of these harmful factors to the inner ear. Anti-apoptotic therapy using a caspase antagonist reduces age-related cochlear neuronal degeneration (Ruan et al., 2014), diminishes noise damage (Wang et al., 2011), and reduces cisplatin ototoxicity (Cooper et al., 2006; Chan et al., 2007; Waissbluth et al., 2012). Cooper et al. (2006) used adeno-associated virus vector to transfect XIAP into mouse cochlea, before injecting cisplatin intraperitoneally after 2 months of target gene expression. *XIAP* gene therapy was found to protect hearing and elevate the survival rate of cochlear outer hair cells by 45%. Chan et al. (2007) also used an adeno-associated viral vector to transfect *XIAP* and successfully prevent cisplatin ototoxicity, resulting in a 68% reduction in hearing impairment, and a 50% reduction in cochlear hair cell damage. Together, these results highlight XIAP as a promising new treatment for cisplatin ototoxicity. However, while several studies have examined viral vector *XIAP* gene transfection in the cochlea, there have been no reports to date of similar studies with non-viral vectors. Boussif et al. (1995) first reported the use of polyethylenimine as a gene vector; however, the success of polyethylenimine alone is limited (Choosakoonkriang et al., 2003). PEI-PEG has been successfully applied to a variety of cell transfection and gene therapies (Okada et al., 2012; Liu et al., 2013), but gene therapy in the inner ear has not, to our knowledge, been reported. Our previous study confirmed that PEI-PEG could transfect cultured spiral ganglion cells *in vitro*, and transfection efficiency and cytotoxicity were better than that obtained with conventional cationic liposomes (Chen and Xu, 2014). In the present study, we built on those findings and successfully performed PEI-PEG transfection of the *XIAP* gene into the cochlea, resulting in elevated local expression of *XIAP* mRNA. Immunohistochemical staining revealed that *XIAP* gene expression was enhanced in the cochlear axis, organ of Corti and stria vascularis, and that it blocked cisplatin ototoxicity, protected spiral ganglion cells, and prevented hearing impairment.

Before inner ear gene transfer is used as a method to treat sensorineural deafness, it is necessary to establish whether the surgery and injection risk damage to the structure and function of the inner ear. Numerous studies have addressed the effects of various methods of gene transfer on the structure and function of the inner ear, including injection through the round window membrane (Wang et al., 2012), scala tympani fenestration surgery (Bogaerts et al., 2008), and semicircular canal fenestration surgery (Gassner et al., 2012). Scala tympani fenestration for viral and liposome vector injection had a small effect on cochlear function and structure (Praetorius et al., 2003). Stöver et al. (1999) injected adenovirus vector into the inner ear by scala tympani fenestration and round window membrane injection; 5 days later, ABR detection showed that the surgery had no notable effect on hearing. Gassner et al. (2012) injected adenovirus vector carrying green fluorescent protein into the cochlea by semicircular canal fenestration surgery, with a slight influence on hearing. Fu et al. (2008) transplanted rat embryonic neural stem cells into the cochlea of naïve rats through cochlear fenestration using recombinant adenovirus carrying green fluorescent protein (Ad-GFP). ABR detection demonstrated that no significant differences in auditory threshold were detected in rats before or after the surgery. Scanning electron microscopy revealed that outer hair cells in the cochlea showed a scattered loss of less than 1%, not significantly different from the untreated group, further demonstrating that transplantation of neural stem cells infected with Ad-GFP into the normal rat cochlea does not affect auditory threshold or cochlear morphology. Our previous study suggested that ABR threshold was temporarily decreased (< 20 dB) but recovered to normal within 28 days after bone marrow mesenchymal stem cells were transplanted in the cochlea through fenestration of the scala tympani (Chen et al., 2010). In the present nanocarrier experiment, we also used scala tympani fenestration, and found no significant difference between ABR thresholds before and after cochlear injection, indicating that cochlear injection did not affect rat ABR thresholds, and further demonstrating that scala tympani fenestration is a safe method by which to administer the PEI-PEG nanocarrier complex.

In summary, we have demonstrated successful PEI-PEG-delivery of XIAP into cochlear tissue cells, which resulted in the prevention of cisplatin ototoxicity. Our results provide a promising new option in gene therapy of sensorineural

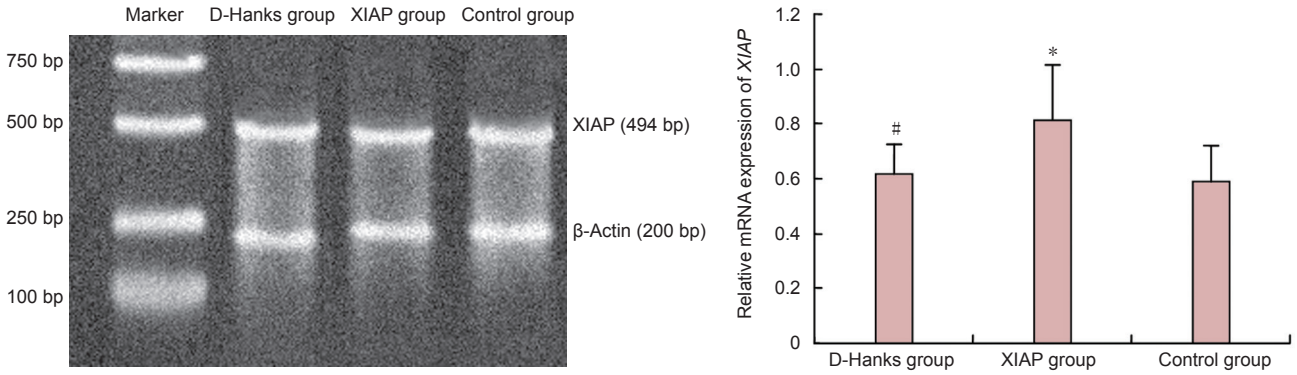

Figure 4 *XIAP* mRNA expression in cochlear tissue of rats with cisplatin-induced ototoxicity after PEI-PEG-mediated *XIAP* gene transfection.
Data are expressed as a ratio of optical density of *XIAP* mRNA to *β-actin* mRNA (mean ± SD). ∗$P < 0.05$, *vs.* control group; #$P < 0.05$, *vs.* XIAP group (one-way analysis of variance and Student-Newman-Keuls *post-hoc* test).

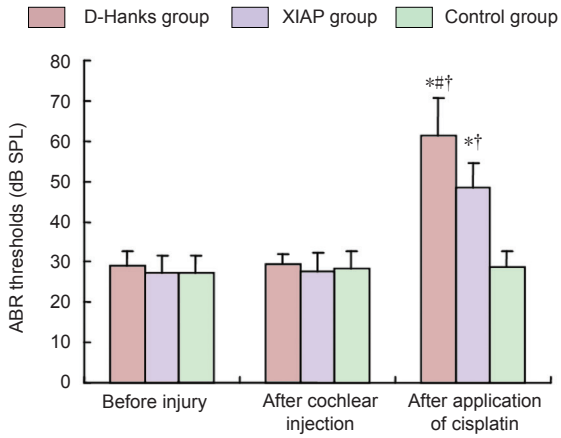

Figure 1 Changes in ABR thresholds after PEI-PEG-mediated *XIAP* gene transfection into the cochlea of rats with cisplatin-induced ototoxicity.
Data are expressed as the mean ± SD. ∗$P < 0.05$, *vs.* control group; #$P < 0.05$, *vs.* XIAP group; †$P < 0.05$, *vs.* after cochlear injection (one-way analysis of variance and Student-Newman-Keuls *post-hoc* test). ABR: Auditory brainstem response; XIAP: X-linked inhibitor of apoptosis protein; PEI-PEG: polyethyleneimine-polyethylene glycol.

deafness and highlight the possibilities for nanomaterial-mediated cochlear-targeted gene therapy.

Author contributions: *GGC participated in study design and experiment implementation, wrote the paper and was in charge of paper authorization. MM conducted the experiment of tissue sections and immunohistochemistry. LZQ participated in audiology detection. QML ensured the integrity of the data and data statistics. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

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