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ORIGINAL ARTICLE

Ameliorative effect of purple bamboo salt-pharmaceutical acupuncture on cisplatin-induced ototoxicity

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Abstract

Conclusion: Our findings demonstrated that purple bamboo salt (PBS)-pharmaceutical acupuncture has an ameliorative effect on cisplatin-induced ototoxicity. *Objectives:* We have previously reported that PBS exhibited anti-allergic and anti-inflammatory actions *in vitro* and *in vivo*. Pharmaceutical acupuncture is a traditional oriental therapeutic technique that combines acupuncture with herbal treatment. The aim of this study was to investigate the protective effect and mechanism of PBS-pharmaceutical acupuncture against cisplatin-induced ototoxicity in the auditory cell line, HEI-OC1, and *in vivo*. *Methods:* The ELISA method, a caspase-3 assay, an MTT assay, Western blot analysis, and a luciferase assay were utilized to investigate the effect of PBS *in vivo* and *in vitro*. *Results:* When it was acupunctured at the Ermen acupoint (triple energizer meridian 21) after an administration of cisplatin, PBS-pharmaceutical acupuncture significantly suppressed interleukin (IL)-6 production and caspase-3 activation induced by cisplatin in the cochlea. In addition, PBS significantly inhibited cisplatin-induced apoptosis and IL-6 production in HEI-OC1 cells. PBS also suppressed cytochrome *c* release and caspase-3 activation, and it inhibited extracellular signal-related kinase and nuclear factor-κB activation in HEI-OC1 cells.

Keywords: *Caspase-3, interleukin-6, extracellular signal-related kinase, nuclear factor-κB*

Introduction

Cisplatin is a highly effective and widely used anticancer agent [1]. It has been shown to induce auditory sensory cell apoptosis [2]. Cisplatin toxicity is also associated with an increase in caspase-3, caspase-8, and caspase-9 activity, cytochrome *c* (cyt *c*) release, apoptosis-inducing factor (AIF) translocation, reactive oxygen species (ROS) generation, and nuclear factor-κB (NF-κB) activation [2–4]. Hearing loss is induced by cisplatin through the activation of ERK [5].

Recently, it was reported that the inner ear has the capacity to generate an active immune response, resulting in hearing loss in some individuals when the immune response in the inner ear is aggravated [6]. Although the immune function in the inner ear is a very important factor in the defense against infectious diseases such as labyrinthitis, immune-related inflammatory responses also cause damage to the delicate tissues of inner ear compartments and can lead to cochlear degeneration and permanent hearing loss [7]. Proinflammatory cytokine is a deteriorating factor associated with cochlear inflammation. It induces secondary inflammatory responses, including leukocyte infiltration and scar formation in injured areas [8]. In particular, IL-6 expression plays an important role in the inflammatory reactions of auditory cells [9]. Other research has reported that cisplatin causes ototoxicity through the apoptotic death of outer hair cells (OHCs) and supporting cells in the organ of Corti accompanied by the secretion and expression of proinflammatory cytokines [10].

Purple bamboo salt (PBS) is known to have various therapeutic effects on diseases such as inflammations

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and allergy [11,12]. However, the protective effect of PBS on hearing loss is not completely understood.

Acupuncture is the insertion of needles into specific cutaneous locations of the body, known as acupoints, for the treatment or prevention of disease [13,14]. Pharmaceutical acupuncture is a traditional oriental therapeutic technique that combines acupuncture with herbal treatment. This technique involves injecting a herbal extract into certain acupuncture points, according to oriental medical theory [15].

Ermen (TB-21) is used mostly as a local point for ear problems (mostly tinnitus and deafness) especially if derived from rising of Liver-Yang [16]. The overall aim of this study was to gain further insight into the protective effect and mechanism of PBS-pharmaceutical acupuncture against cisplatin-induced ototoxicity in the auditory cell line, HEI-OC1, and to assess its effects *in vivo*.

Material and Methods

Reagents

Fetal bovine serum (FBS) and high-glucose Dulbecco's Eagle's medium (DMEM) were purchased from GIBCO BRL (Grand Island, NY, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT), dimethyl sulfoxide (DMSO), 2′-AZINO-bis (3-ethylbenzithiazoline-6-sulfonic acid (ABTS), and other reagents were obtained from Sigma (St Louis, MO, USA). The caspase-3, cyt *c*, NF-κB (p65), and phosphorylate (p)-ERK antibodies (Abs) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The caspase assay kits and mouse IL-6 Abs were supplied by R&D Systems Inc. (Minneapolis, MN, USA).

Preparation of PBS

PBS was provided by Tae-sung Food Inc. (Jeonbuk, Republic of Korea). It contains mostly sodium chloride (about 91.7% of PBS) along with potassium, calcium, iron, copper, manganese, sulfur, zinc, and magnesium in elementary quantities. Powdered PBS was dissolved in distilled water to reach appropriate concentrations filtered through a 0.22 μm syringe filter. It was kept at 4°C.

Animal experiments

All the experiments were performed using male C57BL/6 mice (weighing 19–20 g), which were housed in stainless steel cages in a temperature-controlled room (25°C) equipped to maintain a 12 h light: dark cycle. The animals were treated humanely and with regard for alleviation of suffering. The animals were distributed randomly into three groups containing six animals each, and then fed normal food. Group ($n = 6$) was used as the control. Group ($n = 6$) were administered cisplatin (2 mg/kg) intraperitoneally for 14 consecutive days and at the same time were injected with phosphate-buffered saline (PS) at the Ermen (TB 21). Group ($n = 6$) received cisplatin injections on 4 consecutive days and at the same time were injected with PBS-pharmaceutical acupuncture (20 mg/kg) at the Ermen (TB 21). Acupuncture was performed by acupuncturist Dr Myung, who had been trained for 4 years at the Acupuncture and Meridian Science Research Center; 0.25 × 30 mm soft, spring handle needles (DONG BANG Acupuncture Inc., Chungnam, Korea) were used in same point. The acupuncture provider followed STRICTA guidelines [17]. There was no specific needle stimulation technique and specific response obtained. All of the animals were anesthetized by intraperitoneal administration of 100 mg/kg chloral hydrate (Merck, Germany). The hearts were immediately cannulated and were perfused in a non-recirculating servo pump system (EMKA Technologies, Paris, France) at a constant pressure (100 mmHg) with in PS. The whole temporal bone was removed. The cochlea was dissected out and homogenized. All protocols were approved by the institutional animal care and use committee of Kyung Hee University.

Cell culture

The HEI-OC1 cell line was a gift from Dr Federico Kalinec (House Ear Institute, CA, USA). The establishment of an immortal cell line was facilitated using a transgenic mouse, Immortomouse™ (Charles River Laboratories, Wilmington, MA, USA), which harbors a temperature-sensitive mutant of the SV40 large T antigen gene under the control of an interferon-γ-inducible promoter element. The cochlear half turns from the Immortomice at postnatal day 7 were cultured on uncoated plastic culture dishes under permissive conditions (33°C) in antibiotic-free DMEM. The cochlear explants were placed under non-permissive conditions (39°C) at different times and allowed to differentiate for up to 180 days. The explants and cells growing in the tissue regions formerly associated with the organ of Corti were isolated by lifting them with a micropipette after incubation for 2–5 min with trypsin-EDTA. A cell line, HEI-OC1, was cloned in the absence of antibiotics, using a

limiting dilution method, and characterized. The cells were maintained in a DMEM medium with 10% fetal bovine serum at 33°C under 5% CO_2 in air. The HEI-OC1 cells express several molecular markers that are characteristic of the organ of Corti sensory cells: thyroid hormone, brain-drived neurotrophic factor, calbindin, calmodulin, Connexin 26, Math 1, Myosin 7a, organ of Corti protein 2, tyrosine kinase receptor B and C, platelet-derived growth factor receptor, and prestin. In addition, the HEI-OC1 cells are extremely sensitive to ototoxic drugs.

Assay of caspase-3 activity

The enzymic activity of caspase-3 was assayed using a caspase colorimetric assay kit (R&D Systems) as per the manufacturer's protocol. Briefly, the cells were either untreated or treated with PBS, and then lysed in a lysis buffer. The lysed cells were centrifuged at 14 000 *g* for 5 min. The protein supernatant was incubated with 50 µl of a reaction buffer and 5 µl of a caspase substrate at 37°C for 2 h. The absorbance was measured using a plate reader at a wavelength of 405 nm. Equal amounts of the total protein from each lysate were quantified using a bicinchoninic acid protein quantification kit (Sigma).

Cytokines assay

The levels of IL-6 production were measured using a modification of the enzyme-linked immunosorbent assay (ELISA) as described previously [9]; 96-well plates were coated with 100 µl aliquots of anti-mouse IL-6 monoclonal Abs at 1.0 µg/ml in PS at pH 7.4 and incubated overnight at 4°C. Additional washes, 100 µl of the cell medium or the IL-6 standards were added and incubated at 37°C for 2 h. The wells were then washed and 0.2 µg/ml of biotinylated anti-mouse IL-6 was added and they were incubated again at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 30 min at 37°C. The wells were washed again and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using the recombinant mouse IL-6 in serial dilutions.

MTT assay

Cells were seeded and exposed to cisplatin for 48 h. The cell survival fraction was determined with the MTT dye-reduction assay. In brief, after incubation with cisplatin, MTT solution was added. Plates were further incubated for 4 h at 37°C, and the formazan crystals formed were centrifuged and the pellets dissolved by the addition of DMSO. Absorption was measured by spectrometer at 540 nm.

Preparation of cytoplasmic and nuclear extract

Nuclear and cytoplasmic extracts were prepared as described previously [9]. Briefly, after cell activation for the times indicated cells were washed with ice-cold PS and resuspended in 60 µl of buffer A (10 mM Hepes/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 µl of 10% Nonidet P-40, and centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was collected and used as the cytoplasmic extract. The nuclear pellet was resuspended in 40 µl of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), left on ice for 20 min, and inverted. The nuclear debris was then spun down at 15 000 *g* for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen, and stored at −70°C until analysis was carried out.

Preparation of cytosol fraction

After treatment, cells were harvested by centrifugation at 600 *g* for 10 min at 4°C. Cytosolic fractions were obtained by selective plasma membrane permeabilization with digitonin. Briefly, 1×10^6 cells were lysed for 1–2 min in lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, 0.1 mM PMSF, and 350 mg/ml digitonin). The lysates were centrifuged at 12 000 *g* for 1 min at 4°C, and the supernatant was collected. Equal amounts of protein were separated on a 15% SDS polyacrylamide gel and transblotted onto polyvinylidene difluoride-nitrocellulose filters. Specific cyt *c* bands were detected using anti- cyt *c* antibody.

Western blotting

For an analysis of the levels of caspase-3, p-ERK, and NF-κB, the cells were rinsed with ice-cold PS, and lysed with lysis buffer (1% Triton, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholate in PS). The supernatant was then mixed with an equal volume of 2× SDS

sample buffer, boiled for 5 min, and then separated on 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to nylon membranes by electrophoretic transfer. The membranes were blocked for 2 h in 5% skimmed milk, rinsed, incubated overnight at 4°C with the primary antibodies, and washed in PS/0.5% Tween 20 to remove the excess primary Abs. The membranes were then incubated for 1 h with the HRP-conjugated secondary Abs (against mouse, goat, or rabbit). After three washes in PS/0.5% Tween 20, the protein bands were visualized using an enhanced chemiluminescence assay (Amersham, Piscataway, NJ, USA) as per the manufacturer's instructions.

Transient transfection and luciferase assay

NF-κB luciferase reporter gene constructs (pNF-kB-LUC, plasmid containing NF-κB binding site; STANTAGEN, Grand Island, NY, USA) were transfected into HEI-OC1 cells using transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for each well as per the manufacturer's protocol. After 24 h, the culture medium was changed to fresh culture and the cells were incubated with cisplatin. Cells were harvested after stimulation for 24 h and washed in cold PS buffer. After lysis, luciferase activity was measured using the Dual-Light® System luciferase assay (Promega, Madison, WI, USA) as per the manufacturer's protocol. The luminometer used was Victor light from Perkin Elmer (USA). The results for firefly luciferase activity were normalized to the renilla luciferase activity. All experiments were performed in triplicate and were repeated three times.

Statistical analysis

The results give a summary of the data from at least three experiments and are presented as the mean ± SEM. Statistical evaluation of the results was performed by ANOVA using a Tukey post hoc test and a p value < 0.05 was considered significant.

Results

Effect of PBS-pharmaceutical acupuncture on cisplatin-induced IL-6 production and caspase-3 activation in mice

We acupunctured PBS at the Ermen acupoint (TB 21) with administration of cisplatin for 14 days. Subsequently, the cochlea was isolated and the isolated tissue was homogenized. First, to investigate whether PBS regulates the IL-6 level induced by cisplatin, we performed the ELISA method. As shown in Fig. 1A, the IL-6 level increased in the cisplatin group, but this enhanced level was reduced in the PBS-pharmaceutical acupuncture group.

Caspase-3 is known to play important roles in initiating and executing apoptosis [2]. The next step was therefore to investigate whether PBS affects the caspase-3 activity in the organ of Corti in mice. Western blot analysis and caspase-3 assay were performed. The results showed that the level of pro-

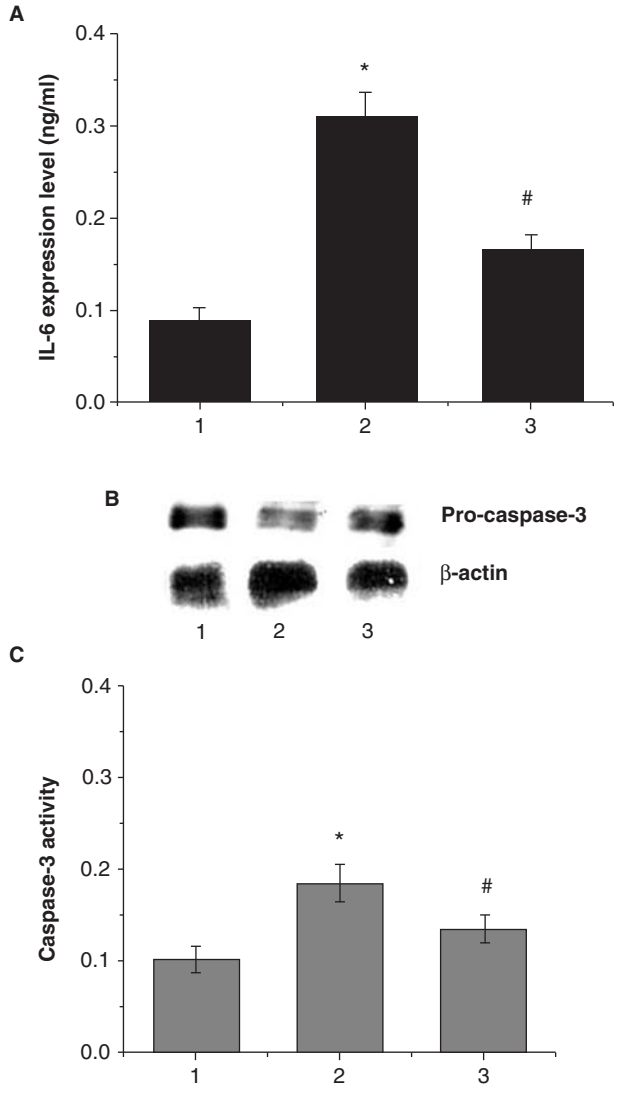

Figure 1. The effect of PBS-pharmaceutical acupuncture on cisplatin-induced IL-6 expression and caspase-3 activity in mice. PBS was acupunctured at Ermen acupoint (TB 21) with administration of cisplatin for 14 days. After that, the cochlea was isolated and isolated tissue was homogenized. (A) Levels of IL-6 were measured using the ELISA method. (B) Levels of pro-caspase-3 were assayed by Western blot analysis. (C) Activities of caspase-3 were determined using a colorimetric kit. All data represent the mean ± SEM of three independent experiments. 1, Control group (n = 6); 2, cisplatin group; 3, cisplatin + PBS group (20 mg/kg). *p < 0.05, significantly different from the cisplatin-untreated group. #p < 0.05, significantly different from the cisplatin-alone treated group.

caspase-3 (an inactive form of caspase-3) was decreased in the cisplatin group. However, this phenomenon was blocked in the PBS-pharmaceutical acupuncture group (Fig. 1B). As shown in Fig. 1C, PBS-pharmaceutical acupuncture inhibited the caspase-3 activity enhanced by cisplatin.

Effect of PBS on cisplatin-induced cell death and IL-6 production in HEI-OC1 cells

The molecular mechanisms underlying the action of PBS were then investigated using the auditory cell line, HEI-OC1, which is highly sensitive to ototoxic drugs. The effects of PBS (0.2 and 2 mg/ml), NaCl (2 mg/ml), $MgCl_2$, $ZnSO_4$, or KCl were initially assessed on the viability of HEI-OC1 cells using the MTT assay. As shown in Fig. 2A, when the cells were treated for 48 h with cisplatin, cell viability decreased significantly compared with media. PBS or NaCl inhibited cisplatin-induced death of HEI-OC1 cells. However, $MgCl_2$, $ZnSO_4$, or KCl did not inhibit cisplatin-induced cell death (data not shown). This indicates that NaCl is an active ingredient of PBS. The protective effect of PBS was higher than that of NaCl. In addition, treatment by PBS or NaCl significantly reduced the IL-6 production induced by cisplatin (Fig. 2B).

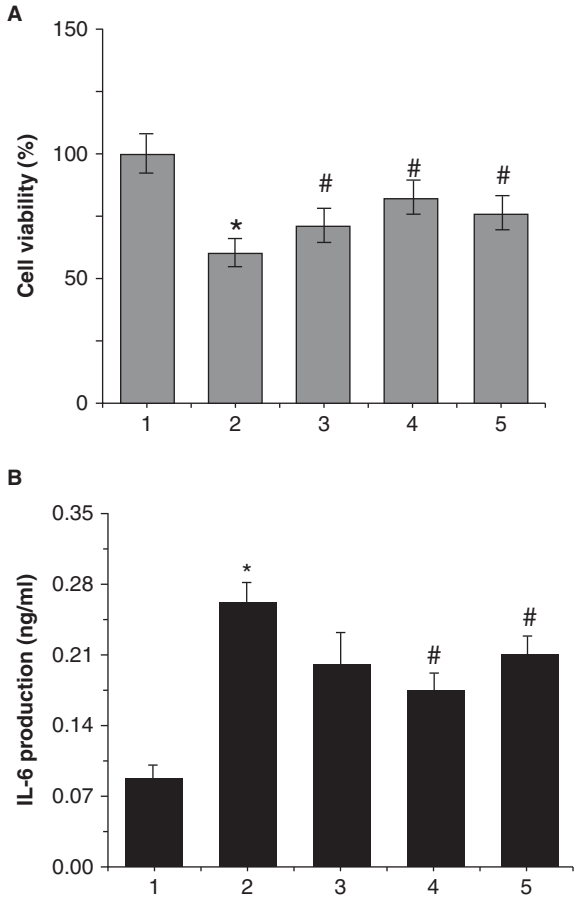

Figure 2. The effect of PBS on cell viability and IL-6 production in HEI-OC1 cells. (A) The cells were pretreated with PBS (0.2–2 mg/ml) and then treated with cisplatin (20 μM) for 48 h. The viability of cells was evaluated by the MTT assay. (B) Levels of IL-6 were measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of three independent experiments. 1, Control; 2, cisplatin; 3, cisplatin + PBS (0.2 mg/ml); 4, cisplatin + PBS (2 mg/ml); 5, cisplatin + NaCl (2 mg/ml). $^{*}p < 0.05$, significantly different from the cisplatin-untreated cells. $^{\#}p < 0.05$, significantly different from the cisplatin-alone treated cells.

Effect of PBS on cisplatin-induced cyt c release and caspase-3 activation in HEI-OC1 cells

The mitochondrial apoptotic cascade requires the release of cyt *c* into the cytosol. To determine if PBS inhibits the release of cyt *c* induced by cisplatin, cells were exposed to cisplatin in the presence or absence of PBS. Cyt *c* release from mitochondria into cytosol was evaluated by Western blot analysis. The results showed that cisplatin induced cyt *c* release into the cytosol and that it was inhibited by treatment with PBS (Fig. 3A). Caspase-3 activity is known to increase in cisplatin-induced apoptosis in HEI-OC1 cells. To determine if PBS inhibits caspase-3 activation induced by cisplatin, caspase-3 assay was performed. As shown in Fig 3C, increased caspase-3 activity induced by cisplatin was significantly inhibited by treatment with PBS (2 mg/ml). NaCl also inhibited the cyt *c* release and caspase-3 activation.

Effect of PBS on cisplatin-induced ERK and NF-κB activation in HEI-OC1 cells

It was reported that hair cell death is associated with an increase of ERK and NF-κB activation [10]. IL-6 expression was also mediated by the activation of NF-κB in HEI-OC1 cells [9]. Based on this research, we investigated if PBS regulates ERK activation to understand the protective mechanism of PBS. As shown in Fig. 4A, the results showed that increased ERK activation induced by cisplatin was significantly inhibited by treatment with PBS (0.2 or 2 mg/ml). Western blot analysis was performed to elucidate the effect of PBS on NF-κB activation. As a result, when the cells were treated with cisplatin, the NF-κB level was significantly increased in nuclear extracts. PBS (0.2 or 2 mg/ml) inhibited the cisplatin-induced increase of the nuclear NF-κB levels in HEI-OC1 cells (Fig. 4B). Next, to investigate the inhibitory effect of PBS on NF-κB activation, we

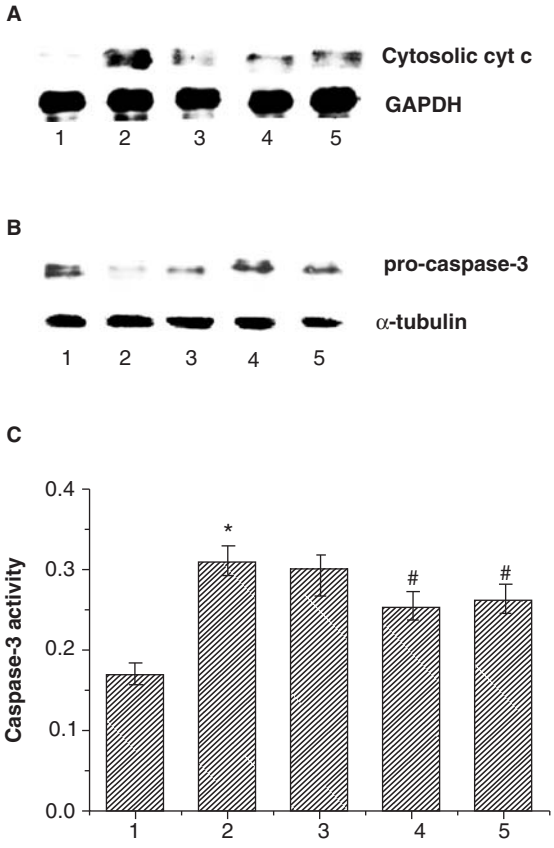

Figure 3. The effect of PBS on release of cyt *c* and activation of caspase-3 in HEI-OC1 cells. (A) The cells were pretreated with PBS (0.2–2 mg/ml) and then treated with cisplatin (20 μM) for 48 h. After isolation of the cytosolic fraction, the protein extracts were assayed for cyt *c* by Western blot analysis. β-Actin was used as the loading control. (B) Levels of pro-caspase-3 after treatment with PBS (0.2–2 mg/ml) were assayed by Western blot analysis. (C) Activities of caspase-3 were determined using a colorimetric kit, as a function of PBS (0.2–2 mg/ml) concentration. 1, Control; 2, cisplatin; 3, cisplatin + PBS (0.2 mg/ml); 4, cisplatin + PBS (2 mg/ml); 5, cisplatin + NaCl (2 mg/ml). All data represent the mean ± SEM of three independent experiments. $^{\star}p < 0.05$, significantly different from the cisplatin-untreated cells. $^{\#}p < 0.05$, significantly different from the cisplatin-alone treated cells.

examined the effect of PBS in an NF-κB-dependent gene reporter assay. Plasmid NF-κB-luciferase and pSV40-luciferase reporter gene constructs were transiently cotransfected into HEI-OC1 cells, which were stimulated by cisplatin. As shown in Fig. 4C, PBS significantly reduced cisplatin-induced luciferase activity. NaCl also inhibited cisplatin-induced NF-κB activation but not ERK activation.

Discussion

Korean ancestors learned a considerable amount of useful knowledge through experience, despite the fact that they did not know how to conduct research as carried out today in modern medicine. However, this knowledge is known to have regulatory effects on various conditions [11,12]. The present study demonstrates that PBS-pharmaceutical acupuncture prevents ototoxicity.

Pharmaceutical acupuncture is a traditional oriental therapeutic technique that combines acupuncture with herbal treatment. The goal is to provide a prolonged mechanical stimulus in the acupoint. Other substances may also be used for this purpose, such as glucose and vitamins. For example, autologous blood (hemopuncture) and bee venom (apipuncture) are injected mainly for anti-inflammatory purposes [18]. The present study found that PBS-pharmaceutical acupuncture prevented cisplatin-induced ototoxicity. Recently, it was reported that cisplatin-induced cochlear injury can occur through the expression of proinflammatory cytokines [8–10]. In addition, flunarizine, known as a T-type Ca^{2+} channel antagonist, has a cytoprotective effect against cisplatin cytotoxicity through the inhibition of proinflammatory cytokine expression in auditory cells [3]. These reports suggest the involvement of proinflammatory cytokines in cisplatin-induced hearing loss. Hence, it was hypothesized that the protective effect of PBS arises through the regulation of proinflammatory cytokines. In this study, we showed that PBS-pharmaceutical acupuncture inhibited IL-6 expression in the organ of Corti in mice. We also showed that caspase-3 activation was inhibited in the PBS-pharmaceutical acupuncture group compared with the control group in the organ of Corti in mice. These results suggest that the protective effect of PBS-pharmaceutical acupuncture against cisplatin-induced cytotoxicity is through the suppression of IL-6 expression induced by cisplatin.

To gain further insights into the molecular mechanisms of PBS in this study, the auditory cell line, HEI-OC1, was used. Apoptosis not only plays an essential role in development and tissue homeostasis but is also involved in a wide range of pathological conditions [19]. Apoptosis is an important mechanism of cochlear hair cell loss following exposure to an ototoxic level of cisplatin. In mammalian cells, there are two major caspase activation pathways: extrinsic and intrinsic pathways. In the extrinsic pathway, binding of the death receptors causes the activation of caspase-8, which is an initiator caspase. In the intrinsic pathway, various forms of cellular stress cause mitochondrial alterations that lead to mitochondrial membrane depolarization and the release of cyt *c*. In the cytosol, cyt *c* binds to and activates Apaf-1, which then activates pro-caspase-9. Active caspase-9 directly cleaves and activates the effector protease, caspase-3. Some studies strongly suggest that caspases play a key

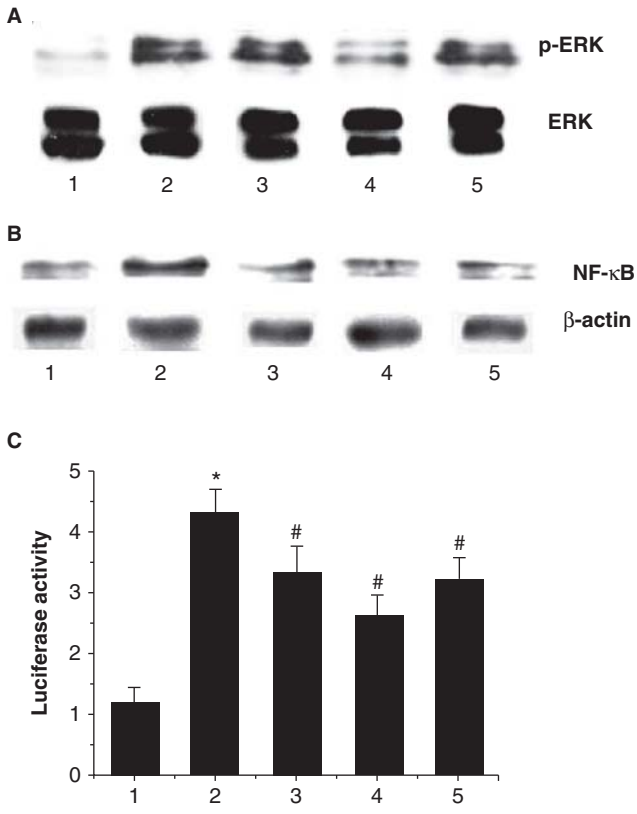

Figure 4. The effect of PBS on activation of ERK and NF-κB in HEI-OC1 cells. (A) The cells were pretreated with PBS (0.2–2 mg/ml) and then treated with cisplatin (20 μM) for 48 h. The protein extracts were assayed for p-ERK by Western blot analysis. (B) After isolation of nuclear fraction, the protein extracts were assayed for NF-κB by Western blot analysis. (C) The cells were transfected with an NF-κB-dependent reporter gene for 24 h, and transfected cells were treated with cisplatin for 48 h. PBS (0.2–2 mg/ml) was added 2 h before treatment with cisplatin. Cells were harvested and luciferase activity was measured as described in Material and Methods. 1, Control; 2, cisplatin; 3, cisplatin + PBS (0.2 mg/ml); 4, cisplatin + PBS (2 mg/ml); 5, cisplatin + NaCl (2 mg/ml). $^{*}p < 0.05$, significantly different from the cisplatin-untreated cells. $^{\#}p < 0.05$, significantly different from the cisplatin-alone treated cells.

role in cisplatin-induced hearing loss [3]. In the present study, treatment with PBS reduced cell death, cyt *c* release, and caspase-3 activation induced by cisplatin in auditory HEI-OC1 cells. These results suggest that the protective effect of PBS arises through the suppression of caspase-3 activation in HEI-OC1 cells.

The MAPK pathways are the central components of the intracellular signaling networks that control many aspects of mammalian cellular physiology including cell proliferation, differentiation, and apoptosis [10]. It was reported that the ototoxicity of cisplatin is associated with ERK activation in HEI-OC1 [10]. The latter report suggested that the ERK pathway is a potential therapeutic target for preventing cisplatin-induced ototoxic damage. Therefore, we examined whether or not PBS affects ERK activation induced by cisplatin. It was found that PBS reduced ERK activation. Although PBS attenuated ERK activation, the effects of PBS on other pathways involving MAPK upstream/downstream and on apoptosis markers were not determined. Therefore, further studies are necessary to clarify the role of PBS on the MAPK pathway in the auditory system.

Activated NF-κB is inducible in most cells. It is implicated in the regulation of proliferation, survival, angiogenesis, apoptosis, and differentiation activities [3]. Acoustic overstimulation causes a marked expression of inflammatory factors through NF-κB activation in the inner ear [20]. Taken together, this suggests that the regulation of the NF-κB signaling pathway can modulate hearing loss. In this study, it was shown that PBS abrogated cisplatin-induced NF-κB activation. These results demonstrate that the protective effect of PBS, at least in part, may be derived through regulation of the NF-κB pathway in auditory cells.

In conclusion, PBS-pharmaceutical acupuncture counteracts ototoxicity by suppressing the expression of IL-6 and the activation of caspase-3 in mice. In addition, PBS inhibited cell death, cyt *c* release, caspase-3 activation, and NF-κB activation as induced by cisplatin in auditory cells. Collectively, these findings show that blocking a critical step in

apoptosis by PBS may be a useful strategy to prevent the harmful side effects of cisplatin ototoxicity in patients who have to undergo chemotherapy. However, histological and physiological investigations are necessary to determine other possible anti-apoptotic mechanisms of PBS.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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