Hwanggunchungyitang Prevents Cadmium-Induced Ototoxicity through Suppression of the Activation of Caspase-9 and Extracellular Signal-Related Kinase in Auditory HEI-OC1 Cells

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Hwanggunchungyitang (HGCT) is a newly designed herbal drug formula for the purpose of treating auditory diseases. A number of heavy metals have been associated with toxic effects to the peripheral or central auditory system. Cadmium (Cd^{2+}) is a heavy metal and a potent carcinogen implicated in tumor development through occupational and environmental exposure. However, the auditory effect of Cd^{2+} is not poorly understood. The purpose of the present study was to investigate whether HGCT prevent the ototoxic effects induced by Cd^{2+} in auditory cell line, HEI-OC1. HGCT inhibited the cell death, reactive oxygen species generation (ROS), activation of caspase-9, and extracellular signal-related kinase (ERK) induced by Cd^{2+} . In addition, we observed that cochlear hair cells in middle turn were damaged by Cd^{2+} . However, HGCT prevented the destruction of hair cell arrays of the rat primary organ of Corti explants in the presence of Cd^{2+} . These results support the notion that ROS are involved in Cd^{2+} ototoxicity and suggest HGCT therapeutic usefulness, against Cd^{2+} -induced activation of caspase-9 and ERK.

Key words Hwanggunchungyitang; cadmium; auditory cell; caspase-9; extracellular signal-related kinase

Cadmium (Cd^{2+}) is a major environment and occupational hazard because of its widespread use in industry and subsequent release into the environment. Irreversible hearing loss is a characteristic effect of a number of heavy metals. The high incidence of hearing loss in human who reside in industrialized countries was increased.^{1–5)} Other experimental study showed that Cd^{2+} also has a dose-dependent deleterious effect on the auditory system.⁶⁾ Cd^{2+} toxicity has been described as *in vitro* and *in vivo* apoptosis but its molecular mechanism in auditory system is not fully understood.

Although Cd^{2+} can activate various signaling pathways, mitochondria are the most pertinent in mediating apoptosis via reactive oxygen species (ROS) generation.^{7,8)} Recent studies suggest that ROS generation associated with Cd^{2+} exposure leads to oxidative stress, which is an important factor involved in Cd^{2+} -induced apoptosis.^{9,10)} Cd^{2+} -induced loss of mitochondrial membrane potential (MMP) may be a spontaneous event at early stages of apoptosis causing membrane perturbations leading to cytochrome (cyt) *c* release.¹¹⁾ Which apoptotic pathway is induced by Cd^{2+} remains controversial. Some studies strongly suggest that caspases play a central role in Cd^{2+} -induced cell death.^{12–14)} Numerous other studies suggest a caspase-independent mechanism.^{15–17)} It is conceivable that Cd^{2+} may induce different apoptotic pathways in different cell types and in dependence on the exposure (cell treatment) conditions.

Mitogen-activated protein kinases (MAPKs) belong to a family of Ser/Thr protein kinases that transmit extracellular signals into the nucleus. There are three subfamilies of

MAPKs including c-Jun NH2-terminal kinase (JNK; also known as stress-activated protein kinase), p38 MAPK, and extracellular signal-related kinase (ERK).¹⁸⁾ In general, the ERK signaling cascade is activated by growth factors and is associated with cell survival and proliferation.^{19,20)} On the other hand, p38 and JNK are mainly activated by cellular stress and are often associated with inflammation and apoptosis. However, cumulative reports have indicated that these signaling pathways exhibit more complex roles in the regulation of distinct cellular effects. The cellular functions regulated by ERK, p38 or JNK seem to depend on the cell type, the stimulus, the duration and strength of kinase activities. For example, the ERK activation is involved in Cd^{2+} -induced G2/M arrest and cell death in CCRF-CEM cells.²¹⁾ But the ERK1/2 pathway was not responsible for Cd^{2+} -induced cytotoxicity in CL3 cells, a lung carcinoma cell line.²²⁾ Also, it was reported that Cd^{2+} -induced apoptosis through the activation of JNK, but no activation of p38 in J774A.1 cells.²³⁾ Taken together, these reports suggest that the discrepancies in MAPKs activation and Cd^{2+} -induced apoptosis may be due to difference in cell type.

Hwanggunchungyitang (HGCT) formula is a newly modified prescription for the purpose of development of auditory disease. HGCT is composed of 9 oriental herbs. In the present study, we investigate the protective effects of HGCT against Cd^{2+} -induced ototoxicity in auditory HEI-OC1 cells. In addition, the protective effects of HGCT against Cd^{2+} -induced hair cell damage have been investigated in organ of Corti.

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MATERIALS AND METHODS

Reagents Fetal bovine serum (FBS), and high-glucose Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO BRL (Grand Island, NY, U.S.A.). SB203580, PD98059 and SP600125 were purchased from Calbiochem (St. Louis, MO, U.S.A.). H2DCF-DA was obtained from Molecular Probes (Leiden, The Netherlands). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other reagents were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Cytochrome *c*, caspase-9, ERK, and cyclooxygenase-2 (COX-2) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.).

Preparation of HGCT An extract of HGCT which is a mixture of 9 traditional drugs as shown in Table 1 was obtained from the College of Pharmacy, Wonkwang University (Iksan, South Korea). Amounts of the 9 traditional drugs studied in this work were shown in Table 1. Extract of HGCT was prepared by decocting the dried description of herbs with boiling distilled water. The extraction decocted for approximately 3 h has been filtered, lyophilized, and kept at 4 °C. The yield of extraction was about 4.85%. Dilutions were made in saline then filtered through 0.45- μ m syringe filter.

Cell Culture The HEI-OC1 cell line was a kind gift from Dr. Federico Kalinec (House Ear Institute, Los Angeles, CA, U.S.A.). Establishment of immortal cell lines was facilitated by a transgenic mouse, ImmortomouseTM (Charles River Laboratories, Wilmington, MA, U.S.A.), which harbors a temperature-sensitive mutant of the SV40 large T antigen gene under the control of an interferon-gamma-inducible promoter element. Cochlear half turns from Immortomice at postnatal day 7 were cultured on uncoated plastic culture dishes under permissive conditions (33 °C) in antibiotic-free DMEM (Gibco BRL, Grand Island, NY, U.S.A.) containing 10% FBS (JRH Bioscience, Lenexa, KS, U.S.A.) and 50 U/ml gamma-interferon. Cochlear explants were placed at different times in non-permissive conditions (39 °C) and allowed to differentiate for up to 180 d. Cultures were monitored on a daily basis by phase-contrast and video microscopy. Explants, cells growing in tissue regions formerly associated with the organ of Corti, were isolated by lifting with a micropipette after 2 to 5 min incubation with trypsin-EDTA. A cell line, named HEI-OC1, was cloned in the absence of antibiotics, using the limiting dilution method and characterized by observation with phase contrast light microscopy, Western blotting and immunofluorescence. Cells

were maintained in DMEM medium with 10% fetal bovine serum at 33 °C under 5% CO₂ in air. HEI-OC1 cells express several molecular markers characteristic of organ of Corti sensory cells: thyroid hormone, brain-derived neurotrophic factor, calbindin, calmodulin, Connexin 26, Math 1, Myosin 7a, organ of Corti protein 2, tyrosin kinase receptors B and C, platelet-derived growth factor receptor, and prestin. In addition, HEI-OC1 cells are extremely sensitive to ototoxic drugs.²⁴⁾

MTT Assay To test the viability of cells, MTT colorimetric assay was performed as described previously.²⁵⁾ Briefly, Cells (3×10^5 cells/well) were exposed to various concentrations of HGCT and then treated with Cd²⁺. After incubation, MTT solution (5 mg/ml in phosphate buffered saline (PBS)) was added (50 μ l/well), and plates were further incubated for 4 h at 33 °C. Precipitated formazan crystals were centrifuged and the pellets were dissolved by the addition of dimethyl sulfoxide (DMSO). Absorption was measured by spectrometer at 540 nm.

Measurement of Caspase-9 Activity The enzymatic activity of caspase-9 was assayed using the caspase colorimetric assay kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's protocol. Briefly, cells were left untreated or treated with Cd²⁺ for 24 h and then lysed in lysis buffer for 10 min on ice. The lysed cells were centrifuged at 14000 rpm for 5 min, and protein was incubated with 50 μ l reaction buffer and 5 μ l caspase-9 substrate at 37 °C for 2 h, and the absorbance was measured at a wavelength of 405 nm on a plate reader. Equal amount of total protein was quantified by bicinchoninic acid (BCA) protein quantification kit (Sigma) in each lysate.

Measurement of Intracellular ROS Generation Intracellular ROS levels were measured using a fluorescent dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA; Eastman Kodak, Rochester, NY, U.S.A.). In the presence of an oxidant, DCFH is converted into a highly fluorescent molecule, 2',7'-dichlorofluorescein (DCF). HEI-OC1 cells were cultured overnight on round coverslips, and then treated with Cd²⁺. Cells were washed twice with serum-free, phenol red-free medium, and incubated for 30 min with 5 μ M DCFH-DA in serum-free, phenol red-free medium medium. Fluorescence intensity was measured in a spectrofluorometer at excitation 485 nm and emission 538 nm.

Western Blot Analysis For analysis of the levels of COX-2, caspase-9, cyt *c*, cells were rinsed twice with ice-cold PBS and were then lysed in ice-cold lysis buffer (1% Triton, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholate in PBS). Cell lysates were centrifuged at 15000 *g* for 5 min at 4 °C; the supernatant was then mixed with an equal volume of 2 $\times$ SDS sample buffer, boiled for 5 min, and then separated through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. After electrophoresis, the protein was transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% skim milk for 2 h, rinsed, and incubated overnight at 4 °C with primary antibodies in PBS/0.5% Tween 20. Excess primary antibody was then removed by washing the membranes four times in PBS/0.5% Tween 20, and the membranes were incubated for 1 h with HRP-conjugated secondary antibodies (against mouse, goat, or rabbit). After three washes in PBS/0.5% Tween 20, the protein bands were visualized by an

Table 1. Prescription of HGCT

Hwanggunchungyitang components	Ratio
1. Glycyrrhizae Radix	2
2. Zingiberis Rhizoma	4
3. Angelicae Gigantis Radix	4
4. Rehmanniae Radix	6
5. Mori Cortex	4
6. Aurantii Fructus	4
7. Citri Pericarpium	4
8. Gardeniae Fructus	4
9. Scutellariae Radix	6

enhanced chemiluminescence assay (Amersham Pharmacia Biotech, NJ, U.S.A.) following the manufacturer's instructions.

Organ of Corti Explant Culture The organ culturing procedure was similar to that described previously.²⁶⁾ Sprague Dawley rats were sacrificed on postnatal day 2 and the cochlea were carefully removed by dissection. The stria vascularis and spiral ligament were removed leaving the organ of Corti. The basal, middle, and apex turns of the cochlea were used for further study. Cochlear explants were treated with high glucose (4.5 g/l) DMEM containing 10% FBS, Cd^{2+} , or the combination of Cd^{2+} and HGCT, and incubated for 24 h at 37 °C, 5% CO_2 . When experimental treatment was completed, the culture was prepared for histological analysis. Organ of Corti explants were fixed at RT for 15 min in 2% paraformaldehyde in PBS. Specimens were rinsed in PBS, incubated in 0.25% Triton X-100 for 2 min and immersed in Tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin (Sigma, 1:100 diluted) in PBS for 20 min. After rinsing three times with PBS, specimens were examined under a fluorescence microscope with appropriate filters appropriate for TRITC (excitation: 510—550 nm, emission: 590 nm). This work was carried out in conformity with all applicable regulations and institutional use rules of Laboratory Animal in Wonkwang University School of Medicine.

Statistical Analysis The experiments shown are a summary of the data from at least-three experiments and are presented as the mean $\pm$ S.E.M. Statistical evaluation of the results was performed by ANOVA with Tukey *post hoc* test. The results were considered significant at a value of $p < 0.05$.

RESULTS

The Effect of HGCT on Cd^{2+} -Induced Cell Viability in HEI-OC1 Cells First, the effect of Cd^{2+} on the viability of HEI-OC1 cells was investigated. Cells were incubated with different concentrations of HGCT (0.01—1 mg/ml) and then treated with Cd^{2+} (10 μM) for 24 h. Cell viability was measured using MTT assay. As shown Fig. 1, when the cells were treated for 24 h with Cd^{2+} , the cell viability significantly decreased. However, HGCT inhibited Cd^{2+} -induced cell death on the HEI-OC1 cells in a dose-dependent manner.

The Effect of HGCT on ROS Generation in HEI-OC1 Cells Mitochondria are a regular source of ROS, but an increase in the rate of ROS production can damage the mitochondrial membrane leading to apoptosis. Thus, we examined whether HGCT affected the Cd^{2+} -induced intracellular ROS generation. Cells were incubated with or without HGCT and loaded with DCF-DA, which is converted into a fluorescent molecule after intracellular oxidation. As shown in Fig. 2, treatment of Cd^{2+} significantly increased the generation of intracellular ROS. However, it was found that HGCT (0.01—1 mg/ml) inhibited these increased ROS level in dose-dependent manner.

The Effect of HGCT on Cyt *c* Release, COX-2, Metallothionein (MT) Expression in HEI-OC1 Cells Cyt *c* is released following mitochondrial membrane permeabilization, and plays a major role in cell death.²⁷⁾ To determine the effect of HGCT on cyt *c* release from mitochondrial into the cytosol, Western blot analysis was performed. As shown in

Fig. 1. The Effect of Cd^{2+} on Cell Viability in HEI-OC1

Cells (3×10^5) were pretreated with HGCT (0.01—1 mg/ml) and treated with Cd^{2+} for various concentrations (10 μM) for 24 h. Cell viability was evaluated by MTT colorimetric assay. Data represent the mean $\pm$ S.E.M. of three independent experiments performed in duplicate. # $p < 0.05$, significantly different from not-treated cells. * $p < 0.05$, significantly different from Cd^{2+} alone-treated cells.

Fig. 2. The Effect of HGCT on ROS Production in HEI-OC1

Cells (3×10^5) were pretreated with HGCT (0.01—1 mg/ml) and treated with Cd^{2+} for concentrations (10 μM) for 24 h. After experiment, cells were loaded with the fluorescent probe DCFH-DA and fluorescence was measured in a spectrofluorometer. Data represent the mean $\pm$ S.E.M. of three independent experiments performed in duplicate. # $p < 0.05$, significantly different from not-treated cells. * $p < 0.05$, significantly different from Cd^{2+} alone-treated cells.

Fig. 3A, treatment of Cd^{2+} significantly induced the release of cyt *c* to the cytosol. We found that the release of cyt *c* was inhibited by pretreatment of HGCT in dose-dependent manner. The relative intensity of the cyt *c* expression level was quantitated by densitometry (Fig. 3B).

Recently, other reports demonstrated that the COX-2 enzyme is constitutively expressed in the organ of Corti and spiral ganglion cells of the guinea pig cochlea.²⁸⁾ In order to gain insight into a possible role of COX-2 in physiological processes, we determined COX-2 expression after Cd^{2+} treatment, and assay the regulatory effect of HGCT. Data in Fig. 3C, the result showed that Cd^{2+} induced the level of COX-2 compared with un-stimulated cells. This enhanced level of COX-2 was inhibited by pretreatment of HGCT in a dose-dependent manner. The relative intensity of the COX-2 expression level was quantitated by densitometry (Fig. 3D).

MT is a ubiquitous metalloprotein found in bacteria, mammals and invertebrates. Although the exact function of MT is unknown, it has been suggested MT is involved in toxic metal detoxification. To determine the effect of HGCT on MT expression, Western blot analysis was performed. As shown in Fig. 3E, treatment of Cd^{2+} induced the MT expression. We found that the expression of MT was not affected by HGCT.

The Effect of HGCT on Caspase-9 Activation in HEI-

Fig. 3. The Effect of HGCT on Apoptosis Associated Genes in HEI-COI Cells

Cells (5×10^6) were pretreated with HGCT (0.01—1 mg/ml) and treated with Cd^{2+} for various concentrations (10 μM) for 24 h. (A) The protein extracts were assayed for cyt *c* by Western blot analysis. (B) The relative levels of the cytosolic cyt *c* were quantitated by densitometry. (C) The protein extracts were assayed for COX-2 by Western blot analysis. (D) The relative levels of the cytosolic COX-2 were quantitated by densitometry. (E) The protein extracts were assayed for MT by Western blot analysis. Figures are representative data of three independent experiments.

Fig. 4. The Effect of Cd^{2+} on Caspase-9 Activity in HEI-COI Cells

(A) Cells (5×10^6) were pretreated with HGCT (0.01—1 mg/ml) and treated with Cd^{2+} for various concentrations (10 μM) for 24 h. The protein extracts were assayed by Western blot analysis for pro-caspase-9. (B) Caspase-9 activities were determined by a colorimetric kit using substrates, as described under Materials and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments performed in duplicate. * $p < 0.05$, significantly different from not-treated cells. ** $p < 0.05$, significantly different from Cd^{2+} alone-treated cells.

CO Cells Pro-apoptotic stimuli induce mitochondrial membrane permeabilization and promote release of cyt *c* in the cytosol leading to activation of pro-apoptotic factors as well as the maturation of caspase-9.²⁹⁾ In the present study, the extent to which HGCT influences the caspase-9 activity was investigated. Cells were pretreated with HGCT (0.01—1 mg/ml) for 1 h, and then treated with Cd^{2+} (10 μM) for 24 h. As shown in Fig. 4A, we observed that Cd^{2+} decreased the pro-caspase-9 expression, an inactive form of caspase-9. However, HGCT inhibited the phenomenon in Cd^{2+} -induced HEI-OC1 cells. The relative intensity of the pro-caspase-9 expression level was quantitated by densitometry.

Next, to confirm whether HGCT inhibited the caspase-9 activation, a caspase assay kit was used. As shown in Fig. 4B, Cd^{2+} induced the caspase-9 activation, these increase level was inhibited by the treatment of HGCT in a dose-dependent manner.

The Effect of HGCT on ERK Activation in HEI-CO Cells In order to explore whether HGCT influences the MAPK pathways in Cd^{2+} -induced apoptosis, inhibitors of PD98059 (ERK inhibitor), SB202190 (p38 inhibitor), and SP600125 (JNK inhibitor) were used. As shown in Fig. 5A, ERK inhibitor was observed to effectively suppress Cd^{2+} -induced the cell death. Based on the results showing the in-

Fig. 5. The Effect of HGCT on ERK Activation in HEI-COI Cells

(A) Cells (3×10^5) were pretreated with PD98059 (2 μM), SB203580 (2 μM), and SP600125 (2 μM) were pretreated for 1 h, and then treated with Cd^{2+} (10 μM) for 24 h. Cell viability was evaluated by MTT colorimetric assay. (B) Cells were pretreated with HGCT (0.01–1 mg/ml) and treated with Cd^{2+} (10 μM) for 24 h. The protein extracts were assayed for phospho-ERK and total ERK by Western blot analysis. Data represent the mean $\pm$ S.E.M. of three independent experiments performed in duplicate. # $p < 0.05$, significantly different from not-treated cells. * $p < 0.05$, significantly different from Cd^{2+} alone-treated cells.

Fig. 6. The Effect of HGCT in the Primary Organ of Corti Explants against Cd^{2+}

The middle turns of the cochlea were used. The organ of Corti explants were pretreated with HGCT (1 mg/ml) for 1 h and then treated with Cd^{2+} . The organ of Corti explants were fixed with paraformaldehyde, and stained with TRITC-conjugated phalloidin, and then observed under fluorescent microscope. 1) Control; 2) Cd^{2+} treatment; 3) HGCT (1 mg/ml) + Cd^{2+} . Figures are representative data of three independent experiments.

hibitory effect of ERK inhibitor on Cd^{2+} -induced cell death, we examined whether HGCT regulate activation of ERK during apoptosis. As shown in Fig. 5B, it was found that Cd^{2+} induced ERK activation, but HGCT inhibited these increased ERK activation in dose-dependent manner.

The Protective Effect of HGCT in the Primary Organ of Corti Explants against Cd^{2+} The following experiments were designed to determine whether HGCT could protect the rat organ of Corti primary explants. The organ of Corti from rats was isolated and treated with Cd^{2+} in the absence or presence of HGCT. Phalloidin, which binds to F-actin, was used as a marker to identify surviving hair cells in cochlear explant cultures which are highly enriched in the stereocilia bundles of hair cells. TRITC-conjugated phalloidin-labeled stereocilia bundles in outer hair cells (OHCs) and inner hair cells (IHCs) facilitated easy identification of hair cells. The orderly arrangement of the three rows of OHCs and a single row of IHCs in a control tissue is shown in Fig. 6. When compared with the control, treatment of Cd^{2+} into culture medium resulted in loss of F-actin staining-posi-

Fig. 7. The Effect of HGCT on the Accumulation of Intracellular Cd^{2+} within Cells

Cells were pretreated with HGCT (1 mg/ml) and treated with Cd^{2+} (10 μM) for 24 h. After experiment, cells were dissolved with diluted HNO_3 . Cd measurements were performed using optical emission spectrometry via ICP/OES. Standards were prepared as increasing amounts of cadmium standards in deionized water each containing 20% of digested cells. Data represent the mean $\pm$ S.E.M. of three independent experiments performed in duplicate. # $p < 0.05$, significantly different from not-treated cells.

tive cells and the disarray of most stereocilia bundles of hair cells, especially OHCs. These data showed that cochlear hair cells were damaged by Cd^{2+} . However, pretreatment with the HGCT prevented the destruction of hair cell arrays of the rat primary organ of Corti explants in the presence of Cd^{2+} .

The Effect of HGCT on Intracellular Cadmium Level in HEI-CO Cells We investigated whether HGCT regulate the accumulation of intracellular Cd^{2+} within cells. Cd^{2+} accumulation was determined in cells by ICP/OES and expressed as g/mg of cell protein. We observed that treatment of Cd^{2+} increased the accumulation of intracellular level of Cd^{2+} within cells (0.055 ± 0.01 g/mg). However, HGCT inhibited the accumulation of intracellular Cd^{2+} compare to Cd^{2+} alone treatment (0.041 ± 0.01 g/mg). The inhibition rate of Cd^{2+} accumulation by HGCT (1 mg/ml) was approximately 23%, but there was no significance. Figure 7 represented the relative level of intracellular Cd^{2+} .

DISCUSSION

The findings herein show that Cd^{2+} induces ROS generation and cyt c release. Cd^{2+} induces apoptosis through activation of caspase-9 and ERK in auditory HEI-OCI cells. In addition, we observed that cochlear hair cells in middle turn were damaged by Cd^{2+} . However, HGCT inhibited ROS generation, and activation of caspase-9 and ERK in auditory cell line. HGCT prevented the destruction of hair cell arrays of the rat primary organ of corti explants in the presence of Cd^{2+} .

HGCT formula is a newly modified prescription for the purpose of development of auditory disease. HGCT consists of 9 different herbs. Other studies reported that each medicine herb has a various effect. For example, *Glycyrrhizae Radix* and *Zingiberis Rhizoma* have been shown to prevent biological toxicity on oxidative stress through potent antioxidant and anti-lipid peroxidation activities,³⁰⁾ *Scutellariae Radix* prevents the injury in PC12 cells.³¹⁾ It was reported that *scutellaria baicalensis* had the scavenging reactive oxygen species.³²⁾ *Aurantii Fructus* has shown anti-shock effect,³³⁾ and *Gardeniae Fructus* has been used for centrally anti-hypertension in cardiovascular system and anti-inflammation, treating parenchyma injuries.³⁴⁾ HGCT composed on the basis of the theory of Korean medicine to maximize

its efficacy. In present study, we demonstrated the pharmacological mechanism of HGCT in auditory cells.

Cadmium (Cd^{2+}) is a nonessential element widely used in industry, and is also found as a contaminant in agricultural products.³⁵⁾ Cd^{2+} contamination of soil and water has raised concern, because this metal is bioaccumulated in the upper levels of the food chain, including in humans, in which its biological half-life is approximately 20 years.³⁶⁾ Given its current rate of release into the environment, Cd^{2+} content in the human body is likely to increase in the future,³⁶⁾ which could lead to a higher incidence of Cd^{2+} -related diseases. Liver and kidney have traditionally been considered the main targets of Cd^{2+} toxicity.^{37,38)} However, recent reports indicate that chronic exposure to low doses of Cd^{2+} may cause neurobehavioral problems in humans and other animals, despite a lack of detectable renal damage.^{39,40)} These observations emphasize the importance of studying the effects of Cd^{2+} in other organs. Recently, it was reported that hair cells are more sensitive to cadmium than kidney tubule cells, and that the cochlear component of hearing is more vulnerable to cadmium toxicity than other parts of the auditory system.⁶⁾ ROS are involved in apoptosis as well as in cell proliferation.⁴¹⁾ ROS may be the causative factor involved in many human degenerative diseases, and antioxidants have been found to have some degree of preventive and therapeutic effects on these disorders.⁴²⁾ ROS may induce cell death directly or act as intracellular messengers during cell death induced by various other kinds of stimuli. Cd^{2+} is reported to cause oxidative stress, *i.e.* increased lipid peroxidation, ROS generation and alterations in glutathione (GSH) and related enzymes, under both *in vitro* and *in vivo* conditions.⁴³⁾ From this, we suggested Cd^{2+} enhanced ROS production might be at the root of Cd^{2+} toxicity. In present study, we showed that Cd^{2+} induced the cell death, and ROS generation. However, HGCT inhibited Cd^{2+} -induced cell death and ROS generation. These results suggested that anti-apoptotic effect of HGCT is through the regulation of ROS in auditory HEI-OCI cells. In addition, we investigated whether HGCT regulate the accumulation of intracellular cadmium within cells. We observed that HGCT inhibited the accumulation of intracellular cadmium within cells. The inhibition rate of cadmium accumulation by HGCT (1 mg/ml) was approximately 23%. This result implicated that HGCT protected against cadmium toxicity.

Mitochondria factors such as cyt *c*, mediate the activation of pro-apoptotic proteases known as caspases. Caspase-9 is the primary initiator caspase in a cascade that activates a number of effector caspases that cleave numerous downstream substrates that result in apoptosis. After mitochondrial outer membrane permeabilization, intermembrane space proteins including cyt *c* are released to activate caspases in the cytoplasm.¹³⁾ Cell death initiated by caspase activation is considered to be the "classic" apoptotic pathway. In some cases, cell death is effectively blocked by caspase inhibition. Taken together, we supposed that the caspase-9 could be an effective target for anti-apoptotic therapy in auditory cells. Therefore, we investigated whether HGCT affects the cyt *c* release, and caspase-9 activation in induced by Cd^{2+} . We showed that HGCT abrogated Cd^{2+} -induced cyt *c* release, and caspase-9 activation. These results demonstrate that the anti-apoptotic effect of HGCT, at least in part, might be de-

rived through the regulation of the mitochondria pathway such as cyt *c*, and caspase-9.

MT is a ubiquitous metalloprotein found in bacteria, mammals and invertebrates. It is induced by various stressors including chemical stressor such as Cd^{2+} . This protein has been implicated in the scavenging of heavy metal by forming linkages. Although the exact function of MT is unknown, it has been suggested MT is involved in toxic metal detoxification. We investigated whether HGCT regulated the MT expression. As shown in Fig. 3E, treatment of Cd^{2+} induced the MT expression. However, we found that HGCT did not affect the expression of MT induced by Cd^{2+} . This result suggested that the protective effect of HGCT could be involved in other mechanisms of MT in HEI-OCI.

MAPKs pathways are central components of the intracellular signaling networks that control many aspects of mammalian cellular physiology including cell proliferation, differentiation, and apoptosis.¹⁹⁾ In general, the ERK cascade is activated by growth factors and is associated with cell survival and proliferation.^{19,20)} On the other hand, p38 and JNK are mainly activated by cellular stress and are often associated with inflammation and apoptosis. However, cumulative reports have indicated that these signaling pathways exhibit more complex roles in the regulation of distinct cellular effects. The cellular functions regulated by ERK, p38 or JNK seem to depend on the cell type, the stimulus, the duration and strength of kinase activities.^{20,44)} Studies recently reported that JNK activation is involved in Cd^{2+} -induced apoptosis in J774A.1. Their study showed that Cd^{2+} increased activation of JNK, but decreased the activation of p38.²³⁾ Iryo *et al.* reported that ERK1/2 was responsible for toxicity of Cd^{2+} , in which experiment U0126 was used as an ERK1/2 inhibitor.²¹⁾ In their study, pretreatment with U0126 suppressed Cd^{2+} -induced ERK activation and the apoptosis, whereas the inhibition of p38 kinase activity with SB203580 did not protect cells from apoptosis. The ERK1/2 pathway was not responsible for Cd^{2+} -induced cytotoxicity of CL3 cells, a lung carcinoma cell line. This discrepancy may be due to the cell type difference. In present study, we observed that ERK inhibitor suppressed the cell death. From this reasons, we speculated that Cd^{2+} induced the apoptosis through the ERK activation, and examined whether Cd^{2+} affected the ERK activation in HEI-OCI. We showed that Cd^{2+} increased the ERK activation. However, HGCT suppressed ERK activation induced by Cd^{2+} . These results suggest that the ERK pathway may be a potential therapeutic target to prevent ototoxic damage from Cd^{2+} . Although HGCT attenuated ERK activation, the effect of HGCT on other pathways involving MAPK upstream and downstream was not elucidated in the present study. Thus, further investigation is necessary to clarify the role of HGCT on the MAPK pathway in the auditory system.

It is reported that Cd^{2+} causes hearing loss in rat and mainly impairs the cochlea without affecting the central auditory pathways.⁶⁾ The cochlea component of hearing is more vulnerable to Cd^{2+} toxicity than other parts of the auditory system. In present study, we investigated whether Cd^{2+} affects the rat primary organ of Corti explants, and HGCT protects the injury of primary organ of Corti explants from Cd^{2+} . The middle turn of the organ of Corti from rats was isolated and treated with Cd^{2+} in the absence or presence of

HGCYT. Incubation media alone did not result in any injury of stereocilia bundles, in which F-actin was intensely labeled with TRITC-conjugated phalloidin. Three rows of outer hair cells and a single row of inner hair cells (IHCs) were clearly observed in control culture of the rat primary organ of Corti explants. We observed that Cd^{2+} destructed the orderly arrangements of the three rows of OHCs and a single row of IHCs in middle turn. These data showed that hair cells were damaged by Cd^{2+} . However, pretreatment with the HGCYT, completely prevented the destruction of hair cell arrays of the rat primary organ of Corti explants in the presence of Cd^{2+} .

In conclusion, we have shown that HGCYT inhibited Cd^{2+} -induced cell death, ROS generation, cyt *c* release, and activation of caspase-9, and ERK in auditory cells. In addition, HGCYT prevented the destruction of hair cell arrays of the rat primary organ of Corti explants in the presence of Cd^{2+} . These data are an important contribution in elucidating the pharmacological mechanism of HGCYT and potential treatment therapies of Cd^{2+} -induced ototoxicity.

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