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Original Article

Effect of Cadmium on Human Middle Ear Epithelial Cells

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OBJECTIVE: Cadmium (Cd^{2+}) exposure can occur through passive smoking, ambient air pollution, and food. Even low exposure can affect hearing and cause lung disease. Here we investigated whether cadmium causes cytotoxicity, induces inflammation, or increases mucin gene expression in immortalized human middle ear epithelial cells (HMEECs).

MATERIALS and METHODS: Cell viability was investigated using the MTT assay following Cd^{2+} treatment. Increases in apoptosis and necrosis were determined, and the production of reactive oxygen species (ROS) was measured. We analyzed the expression of an inflammatory cytokine (COX-2) gene and a mucin gene (MUC5AC) using RT-PCR.

RESULTS: Exposure to >20 μM Cd^{2+} caused a significant decrease in cell viability. Hoechst 33258 staining showed apoptotic morphology of heterogeneous intensity, condensation, and fragmentation after Cd2+ exposure. Cd^{2+} was shown to increase cell death by apoptosis and necrosis by annexin V-FITC/PI double staining. Cd^{2+} exposure increased ROS production and COX-2 and MUC5AC expressions.

CONCLUSION: Our findings suggest that environmental cadmium exposure is related to the development of otitis media.

KEYWORDS: Cadmium, human middle ear epithelial cells, otitis media

INTRODUCTION

Otitis media (OM) is a common inflammatory disease among children. This disease causes the highest number of visits by children to the doctor [1]. More than half of all children will have one or more episodes of OM by the age of 3 years [2]. Annually, OM costs up to $3–5 billion in the United States [1]. OM leads to conductive hearing loss and delays the development of speech, language, balance, and learning. Children with OM have a poor quality of life and suffer from sleep, loss of appetite, otalgia, and behavioral problems [3]. Therefore, the identification and control of risk factors for OM has significant implications for healthcare costs.

Cadmium (Cd^{2+}) is a heavy metal that is considered to be one of the most common environmental and occupational hazards [4]. Groups at high risk of cadmium exposure include people who live in contaminated areas, people who have a higher intake of cadmium through diet, or those who smoke cigarette as well as women [5]. The general population is exposed to cadmium via inhalation (smoking, ambient air) or ingestion (shellfish, offal, vegetables). In the non-smoking population, food is the most common source of cadmium intake [5].

A considerable proportion of non-smokers also have Cd^{2+} levels exceeding 0.5 μg/g, which can affect the kidneys and bone. It is known that even within the legal exposure limits (44.5 nmol/L), cadmium can cause dose-dependent hearing loss and lung disease [6, 7]. Cadmium is considered to be a human carcinogen, and it is related to the development of lung, prostate, breast, kidney, and bladder cancers [8]. In addition, exposure to cigarette smoke and ambient air pollutants containing cadmium is a risk factor for OM [2, 9, 10]. Cadmium-induced ototoxicity via ROS generation and apoptosis has been demonstrated in *in vitro* and *in vivo* experimental models [11]. However, no study has examined the effects of this heavy metal on the middle ear.

Therefore, this study investigated whether cadmium affects cell viability and induces inflammation in human middle ear epithelial cells (HMEECs).

MATERIALS and METHODS

This study was approved by the Soonchunhyang University Bucheon Hospital Institutional Review Board, and written informed consent was not requested.

This study was presented at the 20th Combined Congress of Otorhinolaryngology Head and Neck Surgery, 10 October 2014, Daejeon, Korea.

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Cell Culture

The HMEECs were kindly provided by Dr. David J. Lim [12] and were grown, as previously described [10]. Briefly, we mixed Dulbecco's modified Eagle's medium (DMEM) and bronchial epithelial basal medium (BEBM) (1:1) with other supplements to make growth medium: bovine pituitary extract (52 μg/mL), hydrocortisone (0.5 μg/mL), human epidermal growth factor (hEGF; 0.5 ng/mL), epinephrine (0.5 mg/mL), transferrin (10 μg/mL), insulin (5 μg/mL), triiodothyronine (6.5 ng/mL), retinoic acid (0.1 ng/mL), gentamycin (50 μg/mL), and amphotericin B (50 ng/mL). The incubator was maintained in a humidified atmosphere at 37°C containing 95% air with 5% CO_2. We changed the growth medium every third or fourth day. The doubling time of growth for HMEECs is approximately 3 days; the cells were used for the following studies after 6 days. After approximately 1 week, the cells were stimulated with 10 and 20 μM Cd^{2+} (Sigma; St Louis, MO, USA) suspended in phosphate-buffered saline (PBS) over 24 h. As a control group, some HMEECs were not treated with Cd^{2+}.

Cell Viability Assay

To assess the cell viability, we performed the MTT [3-(4.5-dimethylthiazoyl-2-yl) 2.5 diphenyl tetrazolium bromide; Sigma] assay. HMEECs were seeded in plates, and the culture medium was replaced after 24 h. Subsequently, 40 μL of fresh MTT (5 g/L DW) was added. After the mixture was shaken at room temperature for 30 min, the optical density was measured at 595 nm.

Hoechst 33342 Staining

The nuclei of HMEECs after 24-h treatment with 10 and 20 μM Cd^{2+} were stained using Hoechst 33342. The cells were washed twice with PBS. They were fixed with 3.7% glutaraldehyde for 10 min at room temperature. Then the cells were washed twice with PBS and stained with 10 μg/mL Hoechst 33342 (Sigma-Aldrich; St. Louis, MO, USA) for 10 min at room temperature in the dark. The cells were washed twice and then observed using an inverted fluorescence microscope (BX61; Olympus, Japan). Data were obtained from three repeated experiments.

Annexin V–fluorescein Isothiocyanate (FITC)/propidium Iodide (PI) Flow Cytometry

We seeded cells in six-well culture plates after treatment with 10 and 20 μM Cd^{2+} for 24 h. After collection, the cells were washed using PBS and resuspended in 1×binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$]. The cells were observed using an annexin V–FITC apoptosis detection kit according to the manufacturer's protocol (BD, San Diego, CA, USA). The cell cycle was evaluated using a flow cytometer (Beckman Coulter; Fullerton, CA, USA). Data were obtained from three repeated experiments.

ROS Activity Assays

HMEECs ($7\acute{}10^3$) were treated with Cd^{2+} for 6 and 24 h. ROS activity in the cells was quantified using an OciSelect ROS assay kit (Cell Biolabs; San Diego, CA, USA). Briefly, the cells were cleaned twice with PBS and then incubated with 100 μL of 2',7'-dichlorofluorescein-diacetate (DCFH-DA) in culture medium at 37 °C for 1 h. The cells were washed twice with Dulbecco's phosphate buffered saline (DPBS), harvested, and analyzed by flow cytometry with a fluorometric plate reader at 480 nm/530 nm.

Real Time-RT Polymerase Chain Reaction

RNeasy® Mini Kits (QIAGEN; Hilden, Germany) were used to extract RNA from the HMEECs. The primer sequences of the genes were as follows: COX2, 5'GTCAAAACCGAGGTGTATGT3'; Muc5AC, 5'CAGCACAACCCCTGTTTCAAA-3'; β-actin, 5'GGACTTCGAGCAAGAGATGG3'. PCR amplification was performed using the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 20 s, 55°C for 10 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 min. Results were obtained from three repeated experiments using triplicate samples.

Statistical Analysis

The statistical significance of differences among the experimental groups was determined using one-way analysis of variance (ANOVA). For multiple comparisons, Scheffe's F-test was used. A *P*-value of <0.05 for the null hypothesis was considered to indicate a statistically significant difference. SPSS for Windows version 20.0 (IBM SPSS; Chicago, IL, USA) was used for the statistical analyses. This study was approved by the institutional review board of Soonchunhyang University [SCHBC_IRB_2012-157].

RESULTS

Cd^{2+} Reduced Cell Viability of HMEECs

The results of the cell viability assays showed that exposure to more than 20 μM Cd^{2+} for 6~24 h significantly decreased HMEEC viability as compared with the controls ($p<0.05$; Figure 1).

Cd^{2+} Induced Apoptosis and Necrosis in HMEECs

The nuclei of the normal control cells were round and stained homogeneously. However, the addition of 10 μM Cd^{2+} resulted in nuclei with the characteristic apoptotic morphology of heterogeneous intensity, condensation, and fragmentation. These findings were observed more frequently at 20 μM Cd^{2+} (Figure 2).

Flow cytometry analysis with annexin V–FITC/PI double staining showed that 20 μM Cd^{2+} increased apoptosis and necrosis in HMEECs. Following incubation with 20 μM Cd^{2+} for 24 h, approximately five-fold more apoptotic and necrotic cells showed positive staining compared with the controls (Figure 3).

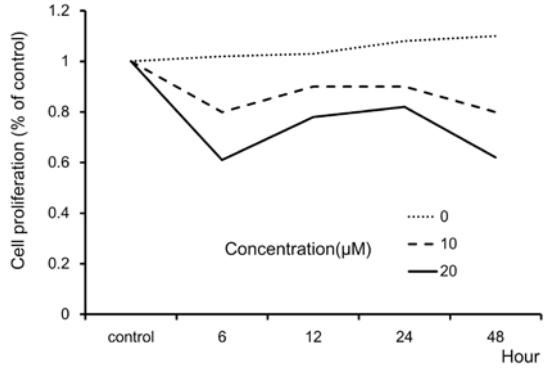

Figure 1. Cell viability of human middle ear epithelial cells (HMEECs) following exposure to cadmium. Exposure to 20 μg/mL cadmium for 24 h caused significant decreases in HMEEC viability compared with controls ($p<0.05$). The data shown represent the mean ± SD of three repeated experiments with six samples. *: $p<0.05$ compared with controls at each dosage, determined by ANOVA

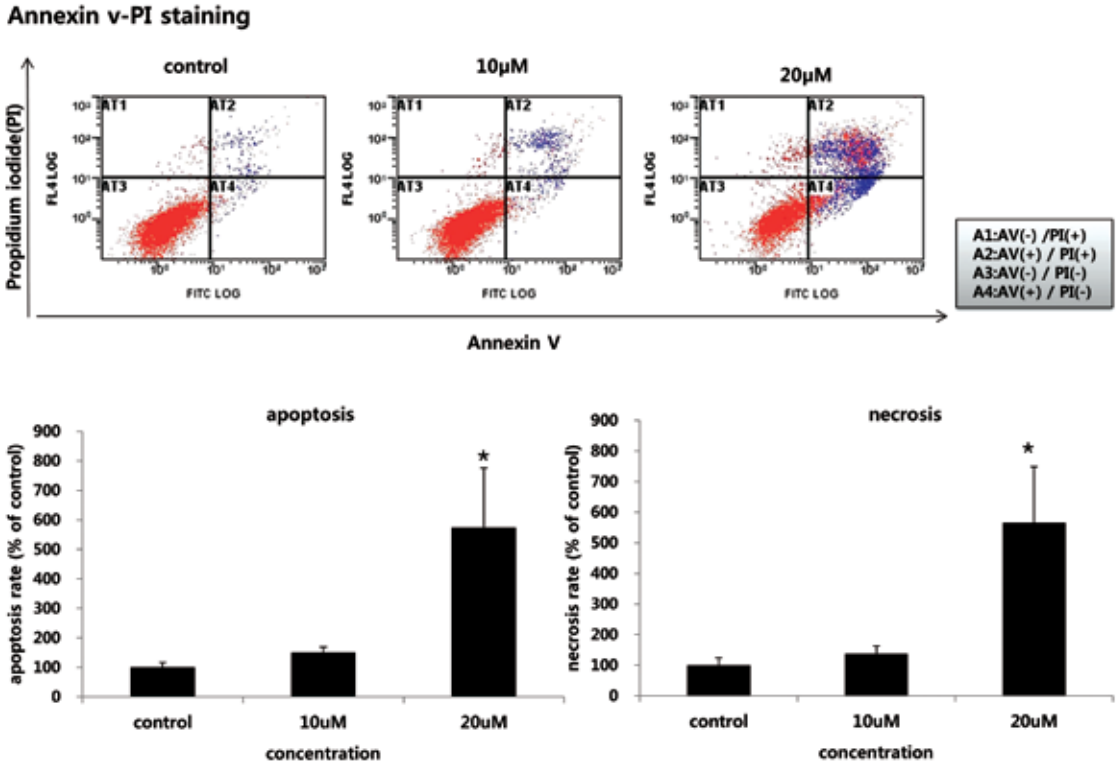

Figure 2. Flow cytometry analysis of HMEECs following exposure to acrolein with annexin V–FITC/PI double staining. After treatment with 10 μM cadmium for 24 h, the number of apoptotic or necrotic cells stained by annexin V–FITC or PI did not increase. Following treatment with 20 μM cadmium, the number of apoptotic or necrotic cells increased by approximately five-fold. Data are presented as the mean±SD from triplicate determinations and three independent experiments. Two other independent experiments produced similar results. *: p<0.05 compared with controls at each dosage, determined by ANOVA

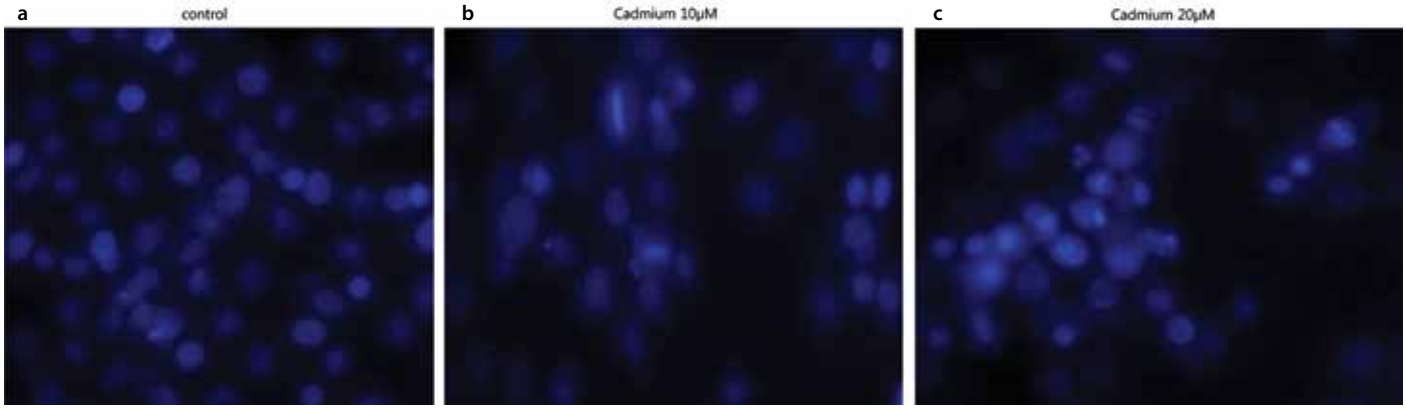

Figure 3 a-c. Nuclear morphology of HMEECs following exposure to cadmium by Hoechst 33342 staining. Exposure to 10 and 20 μM cadmium (b, c) showed the typical apoptotic nuclear morphology (i.e., heterogeneous intensity, condensation, and fragmentation) when compared with control cells (a)

Cd^{2+} Increased ROS Production in HMEECs

The effect of oxidative stress injuries caused by Cd^{2+} was measured using intracellular reactive oxygen species (ROS) generation using flow cytometry. The results showed that exposure of HMEECs to 20 μM Cd^{2+} for 6 and 24 h significantly increased the intensity of dichlorofluorescein (DCF) fluorescence, which is an indicator of ROS formation (Figure 4).

Cd^{2+} Increased Inflammatory Cytokine and Mucin Gene Expression in HMEECs

When the cells were stimulated with Cd^{2+} concentrations of 20 and 30 μg/mL for 24 h, the expression of COX-2 increased by 8.23±0.81 times and 18.56±0.82 times, respectively (p<0.05; Figure 5a). The MUC5AC expression in HMEECs increased on stimulation with >20 μg/mL Cd^{2+} for 24 h (p<0.05; Figure 5b).

DISCUSSION

In this study, we demonstrated that Cd^{2+} affects the cell viability of HMEECs and increases apoptosis. Cd^{2+} increased ROS production and induced inflammation and mucin gene expression. Cadmium can reduce cell viability in the inner hair cells, central nervous system, lung, bone, kidney, and liver. Cadmium affects the proliferation and differentiation of cells. Cadmium damages DNA, membranes, and proteins, and inhibits DNA repair in different ways [4, 12-14]. In this study,

we demonstrated that Cd^{2+} also decreases the cell viability of middle ear epithelial cells.

Cadmium increases apoptosis of various cells, such as HeLa cells, endothelial cells, kidney tubular epithelial cells, glioma cells, and fibroblasts. Cadmium induces both extrinsic and intrinsic apoptosis pathways. Cadmium-mediated apoptosis is related to Fas, Capase-8,9, and p53 [12]. It is known that cadmium reduces cell viability by increasing oxidative stress [4]. Cadmium decreases microvessel antioxidant defense systems and increases lipid peroxidation. In addition, cadmium increases ROS production [4, 15]. ROS production has a greater effect in acute poisoning [16]. The free radical scavenger, N-acetylcysteine, protects against cadmium-induced apoptosis in human breast cancer cells; the cytotoxic effect of cadmium is mediated by oxidative stress [17]. In this study, we demonstrated that Cd^{2+} also increases apoptosis and ROS production in HMEECs. Interestingly, Cd^{2+} produced more ROS at 6 h than at 24 h. Several antioxidants have protective effects on Cd^{2+}-induced toxicity and ototoxicity by decreasing ROS generation and inhibiting apoptosis pathways [11, 14, 17, 18].

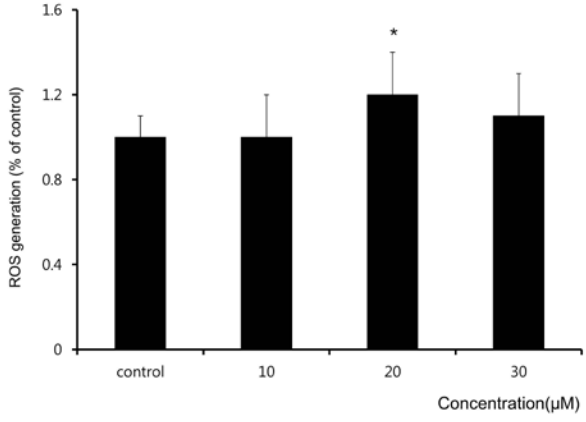

Figure 4. Effect of cadmium on oxidative stress damage in HMEECs. Exposure to 10 or 20 μM cadmium increased ROS generation. Data are presented as the mean±SD from triplicate determinations and three independent experiments. Two other independent experiments produced similar results.
*: p<0.05 compared with controls at each dosage, determined by ANOVA.

Cadmium also induces cell injury through epigenetic changes in DNA expression or inhibition of cellular transport pathways. In addition, cadmium competes with zinc and magnesium, which are needed for heme synthesis or mitochondrial function [12, 19]. Cadmium also impairs immune function, arrests the cell cycle, and induces apoptosis in splenocytes; furthermore, it increases the rates of autoimmunity and production of non-specific antibodies. It also decreases lymphocyte proliferation and natural killer cell activity [19-21].

Inflammatory cytokines and mucin secretion have important roles in the development of OM. Inflammatory cytokines, including COX-2, TNF-α, NF-κB, IL-1, IL-6, and IL-8, play a critical role in immune regulation, the initiation of mucosal changes, the inflammatory response in the middle ear, and stimulating mucin secretion [22, 23]. Increased inflammatory cytokine and mucin gene expression have been demonstrated in cigarette smoke- and diesel-induced OM [9, 10]. Inhalation of cadmium resulted in increased TNF- α, IL-6, and macrophage inflammatory protein (MIP-2) in rat lungs [24]. Cadmium oxide nanoparticles are used in optoelectronic devices and medical imaging devices; by one day post-exposure, decreased lavagable cell viability, and increased proinflammatory cytokines such as IL-1β, TNF- α, and interferon-γ were detected [25]. To our knowledge, our study is the first to report induced mucin gene expression by Cd^{2+}. We also demonstrated increased expression of COX-2.

Human exposure to cadmium occurs mainly through air pollutants or food. We believe that cadmium can access the middle ear space through the Eustachian tube, like other air pollutants or smoke, or can enter the middle ear space via systemic circulation. Cadmium has been found in ossicles after systemic exposure [13], and its uptake has also been demonstrated in nasal mucosa or olfactory pathways [26]. There is no treatment for cadmium toxicity. EDTA and other chelators show limited therapeutic effects on humans and animals.

In conclusion, our study provides a link that helps to explain the causal contribution of environmental cadmium exposure to otitis media. This *in vitro* study has the limitation of applying the results to the human middle ear. In future, *in vivo* studies and clinical trials are needed to confirm the effects of cadmium in the middle ear.

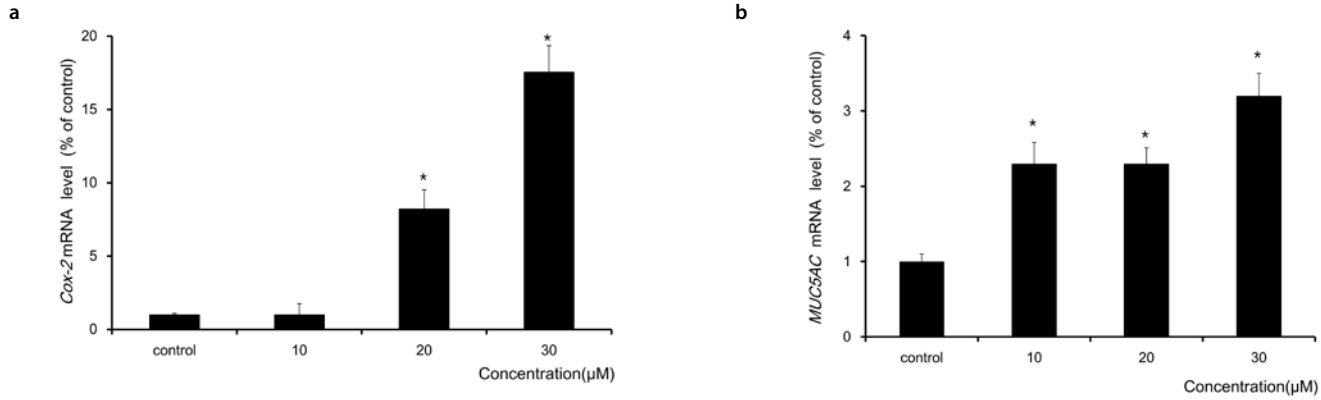

Figure 5 a, b. Upregulation of COX-2 and MUC5AC by cadmium exposure in HMEECs. RT-PCR was used to measure COX-2 and MUC5AC mRNA in cadmium-exposed HMEECs (a) The expression of COX-2 mRNA was upregulated by treatment with 20 and 30 μg/mL cadmium for 24 h (b) The expression of MUC5AC mRNA was upregulated by treatment with 10 to 30 μg/mL cadmium for 24 h. The data shown represent the mean±SD from three repeated experiments with triplicate samples. *: p<0.05 compared with controls at a specific time or dose (determined by ANOVA).

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Soonchunhyang University Bucheon Hospital Institutional Review Board.

Informed Consent: Written informed consent was not requested for this basic study since we used immortalized human middle ear cells.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - J.J.S., A.S.J., M.K.P.; Design - J.J.S., A.S.J., M.K.P.; Supervision - J.J.S., A.S.J., M.K.P.; Resources - J.Y.K., Y.C.R., M.P., M.K.P.; Materials - J.Y.K., Y.C.R., M.P., M.K.P.; Data Collection and/or Processing - J.Y.K., S.H.K., Y.C.R., M.P., M.K.P.; Analysis and/or Interpretation - J.Y.K., S.H.K., Y.C.R., M.P., M.K.P.; Literature Search - M.K.P., S.H.K., M.P.; Writing Manuscript - J.Y.K., S.H.K., Y.C.R., M.K.P.; Critical Review - J.J.S., A.S.J., M.K.P.

Conflict of Interest: No conflict of interest was declared by the authors.

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