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Protective effects of caffeic acid phenethyl ester (CAPE) against neomycin-induced hair cell damage in zebrafish

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ABSTRACT

Objective: Caffeic acid phenethyl ester (CAPE) is known to reduce the generation of oxygen-derived free radicals, which is a major mechanism of aminoglycoside-induced ototoxicity. The objective of the present study was to evaluate the effects of CAPE on neomycin-induced ototoxicity in zebrafish (Brn3c: EGFP).
Methods: Five-day post-fertilization zebrafish larvae ($n = 10$) were exposed to 125 μM neomycin and one of the following CAPE concentrations for 1 h: 50, 100, 250, 500, or 1000 μM. Ultrastructural changes were evaluated using scanning electron microscopy (SEM). The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) assay and 2-[4-(dimethylamino)styryl]-*N*-ethyl-pyridiniumiodide (DASPEI) assay were performed for evaluation of apoptosis and mitochondrial damage.
Results: CAPE decreased neomycin-induced hair cell loss in the neuromasts (500 μM CAPE: 12.7 ± 1.1 cells, 125 μM neomycin only: 6.3 ± 1.1 cells; $n = 10$, $P < 0.05$). In the ultrastructural analysis, structures of mitochondria and hair cells were preserved when exposed to 125 μM neomycin and 500 μM CAPE. CAPE decreased apoptosis and mitochondrial damage.
Conclusion: In the present study, CAPE attenuated neomycin-induced hair cell damage in zebrafish. The results of the current study suggest that neomycin induces apoptosis, and the apoptotic cell death can be prevented by treatment with CAPE in zebrafish.

1. Introduction

Aminoglycosides are clinically important antibiotics with a broad antibacterial spectrum. They induce no allergic response and are easy to use in an emergency situation. Although aminoglycosides are notorious for their ototoxicity and nephrotoxicity, they are still widely used for treatment of aerobic, gram-negative and some gram-positive bacterial infections. In addition, the use of aminoglycosides is recommended in cases of multidrug-resistant tuberculosis. They are more widely used in developing countries due to their low cost [1,2].

Hair cell death by aminoglycosides is due to both apoptosis and necrosis. The overproduction of reactive oxygen species (ROS) is an important toxic action of aminoglycosides toward hair cells [2,3]. Although the details of ROS formation differ depending on the circumstances, ROS overproduction is a mechanism of hair cell damage in noise, aging, and ototoxicity. Many antioxidants have shown a protective effect on hair cells in various models of damage both *in vitro* and *in vivo* [1–4].

Caffeic acid phenethyl ester (CAPE) is an active component of honey bee propolis extracts. It is a natural phenolic chemical compound and superior antioxidant. In addition, it has anti-inflammatory, antimicrobial, immunomodulatory, and anticarcinogenic effects. It has been used in folk medicine for many years, and many experimental studies have recently shown its antioxidative effect [5,6].

These findings lead us to believe that CAPE may be a good candidate for prevention of ototoxicity by aminoglycosides. Thus, the aim of the current study was to investigate whether CAPE has protective effects on neomycin-induced hair cell damage in transgenic zebrafish.

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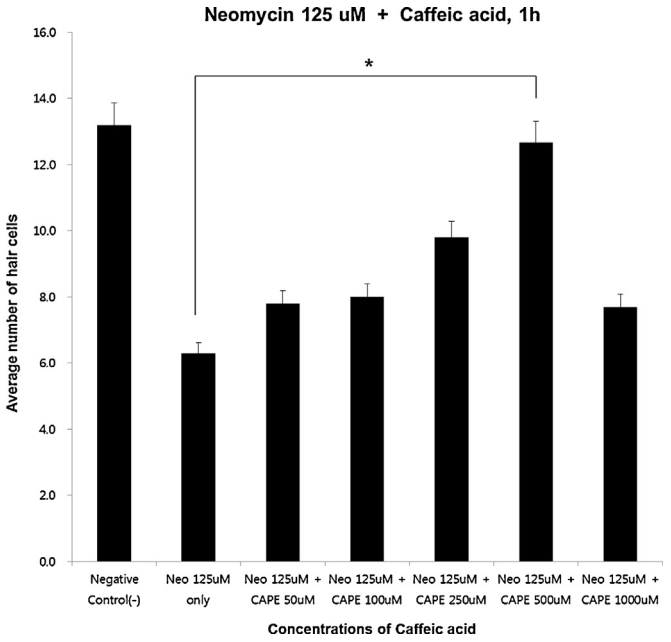

Fig. 1. Quantitative assay of neuromasts in zebrafish. Hair cells from four neuromasts (SO1, SO2, O1, and OC1) were counted. Hair cell survival was calculated as a percentage of the control group. Treatment of the zebrafish with 125 μM neomycin (neo) for 1 h significantly decreased the number of hair cells in neuromasts. Caffeic acid phenethyl ester (CAPE) protected against neomycin-induced hair cell loss of the neuromasts in zebrafish ($n = 10$, $P < 0.05$).

2. Methods

2.1. Chemical materials

Neomycin and caffeic acid phenethyl ester (CAPE) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Zebrafish model

Zebrafish larvae (Brn3c: EGFP) were produced by pairwise matings, raised at 28.5 °C in egg water or embryo medium (EM; 15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM NH_2PO_4, and 0.7 mM $NaHCO_3$) [7], and staged according to days post-fertilization (dpf) and morphological criteria [8]. The larvae were collected and raised at 28.5 °C. The current study was approved by the Korea University Institutional Animal Care and Use Committee (Approval No. KUIACUC-2012-116). All methods were performed in accordance with the guidelines of the Animal Care Ethics Committee of Korea University Medical Center and National Institutes of Health (NIH) guidelines.

2.3. Chemical administration and zebrafish preparation

Neomycin solutions were prepared by adding the neomycin powder to the embryo medium. Neomycin has been shown to decrease the viability of neuromasts in a dose-dependent manner. We previously reported that neuromasts treated with 125 μM neomycin for 1 h show a viability rate of 50% [9]. Thus, that concentration was chosen for the following study. For the current experiment, 10 fish were tested at each of the following concentrations of caffeic acid and repeated three times. The 5-dpf zebrafish larvae were exposed to 125 μM neomycin and 50, 100, 250, 500, and 1000 μM CAPE for 1 h. The larvae were then washed with embryo medium three times. The fish were anesthetized with tricaine (3-aminobenzoic acid 0.4 g/ethyl ester; 100 mL; pH 7, adjusted using Tris buffer) for 5 min [10–12].

2.4. Evaluation of zebrafish hair cells

The zebrafish were placed with methylcellulose on a depression slide for evaluation of hair cell damage under a fluorescence microscope. Hair cells within neuromasts of the supraorbital (SO1 and SO2), otic (O1), and occipital (OC1) lateral lines on one side of each fish were analyzed [10–15]. The average numbers of hair cells of the SO1, SO2, O1, and OC1 neuromasts were evaluated in each zebrafish for all experimental and control conditions ($n = 10$) using fluorescence microscopy (AxioCam MRc5; Carl Zeiss).

2.5. Terminal deoxynucleotidyl transferase (TdT)-Mediated dUTP-biotin nick end labeling (TUNEL) assay for apoptosis evaluation and 2-[4-(dimethylamino)styryl]-N-ethylpyridiniumiodide (DASPEI) assay for mitochondria

Apoptotic cells in zebrafish were identified by the TUNEL method using an *in situ* cell detection kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. The larvae were exposed to medium containing 125 μM neomycin and 500 μM CAPE for 1 h. The larvae were then washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde. Next, the larvae were incubated with 50 μL of TUNEL reaction mixture (TdT and fluorescein-dUTP) at 37 °C for 60 min in a humid atmosphere. The 5-dpf zebrafish larvae were evaluated using a fluorescence microscope. In the current study, the fluorescent dye 2-[4-(dimethylamino)styryl]-*N*-ethylpyridinium iodide (DASPEI;

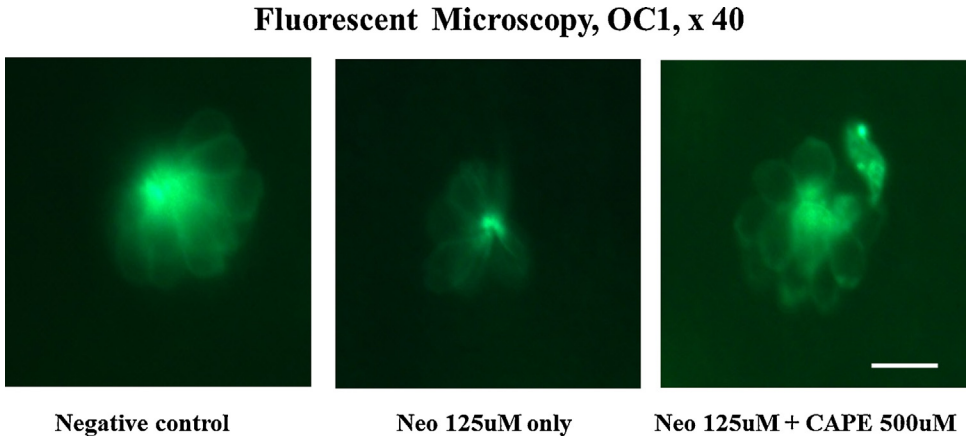

Fig. 2. Fluorescence microscopy images (OC1, ×40). Zebrafish (5 dpf) were treated with 125 μM neomycin (neo) and 500 μM CAPE for 1 h. Treatment with neomycin resulted in a significant decrease in the number of hair cells of the neuromasts. CAPE attenuated the neomycin-induced hair cell damage. Bar = 10 μm.