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Protective effect of an astaxanthin nanoemulsion against neomycin-induced hair-cell damage in zebrafish

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1. Introduction

Degeneration of the mechanosensory hair cells of the inner ear sensory epithelia is the main cause of deafness. Hair cells are commonly lost through drug-and noise-induced trauma, as well as aging. Many recent studies have reported that the cause of damage to these hair cells is oxidative stress due to free radicals. In particular, hair cells are very susceptible to aminoglycosides [1]. Antioxidants reduce the generation of oxygen-derived free radicals, and various antioxidants have been marketed, with different dosage forms available for each agent. We focused on astaxanthin as a strong antioxidant.

Astaxanthin is an oceanic carotenoid that is distributed in marine bacteria, shellfish, algae, and fish [2]. It provides the best protection against free radicals in rats, followed by lutein and β-carotene [3]. The antioxidant activity of astaxanthin has been shown to be 10 times that of lutein, canthaxanthin, and β-carotene. However, astaxanthin is fat soluble, which hinders its absorption from the intestinal tract and skin [4]. Therefore, a nanopreparation of astaxanthin has been developed that is more stable and well absorbed [4]. Using zebrafish lateral-line hair cells, we investigated the protective effects of astaxanthin against neomycin-induced hair-cell death.

2. Materials and methods

2.1. Animals

Zebrafish (*Danio rerio*) embryos of the wild-type strain were produced by paired matings of adult fish. Embryos were maintained at a density of 50 per 100-mm^2 petri dish in an embryo medium (1 mM $MgSO_4$, 120 μM KH_2PO_4, 74 μM Na_2HPO_4, 1 mM $CaCl_2$, 500 μM KCl, 15 μM NaCl, and 500 μM $NaHCO_3$ in dH_2O) at 28.5 °C until 4 or 5 days post fertilization, when the experiments were conducted. This study was reviewed by the Committee for the Ethics of Animal

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Experiments of the Yamaguchi University School of Medicine and was carried out according to the Guidelines for Medicine and the Law (No. 105) and Notification No. 6 of the Japanese government.

2.2. Chemicals

Neomycin sulfate solution at a concentration of 10 mg/mL was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The nanoemulsion of astaxanthin was obtained from FUJIFILM Co. (Kanagawa, Japan). The astaxanthin suspension was prepared using conventional astaxanthin powder obtained from Sigma Chemical Co.

2.3. Chemical interventions

2.3.1. Dose-response testing of neomycin

Zebrafish (5 days post fertilization (dpf)) were exposed to neomycin at concentrations of 0, 10, 50, 100, 200, and 400 μ for 1 h.

2.3.2. Protective effects of astaxanthin

Zebrafish larvae (n = 10) were exposed to the astaxanthin nanoemulsion (0.01, 0.1, 1, or 10 μM) or the suspension for 1 h, or were left unexposed. Subsequently, the larvae were exposed to neomycin for 1 h by adding the neomycin solution to make up a concentration of 200 mM.

2.4. Immunohistochemical examination

The larvae were washed with the embryo medium, then anesthetized with MS222 (0.5 mM 3-aminobenzoic acid ethyl ester) for 1 min. After fixation in 4% paraformaldehyde, the zebrafish larvae were rinsed three times in phosphate–buffered saline (PBS), then placed in a blocking solution (1% Triton-X and 5% normal goat serum [NGS] in PBS) for 1–2 h at room temperature. The larvae were then incubated with an anti-parvalbumin antibody obtained from Sigma Chemical Co. (monoclonal, 1:400 in 1% Triton-X and 1% NGS in PBS) at 4 °C overnight, then rinsed three times in 1% Triton-X in PBS (PBS-T) and incubated with Alexa 594 goat anti-mouse fluorescent antibody (1:500 in 1% Triton-X and 1% NGS in PBS) for 3–4 h. Following secondary antibody labeling, the larvae were rinsed three times in PBS followed by three rinses in PBS-T and mounted between two coverslips for imaging with a fluorescence microscope (XF- EHD2; Nikon, Tokyo, Japan). Hair cells from supraorbital line 1 (SO1), supraorbital line 2 (SO2), orbital line 1 (O1), and occipital line (OC1) neuromasts were counted [5] (Fig. 1). Fig. 2 shows the protocol used for these experiments. Results were calculated as the mean hair-cell survival and are expressed as a percentage of the control.

2.5. Quantification of anti-oxidant capacity

The OXY-Adsorbent test (FREE Carpe Diem; Diacron International, Grosseto, Italy) was used to quantify the antioxidant capacity of zebrafish larvae exposed to astaxanthin nanoemulsion by evaluating the capacity of each sample to inactivate the oxidant solution (HClO) added in excess. Thirty larvae were exposed to astaxanthin for 2 h, then rinsed three times in the embryo medium. The larvae only were then placed in a micro tube and homogenized, then centrifuged for 5 min. The OXY-adsorbent test was performed using the supernatant as the sample. All standards and samples were diluted 1:30 prior to analysis. During the automated procedure, 10 μL sample was added to 1 mL HClO solution, followed by 10 μL *N*,*N*-diethyl-*p*-phenylendiamine [6] after 10 min incubation. This amine is oxidized by the residual HClO, and the reaction produces a pink-colored product, which can immediately be measured at 520 nm [6]. The antioxidant capacity is expressed indirectly as the quantity of HClO in micromoles consumed by 1 mL of the sample (μmol HClO/mL) [6].

2.6. Detection of reactive oxygen species (ROS) with 2′,7′-dichlorodihydrofluorescin diacetate (H2DCFDA)

We measured ROS generation using H2DCFDA, a membrane-permeable fluorescent dye (Molecular probes, D-399). Neuromast hair cell nuclei were labeled by incubating with 0.1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) (Sigma D-9542) dissolved in E3 medium for 5 min. Larvae were treated with 200 mM neomycin sulfate (Sigma, N-1876). After the treatments, larvae were anesthetized and mounted for observation under a fluorescent microscope.

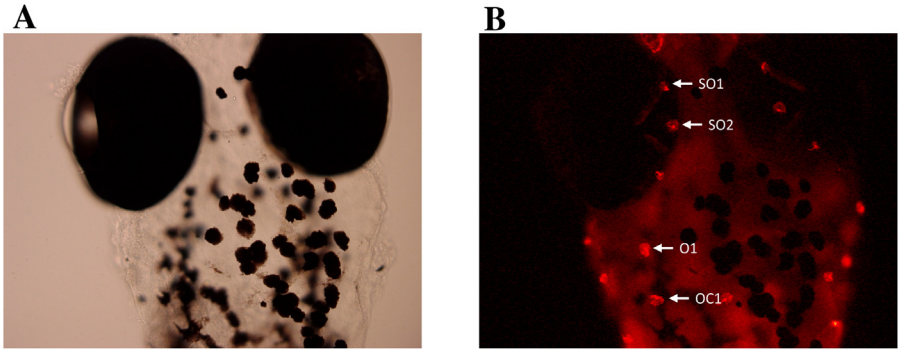

Fig. 1. (A) Optical microscopy images of zebrafish. (B) Fluorescent microscope images of zebrafish lateral-line neuromasts. Neuromast hair cells from the supraorbital line 1 (SO1), supraorbital line 2 (SO2), orbital line 1 (O1), and occipital line (OC1) were counted.

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