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Diosmetin protects against retinal injury via reduction of DNA damage and oxidative stress

Zeren Shen[a,b,1], Jinjin Shao[a,1], Jiabin Dai[a], Yuchen Lin[a,b], Xiaochun Yang[a], Jian Ma[c], Qiaojun He[a], Bo Yang[a], Ke Yao[b], Peihua Luo[a,*]

[a] Institute of Pharmacology & Toxicology, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, P.R. China
[b] Eye Center, Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, P.R. China
[c] Center for Drug Safety Evaluation and Research of Zhejiang University, Hangzhou, P.R. China

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ABSTRACT

Visual impairment is a global public health problem that needs new candidate drugs. *Chrysanthemum* is a traditional Chinese drug, famous for its eye-protective function, with an unclear mechanism of action. To determine how *chrysanthemum* contributes to vision, we identified, for the first time, the component of *chrysanthemum*, diosmetin (DIO), which acts in protecting the injured retina in an adriamycin (ADR) improving model. We observed that DIO could attenuate the apoptosis of retinal cells in Sprague–Dawley rats and verified this effect in cultured human retinal pigment epithelium (RPE) cells, ARPE-19. Our further study on the mechanism revealed the counteractive effect of DIO on the attenuation of DNA damage and oxidative stress, which occurs in a wide range of retinal disorders. These results collectively promise the potential value of DIO as a retinal-protective agent for disorders that lead to blindness. In addition, we identified, for the first time, the component of *chrysanthemum*, DIO, which acts in protecting the injured retina.

1. Introduction

Recently, it was observed that there is a trend of people having a greater risk of visual impairment as a result of aging, light pollution and greater propensity for visually disabling conditions. To address the issue of decreased vision, more drugs that can improve eyesight need to be made available. Turning to Chinese medicine, the *chrysanthemum*, cultivated in China as a flowering herb dates back to the 15th Century B.C [33] http://www.mums.org/history-of-the-chrysanthemum/, December 10, 2014). It has long been prescribed for the treatment of eye diseases in Chinese traditional preparations. Recent studies have shown that flavonoids in *chrysanthemum* are more likely to contribute to curing eye diseases [1,21,23,30,41]. Despite of recent progress, the definite component of *chrysanthemum* and the mechanism of action in the way *chrysanthemum* contributes to vision remains to be elucidated.

Diosmetin (3′,5,7-trihydroxy-4′-methoxyflavone) is the aglycone of the flavonoid glycoside diosmetin-7-O-β-D-glucoside, which occurs naturally in the *chrysanthemum* flower heads. This glucoside is hydrolyzed to its aglycone diosmetin (DIO) by intestinal microflora enzymes prior to its absorption into the body. Pharmacologically, it has been established that DIO possesses strong antioxidant properties [7,25,50]. In addition, the latest study demonstrated that DIO could significantly enhance the adenosine triphosphate (ATP) levels in cells [36]. Because many retinal diseases are related to excessive oxidant stress and limited ATP release [3,14,26,35,38], we hypothesized that DIO is the key factor by which *chrysanthemum* improves eye function based on the analysis of its protective effect related to its potent antioxidant and increased ATP in the retina.

Abbreviations: ADR, adriamycin; AMD, age-related macular degeneration; ATP, adenosine triphosphate; CNV, choroidal neovascularisation; DIO, diosmetin; H&E, hematoxylin and eosin; IC_{50}, inhibition for 50% of the cells; IVI, intravitreal injection; PVR, proliferative vitreoretinopathy; ROS, reactive oxygen species; RPE, retinal pigment epithelium.

* Corresponding author at: Institute of Pharmacology & Toxicology, College of Pharmaceutical Sciences, Zhejiang University, 866 Yuhangtang Road, Zijingang Campus, Zhejiang University, Hangzhou, Zhejiang, P.R. China. Fax: +86 571 88208400.
E-mail address: peihualuo@zju.edu.cn (P. Luo).
[1] These authors contributed equally to the manuscript.

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Adriamycin (ADR), also called doxorubicin, is an antibiotic anthracycline that is widely applied in ophthalmology against several proliferative and angiogenic eye diseases, such as proliferative vitreoretinopathy (PVR) [32], choroidal neovascularization (CNV) [46] and some ocular tumors [18,34], because of its anti-angiogenic and anti-proliferative properties. However, the efficacy of ADR for eye diseases is limited by its toxic retinal effects [13,46] at two fundamental levels, altering DNA and producing free radicals [11]. These events are observed in many potentially blinding eye conditions, such as AMD [3,22], diabetic retinopathy [26], hypertensive retinopathy [32], light-induced retinal damage [27], and more. Therefore, we used ADR-induced retinal toxicity as an example for investigating how DIO protects the retina.

In this study, we investigated the role and mechanism of DIO cytoprotection in the retina. First, we demonstrated that this flavonoid from *chrysanthemum* could protect the retina from apoptosis via reducing DNA damage and oxidative stress. Moreover, this finding favors DIO as a potential retinal-protective drug candidate for alleviating the severity of eye diseases in the clinic, and it can be developed as a complementary medicine.

2. Materials and methods

2.1. Drugs and chemicals

Diosmetin (purity: 99.0%) was purchased from Nanjing Zelang Medical Technology Company (Nanjing, China). Adriamycin was a generous gift from Zhejiang Cancer Hospital (Hangzhou, China). A stock solution of Adriamycin (50 mM) and Diosmetin (50 mM) was prepared with dimethyl sulfoxide (DMSO) and stored at −20 °C. The stock solution was further diluted with the appropriate assay medium immediately before use. The final DMSO concentration did not exceed 0.2% throughout the study. Antibodies for procaspase-3, cleaved caspase-3, PARP, β-action, Bcl-2 and γ-H2AX were purchased from Santa Cruz Biotechnology (CA, USA). Secondary anti-mouse, anti-goat and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (CA, USA). The western blot detection reagent ECL was purchased from Pierce Biotechnology (Rockford, USA). The TUNEL cell apoptosis detection kit was purchased from Beyotime Institute of Biotechnology (Haimen, China). The Annexin V FITC-Propidium Iodide (PI) kit was purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Animal treatment and drug administration

Animal care procedures were approved by National Institute of Health Guide for the Care and Use of Laboratory Animals and were in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Sprague–Dawley male rats (body weight of 190–220 g, 6 weeks old) were supplied by the Shanghai Laboratory Animal Center, Chinese Academy of Sciences and housed in a clean grade room at 21 ± 1 °C and 60 ± 5% humidity under a 12-h light/dark cycle. Rats were fed sterile tap water and chow diet ad libitum from Shanghai SLAC Laboratory Animal Co., Ltd.

Three groups of rats ($n = 8$ in each group) received 5 μl intravitreal injections in the right eye with ADR, DIO, or both, through a Hamilton syringe with a 30-gauge needle into the inferotemporal part of the eye. The other eye was injected with the same volume of vehicle (0.015% DMSO in saline, highest DMSO concentration among drug groups) and served as a control. According to the drug concentration in vitro, rats were treated with ADR (1.5 μM/eye) and DIO (6 μM/eye) through IVI 5 days before they were euthanized. The eyeballs were surgically excised and fixed in phosphate-buffered 10% formaldehyde. The fixed eyes were sectioned at the pars plana area and the posterior segment was dissected into tissue samples for microscopic observation or stored in tissue protein extraction reagent for western blot analysis.

2.3. Histopathological analysis

Retina samples were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) for histopathological analysis and the central parts of the lesions were examined by light microscopy.

2.4. TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assays were performed with a one-step TUNEL kit according to the manufacturer's instructions. Retina samples were treated as mentioned in the histopathological analysis. Briefly, retina tissue sections were pretreated with proteinase K, washed with PBS, and then stained by TUNEL reaction mixture (label and enzyme solutions) for 1 h at 37 °C. The FITC-labelled TUNEL-positive cells were imaged under a fluorescent microscope (DMI 4000 B, Leica, Germany) using 488 nm excitation and 530 nm emission. The cells with green fluorescence were defined as apoptotic cells.

2.5. Western blot analysis

The protein samples of the dissected retina or ARPE-19 cells were extracted in lysate buffer and the total protein concentration of whole cell lysates was determined using the Bradford method (BioRad, Hercules, CA, USA). 40.0–80.0 μg of total proteins were loaded per lane and fractionated on 10–15% Tris glycine precast gels, transferred to PVDF membrane (Millipore, Bedford, MA), and probed with primary antibodies and then HRP-labeled secondary antibodies. Proteins were visualized using ECL.

2.6. Cell lines and cell culture

ARPE-19 cells (from ATCC cell line) were cultured in DMEM/F12 medium supplemented with 10% heat-inactivated foetal serum. The cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere, and the medium was changed every other day.

2.7. Cytotoxicity assay

ARPE-19 cells were seeded in 96-well plates (3×10^4/well). After treatment with varying concentrations of ADR (0–1.5 μM) and DIO (0–8.0 μM), viable cells were determined using an MTT assay. MTT was added (30.0 μl/well), and plates were incubated for an additional 4 h at 37 °C. The purple formazan crystals were dissolved in 100 μl of DMSO. After the crystal dissolved, the plates were read on an automated microplate spectrophotometer (ThermoMultiskan Spectrum, Thermo Electron Corporation, USA) at 570 nm. The concentration of drug inhibition for 50% of the cells (IC_{50}) was calculated using the PrismPad computer program (GraphPad Software Inc., USA) with Microcomputers.

2.8. Flow cytometry analysis of cell apoptosis

The Annexin V FITC-Propidium Iodide (PI) kit was used to detect cell apoptosis. The cells were grown on a six-well plate at 1×10^5 cells/well and treated with drugs for 48 h at 37 °C. The cells were washed twice and collected with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4). Staining for apoptosis was performed according to the manufacturer's instructions. PI-negative, Annexin V-negative staining cells are considered to be live