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Research article

Neuroprotective effect of epigallocatechin-3-gallate in a mouse model of chronic glaucoma

Ceying Shen[a,1], Lanlan Chen[a,1], Libin Jiang[a,*], Timothy Y.Y. Lai[b]

[a] Beijing Ophthalmology and Visual Sciences Key Laboratory, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing, China
[b] Department of Ophthalmology & Visual Sciences, The Chinese University of Hong Kong, Hong Kong Eye Hospital, Kowloon, Hong Kong; Retina & Macula Centre, Kowloon, Hong Kong

HIGHLIGHTS

- Consumption of EGCG plays a neuroprotective role on RGCs in a mouse model of chronic high IOP.
- Consumption of EGCG had no effect on the IOP value of mice.
- It is the first report studied in ophthalmologic neuroprotective effects of EGCG by oral administration in mice.

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ABSTRACT

Epigallocatechin-3-gallate (EGCG) is a powerful antioxidant with suggested neuroprotective action. This study investigated the protective effects of EGCG against retinal ganglion cells (RGCs) degeneration in an animal model of glaucoma. C57BL/6J mice ($n = 54$) were divided randomly into four groups: normal control group (group A, $n = 12$); EGCG control group with EGCG in drinking water (group B, $n = 12$); microbeads control group with anterior chamber microbeads injection to induce elevation in intraocular pressure (IOP) plus normal drinking water (group C, $n = 18$); and EGCG study group receiving an anterior chamber microbeads injection plus EGCG in drinking water (group D, $n = 12$). Animals were treated orally with either vehicle or EGCG (50 mg/kg × d). IOP was measured and animals were sacrificed at days 15 and 27. Neurons were retrograde-labeled by fluorogold and immnunolabeled by class III β-tubulin to quantify RGCs in the retinal ganglion cell layer on flat mounts histologically and compared. All mice that received microbeads injections (groups C and D) developed IOP elevation higher than un-injected control mice. At days 15 and 27, progressive loss of RGCs was observed after microbeads injection in group C ($P < 0.01$). In contrast, the fluorogold-labeled RGC density and class III β-tubulin-positive RGC density were significantly higher in group D as compared to group C ($P < 0.01$) but significantly lower than group B ($P < 0.01$). These parameters did not differ significantly between groups A and B ($P > 0.05$). The findings suggest the consumption of EGCG plays a neuroprotective role on RGCs in a mouse model of elevated IOP.

1. Introduction

Glaucomatous optic neuropathy is a chronic neurodegenerative disease characterized by progressive loss of retinal ganglion cells (RGCs), atrophy of optic nerve, and eventual loss of vision [1,2]. Lowering of intraocular pressure (IOP) in glaucoma is the main therapeutic strategy. However, despite lowering IOP, it is difficult to stop the disease progression and many glaucoma patients experience progressive optic nerve damage with visual loss.

A potential area for glaucoma treatment is neuroprotection, which aims to slow or prevent death of RGCs [3]. Epigallocatechin-3-gallate (EGCG), a flavonoid in various green tea plants has gained increased attention in recent years for its suggested health benefits [4]. EGCG is a powerful antioxidant exhibiting multifunctional properties [5], including anti-inflammatory [6,7] and vasodilator effects [8], which all contribute to its neuroprotective action [9,10]. In our previous study, it was shown that EGCG led to a protective effect on RGCs in experimental optic nerve crush and *N*-methyl-D-

Abbreviations: EGCG, epigallocatechin-3-gallate; IOP, intraocular pressure; RGC, sretinal ganglion cells.

* Corresponding author at: Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing 100730, PR China. Tel.: +86 10 5826 9920; fax: +86 10 5826 9920.
E-mail address: jlbjlb@sina.com (L. Jiang).

[1] These authors contributed equally and both qualify as first author.

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aspartate toxicity model [11,12]. With these preliminary findings, we investigated the effects of EGCG on counteracting the neurodegenerative damage induced by high IOP. This study aims to evaluate the efficacy of orally administered EGCG on the neuroprotective effects upon RGCs in mice with chronic high IOP.

2. Material and methods

2.1. Animal use

The experimental study included 54 female C57BL/6J mice (8 weeks old, weighing 20–30 g). The protocol was approved by the University Institutional Animal Care and Use Committee of Capital Medical University. All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were kept in room temperature and in a 12-h/12-h light–dark cycle with free access to food and water. All mice were examined with ophthalmoscope to rule out any ocular abnormality and then randomly divided into four groups: a normal control group (group A, $n = 12$) without any procedure performed; a control group with EGCG in drinking water (group B, $n = 12$); a microbeads control group with anterior chamber microbeads injection and normal drinking water (group C, $n = 18$); and an EGCG study group with anterior chamber microbeads injection plus EGCG in drinking water (group D, $n = 12$). Each group was divided into two subgroups (each subgroup contains 9 animals in group C and 6 animals in the other three groups) with follow-up periods of 15 and 27 days after the microbeads injection. All animals were sacrificed at the end of each follow-up period. Animals of groups B and D were treated orally with EGCG (Sigma Chemical, St. Louis, MO) at a dosage of 50 mg/kg dissolved in fresh drinking water daily from 2 days prior to the microbeads injection until the end of the follow-up. The fluid consumption per day (up to 9 mL/day, taking into account for the volume not consumed by the mouse and minimal potential leakage from the water bottle) was similar as for animals which did not have EGCG in their drinking water.

2.2. Induction of IOP elevation

Polystyrene microbeads (diameter = 10 μm, Invitrogen, Carlsbad, CA) were re-suspended in sterile phosphate-buffered saline (pH 7.4) at a final concentration of 9.0×10^6 beads/mL. Two days after the first EGCG orally administration, mice in groups C and D were anesthetized by intraperitoneal injection of 10% chloral hydrate (8 mL/kg body weight, Sinopharm Chemical Reagent, China). Elevated IOP was induced in the left eyes by anterior chamber injection of 2 μL microbeads using a glass micropipette connected to a Hamilton syringe (Hamilton Company, Reno, NV) linked with a 30-gauge needle under operating microscope. Levofloxacin (Cravit, Santen Pharmaceuticals, Osaka, Japan) was applied topically after the injections. Mice with complications such as cataract, hyphema or signs of inflammatory responses were excluded and replaced to keep six mice per subgroup constant.

2.3. IOP Measurement

IOP was measured in both eyes using a tonometer (TonoLab, Colonial Medical Supply, Espoo, Finland) [13] every three days at the same time in the morning to avoid diurnal variation. Mice were anesthetized by intraperitoneal injection of 10% chloral hydrate. Measurement was initiated within 5 min after animals lost consciousness. The tonometer recorded six measurements and after elimination of high and low readings, generated an average. The means of six readings were calculated to determine the IOP.

2.4. Retrograde labeling of RGCs by FG

Five days prior to sacrifice, the mouse was placed in a stereotactic apparatus and the skull was exposed. Based on the marked bregma, two small windows (~2 mm diameter) were drilled as the designated coordinates: 4.0 mm behind the bregma, on the anteroposterior axis, ±0.5 mm lateral to the midline on both hemispheres. Using a Hamilton syringe, 1 μL 4% Fluoro-Gold (FG, Biotium Corporation) was slowly injected bilaterally into the superior colliculi. The tip of the syringe was advanced perpendicular to the skull by 1.2 mm under the endocraniumdura mater ancephali. After each injection, the syringe was kept unmoved for 5 min. Following wound suturing, antibiotic ointment was applied.

2.5. Histology and immunohistochemistry

Five days after FG application, the mice were transcardially perfused under deep anesthesia with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), at a flow rate of 20 mL/min. Eyes were enucleated and fixed in 4% paraformaldehyde for 15 min, with the 12 O'clock position was marked on the limbus. Every globe was bisected at the equator with the lens removed and whole flat-mounted retinas were assayed for RGC density. To prepare the flat-mount, the retina was dissected from the sclera/choroid and flattened by making four radial cuts on the peripheral retina. The procedures were processed on ice and protected from light. After fixed in 4% paraformaldehyde for 1 h, retinal flat-mounts were incubated with a primary antibody against a marker of neuronal lineage cells that is highly expressed in the RGC layer, class III β-tubulin (1:50, Tuj1; Cell Signaling USA) at 4 °C overnight, followed by an Alexa Fluor 488-conjugated secondary antibody (1:1000, Cell Signaling USA) at 37 °C for 2 h.

Retinal flat-mounts were spread on a gelatin-coated glass slide and observed under fluorescence microscope (LEICA DM6000B, Germany). Fluorescence micrographs were taken from each retinal quadrant at a distance of 1–1.5 mm from the optic disc centre. Cells positively labeled by FG and class III β-tubulin were counted under different filter sets. Cell counting was performed by two masked investigators and the results were averaged. To measure the inter-observer variation, 20 fields were counted independently by two masked observers. Changes in RGC densities were expressed as RGC survival percentage, which was calculated by dividing the RGC density of the surgical induced high IOP eye by the RGC density of the contralateral control eyes.

Three animals remaining in each group C were killed by intraperitoneal overdose injection of 10% chloral hydrate at 15 and 27 days after microbeads injection. Eyeballs were removed immediately and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at 4 °C, transferred into graded sucrose solutions and finally frozen in embedding compound (Tissue-Tek; Sakura Fine-technical, Tokyo, Japan). Sections with thickness of 10 μm were prepared parallel to the vertical sagittal plane and stained with hematoxylin/eosin. Microbeads distribution and anterior chamber morphology were assessed and photographed under light microscope (LEICA DM 4000B; Leica Camera AG, Germany).

2.6. Statistical Analysis

Statistical analysis was performed using a commercially available statistical software package (SPSS for Windows, version 13.0; IBM-SPSS Inc. Chicago, IL, USA). IOP measurements in each group at different time points were compared using two-factor repeated measures analysis of variance. Differences between FG-labeled RGCs and class III β-tubulin-positive RGCs were analyzed by two-way ANOVA; multiple comparisons are made by SNK-q test. The