Protective effects of quercetin in a rodent model of anterior ischemic optic neuropathy (rAION)

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KEYWORDS
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Abstract
Background: Anterior ischemic optic neuropathy (AION) is the leading cause of sudden optic nerve-related (ON-related) vision loss in elderly people. However, no considerable treatments are available for the neuroprotection of NAION. The purpose of this study was to detect the effects of intravitreal injection of quercetin (Qcn) in a rodent model of anterior ischemic optic neuropathy (rAION). Methods: The rAION model was established using verteporfin and laser in a photodynamic procedure on the optic discs (ON) of rats. The rats received intravitreal injection of Qcn 2 days before the injury and once/week for 4 weeks after the infarct on optic neuropathy. Flash-visual evoked potential (VEP) were recorded to assess the visual function. TUNEL and retrograde Fluorogold labeling assessed the apoptosis and density of retinal ganglion cells (RGCs). ED-1 and Iba-1 staining of the optic nerves displayed the inflammatory response. Results: At 14 days post-infarct, Qcn treatment significantly reduced the number of apoptotic RGCs, as well as, ED1/Iba-1-positive cells/high power field (HPF) in the ON (p<0.01) as compared to the rAION group. At week 4 after rAION, 28.4% VEP amplitudes were estimated in the treated eyes of the fellow eyes in the rAION group and 64.7% in the rAION+Qcn group (p<0.01). In addition, Qcn saved the RGCs in the central retinas as compared to those of the rAION group (1967.5±162.1 and 2868±325.3 mm², respectively (p<0.01), and the corresponding densities were 1654.8±104.8 and 2208±272.9 mm² in the mid-peripheral retinas, respectively (p<0.01). Conclusion: The intravitreal injection of Qcn could protect the RGCs from injury in the rAION animal model, as demonstrated anatomically by RGC density and functionally by F-VEP. Moreover, Qcn might exert an anti-apoptosis role in the survival of RGCs and anti-inflammatory in the optic nerves.

Background
Non-arteritic anterior ischemic optic neuropathy (NAION) is the most common cause of vision loss in individuals >55 years of age. The incidence rate of NAION was 0.03±0.03% (mean ± standard deviation) per 5 years in China alone[1, 2], among which, the central vision was affected in 11.7% cases[3]. The pathophysiology was unclear; however, there is inadequate circulation in the small vessels to the optic nerve head (ONH) [4][3], resulting in the secondary loss of retinal ganglion cells...
(RGCs) in the end-stage. The ONH is susceptible to vascular insufficiencies, and the breakdown of the blood-ON barrier was detected within hours post-injury in rat models of anterior ischemic optic neuropathy (rAION) [5, 6], followed by the early recruitment of extrinsic macrophages and activation of resident microglia at the location of ischemic optic discs (ON). In addition, the aggregation of inflammatory cells results in harmful substances, including pro-inflammatory cytokines, proteases, and free radicals that can aggravate the neuronal damage[7].

Hitherto, no considerable treatments are available for the neuroprotection of NAION. Therefore, several neuroprotective agents have been explored in rat models of rAION, including granulocyte colony-stimulating factor (G-CSF)[8], ciliary neurotrophic factor (CNTF)[9], prostaglandin J2 (PGJ2)[10], and methylprednisolone[11]. However, these agents have not been used widely in the clinic. Thus, identifying the effective neuroprotective agents for preventing or retarding RGC loss as well as for maintaining the function of RGC is essential. Recently, rAION resulting from laser photoactivation has been used widely, according to the generation of superoxide radicals by a photochemical reaction effectuated on the ON, resulting in the secondary loss of RGCs.[2, 8] In this study, base on the previous establishment of an alternative model of rAION resulting from laser photoactivation of verteporfin[12], we assessed a potential benefits of neuroprotective strategies.

Quercetin (Qcn) is an omnipresent flavonoid compound that can be extracted from various fruits and vegetables, such as onions, cranberries, fennel, dark grapes, and cocoa. Previous studies have shown that Qcn serves as a free radical scavenger and with properties, such as anti-apoptosis, anti-oxidant, anti-inflammatory, and anti-cancer [13]. In the retina, we found that Qcn protected ARPE-19 cells from oxidative stress injury [14]. Other studies reported that Qcn has protective effects in the retina ischemia-reperfusion injury[15], ocular inflammation [16], and neuroprotective effects on RGC in glaucoma[17]. However, whether Qcn protects the RGCs in rAION is poorly understood. In NAION, the factors concerning the RGC injury, such as oxidative stress, ischemia-hypoxia, and inflammatory response, are the targeted mechanisms underlying the protective effects exerted by Qcn. Thus, it can be speculated that Qcn protects RGC loss due to laser injury in rAION, which warranted investigation with respect to RGC function, viability, and apoptosis with or without Qcn in rAION.
Methods

Animals

Adult male Sprague Dawley (S–D) rats (180-200 g), were obtained from Shanghai Laboratory Animal Center, Chinese Academy Sciences. All rats were kept in a standard animal facility with ad libitum access to food and water, with a day-night rhythm of 12-hour at constant temperature. All manipulations were performed under general anesthetization, induced by an intraperitoneal injection of 10% (w/v) chlortal hydrate (3.5 mL/kg). The entire experiment was performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO).

S–D rats were randomly divided into four groups (n = 6/group): (1) control, (2) Qcn; normal rats were administered intravitreal injection of 2 µL at a dose 10 µM Qcn (Sigma–Aldrich, St. Louis, MO, USA) 2 days before the injury of ONH and then once a week for 4 weeks[17], (3) rAION, and (4) rAION + Qcn; treated with Qcn as described for group 3. The right eyes of the rats in groups 3 and 4 received the same volume of PBS as sham control.

Induction of AION

The details of rAION induction were the same as our previous reports [12]. Briefly, verteporfin (6 mg/m²) (Novartis Ophthalmics Europe Ltd.) was injected into the veins of the tails, followed by laser application on the ONH of the left eye of each rat. A 689 nm laser with 600 mW/cm² energy was used to irradiate the ONH for 158 s. The irradiation area had a diameter of 500-μm. The fellow eye was only aimed at ONH by the aiming beam without laser emission, while the other operations were identical.

Flash-visual evoked potentials (F-VEPs)

In our previous study[12], the VEP amplitudes after treatment were estimated as 87.3±11%, 67.6±11.5%, and 35.9±13.6% of the fellow control eyes in week 1, 2, and 3, respectively (p<0.05), suggesting that the lowest amplitude in rAION was estimated in week 4. Therefore, the F-VEP was performed on day 28 after intervention, using a Ganzfeld system (RetiPort, Roland Consult, Brandenburg, Germany). The detailed procedure and settings for recording FVEPs has been described in our previous reports [12]. P₁-N₂ amplitude was measured and analyzed.

Immunohistochemistry
In our previous study[12], on day 14, ED(+) and lba1(+) cells were widely distributed on the ON, and both reached a maximum number (65.5±8.7 and 76.8±10.8 respectively, p<0.01) during the study. Fourteen after laser application, animals were humanely euthanized with Euthasol (> 150 mg/kg sodium pentobarbital), and the eyes were enucleated. The eyes were fixed with PBS containing 4% paraformaldehyde (PFA) overnight. After dissecting foreparts and lens, the eye cups were dehydrated and embedded using paraffin. Then, 5-mm thick retinal cross sections were sliced, stained using a standard hematoxylin eosin (H&E) (Sigma, MO, USA) protocol. The slices were selected and incubated for 24 h with mouse anti-CD68 monoclonal antibody (Serotec Ltd, Oxford, UK) or rabbit anti-lba1 monoclonal antibody (Abcam Inc. Cambridge, MA, USA) at 1:100 under 4 °C and subsequently stained with FITC-conjugated secondary antibodies (Jackson ImmunoResearch, West Point, PA, USA) at room temperature for 1 h. All staining was examined under a laser scanning confocal microscope (Zeiss LSM 510, Carl Zeiss, Germany) after counterstaining with 4’,6-diamidino-2-phenylindole (DAPI).

**TdT-mediated dUTP nick-end labeling (TUNEL) assay**

In our previous study[12], the number of TUNEL-positive cells increased significantly on day 7 post-AION and peaked (5.71±0.76, p<0.01) by day 14. Thus, the apoptosis of RGC was measured at day 14 after laser application. The method was the same as in our previous reports [12]. DeadEnd™ Fluorometric TUNEL System was used to detect tissues apoptosis. The procedures of TUNEL assay were the same as our previous reports[12]. The ratio of TUNEL-positive cells to DAPI-positive cells was used to describe the apoptosis level[20].

**Retrograde labeling of RGCs with fluorogold and morphometry of the RGCs**

In our previous study[12], at 4 weeks post-AION, the densities of RGCs in the central retinas increased significantly: 3,075±298 and 2,078±141/mm² in the normal and AION eyes, respectively; thus, the RGCs were assessed. The detailed procedure was described in our previous reports [12]. In brief, rats were placed on Stereotaxis (Stoelting Kiel, Germany) after deep sedation, and FluoroGold (FG; Biotium, Hayward, CA, USA) (4%; 2 µL each) injection was performed in the superior colliculus bilaterally. One week after labeling, eyes were enucleated following sacrifice of the animals. After anterior segments were removed, the eyes were transferred to 4% PFA for 1 h. By dividing the retina
into four quadrants: superior, inferior, temporal, and nasal, the RGCs were evaluated. And each quadrant was further divided into central, middle, and peripheral regions (0.8–1.2 mm from the optic disc, 1.8–2.2 mm from the optic disc, 0.8-1.2 mm from the retinal border, respectively). The retina was viewed using the Olympus BX53 fluorescence microscope (Olympus, Tokyo, Japan) equipped with UV excitation (excitation filter, 350–400 nm; barrier filter, 515 nm), as well as a digital imaging system. Two 200 × 200 μm² standard square areas were examined in each area. Labeled RGCs/mm² of the counted retinal region was used to represent the density of RGCs per group[21].

Statistical analysis

Statistical analysis was performed with SPSS version 20 (SPSS, IL, USA). Data were expressed as mean±SD. We used unpaired Student’s t-test and one-way analysis of variance for two-group data. And post-hoc Bonferroni’s multiple comparison test was used for three or more groups. A value of P < 0.05 was considered to be statistically significant. Each experiment was repeated three times.

Results

F-VEPs

In order to eliminate the interanimal variations, at 4 weeks after infarct, we compared the VEP amplitudes from the study eyes and the fellow eyes of the same animal rather than from different animals and found that the VEP amplitudes in study eyes were 97.8±1.26% of the fellow eyes in the control group, 95.9±3.87% in the Qcn group, 28.4±5.51% in the rAION group, and 64.7±4.86% in the rAION + Qcn group (p<0.05; Fig. 1). There were obvious improvements in the VEP amplitudes after Qcn injection as compared to PBS injection.

Apoptotic cells in the RGC layer

On day 14 after AION induction, the RGC layer showed a negligible induction of apoptotic cells based on the number of TUNEL-positive cells/HPF in ganglion cell layer(GCL) in the control and Qcn groups (0.4±0.55 and 0.6±0.55, respectively) as compared to 5±0.82 and 1.86±0.69 positive cells/HPF in the rAION and rAION+Qcn groups, respectively (Fig. 2). Although the number of apoptotic cells did not differ significantly between the control and Qcn groups (p=0.5), it decreased obviously in the rAION+Qcn group as compared to the rAION group (p<0.01).
Altered inflammatory response in retina and ON

On day 14 after treatment, occasional ED1(+) cells and Iba-1(+) cells were distributed in the control and Qcn ONs (Fig. 3). Nevertheless, ED(+) as well as Iba1(+) cells were widely distributed on ONs (67.5±9.57 and 77.5±12.58, respectively) in rAION group and exhibited a considerable decrease (19.75±7.32 and 40±8.16, respectively) in the rAION+Qcn group. The differences in microglial infiltration were statistically significant in the rAION group as compared to the Qcn group (p<0.01).

RGC densities following AION induction

At 4 weeks after infarct, the densities of RGCs in the central retinas were 3125±348.2, 3158±302.3, 1967.5±162.1, and 2868±325.3/mm² in the control, Qcn, rAION, and rAION+Qcn groups, respectively, while the corresponding densities were 2645.3±167.9, 2602.5±226.2, 1654.8±104.8, and 2208±272.9/mm² in the mid-peripheral retinas (Fig. 4). These data indicated that the toxicity was absent after Qcn treatment, and the densities of RGCs showed a significant variation in the rAION group as compared to the rAION+Qcn group (p<0.01).

Discussion

The present study demonstrated that Qcn does not exert a toxic effect on the retina of normal rat. Also, the potential effect of Qcn against RGC damage was investigated using a rAION model. Furthermore, Qcn preserved the visual function, prevented apoptosis of RGC, decreased the macrophage/microglial infiltration in the ONs, and impeded the secondary RGC death. Qcn was administered by intraperitoneal, oral, or intravitreal injection on the retina as described previously[17, 22, 23]. However, based on the pharmacodynamic maintenance and prolonged exposure of Qcn on the retina, we selected the intravitreal injection as the mode of Qcn administration. A previous study[17] described the neuroprotective role of Qcn on RGC in a rat chronic ocular hypertension (COHT) model as a reference for the determination of the dose and frequency of Qcn administration. Furthermore, laser photoactivation of verteporfin on rats leads to a similar response on patients affected with AION. In the rAION group, the amplitude of F-VEPs was reduced distinctly as compared to the control eyes, and the number of apoptotic RGCs, as well as the infiltration of inflammatory cells, peaked on day 14 after PDT[12]. Besides, on day 28 post-infract, the densities of surviving RGCs...
showed a remarkable reduction. Together, these results suggested that inflammation participated in this rAION model with the evident death of RGCs in the late stages, which was in agreement with the previous models[2]. Thus, this kind of animal model of AION is feasible for studying the mechanism underlying the ON injury and exploring the neuroprotective measures. Strikingly, RGC dysfunction occurs early, following optic disc edema, and is generally damaged until the loss in AION. Therefore, in the early stages, the functional protection is essential for alleviating the optic neuropathy. Hence, in the current study, Qcn was injected intravitreally immediately post-injury. As detected on F-VEP, the functional deficits were indicated by a decreased amplitude of P1 in the rAION group; however, a rebound amplitude of P1 was detected in the rAION+Qcn group, suggesting that Qcn administration coupled with rAION, could alleviate the early RGC functional damage. On the other hand, the VEP latency was not analyzed due to the considerably minor changes in different groups in the state of dark adaptation as stated previously[10]. FG retrograde labeling revealed that Qcn effectively preserved the RGCs from loss either in the center or the mid-peripheral retinas, which was consistent with the subsequent verification by TUNEL. However, a small proportion of RGCs is yet missed from the protective effect of Qcn, which is similar to those of previous studies on the protective effect of AION drugs[9, 11, 18]. Thus, this concern necessitates further exploration following the identification of the potential neuroprotective effect of Qcn in rAION.

In the acute phase cytotoxic edema is rapidly followed by vasogenic edema, which can further damage the ON and surrounding retina. Recruitment of extrinsic macrophages (ED-1) and activation of resident microglia (Iba-1) within 3 days post-ischemia at the core of ischemic ON suggest the damage of the blood-ON barrier[11, 24]. Macrophages have been reported to accumulate within 35 days, suggesting that the inflammatory response was persistent in rAION[25, 26]. Activated macrophages play a dual role[27]. On the one hand, they can improve regeneration and remyelination. On the other hand, they could secrete harmful substances including pro-inflammatory mediators, it may aggravate the neuronal damage. The current results demonstrated that Qcn decreased the expression of ED-1 and Iba-1, i.e., decreased the extrinsic macrophage and resident
microglia infiltration in ONs. Qcn treatment has also been reported to stabilize the blood-retinal barrier and prevent inflammation in diabetic rat retina and ARPE-19 cells[15, 23]. Notably, Qcn exhibits the potential to stabilize the blood-ON barrier and restrict the infiltration of the inflammatory response at the ischemic ONs.

Conclusions
In summary, early intravitreal injection of Qcn exerts a neuroprotective role in rAION, as verified by decreased RGC apoptosis, restored RGC density counts, improved electrophysiological visual function, and restricted inflammatory response. Nevertheless, although the treatment of Qcn is effective in this rAION model, further studies as well as further clinical explorations are required to obtain a suitable concentration and dosing frequency of Qcn and investigate the difference between the outcomes of the models and the clinical applications.

Abbreviations
AION  anterior ischemic optic neuropathy
DAPI  4′,6-diamidino-2-phenylindole
F-VEP Flash-visual evoked potential
ON  Optic nerve
ONH  Ptic nerve head
Qcn  Quercetin
rAION  rat models of anterior ischemic optic neuropathy
RGCs  Retinal ganglion cells

Declarations

Ethics approval and consent to participate
All animal experiments adhered to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Ethical approval for this investigation was obtained from the Research Ethics Committee, Shanghai jiaotong University School of Medicine.

Consent for publication
Not applicable

Acknowledgements
Not applicable

**Availability of data and materials**

All the summarized data is presented in paper. The raw data of the present research is available upon reasonable request from the corresponding author.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

LYN, MJY and GYY were primarily responsible for experimental concept and design, and was major contributors in writing the manuscript, the first two authors contributed equally to this paper. LYN raised all the experimental rats and helped to operate on them. ML performed the histological examination of the retina and optic nerve. GQ and WF performed data acquisition and analysis. All authors reviewed and approved the final manuscript.

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Figures
Figure 1

(A) Changes in the amplitude of P1 (mean±SD) in FVEPs in different groups. (B) 28 days post-rAION, the amplitude of P1 in treated eyes measured 97.8% of the fellow eyes in the control group, 95.9% in the Qcn group, 28.4% in the rAION group, and 64.7% in the rAION+Qcn group. (*p<0.05 in the rAION+Qcn group compared to the rAION group, *p<0.05 in the rAION group compared to the control group, n=6 in each group).
Figure 2

TUNEL-positive cells in the RGC layer. (A) A decrease in the number of apoptotic cells (mean ± SD) with the treatment of Qcn. (B) 14 days post-rAION, the number of apoptotic cells was 0.4±0.55, 0.6±0.55, 5±0.82, and 1.86±0.69 positive cells/HPF in the control, Qcn, rAION, and rAION+Qcn groups, respectively. Blue indicated nucleus and green indicated the positive cells (**p<0.01, n=6 in each group; field of 200×200 μm²).
Infiltration of ED1(+) cells and Iba1(+) cells in the ONs. (A) There was less infiltration of ED1- and Iba-1-positive cells (mean±SD) with the treatment of Qcn at 14 days post-rAION. (B) In the control and Qcn groups, occasional ED1(+) cells and Iba-1(+) cells were distributed. The positive ED1 and Iba-1 cells at the ON lesion sites in rAION group were prominent (67.5±9.57 and 77.5±12.58, respectively), whereas this proportion decreased significantly in the rAION+Qcn group (19.75±7.32 and 40±8.16, respectively). Blue indicated nucleus and green indicated positive cells (**p<0.01, n=6 in each group).
Figure 4

FG labeling of surviving RGC in the retinas at 4 weeks after infarct. (A, B) The densities of RGCs in the central retinas were 3125±348.2, 3158±302.3, 1967.5±162.1, and 2868±325.3/mm² in the control, Qcn, rAION, and rAION+Qcn groups, respectively, while the corresponding densities were 2645.3±167.9, 2602.5±226.2, 1654.8±104.8, and 2208±272.9/mm² in the mid-peripheral retinas. (C) Schema of the whole flat-mounted retina, the circles with different colors represent the different partitions of the retina, and the RGCs between the same color circles were counted. Blue indicated nucleus and green indicated positive cells (**p<0.01, n=6 in each group, field of 200×200 μm²).

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