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

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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.

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) chloral 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 ($n = 6/\text{group}$): (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 Iba1(+) 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-Iba1 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: $3,075 \pm 298$ and $2,078 \pm 141/\text{mm}^2$ 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 $200 \times 200 \mu\text{m}^2$ standard square areas were examined in each area. Labeled RGCs/ mm^2 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 $\pm$ 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 $97.8 \pm 1.26\%$ of the fellow eyes in the control group, $95.9 \pm 3.87\%$ in the Qcn group, $28.4 \pm 5.51\%$ in the rAION group, and $64.7 \pm 4.86\%$ 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 $2868 \pm 325.3/\text{mm}^2$ 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 $2208 \pm 272.9/\text{mm}^2$ 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 ($p < 0.01$).

Abbreviations

AION anterior ischemic optic neuropathy

DAPI 4',6-diamidino-2-phenylindole

F-VEP Flash-visual evoked potential

ON Optic nerve

ONH Optic 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 $\pm$ 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 $\pm$ SD) with the treatment of Qcn. (B) 14 days post-rAION, the number of apoptotic cells was 0.4 $\pm$ 0.55, 0.6 $\pm$ 0.55, 5.0 $\pm$ 0.82, and 1.86 $\pm$ 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 $\times$ 200 μ m²).

Figure 3

Infiltration of ED1(+) cells and Iba1(+) cells in the ONs. (A) There was less infiltration of ED-1- 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 $2868 \pm 325.3/\text{mm}^2$ 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 $2208 \pm 272.9/\text{mm}^2$ 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 $200 \times 200 \mu\text{m}^2$).

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