

Houttuynia cordata Thunb rescues retinal ganglion cells through inhibiting microglia activation in a rat model of retinal ischemia-reperfusion

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Abstract

• **AIM:** To determine whether *Houttuynia cordata* Thunb (HCT) can increase the survival of the retinal ganglion cells (RGCs) and inhibit microglia activation following retinal ischemia-reperfusion (RIR) injury.

• **METHODS:** Rat model of RIR was induced by transient elevation of the intraocular pressure (IOP). HCT was orally administered for 2d before the performance of retinal RIR model and once a day for the next 14d. After 14d of RIR injury, the rats were sacrificed for further analysis. Survival RGCs were stained with haematoxylin and eosin (H&E). Apoptosis of RGCs was detected by TUNEL staining. Retinal function was examined by flash-electroretinography (F-ERG). Retinal microglia were labeled using Iba-1, one specific marker for microglia. The mRNA expression levels of inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF- α), and interleukin 1 beta (IL-1 β) were assessed by quantitative real time reverse transcription polymerase chain reaction (qRT-PCR).

• **RESULTS:** Systemic HCT treatment significantly reduced RGCs death by H&E staining and exhibited an anti-apoptotic effect as assessed by TUNEL staining at day 14 after RIR injury. HCT greatly improved the retinal function as examined by F-ERG. The number of activated microglia significantly increased after RIR injury, which was significantly attenuated by HCT treatment. Besides, RIR injury induced a strong upregulation of pro-inflammatory genes TNF- α , iNOS and IL-1 β mRNAs at day 14 post injury, which was suppressed by HCT.

• **CONCLUSION:** Neuroprotective effects of HCT encourage the survival of RGCs through inhibiting microglia activation due to RIR injury. Together these results support the use of HCT as promising therapy for the ischemic events of the retina diseases.

• **KEYWORDS:** *Houttuynia cordata* Thunb; retinal ganglion cell; microglia activation; retinal ischemia-reperfusion

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INTRODUCTION

Retinal ischemia-reperfusion (RIR) is an usual complication of various ocular diseases, such as diabetic retinopathy, acute glaucoma, and nonarteritic anterior ischemic optic neuropathy, which can lead to visual dysfunction^[1]. Effective treatments for the ischemic events of the retina and optic nerve remain elusive. Apoptosis of retinal ganglion cells (RGCs) is the hallmark of the pathologic change after retinal RIR injury. RGCs, locating in the innermost layer of retina, are the most important subtypes of neurons in the retina and undertake the sole responsibility of transmitting the visual stimuli to the brain. Severe damage on RGCs will result in visual impairments and eventually blindness. RGCs are highly sensitive to ischemic insults, therefore, numerous of neuroprotective therapeutic approaches to improve RGCs survival have been developed for the management of all ocular pathologies that share the death of RGCs^[2-4].

The specific mechanisms of RGCs death have not yet been identified, but it is extensively related to retinal microglia activation due to retinal ischemia/hypoxia. Activation of microglia has been described in RIR injury of the retina^[5-8]. The excessively activated retinal microglial cells release a variety of neurotoxic mediators, including inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF- α), and interleukin 1 beta (IL-1 β), which cause more RGCs death

and further worsen the injury of retinal tissues. Accumulating evidence have indicated that inhibition of the excessively activated microglia induces neuroprotective effect to RIR-induced RGCs death^[9-12].

Houttuynia cordata Thunb (HCT, Family: *Saururaceae*) is well known because of its medicinal properties in China, which has been traditionally used for treating the oxidative stress and inflammation related diseases, through against the oxidative stress and inflammation^[13-14]. In our previous study, we firstly reported that HCT was an inhibitor of lipopolysaccharide (LPS)-induced retinal microglial activation. HCT significantly suppressed the activation of retinal microglia *via* suppression of p38-MAPK signaling pathway and might be used as novel therapeutic strategy for part of ocular diseases characterized by over-activated microglia^[15]. Because of the excessive activation of microglia in the retina after RIR injury, we hypothesized that HCT treatment might increase the survival of the RGCs though inhibiting microglia activation. In order to test this hypothesis, we performed the animal model of retinal RIR through transient elevation of the intraocular pressure (IOP) and determined that HCT elicited neuroprotective effects on RGCs though inhibiting microglia activation.

MATERIALS AND METHODS

Ethical Approval The experimental protocol was approved by the Ethical Review Committee of the Second Hospital of Shandong University and all experimental procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmologic research.

***Houttuynia cordata* Thunb Preparation** HCT ethanol extract was acquired according to our previously published article and stored at -80°C in aliquots until later use^[15].

Experimental Animals All experiments were performed in adult female SPF male Sprague-Dawley rats (2-3mo, 200-250 g). The animals were housed with an alternating 12-hour light/dark cycle in temperature-controlled room and unlimited access to food and water.

Retinal Ischemia-Reperfusion Model The detailed protocol for inducing retinal RIR injury was consistent with previously published^[2,16]. In brief, a 30-gauge needle attached with 500 mL saline-filled bottle was inserted into the anterior chamber through the cornea of the randomly selected eye. Before that, the rats were generally anesthetized with intraperitoneal injection of a mixture of ketamine (50 mg/kg) and xylazine (2 mg/kg). One drop of 1% tropicamide and 0.5% proparacaine hydrochloride were administered into each eye for pupil dilation and local anesthesia, respectively. IOP was increased to 110 mm Hg for 60min by keeping the saline bag to a height of 1498 mm above the eye. Tono-Pen (Medtronic Co., Dublin, Ireland) was used to monitor the IOP. After 60min elapsed,

gently pull the needle from the anterior chamber. Only the rats with intact iris and unimpaired lens were used for further analysis.

Systemic HCT Treatment HCT treatment regimen was employed as previously described^[17-18]. The rats were randomly divided into 4 equal groups (6 rats per group), as follows: Group 1: rats received the normal diet; Group 2: rats received retinal RIR injury+oral administration just normal diet; Group 3: rats received RIR injury+oral administration HCT (400 mg/kg·d); Group 4: rats received retinal RIR injury+oral administration HCT (1000 mg/kg·d). HCT was administered for two days before the performance of retinal RIR model and once a day for the next 14d. At 14d after RIR injury, the rats were sacrificed by an overdose of 10% chloral hydrate. Significant loss of RGCs and activation of microglia take place in a delayed fashion in the course of retinal RIR injury. The number of RGCs decreased to less than 50%, meanwhile, the retinal microglia displayed an activated morphology and increased obviously in number on day 14 in an acute model of ocular hypertension. Therefore, we determined the time of 14d after RIR injury as potential optimum to study the neuroprotective effect of HCT in this model^[19].

Analysis of Haematoxylin and Eosin The removed eyes were enucleated and fixed in 4% paraformaldehyde in phosphate buffer saline (PBS) for 24h. The orientation was labeled at 12 o'clock of the corneal limbus. The eyes were embedded in paraffin and then 5 µm thin sections were cut with a microtome (CM 1900; Leica Microsystems, Wetzlar, Germany). Sections, running through the pupil and optic nerve head, were stained with haematoxylin and eosin (H&E). The staining procedure was performed according to the manufacturer's instructions and could be found anywhere. Images of slides were captured using an Olympus BX51 microscope (Tokyo, Japan). The numbers of survival RGCs in the central retinal region, 2 mm above the optic nerve head, were manually counted by two independent individuals. The numbers were averaged and compared between different groups. Density of survival RGCs was recorded as cell number/high-power microscopic field (HPF).

TUNEL Analysis The terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine (dUTP)-biotin nick end labeling (TUNEL) method was used to evaluate the presence of apoptotic RGCs at 14d after model establishment^[20]. Frozen sections were prepared and one commercial TUNEL kit (*in situ* cell death detection with fluorescein; Roche Biochemicals, Mannheim, Germany) was performed according to the manufacturer's instruction. Retinal sections were observed by confocal laser scanning microscopy (Zeiss, Germany) and images were compiled using an image analysis system (ImagePro Plus 6.0 software, Cybernetics, Bethesda, MD).

Numbers of TUNEL-positive RGCs in the central retina were imaged at $\times 40$ magnification and 6 different square regions with clear visualization were acquired in each section and at least 3 sections in each eye. The value, TUNEL-positive RGCs to the total number of RGCs represented the ratio of apoptotic RGCs.

Examination of Flash-Electroretinography Retinal functions were tested by flash-electroretinography (F-ERG) at 14d post surgery^[21]. The F-ERG responses were recorded using the RetiPort system (ROLAND, Consult, Germany). Before the F-ERG recording, the rats were initially acclimatized to dark overnight. Under dim red illumination, the rats were generally anesthetized and 1% tropicamide was administered to dilate the pupils. SD rats were kept warm and placed facing the stimulus at a distance of 20 cm. Loops were placed on the center of the rat's cornea. The reference electrode was placed subcutaneously on the temporal canthus, and the grounding electrode was laid near the tail.

Immunofluorescent Labeling for Retinal Microglia The fixed eyes were embedded and sectioned in 7 mm slices with a microtome. Immunostaining was performed using standard procedures. The sections were washed 3 times with 0.1 mol/L PBS and then blocked in 3% bovine serum albumin (BSA) and 0.2% Triton-X in PBS for 1h at room temperature (RT). After incubating overnight at 4°C with the primary antibody IBA-1 (1:500, WAKO) for microglia staining, the slides were subsequently washed and incubated for 1h with secondary Alexa Fluor-conjugated antibodies (Cell Signaling Technology, USA) in the dark at RT. Nuclei were counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI). After triple washing with phosphate buffered solution with Tween (PBST) buffer, the samples were mounted and captured by confocal laser scanning microscopy (Zeiss, Germany). Numbers of microglia were calculated from counting Iba1-positive cells with an identifiable Hoechst-stained nucleus. The activated microglia were counted as cells exhibiting an amoeboid shape, with marked cellular hypertrophy and retraction of processes^[9].

RNA Extraction and RT-PCR The mRNA levels of iNOS, TNF- α and IL-1 β were detected using RT-PCR^[15]. At 14d post injury, the rats were sacrificed and retinal tissues were fully dissected and collected under microscope. The total retinal RNAs were homogenized and isolated using a PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was generated using the High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR detection was performed using the SYBR Green Master (Invitrogen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers sequences were

listed below: TNF- α , 5'-CAGGTTCCGTCCCTCTCATA-3' and 5'-TGCCAGTTCCACATCTCG-3'; iNOS, 5'-GCAAGCCCTCACCTACTTCC-3' and 5'-AACCTC TGCCTGTGCGTCT-3'; IL-1 β , 5'-CCTTCTTTTCCTTC ATCTTTG-3' and 5'-ACCGCTTTTCCATCTTCTTCT-3'; GAPDH, 5'-GATGCTGGTGTGCTGAGTATGRCG-3' and 5'-GTGGTGCAGGATGCATTGCTCTGA-3'. Accompanying software was used for the acquisition of threshold cycle (Ct) values. The relative gene expression calculated by the $2^{-(\Delta Ct)}$ method. Each group was normalized in the same way and compared to control group and then set at 100%.

Statistical Analysis All the cells counting were performed by a blinded observer and all data were expressed as mean \pm standard deviation (SD). Data analysis was calculated by SPSS software (version20; SPSS Inc., Chicago, IL, USA). Differences among groups were assessed by a one-way analysis of variance (ANOVA) followed by Dunnett's test or the Student's *t*-test. Nonparametric data were evaluated using the Mann-Whitney *U* test. A *P* value <0.05 was regarded as statistically significant.

RESULTS

HCT Treatment Prevents RGCs Loss Following RIR Injury To compare the influence of HCT on the apoptosis of RGCs, the numbers of survival RGCs in the central retinal region were evaluated through H&E staining. Representative photomicrographs of H&E staining images from 2 mm above the optic nerve head are shown in Figure 1. The average number of survival RGCs in the central retina was 24.4 ± 5.4 /HPF in the control group ($n=6$), which obviously decreased to 9.7 ± 2.0 /HPF on day 14 after retinal RIR injury in the rats with normal diet. Two independent methods of systemic HCT treatment were utilized to assess the effect of HCT on the loss of RGCs after RIR injury. In the rats receiving RIR injury+oral administration HCT (400 mg/kg·d), the number of RGCs was 11.9 ± 3.3 /HPF, while it was significantly higher than the rats with normal diet ($P < 0.01$). On the other hand, the number of RGCs increased to 15.1 ± 3.7 /HPF after prior treatment of HCT (1000 mg/kg·d) compared with the rats with normal diet ($P < 0.001$, Figure 2). The result showed that systemic HCT treatment had a greater neuroprotective effect on RGCs in the model of retinal RIR injury.

HCT Treatment Inhibits Apoptosis of Survival RGCs due to RIR Injury In order to further confirm the neuroprotective effect of HCT on RGCs, TUNEL assay was performed 14d after RIR injury. As shown in Figure 3, there were notably more TUNEL-positive RGCs in the RIR injury group when compared with the control group. Average 64.1% of RGCs showed strong positive reaction to TUNEL. This increase in the number of apoptotic RGCs was significantly inhibited by systemic HCT treatment. Average 48.06% and 21.94%

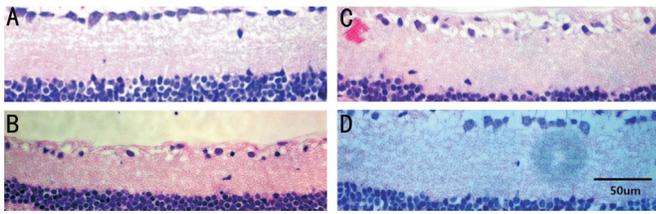


Figure 1 H&E staining of the retina Representative images showed the neuroprotective effect of HCT treatment on the RGCs at 14d after RIR injury. A: Control; B: RIR+normal diet; C: RIR+400 mg/kg·d HCT; D: RIR+1000 mg/kg·d HCT. Scale bar=50 μm.

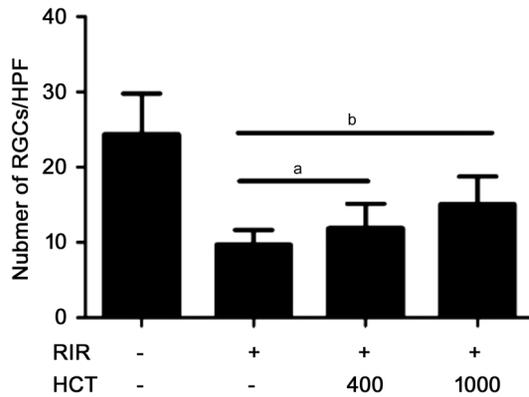


Figure 2 The numbers of survival RGCs in the rats receiving HCT treatment were significantly higher than the rats with normal diet after retinal RIR injury ^a $P<0.01$, ^b $P<0.001$. $n=6$ rats per group.

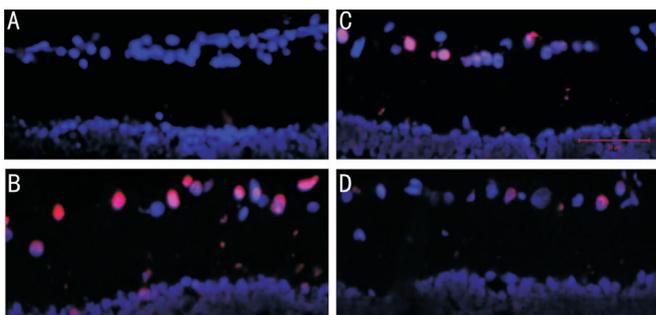


Figure 3 TUNEL staining in the retinal sections of four groups 14d after RIR injury A: Control. Very few TUNEL-positive RGCs were detected in the control group. B: RIR+normal diet. Notably more TUNEL-positive RGCs were found in the rats with normal diet after RIR injury. C, D: Significantly reduced TUNEL-positive RGCs were seen in the rats receiving oral administration HCT after RIR injury. RIR+400 mg/kg·d HCT (C). RIR+1000 mg/kg·d HCT (D). Scale bar=50 μm.

of RGCs were labeled by TUNEL in the rats receiving oral administration HCT 400 mg/kg·d and 1000 mg/kg·d respectively after retinal RIR injury (Figure 4).

Effect of HCT on Alteration of ERG in Rats After RIR Injury To study the retinal functions, we performed F-ERG examination in the rats at baseline and 14d after retinal RIR injury (Figure 5). At baseline, one normal and typical ERG was recorded. The mean amplitude of a-wave and b-wave

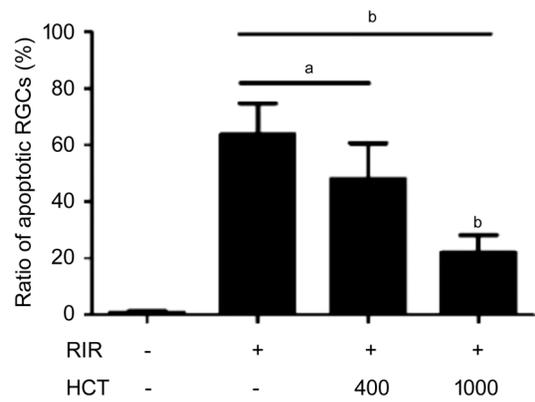


Figure 4 The numbers of TUNEL-positive RGCs in the rats receiving HCT treatment were significantly lower than the rats with normal diet after retinal RIR injury ^a $P<0.01$, ^b $P<0.001$. $n=6$ rats per group.

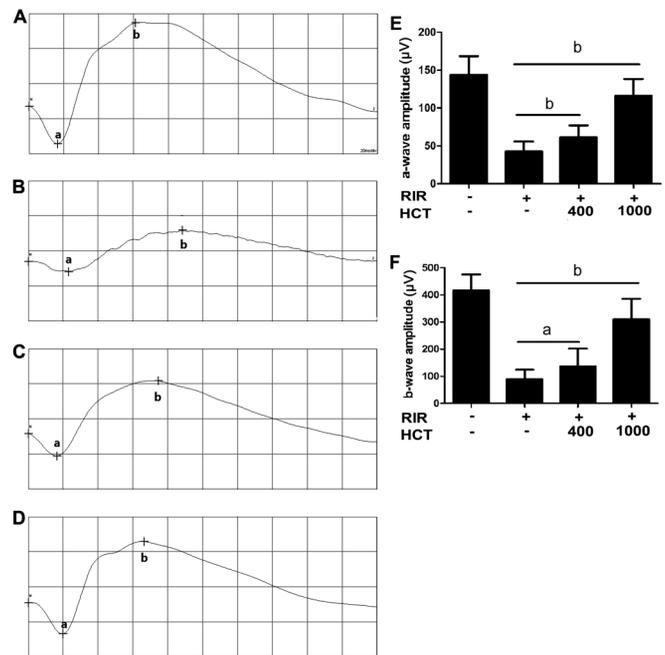


Figure 5 F-ERG examination was performed to evaluate retinal function Individual typical ERG records showed that HCT treatment significantly inhibited the reduction of the a- and b-wave amplitudes induced by RIR injury. A: Control; B: RIR+normal diet; C: RIR+400 mg/kg·d HCT; D: RIR+1000 mg/kg·d HCT. ^a $P<0.01$, ^b $P<0.0001$. $n=6$ rats per group.

were 143.8 ± 24.9 μV and 416.3 ± 58.4 μV, respectively. After the retinal RIR injury, there was a substantial decrease in both the a- and b-wave amplitudes. Notably, a significant recovery in between the a-waves and b-waves was detected in the rats receiving HCT treatment.

HCT Treatment Attenuated the Activation of Retinal Microglia Induced by RIR Injury In our prior study, we found that HCT could inhibit LPS-induced retinal microglial activation *in vitro*. In order to verify the effect of HCT on the microglia activation, the immunofluorescent labeling of Iba-1, a specific marker of microglia, was explored in the retinal

RIR model. The result showed that retinal microglia exhibited ramified morphology in the control group and noticeably changed after injury as cells showing soma enlargement with shortening and thickening of their processes. At the same time, the number of microglia significantly increased following RIR injury. The mean density of microglia in the ganglion cell layer (GCL) was 0.5 ± 0.6 cells/HPF in the control group. However, RIR injury induced a strong upregulation of Iba-1 in the GCL, the mean number of microglia obviously increased to 8.6 ± 2.1 cells/HPF in the rats receiving normal diet after retinal RIR injury, which was significantly attenuated by systemic HCT treatment. At 14d post-injury, the number of Iba-1 positive microglia reduced to 6.3 ± 1.9 cells/HPF and 4.7 ± 1.8 cells/HPF in the rats receiving HCT treatment at 400 mg/kg·d and 1000 mg/kg·d (Figures 6 and 7).

HCT Treatment Suppressed RIR-Induced Inflammatory Cytokine Secretion Previous studies have shown that microglia activation is associated with the production of inflammatory cytokines, which leads to apoptotic cell death of RGCs. We then investigated the effect of HCT on the secretion of inflammatory cytokines using RT-qPCR. As shown in Figure 8, RIR injury induced a remarkable upregulation of iNOS, TNF- α , and IL-1 β mRNAs at day 14 post-injury, which was significantly suppressed by HCT treatment.

DISCUSSION

HCT has a wide range of biological activities, including anti-inflammation, anti-cancer, anti-obesity, anti-oxidative stress, and immune regulation^[13-15]. A *in vitro* model of LPS-induced mouse peritoneal macrophages, HCT could mediate inhibition of cyclooxygenase-2 enzyme activity and can affect related gene and protein expression^[13]. HCT could decreased the serum levels of interleukin-6 (IL-6) and macrophage inflammatory protein-1 α (MIP-1 α) in the rat model of oxaliplatin-induced neuropathy and suggested that HCT is useful as a therapeutic drug for neuropathic pain. HCT also has a therapeutic effect on allergic inflammatory disease through inhibiting the production of pro-inflammatory cytokines^[14]. Retinal RIR injury is very common among a group of major vision-threatening diseases, which characterized by the apoptosis of RGCs. However, no report has been elucidated on the effect of HCT on the RGCs apoptosis after RIR injury.

In the present study, we evaluated the effect of HCT on RGCs death using H&E staining, TUNEL, and ERG to comprehensively evaluate the histologic and functional changes of the retina after RIR injury. Our results showed that systemic HCT treatment could reduce the apoptosis of RGCs and improve retinal functions. Moreover, HCT showed an anti-inflammatory effect *via* inhibiting retinal microglia activation. To the best of our knowledge, the present study is the first to prove a neuro-protective effect of HCT on RGCs in a rat

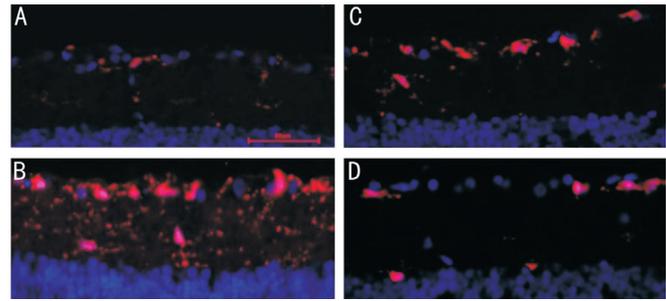


Figure 6 The immunofluorescent labeling of Iba-1, one specific marker of microglia, in the retinal sections A: Control. Confocal images of retinal sections showing the distribution of microglia under resting in the GCL. B: RIR+normal diet. RIR injury induces a dynamic increased number of Iba1-positive microglia cells in the GCL and most of the microglia cells exhibited activated morphology, showing soma enlargement with shortening and thickening of their processes; C, D: Oral administration HCT significantly inhibited the microglia accumulation in the GCL in the rats after RIR injury. RIR+400 mg/kg·d HCT (C); RIR+1000 mg/kg·d HCT (D). Scale bar=50 μ m.

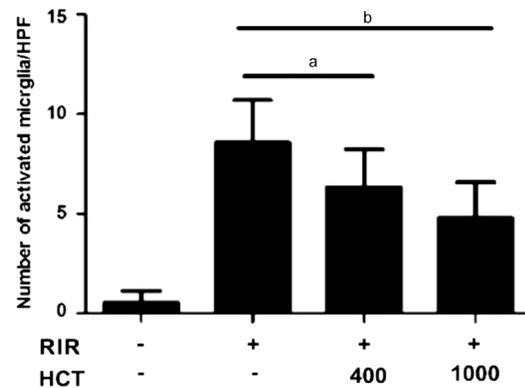


Figure 7 Oral administration HCT significantly inhibited the microglia accumulation in the GCL in the rats after RIR injury ^a $P < 0.01$, ^b $P < 0.0001$. $n = 6$ rats per group.

model of retinal RIR injury. Our findings provided compelling evidence that HCT might be a promising therapy for the ischemic events of the retina diseases.

Microglia are resident macrophages of the retina and play a pivotal role in immune defense against pathogens^[22]. Activation of the resident retinal microglia widely occurs in animal model of retinal RIR, as well as acute glaucoma, retinal vascular occlusion, diabetic retinopathy, which leads to death of RGCs, initiating further retinal microglia activation^[5,23]. It is well known that the activated microglia can elevate the secretion of inflammatory cytokines, for example TNF- α , TGF- β , IL-1 β , and iNOS, which can remarkably magnify the local inflammatory response and in turn contribute to RGCs death. Therefore, therapeutic strategies targeting activated microglia may be beneficent to retinal diseases^[9,11-12,23-24]. We have previously reported that pretreatment with HCT could inhibit the LPS-induced microglia activation and HCT is a pharmacological inhibitor of microglial neurotoxicity^[15]. In

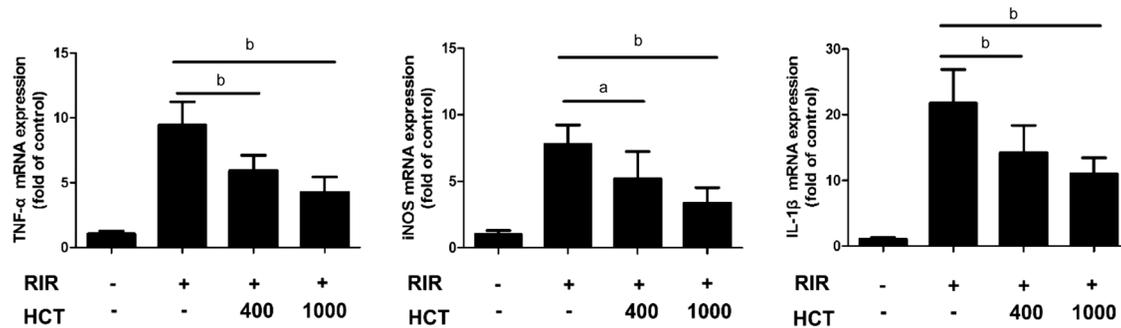


Figure 8 The effects of HCT on the secretion of inflammatory cytokines, TNF- α (A), iNOS (B), and IL-1 β (C), in the retina tissues were determined by qRT-PCR. Histograms showed that HCT treatment obviously suppressed RIR injury-induced inflammatory cytokine secretion. ^a $P < 0.001$, ^b $P < 0.0001$. $n = 6$ rats per group.

the present study, using immunofluorescence and qRT-PCR, we showed that HCT treatment could obviously attenuate the activation of retinal microglia induced by retinal RIR injury. Furthermore, qRT-PCR analysis demonstrated that RIR-induced inflammatory cytokines in the retina tissues were considerably suppressed by HCT treatment. These results suggest that HCT exhibited strong antioxidant activities through inhibiting microglia activation in the ischemic retina. In summary, while the data presented here support our hypothesis that HCT treatment increased the survival of the RGCs through inhibiting the activation of retinal microglia due to RIR injury. Our results demonstrated that neuroprotective effects of HCT encouraged the survival of RGCs after retinal RIR injury might be due to its prevention of microglia activation and the associated inflammation. HCT has anti-neuroinflammatory properties and could be used as a therapeutic agent applicable to microglia-related neuroinflammation.

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Conflicts of Interest: Ren LM, None; Zhang YH, None.

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