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Neuroprotective effect of ciclopirox olamine in retinal ischemia/reperfusion injury

Enming Du[1†], Xiaolin Jia[2†], Xiaoli Li[1], Beibei Zhang[1], Yaping Zhai[1] and Fangyuan Qin[1*]

Abstract

Retinal ischemia–reperfusion (IR) injury is a basic pathological procedure in clinic and associated with various ischemic retinal diseases, including glaucoma, diabetic retinopathy, retinal vascular occlusion, etc. The purpose of this work is to investigate the effect of ciclopirox olamine (CPX) on retinal IR injury and further explore the underlying mechanism. In vitro assay exhibited that CPX exhibited significant neuroprotection against oxygen glucose deprivation (OGD) and oxidative stress-induced injuries in 661W photoreceptor cells. OGD injury showed a proinflammatory phenotype characterized by significantly increased production of cytokines (IL-6, IL-23 and TNF-α), while CPX significantly inhibited their secretion. In addition, the in vivo experiment demonstrated that CPX significantly preserved the normal thickness of the retina. Therefore, we suggest that CPX is identified in our research as a prospective therapeutic agent for retinal IR injury.

Keywords Ciclopirox olamine, Retina, Ischemia/reperfusion injury, Neuroprotection

Introduction

Ischemia–reperfusion (IR) injury refers to an initial restriction of blood supply, followed by the subsequent restoration of perfusion, which is involved in the pathophysiology of multiple degenerative diseases [1, 2]. In general, ischemia cuts the supply of the oxygen and various nutrients to a particular tissue or organ, triggering a cascade of events that eventually ends with cell death. Blood reperfusion of the ischemic tissue is needed, but also causes more destruction by the severe free radical burst and excessive activated inflammatory responses [3]. Retina being an extension of central nervous system, it makes retina to convert external visual stimuli into electrical signals and then transport to the brain. Therefore, retina exhibits high activity in metabolism and its blood flow needs to be maintained at a higher level, which render the retina very susceptible to IR injury [4]. Retinal IR injury has been extensively studied due to its impact on glaucoma, diabetic retinopathy, retinal vascular occlusion, ischemic optic neuropathy and other ocular disorders [5]. Multiple factors, such as intracellular reactive oxygen species (ROS), sterile inflammation, vascular permeability, leukocyte aggregation and apoptotic retinal cell death, contribute to the pathogenesis of retinal IR injury, which eventually leads to visual loss or blindless [6–8]. Currently, the molecular mechanism underlying retinal IR injury remains obscure. Owing to the relative lack of effective therapeutic options, retinal IR injury remains a major challenge of blindness worldwide. Thus, exploring innovative drugs is urgently needed to efficiently avoid visual loss related with retinal ischemic disorders [9–17].

Ciclopirox olamine (CPX) is a broad-spectrum antifungal agent and has been clinically used for decades [18]. Increasing evidence has demonstrated that CPX is a very promising agent in the treatment of multiple diseases, including cancer, diabetes, cardiovascular disorders, and HIV infection [19–24]. More recently, CPX has been

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investigated in ischemic brain injury, showing good efficacy and excellent tolerability. The results systematically demonstrated that treatment with CPX protected the rat brain against IR injuries including brain infarction, neurological dysfunction, inflammation, apoptosis as well as blood–brain barrier disruption [25, 26].

In this study, we investigated the therapeutic potential of CPX in retinal IR injury. We employed oxygen–glucose deprivation (OGD) and oxidative stress-induced injuries in 661W cells as in vitro model and high intraocular pressure (IOP)-induced retinal IR rats as in vivo model, and found CPX can significantly inhibit OGD and H_2O_2 induced 661W cell apoptosis as well as alleviate oxidative stress and inflammatory change. In vivo, systemic treatment with CPX preserved the normal thickness of the retina after IR injury. All these results proved CPX was effective for amelioration of retinal IR injury, and proposed CPX as a novel pharmacological intervention for retinal IR associated ocular disorders.

Results

In vitro biocompatibility evaluation of CPX

Biocompatibility is a prerequisite for therapeutic agents in the treatment of retinal IR injury, so the influence of CPX (Fig. 1A) on 661W cells (a photoreceptor cell line) proliferation was tested by CCK-8 assay at the first step. As shown in Fig. 1B, no remarkable changes of cell viability were observed under treatment with CPX at the concentrations of 0.2, 0.5 and 1 μM. Cell viability showed a slight decrease at 2 μM (86.3%). However, the viability of 661W cells was significantly suppressed when the CPX was higher than 2 μM. With the increase of CPX, the apoptosis of the 661W cells became more prominent.

CPX protecting 661W cells against OGD damage

First, the neuroprotective effect of CPX was evaluated by the in vitro oxygen–glucose deprivation (OGD) model, which was widely used in the study of ischemic stroke. The results are summarized in Fig. 2. As shown in Fig. 1B, CPX showed little toxicity up to 2 μM, which was selected to evaluate its potential neuroprotective effects. The 661W cells were subjected to OGD as an in vitro retinal IR injury model. After 24 h of reoxygenation, OGD induced 661W cells showed prominently decreased cell viability (70.0%), while CPX treatment restored this reduction caused by OGD damage. CCK-8 analysis revealed that CPX both showed good neuroprotective activity at 1 and 2 μM, and the cell viabilities were increased by 15% and 26%, respectively, compared with OGD model group (Fig. 2A). For further investigation, we performed Hoechst-PI staining fluorescence imaging to monitor cell morphological changes during apoptosis after reperfusion. With the aid of fluorescence microscopy, we observed OGD treatment induced obvious morphological changes of 661W cells, including bright and shrinking cell body, and reduced cell number, while CPX treatment remarkably attenuated the cell morphological changes (Fig. 2B). In addition, we also performed Annexin V-FITC and PI staining to examine whether the neuroprotective effects of CPX on 661W cells correlated with reduced apoptosis. OGD treatment induced obvious increases in the number of apoptotic cells, which was increased from 0.6 to 24.3% in the OGD group compared to the control group. Treatment with CPX at 2 μM significantly reduced the apoptotic ratio to 6.7% (Fig. 2C). These results mentioned above indicated that CPX treatment not only protects the cell morphology but also reduces the cell apoptosis against OGD injury.

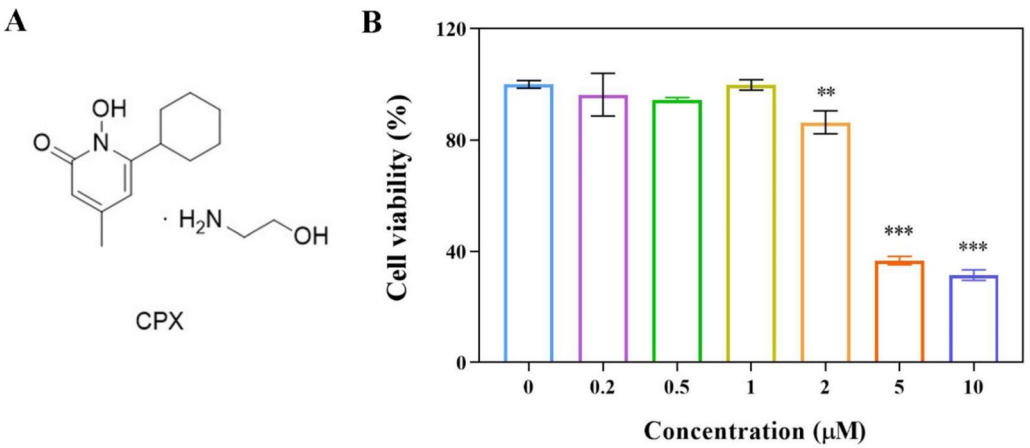

Fig. 1 (**A**) Structure of CPX; (**B**) Cell viability analysis of 661W cells incubated with CPX at indicated concentrations for 24 h ($^{**}p < 0.01$, $^{***}p < 0.001$)

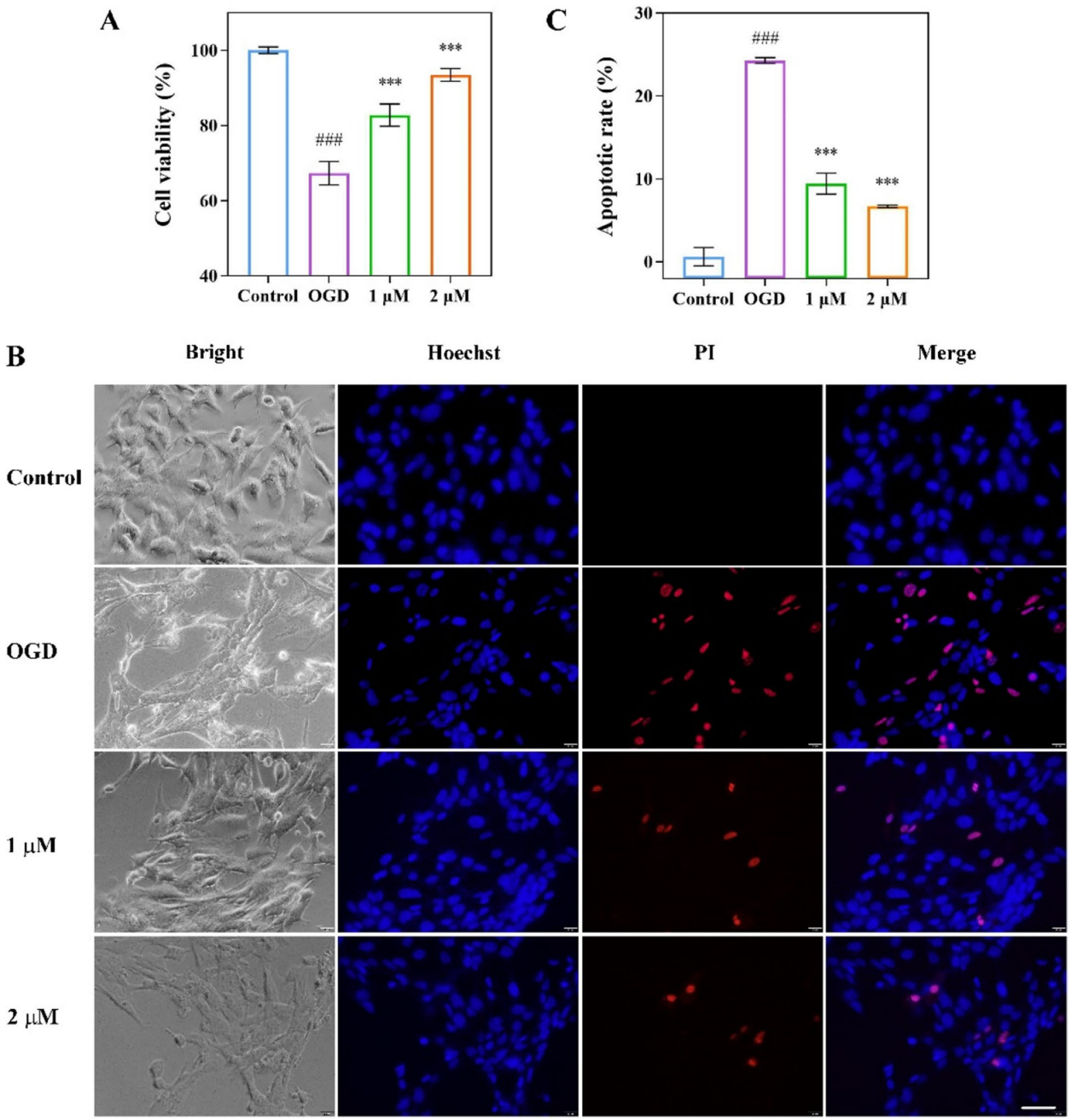

Fig. 2 **A** CPX improved 661W cells survival after OGD damage; Representative Hoechst-PI staining fluorescence images (**B**) and the percentages of apoptotic cells analysis (**C**) of 661W cells (scale bar = 20 μm, $^{###}p < 0.001$ *vs* control, $^{***}p < 0.001$ *vs* OGD group)

CPX attenuates H_2O_2-induced apoptosis in 661W cells

Considering that oxidative damage caused by the retinal IR injury can lead to neuronal apoptosis which results in neuron death, reduction of oxidative damage is another important indicator of neuroprotective capability. Thus, in vitro model using H_2O_2 stimulus is widely used to mimic ischemic stroke injury. In the present study, we evaluated the neuroprotection of CPX on H_2O_2-stimulated cellular models. As shown in Fig. 3A, 400 μM H_2O_2 reduced cell viability to ~ 50% of the control. CPX at 1 and 2 μM promoted cell viability up to ~ 80% and the cell viabilities were increased by 30% compared to the control group (Fig. 3B). As shown in Fig. 3C, the distribution of cells stained with Annexin V-FITC and PI to the lower left quadrant indicated that these cells were viable. H_2O_2 induced obvious increases

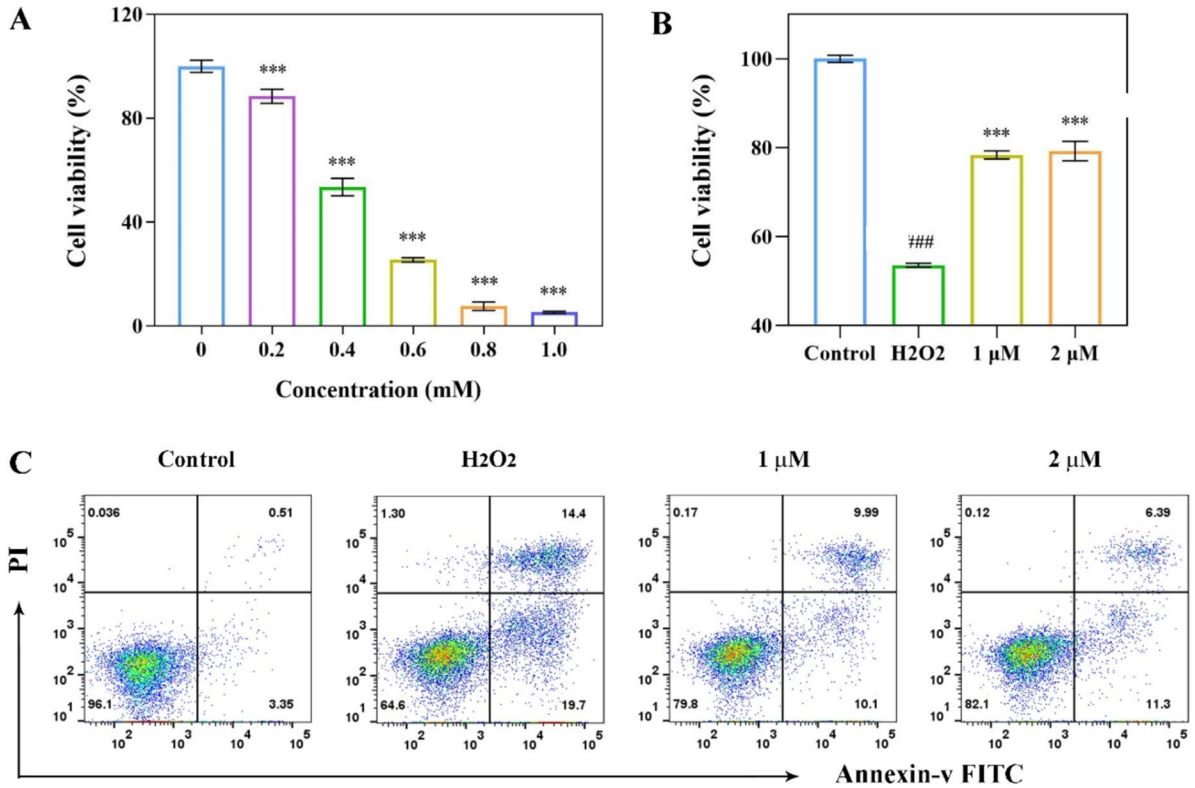

Fig. 3 CPX attenuated H_2O_2-induced oxidative stress. (**A**) H_2O_2-induced apoptosis in 661W cells; (**B**) statistical results of the apoptotic rate; (**C**) Representative plot of Annexin V-FITC/PI staining ($^{###}p < 0.001$ *vs* control, $^{***}p < 0.001$ *vs* OGD group)

in cell numbers at early and late stages of apoptosis. However, CPX treatment significantly reduced the apoptotic ratio compared to the H_2O_2 group. These above results suggested that CPX could attenuate oxidative damage in 661W cells.

CPX inhibited inflammatory responses in OGD induced cell damage

To check whether CPX inhibits the production of inflammatory cytokines, 661W cells were pre-treated with CPX before being stimulated with OGD. As shown in Fig. 4, we observed a marked increase in the production of inflammatory cytokines as measured by the flow cytometry (IL-6: 11793.7 *vs* 2.5 pg/mL; IL-23: 6471.5 *vs* 0 pg/mL and TNF-α: 69.7 *vs* 0 pg/mL), suggesting significant inflammatory responses induced by OGD injury in 661W cells. However, the inflammatory responses were significantly but incompletely reversed by the pre-treatment with CPX, which showed a higher inhibition at 2 μM (IL-6: 565.9 pg/mL; IL-23: 140.0 pg/mL and TNF-α: 0.6 pg/mL). Collectively, these findings indicated that the aberrant change on inflammatory responses in OGD induced cell damage was significantly suppressed by CPX treatment.

CPX preserved the normal thickness of the retina after I/R injury

To further determine whether CPX treatment could prevent retinal damage in a rat model of retinal IR injury, CPX solution was administrated intravenously via the tail 2 h before retinal IR injury. After intraocular pressure (IOP) elevations at 110 mm Hg for 90 min, the needle was carefully removed to allow the IOP to return to normal. CPX was then continuously intravenously injected into the rats every day for 7 days. The dose of CPX (3 mg/kg) was determined based on previous studies, which was evaluated various effects of CPX treatment [26]. Ten days after natural reperfusion, the retinas suffered morphological alterations in a mouse model of retinal IR injury. The thickness of the inner retina and whole retina exposed to retinal IR in H&E staining decreased by 21.7 and 51.8 μm, respectively. However, retinal damage was significantly ameliorated by CPX, with the inner and whole retinal thickness increases of 9.8 μm and 22.6 μm after treatment with 3 mg/kg CPX, respectively (Fig. 5A, D and E). Similar results were observed in the retinal thickness monitored by optical coherence tomography (OCT) and the changes in the thickness of inner and whole retina also demonstrated the neuroprotective effect of CPX in

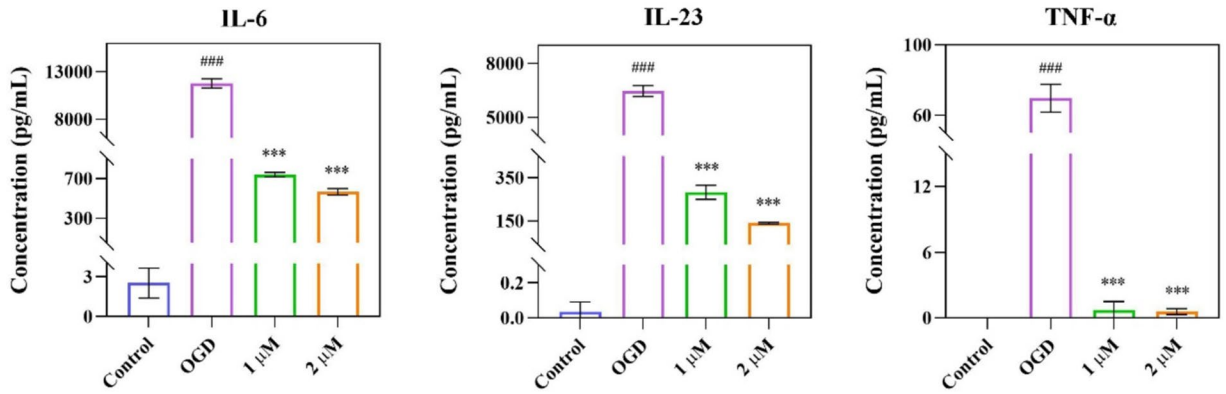

Fig. 4 CPX inhibited the production of inflammatory cytokines in the OGD induced 661W cell injury ($^{###}P < 0.001$ *vs* control group, $^{***}P < 0.001$ *vs* OGD group)

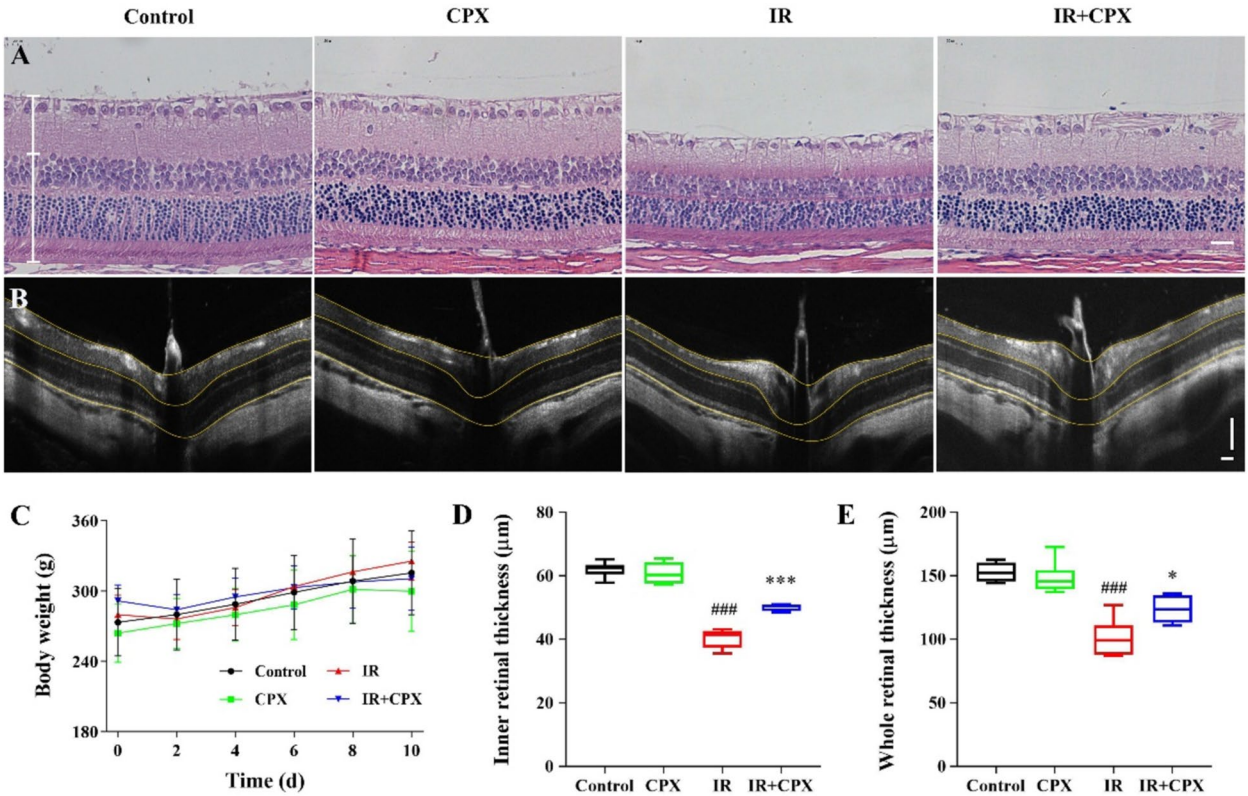

Fig. 5 CPX administration ameliorated retinal thickness damage. (**A**) Representative H&E images of retinal sections after retinal IR injury (scale bar: 50 μm); (**B**) Representative OCT scans for the retinas immediately after IR in living rats (scale bars: 200 μm); (**C**) Body weights of the Sprague–Dawley (SD) rats after CPX administration; Thickness of the inner retina (**D**) and whole retina (**E**) decreased under retinal IR injury, whereas CPX pretreatment rescued the deterioration of the above indicators. Data are presented as the mean ± SD ($^{###}P < 0.001$ *vs* control group, $^{*}P < 0.05$, $^{***}P < 0.001$ *vs* IR group)

retinal IR (Fig. 5B). H&E staining on retinal cryosections and thickness quantification of retinal layers revealed no differences in histologic morphology or retinal thickness between the control and CPX administered eyes (Fig. 5A, D and E). In addition, body weight was unchanged in the CPX group compared to the control and retinal IR groups (Fig. 5C). These data suggested that CPX intravenous administration caused no obvious intraocular and systemic toxicity.

Discussion

In this work, we explored the protective effect of CPX against retinal IR injury and its underlying mechanism. Our main findings are as follows: (1) CPX prevented cell loss in the OGD and H_2O_2 induced 661W cell injuries (Figs. 2 and 3); (2) CPX ameliorated the microenvironment of inflammation by downregulating the levels of pro-inflammatory cytokines (IL-6, IL-23 and TNF-α) under OGD-induced inflammation (Fig. 4); (3) Short-term administration of CPX at 3 mg/kg helped maintain a relatively normal morphology of the retina (Fig. 5).

The retinal IR injury is a basic pathological procedure in clinic and associated with various ischemic retinal diseases, which are major causes of irreversible vision loss globally. Multiple factors, such as oxidative stress, pro-inflammatory processes and death of retinal cells, can lead to retinal IR injury [10, 27]. Unlike other antifungal compounds, CPX has good blood–brain barrier permeability, superior antioxidant capacity and anti-inflammatory activity in cell and animal studies [26], indicating that it possesses development potential as a retinal anti-ischemic agent. In this study, treatment with CPX at 1 and 2 μM significantly suppressed the apoptotic ratio in 661W cells after OGD exposure (Fig. 2). Besides, CPX exerted ameliorative effects against H_2O_2-induced 661W cell damage (Fig. 3). These results further verify the neuroprotective activity of CPX, which might be associated with its anti-apoptosis and anti-oxidative stress properties. In addition, ischemic injury is associated with inflammation cytokines and overmuch release of inflammatory mediators by necrotic cells further intensifies the inflammatory level [4, 11]. In middle cerebral artery occlusion (MCAO) rat model, CPX treatment markedly inhibited microglial activation and reduced the levels of pro-inflammatory cytokines [25, 26]. Our results also showed that OGD induced overproduction of pro-inflammatory cytokines was also significantly suppressed by CPX treatment.

The Akt/GSK3β signaling pathway has a significant impact on maintaining cell survival after ischemic retinal injury [28, 29]. Recently published studies show that pregabalin-mediated retinal ganglion cell (RGC) neuroprotection from retinal IR injury was attributed to upregulation of the Akt/GSK3β pathway [30]. Feng et al. also found CPX treatment further enhanced the phosphorylation of Akt and GSK3β under OGD insults and targeting Akt-GSK3β pathway by CPX could effectively contribute to its potent anti-ischemic effects [26]. Therefore, the potential mechanisms of CPX-mediated Akt/GSK3β pathway activation may also contribute to its neuroprotective effects on retinal IR injuries.

To accommodate the high oxygen consumption rate, the retina possesses a unique dual blood supply that divides the retina into inner and outer layers for more efficient oxygenation [31]. Inner retinal layers receive supply from branches of the central artery of the retina and are more susceptible to hypoxic challenges [32, 33]. Previous studies reported that CPX treatment at 3 mg/kg i.v. not only reduced the brain infarction and neurological deficits but also ameliorated the blood–brain barrier in MACO rats [26]. In this study, the retinal IR injury model was established by increasing intraocular pressure (IOP) [34]. After ten days of reperfusion, significant morphological changes were observed in retinal tissue sections, particularly the retinal thinning following retinal IR injury. Moreover, we found that CPX remarkably thickened the retina, especially in the inner retina, showing a protective effect on retinal morphology. One potential limitation of this study is that the protective effect of CPX at a single agent dose was evaluated, further research with multiple doses is needed to provide the dose–response relationship and identify the ideal dosage. Additionally, we only examined early outcomes in the first 10 d after retinal IR injury. The long-term effects of CPX and its functional assessment still need to be addressed.

Conclusions

In summary, our study demonstrated that CPX significantly prevents OGD and oxidative stress induced 661W cell death, and ameliorates retinal morphology. Moreover, our studies provide strong evidence that CPX exerts powerful anti-oxidative, anti-inflammatory and anti-apoptotic effects, acting as a neuroprotective agent against retinal IR injury. Although systematic studies will be necessary to further illustrate the specific mechanism of action and translate to clinical usage, our results provide compelling evidence that CPX has great prospects for application in treating diseases associated with retinal IR injuries.

Methods

Cell culture

The 661W cells, a mouse photoreceptor cell line, were purchased from ATCC. The cells were cultured in high-glucose DMEM (4.5 g/mL, Solarbio, China) supplemented with 10% FBS (Vigonob, UY), 100 μg/mL streptomycin (Solarbio) and 100 U/mL penicillin (Solarbio) in a humidified atmosphere under 5% CO_2 at 37 °C conditions. When cells reached 90% confluence, they were seeded into 6- or 96-well plates for subsequent experiments.

Cell viability assay

The 661W cells were grouped and cultured at 1×10^4 cells per well in a 96-well plate for 12 h to allow them to attach.

Then, the cells were incubated with CPX (> 99.9%, Macklin Inc., China) at the indicated concentrations for 24 h. Cell Counting Kit-8 (CCK-8, APExBIO Technology LLC, USA) solution was used as manufacturer's instructions.

OGD injury

661W cells in exponential growth phase were seeded into 96-well plates at a density of 1×10^4 cells per well for 12 h to allow them to attach. To mimic IR injury, OGD injury was induced in 661W cells. Briefly, cells were pretreated with CPX at the indicated concentrations for 2 h, then the medium was changed to glucose and serum-free DMEM with CPX and the cells were cultured in a hypoxia chamber containing 1% O_2 for 8 h. After hypoxia injury, the complete culture medium with CPX was changed and continued to be cultured under normal culture conditions for 24 h. Finally, cell viability was detected by CCK-8 assay. The cells of OGD group were cultured without CPX and the control cells were cultured in a complete medium.

H_2O_2 injury

661W cells in exponential growth phase were seeded into 96-well plates at a density of 1×10^4 cells per well for 12 h to allow them to attach. The cells were pre-incubated with CPX at the indicated concentrations for 2 h, followed by exposure to H_2O_2 freshly prepared for 24 h. The control group without H_2O_2 was cultured in a complete medium. Cell viability was detected by CCK-8 assay.

Analysis of apoptosis

661W cells in exponential growth phase were seeded into 96-well plates at a density of 1×10^4 cells per well. After OGD injury, cells were incubated with Hoechst 33342/PI (Beyotime, China) at 4 °C for 30 min in the dark, and then washed with PBS. Images were taken using using a fluorescence microscopy (IX73, Olympus, Japan) immediately. For OGD or H_2O_2 injury, 661W cells were collected by centrifugation (1000 r/min, 5 min). After PBS washing, cells were stained with Annexin V-FITC and PI (Beyotime, China), and then were analyzed on a FACSCanto flow cytometer (BD Biosciences, USA) within 1 h.

ELISA

661W cells in exponential growth phase were seeded into 96-well plates at a density of 1×10^4 cells per well. After OGD injury, 45 μL medium was collected at 4 h after reperfusion and centrifuged for 5 min at 3,000 rpm. The expressions of IL-1β, IL-6, IL-10, IL-17A, IL-22, IL-23, IFN-γ and TNF-α in the supernatant of cultured 661W cells were detected by flow cytometry with AimPlex multiple immunoassay Flow.

Animals

Animal protocols were approved by the Institutional Animal Care and Use Committee of Henan Eye Hospital and performed according to the welfare of experimental animals. Adult male Sprague–Dawley (SD) rats (6–8 weeks, 180–200 g) were purchased from Huaxing Experimental Animal Farm of Huiji District (zhengzhou, China). Animals were maintained in temperature and humidity-controlled rooms on a 12:12 light/dark cycle with freely accessible to food and water. The rats were randomly assigned into four groups ($n=6$/group) before surgery: (i) Control group; (ii) CPX group was administered intravenously with CPX every 24 h for 7 days at 3 mg/Kg; (iii) IR group was treated by anterior chamber perfusion and intravenously injected with normal saline; (iv) IR + CPX group was conducted similar surgical procedures and injected with CPX.

Retinal IR injury model

The retinal IR injury was performed by acute elevation of intraocular pressure (IOP) via anterior chamber perfusion as previously described [12]. Briefly, rats were anesthetized, and then ischemia was applied to the left eyes by increasing IOP to cut off the blood supply from the retinal artery. A 33-gauge needle penetrated into the anterior chamber and its attached saline reservoir was elevated to achieve an IOP of 110 mmHg for 90 min, and then removal of the needle allowed the release of pressure and natural reperfusion. Finally, a topical antibiotic and steroid ointment was applied to the conjunctival sac.

Hematoxylin and eosin (H&E) staining

The rats were generally anesthetized and sacrificed, and then the eye from each rat was removed for histology. The eyes were fixed in 4% paraformaldehyde fix solution and further embedded in paraffin, which were cut into 5 μm slices parallel to the central part of the eyeballs through the optic nerve and performed H&E staining. Finally, the sections were dehydrated and sealed by neutral balsam. Images were obtained using a multispectral panoramic tissue scanning microscope (TissueFAXS Spectra, Zeiss). CaseViewer software (3DHISTECH CaseViewer, Budapest, Hungary) was used to label the thickness of ganglion cell layer.

Statistical analysis

Statistical analysis was performed on GraphPad Prism Version 8.0, which were reported as the mean ± SD. Between-group comparison was carried out by one-way ANOVA. The significant level was $p<0.05$.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12860-024-00520-w.

Supplementary Material 1.

Acknowledgements
Not applicable.

Authors' contributions
Fangyuan Qin contributed to the conception of the study; Enming Du, Xiaolin Jia and Xiaoli Li performed the experiment; Fangyuan Qin and Beibei Zhang contributed to wrote the manuscript; Beibei zhang contributed to the analysis of the experimental data; Yaping zhai contributed to the Writingoriginal draft and Writing–review editing; Enming Du and Beibei zhang helped perform the analysis with constructive discussions. The author(s) read and approved the final manuscrip.

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Availability of data and materials
Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate
All experimental animal procedures were approved by the Animal Care and Ethical Committee of Henan Eye Hospital, and strictly followed the ethical guidelines of the Basel Declaration and the International Council for Laboratory Animal Science (ICLAS).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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