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Neuroprotective Properties of Chlorogenic Acid and 4,5-Caffeoylquinic Acid from Brazilian arnica (*Lychnophora ericoides*) after Acute Retinal Ischemia

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Key words

Lychnophora ericoides, compositae, Asteraceae, 4,5-di-*O*-[E]-caffeoylquinic acid, chlorogenic acid, acute rat retinal ischemia, neuroprotection

received	March 28, 2022
accepted after revision	June 27, 2022
published online	October 11, 2022

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Planta Med 2023; 89: 183–193
DOI 10.1055/a-1903-2387
ISSN 0032-0943

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

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ABSTRACT

Lychnophora is a genus of South American flowering plants in the daisy family, popularly known as “Brazilian arnica”. It is used in traditional medicine as an anti-inflammatory and analgesic agent, whose active components are derived from chlorogenic acid (CGA) and C-flavonoids. Since the drugs currently used are ineffective to treat glaucoma, agents with antioxidant and anti-inflammatory properties may represent new alternatives in preventing cellular lesions in retinal ischemia. In this study, we report the neuroprotective effects of CGA and 4,5-di-*O*-[E]-caffeoylquinic (CQA) acid, isolated from *Lychnophora* plants, in a rodent glaucoma model. Wistar rats were administered intravitreally with 10 µg CGA or CGA, and then subjected to acute retinal ischemia (ISC) by increasing intraocular pressure (IPO) for 45 minutes followed (or not) by 15 minutes of reperfusion (I/R). Qualitative and quantitative analyses of neurodegeneration were performed using hematoxylin-eosin or Fluoro-Jade C staining protocols. All retinas submitted to ISC or I/R exhibited matrix disorganization, pyknotic nuclei, and pronounced vacuolization of the cytoplasm in the ganglion cell layer (GCL) and inner nuclear layer (INL). Pretreatment with CGA or CQA resulted in the protection of the retinal layers against matrix disorganization and a reduction in the number of vacuolized cells and pyknotic nuclei. Also, pretreatment with CGA or CQA resulted in a significant reduction in neuronal death in the GCL, the INL, and the outer nuclear layer (ONL) after ischemic insult. Our study demonstrated that CGA and CQA exhibit neuroprotective activities in retinas subjected to ISC and I/R induced by IPO in Wistar rats.

Introduction

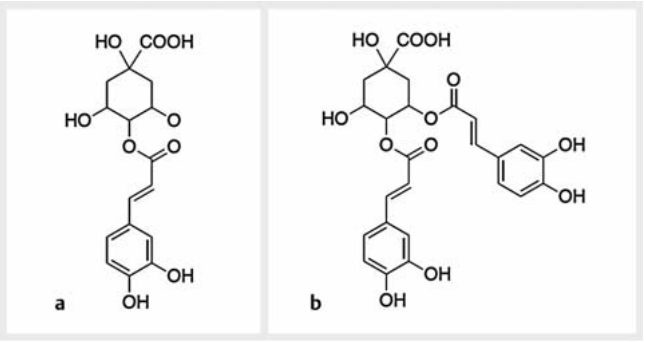
Lychnophora ericoides Mart., identified in this study by Dr. João Semir from the Biology Institute of the State University of Campinas (UNICAMP, Campinas, SP, Brazil), is one of several species from *Lychnophora* (subtribe *Lychnophorinae*, Asteraceae), popularly known as “false arnica” or “Brazilian arnica”. Plants of the genus Lychnophora are used in traditional medicine as analgesic and anti-inflammatory agents [1]. Moreover, Gobbo-Neto et al. (2005) [2] also observed an anti-inflammatory activity from *L. ericoides*. The main compounds in the “arnicas” with significant biological activity are phenolic compounds, including chlorogenic

ABBREVIATIONS

ANOVA	analysis of variance
AP-1	Activating protein-1
Bax	Bcl-2-associated X-protein
BCG	Mycobacterium bovis Calmette-Guérin Bacillus
BDNF	brain-derived neurotrophic factor
CAPE	caffeic acid phenethyl ester
CAT	catalase
CGA	chlorogenic acid
CQA	4,5-di-*O*-[E]-caffeoylquinic acid
EMR	electromagnetic radiation
FJC	Fluoro-Jade C
GCL	Ganglion cell layer
GSH-Px	phospholipid hydroperoxide glutathione peroxidase
INL	inner nuclear layer
IOP	intraocular pressure
LCA	licarin A
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
n-BuOH	*n*-butanol
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
Nrf2	Nuclear factor-erythroid 2 related factor 2
ONL	outer nuclear layer
PGE2	Prostaglandin E2
PTEN	phosphatase and tensin homolog
RGC	retinal ganglion cell culture
RGC	retinal ganglion cells
ROS	reactive oxygen species
SEM	standard error of the mean
SOD	superoxide dismutase
TGF-β	transforming growth factor-beta
Thy-1	Thy-1 cell surface antigen
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

acid (CGA) and its derivatives such as 4,5-di-O-caffeoylquinic acid (CQA), both compounds present at ▶ **Fig. 1** (**a** and **b**, respectively) [2, 3]. Compound CQA has been shown to have neuroprotective potential in an acute seizure model [4]. Furthermore, recently our group described an anti-inflammatory effect of another phenolic compound, licarin A (LCA), in a model of uveitis induced by *Mycobacterium bovis* Calmette-Guérin Bacillus (BCG). Intravitreal injection of LCA significantly reduced the levels of inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 [5]. These studies encouraged the investigation of these compounds from "*Brazilian arnica*" in ocular disease models.

In 2040, it is estimated that almost 111.8 million people worldwide will suffer from glaucoma, which causes irreversible blindness [6]. The primary cause of glaucoma is unknown, but several risk factors have been identified, including elevated intraocular pressure (IOP) and aging. Loss of vision in glaucoma is caused



▶ **Fig. 1** Chemical structures of chlorogenic acid (**a**) and its derivatives such as 4,5-di-*O*-caffeoylquinic acid (**b**).

mainly by the death of retinal ganglion cells (RGC), the neurons that convey visual information from the retina to the brain. Lowering IOP is not enough to protect the retina neurons. Therefore, therapeutic strategies aimed at delaying or halting RGC loss, which would be valuable to save vision in glaucoma, are needed [7]. The ischemic zone, or *penumbra*, is an area where tissue does not immediately die, hence the surrounding neurons can still be protected from neurodegeneration, halting the injury process [8].

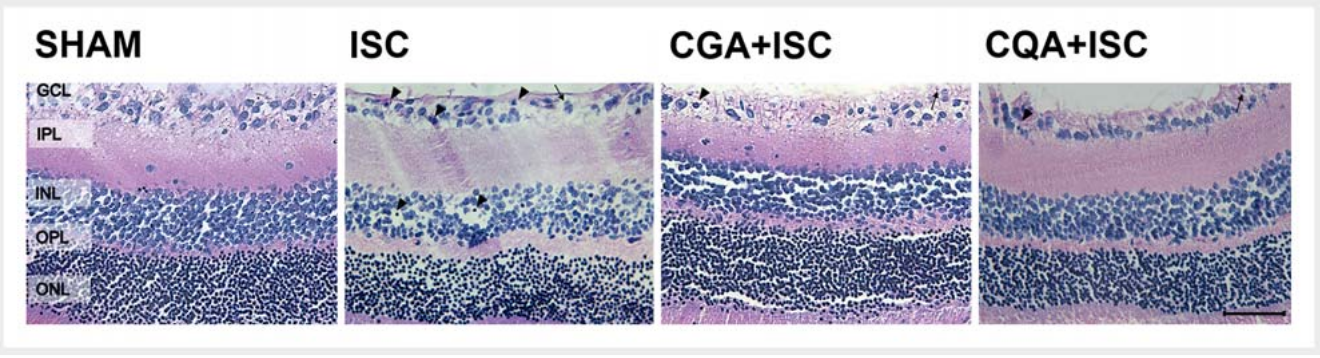
Some potential therapies, such as anti-inflammatory and antioxidant drugs [9, 10], both developed from synthetic [11] and natural compounds [4] have been studied in glaucoma experimental models. In this regard, natural compounds with antioxidant and anti-inflammatory properties may represent an attractive approach to preventing neuronal death progression in the area surrounding the ischemic zone deeply related to inflammatory process. *In vitro* and *in vivo* studies demonstrated that CGA, which is the major component of coffee, reduces apoptosis of retinal cells induced by hypoxia and nitric oxide (NO) and that coffee consumption may help prevent retinal degeneration [12]. Previous studies suggested that CGA and its derivates have significant anti-inflammatory effects [2, 3]. Additionally, CGA derivatives had significant effects on reducing prostaglandin-E2 (PGE_2) and (TNF-α) production [13], factors closely related to excitotoxicity in glaucoma [14].

Collectively, these studies encouraged us to investigate the potential properties of CQA and CGA extracted from the "*Brazilian arnica*" in a glaucoma experimental model of acute retinal ischemic injury in Wistar rats.

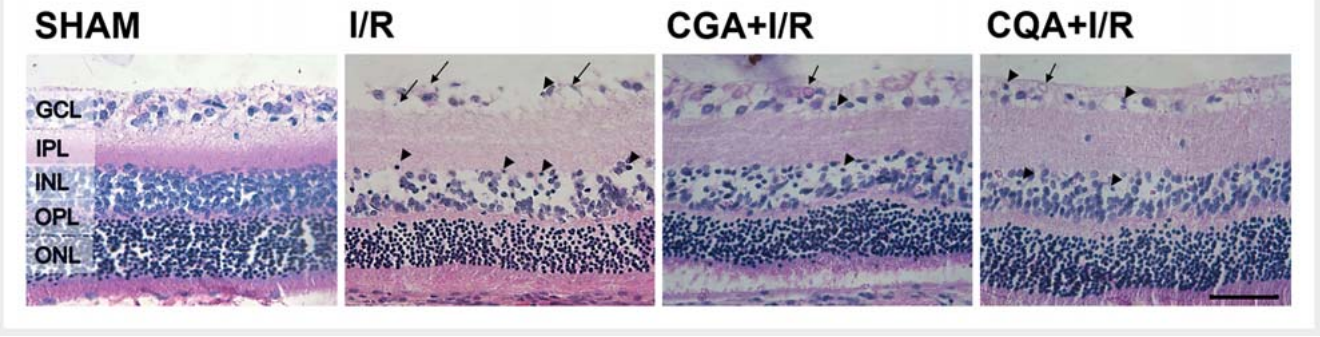
Results

A comparison of previous isolated standards and the high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis [15] confirmed the identification of the major isolated secondary metabolites as CQA (not shown).

▶ **Fig. 2** shows micrographs of the retinas in the following groups: Sham contralateral retinas (SHAM), ischemic retinas (ISC); chlorogenic acid-treated ischemic retinas (CGA+ISC); 4,5-di-*O*-[E]-caffeoylquinic acid-treated ischemic (CQA+ISC). As illustrated, the ganglion cell layer (GCL) and inner nuclear layer (INL) from retina submitted to ISC contained pyknotic nuclei (head ar-



▶ **Fig. 2** Neuroprotective effects of chlorogenic acid (CGA) and 4,5-di-*O*-[E]-caffeoylquinic acid (CQA) treatments on acute retinal ischemia (ISC). Representative photomicrographies of transverse sections of rat retinas stained with hematoxylin-eosin: Sham contralateral retinas (SHAM), ischemic retinas (ISC); chlorogenic acid-treated ischemic retinas (CGA+ISC); 4,5-di-*O*-[E]-caffeoylquinic acid-treated ischemic (CQA+ISC). Arrows point to vacuolization and head arrows to pyknotic nuclei. Scale bar = 50 µm (5 × magnification).



▶ **Fig. 3** Neuroprotective effects of chlorogenic acid (CGA) and 4,5-di-*O*-[E]-caffeoylquinic acid (CQA) treatment on acute retinal ischemia-reperfusion (I/R). Representative photomicrographies of transverse sections of rat retinas stained with hematoxylin-eosin: Sham contralateral retinas (SHAM), ischemic-reperfused retinas (I/R); chlorogenic acid-treated ischemic-reperfused retinas (CGA+I/R); 4,5-di-*O*-[E]-caffeoylquinic acid-treated ischemic-reperfused retinas (CQA+I/R). Arrows point to vacuolization, and head arrows point to pyknotic nuclei Scale bar = 50 µm (5 × magnification).

rows) and cytoplasm vacuolization (arrows). All retinas of the acute retinal ischemia-reperfusion (I/R) group exhibited matrix disorganization, vacuolization, pyknotic nuclei, and pronounced vacuolization of the cytoplasm in GCL and INL compared to the sham-control retinas (▶ **Fig. 3**). Retinas treated with CGA or CQA acids were qualitatively protected against histological lesion features in different levels (▶ **Fig. 3**). These histological alterations were not observed in the contralateral eyes (SHAM group).

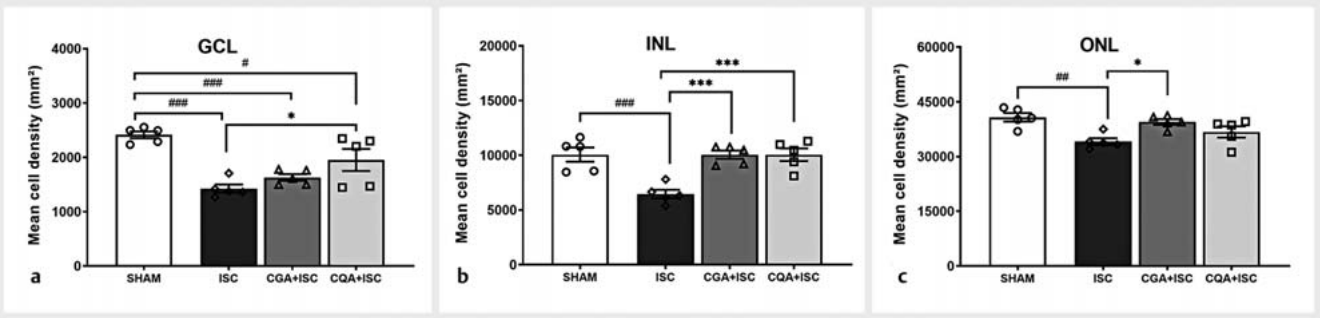
The quantitative histological analysis of retinas showed a significant reduction of 41% in the average density of GCL neurons in the ISC group compared to the SHAM group ($F_{(3,16)} = 13.6$; $p = 0.0001$) (▶ **Fig. 4a**).

Pretreatment with CGA, although it slightly reduced retinal ganglion cell culture (RGC) loss, had no significant protective effect (▶ **Fig. 4a**). Conversely, CQA effectively reduced the retinal damage in the CQA+ISC group (p = 0.0001). Compared to SHAMs, CQA treatment significantly decreased the RGC death to 20% (p = 0.0001).

As shown in ▶ **Fig. 4b**, the density of RGC also was decreased in the INL of the ISC groups by 36% compared to the SHAM group (p = 0.0002). The pretreatment with CGA and CQA effectively reduced neuronal loss in the INL. There were significant differences in average density of cells among CGA+ISC and CQA+ISC (as compared to untreated ischemia ($F_{(3,16)} = 12.3$; p = 0.0002). We observed a reduction of 36% cell density in the INL of the ISC group, whereas, in the CGA+ISC and CQA+ISC groups, only 0.5% of neuronal loss was observed (▶ **Fig. 4b**).

The quantitative analyzes of average cell density of in the outer nuclear layer (ONL) showed a decrease of 16% in the ISC group compared to SHAM group ($F_{(3,16)} = 6.8$, p = 0.0037; ▶ **Fig. 4c**). Pretreatment with CGA effectively protected the ONL from cell loss, as we observed a reduction of 3% in the average density of cells in the ISC group (p = 0.0037). The CQA+ISC group was not statistically different from the ISC group, although the pretreatment with CQA reduced the cell loss to 10% (p > 0.05) in comparison to the SHAM group (▶ **Fig. 4c**). All means ± SEM values for the experimental groups analyzed are shown in ▶ **Table 1**.

Next, we investigated the effect of CGA and CQA on the ischemia-reperfusion protocol. The data shown a cell loss of 60% in the

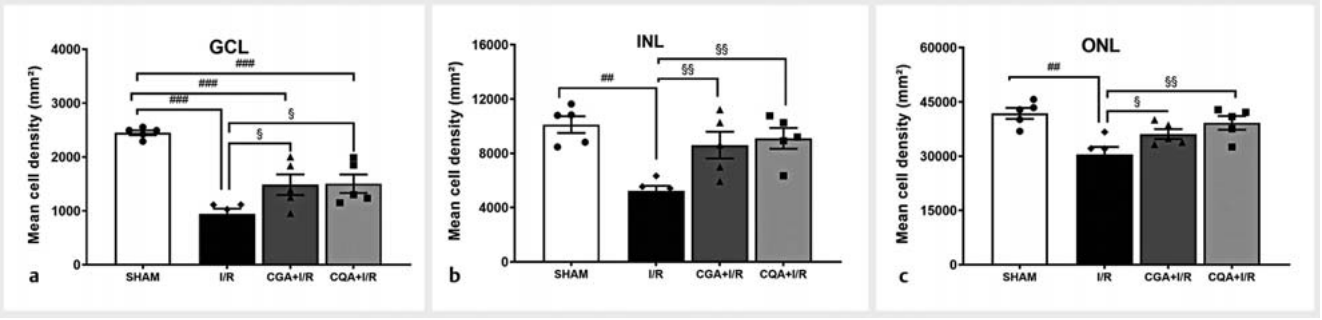


▶ **Fig. 4** Chlorogenic acid (CGA) and 4,5-di-*O*-[E]-caffeoylquinic acid (CQA) treatment on acute retinal ischemia (ISC). Histograms representing cell density (cells/mm^2) (**a**) in the ganglion cell layer (GCL); (**b**) in the inner nuclear layer (INL); (**c**) cellular density in the outer nuclear layer (ONL). Data are expressed as mean ± SEM (n = 5). # p < 0.05; # # p < 0.001; # # # p < 0.0001 vs. SHAM group; * p < 0.05; * * * p < 0.0001 vs. ISC group.

▶ **Table 1** Cell density values for each layer of rat retina stained with hematoxylin-eosin on acute retinal ischemia (ISC) model.

RETINAL LAYERS	SHAM	ISC	CGA + ISC	ISC+CQA
GCL	2,414 ± 63.2	1,424 ± 75.1$^{\#\#\#}$	1,630 ± 60.2$^{\#\#\#}$	1,953 ± 203.5$^{\#*}$
INL	10,056 ± 648.3	6,453 ± 395.2$^{\#\#\#}$	10,051 ± 377.8***	10,048 ± 579.1***
ONL	40,761 ± 1,153	34,098 ± 914.5$^{\#\#}$	39,494 ± 776.3*	336,747 ± 1,557

Data are expressed as mean cell density (cells/mm^2) ± SEM. One Way ANOVA followed by Student-Newman-Keuls (n = 5). $^{\#}$ p < 0.05; $^{\#\#}$ p < 0.001; $^{\#\#\#}$ p < 0.0001 vs. SHAM group; * p < 0.05; *** p < 0.0001 vs. ISC group. Sham contralateral retinas (SHAM), ischemic retinas (ISC); chlorogenic acid-treated ischemic retinas (CGA+ISC); 4,5-di-*O*-[E]-caffeoylquinic acid-treated ischemic (CQA+ISC).



▶ **Fig. 5** Chlorogenic acid (CGA) and 4,5-di-*O*-[E]-caffeoylquinic acid (CQA) treatment on acute retinal ischemia-reperfusion (I/R). Histograms representing cell density (cells/mm^2) (**a**) in the ganglion cell layer (GCL); (**b**) in the inner nuclear layer (INL); (c) cellular density in the outer nuclear layer (ONL). Data are expressed as mean ± SEM (n = 5). $^{\#\#}$ p < 0.001; $^{\#\#\#}$ p < 0.0001 vs. SHAM group; § p < 0.05; §§ p < 0.001 vs. I/R group.

GCL of the I/R group, when compared to SHAM group ($F_{(3,16)}$ = 20.1; p = 0.0001) (▶ **Fig. 5a**).

Pretreatment with CGA had no significant protective effect on I/R model. Conversely, CQA administration effectively reduced the RGC loss to 35% in the CQA+I/R group when contrasted to I/R group (p = 0.0001) (▶ **Fig. 5a**).

A reduction of 48% in the INL cell density was observed in the I/R group in comparison to the SHAM group. Pretreatment with CGA and CQA effectively protected the INL against the neuronal loss in the retinal ischemia-reperfusion protocol in 15% and 10%, respectively ($F_{(3,16)}$ = 8.6; p = 0.0012) (▶ **Fig. 5b**).

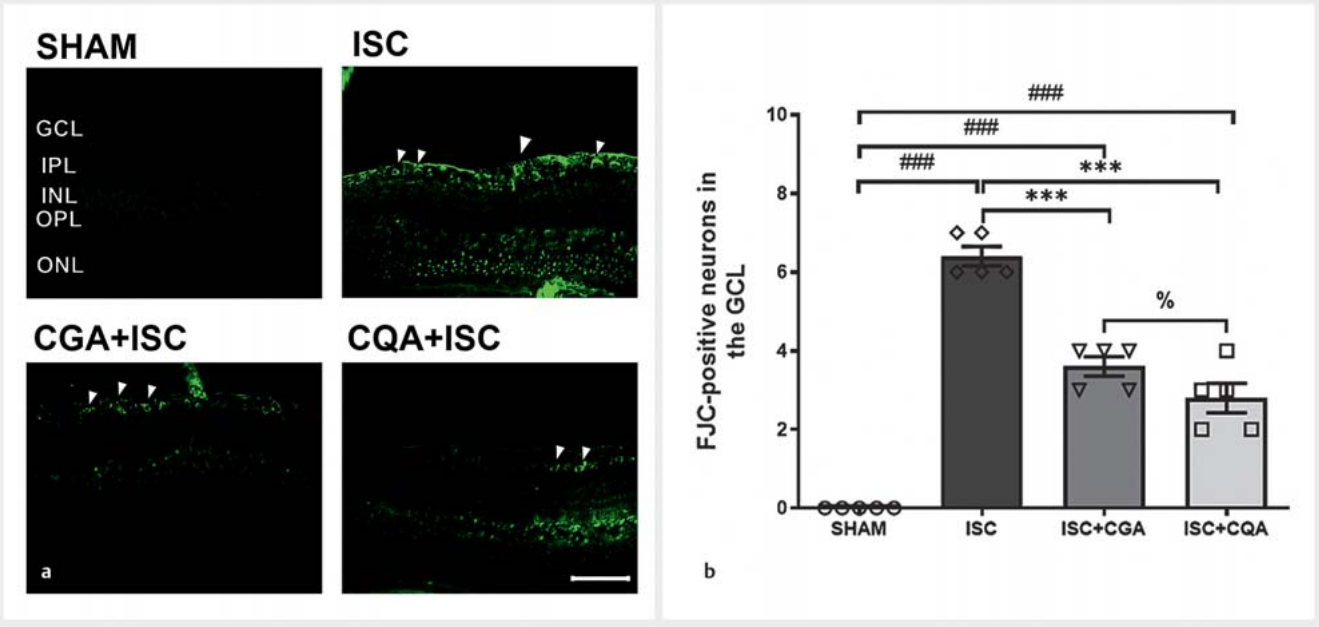
The I/R group resulted in a decrease of 25% in the neuronal density in the ONL compared to the SHAM group ($F_{(3,16)}$ = 6.791, p = 0.0037; ▶ **Fig. 5c**). Pretreatment with CGA and CQA reduced the cellular loss in the retinal ischemia-reperfusion model to 11% and 4%, respectively, in comparison to the I/R group (p = 0.0019) (▶ **Fig. 5c**).

All means ± SEM values for the experimental groups analyzed are shown in ▶ **Table 2**.

▶ **Table 2** Cell density values for each layer of Wistar rat retina stained with hematoxylin-eosin on acute ischemia-reperfusion (I/R) model.

RETINAL LAYERS	SHAM	I/R	ICGA+I/R	ICQA+I/R
GCL	2,451 ± 45.3	942.8 ± 97.8$^{###}$	1,486 ± 191.7$^{###*}$	1,504 ± 172.2$^{###*}$
INL	10,110 ± 618.8	5,230 ± 371.9$^{##}$	8,598 ± 981.9**	9,093 ± 766.3**
ONL	41,794 ± 1,511	30,464 ± 2,105$^{##}$	36,108 ± 1,349§	39,197 ± 1,893§§

Data are expressed as mean cell density (cells/mm^2) ± SEM. ANOVA one way followed by Student-Newman-Keuls. (n = 5). $^{##}$ p < 0.001; $^{###}$ p < 0.0001 vs. SHAM group; § p < 0.05; §§ p < 0.001 vs. I/R group. Sham contralateral retinas (SHAM), ischemic retinas (I/R); chlorogenic acid-treated ischemic retinas (CGA+I/R); 4,5-di-*O*-[E]-caffeoylquinic acid-treated ischemic (CQA+I/R).



▶ **Fig. 6** Effects of chlorogenic acid (CGA) and 4,5-di-*O*-[E]-caffeoylquinic acid (CQA) treatments against ganglion cells layer (GCL) neurodegeneration on acute retinal ischemia (ISC) model. **a** representative photomicrographies of transverse sections of rat retinas stained with Fluoro-Jade C (FJC). Sham contralateral retinas (SHAM), ischemic retinas (ISC); CGA-treated ischemic retinas (CGA+ISC); CQA-treated ischemic (CQA+ISC). White arrows point to FJC-positive neurons. No FJC-positive neurons were detected in the SHAM group. Scale bar: 50 µm (5 × magnification). **b** The histogram represents FJC-positive neurons in the ganglion cell layer (GCL) from the Wistar rat retina. Data are expressed as mean ± SEM (n = 5). $^{###}$ p < 0.0001 vs. SHAM group; *** p < 0.0001 vs. ISC group; $^{\%}$ p < 0.05 vs. CQA+ISC group.

FJC staining was used to evaluate neuronal degeneration after retinal ischemia and retinal ischemia-reperfusion protocols to validate the H-E staining analysis. In this study we prioritized analysis of the RGC in the GCL, as it is the most susceptible to death by ischemic injury, although the degenerative process can be found in the other layers of the retina (▶ **Fig. 6a** and **7a**).

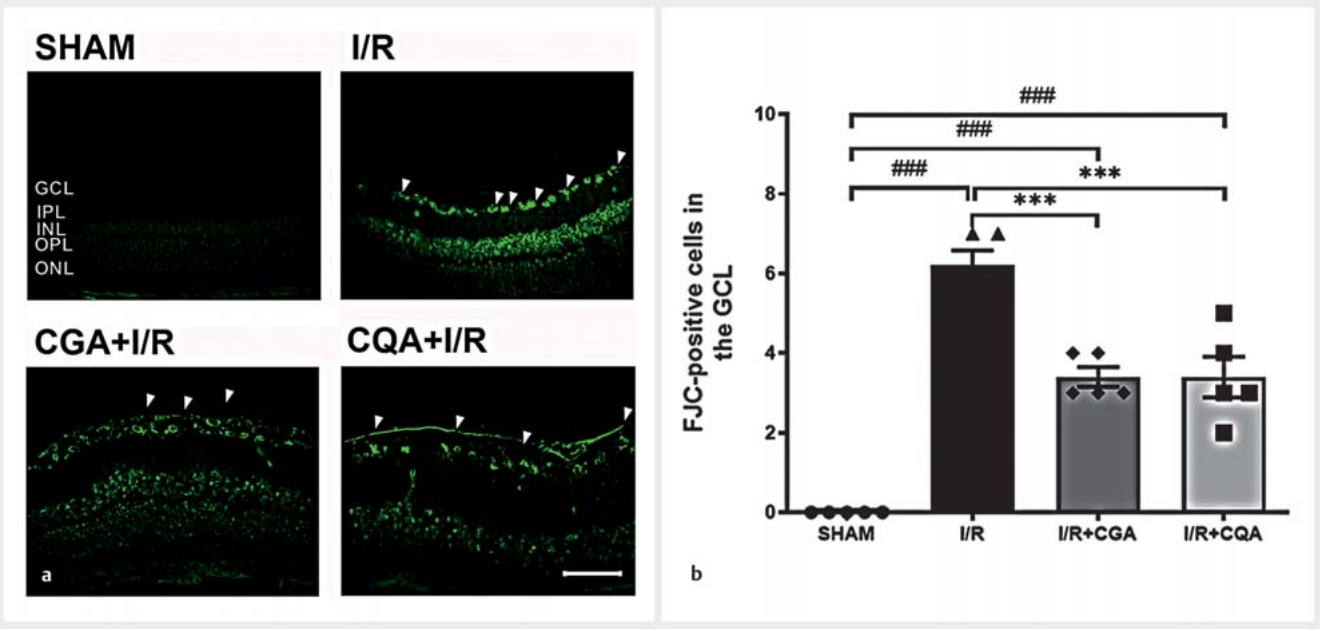
The average of FJC-positive neurons in the GCL was 6.4 in the ISC group (6.4 ± 0.2). Retinas pretreated with CGA and CQA exhibited a significant reduction on the average of FJC-positive neurons in the CGA+ISC and CQA+ISC groups ($F_{(3,16)} = 74.5$; p < 0.0001), as illustrated in ▶ **Fig. 6b**.

The number of neurodegenerating neurons in the I/R group is 6.2 ± 0.4 (p < 0.0001) in comparison to 0.0 ± 0.0 in the SHAM group (▶ **Fig. 7b**).

Pretreatments with CGA and CQA resulted in a significant reduction in the number of FJC-positive neurons in the I/R group, with an average of 3.4 neurons in the CGA+I/R group and 4.7 in the CQA+I/R group in comparison to I/R group ($F_{(3,16)} = 55.97$, p < 0.0001) (▶ **Fig. 7b**).

Discussion

Treatments for acute and chronic neurological diseases like Alzheimer's, Parkinson's, epilepsy, stroke, and retinal neuropathologies such as glaucoma have had limited success and often exhibit undesired side effects. Consequently, there is a pressing unmet need for effective treatments for these diseases. In this context, pharmacological studies of natural products with the goal of developing new drugs are crucial. Natural products are an abundant



▶ **Fig. 7** Effects of chlorogenic acid (CGA) and 4,5-di-*O*-[E]-caffeoylquinic acid (CQA) treatment against ganglion cells layer (GCL) neurodegeneration on acute retinal ischemia-reperfusion (I/R) model. **a** representative photomicrographies of transverse sections of rat retinas stained with Fluoro-Jade C (FJC). Sham contralateral retinas (SHAM), ischemic retinas (ISC); chlorogenic acid-treated ischemic retinas (CGA+I/R); 4,5-di-*O*-[E]-caffeoylquinic acid-treated ischemic (CQA+I/R). White arrows point to FJC-positive neurons. No FJC-positive neurons were detected in the SHAM group. Scale bar: 50 µm (5 × magnification). **b** The histogram represents FJC-positive neurons in the ganglion cell layer (GCL) from the Wistar rat retina. Data are expressed as mean ± SEM (n = 5). ### $p < 0.0001$ vs. SHAM group; §§§ $p < 0.0001$ vs. I/R group.

source of new bioactive molecules that can be helpful to identity new compounds with neuroprotective activity. In this context, Brazil stands out as a rich source of biodiversity globally, which can provide an arsenal of potential neuroactive compounds from both animals and plants [16].

Acute experimental glaucoma, a model of ischemic insult, simulates the enhancing of IOP for a limited period and it has been employed in many studies [17]. There are many advantages of this model, such as being a relatively simple procedure, allowing for good control over the ischemic injury area, and for studying details of the I/R period [17–19].

Ischemia and I/R injury trigger deadly pathways induced by glutamate-excitotoxicity, free radical-mediated injury, and inflammatory mechanisms [20]. Consequently, a potential effective neuroprotective molecule should derail some of these cascade pathways [21]. In this work we investigated natural compounds like CGA and CQA, as they have previously shown to have anti-inflammatory, antioxidant, and neuroprotective effects. CGA is an ester of caffeic acid and quinic acid [22], and are known to have anti-inflammatory [23], and antioxidant [24]. Hence, further investigation of CGA and CQA may be helpful in the search for novel therapies. To the best of our knowledge, this is the first report evaluating CGA and CQA in models of acute retinal ischemia and – acute retinal I/R induced by increased intraocular pressure in Wistar rats.

Our data demonstrated that acute retinal ischemia and I/R induced significant cellular disorganization of the GCL, INL, and ONL. Additionally, we observed an increase of pyknotic nuclei and pronounced vacuolization of the cellular cytoplasm in all retinal layers. Such results are similar to data previously reported by others [18, 19]. In addition, the neuronal density was greatly reduced in the GCL (40% in ISC and 60% in I/R groups), INL (36% in ISC and 45% in I/R groups), and ONL (16% in ISC and 25% in I/R groups), with the group that went through reperfusion (I/R group) showing more damage than the ischemic retinas group (ISC). It is expected that I/R results in more damage than ischemia [25].

Intravitreal pre-treatments with CGA or CQA resulted in significant preservation of the structural organization of retinal cells in GCL, INL, and ONL in both ISC and I/R groups. Our data demonstrated that CQA, but not CGA, protected the RGC against the injury after ischemia. However, CGA effectively protected the ONL layer from cell loss, whereas CQA did not have this effect. This result was unexpected, and further studies are needed to understand this effect thoroughly. Furthermore, both treatments were neuroprotective in the INL layer. This neuroprotective potential was also demonstrated with CGA and CQA in the ischemia-reperfusion protocol. The compounds significantly reduced neuronal loss in the CGL, INL, and ONL, even though the most severe retinal damage occurred after reperfusion. We suggest that this robust neuroprotective effect against an insult of greater magnitude is due, in addition to the intrinsic effects of CGA and CQA, to the prior initiation of treatment against damage caused by reperfusion. Thus, this pre-treatment would act in an early stage of the signaling cascade that culminates in cell death, minimizing the damage caused by ischemia and I/R.

Flavones and flavonols, the most important and common classes of natural products with anti-inflammatory and antioxidant activities [26, 27], share structural similarity with CGA and CQA, as they are phenols derivatives of caffeic acid. This led us to speculate they might act through similar mechanisms to offer neuroprotection. Some previous studies corroborate with this possibility; one study showed that these compounds have antioxidant properties by acting directly on reactive oxygen species (ROS), scavenging or metal ion chelation, since both terminate in redox homeostasis processes for cell survival along with the anti-inflammatory activity [28]. Other study reports that CQA had a higher antioxidant ability in comparison to other di-O-caffeoylquinic acids in hydroxyl radical (OH)-damaged bone marrow-derived mesenchymal stem cells, which could be due to electron transfer, H^+-transfer, and Fe^{2+}-chelating due to relative positions of two caffeoyl moieties and to the conformational effect from the cyclohexane skeleton [29].

In vitro and *in vivo* studies demonstrated that CGA, which is a major component of coffee, significantly reduces apoptosis of retinal cells induced by hypoxia and nitric oxide (NO), and that coffee consumption may help prevent retinal degeneration [12], which is further supported by the fact that CGA crosses the Brain Blood Barrier [30]. Also, it has been reported that CGA prevents the downregulation of Thy-1 protein (Thy-1 cell surface antigen), an early marker of retinal ganglion cell culture (RGC) stress [12]. These previously reported mechanisms could be explored in the context of retinal glaucoma in future studies.

There are several examples of caffeic acid and its analogs with translational potential. For example, these compounds have been shown to offer tissue-protection against several models of ischemic lesion [31]. Caffeic acid phenethyl ester (CAPE) increased the activities of three antioxidant, including superoxide dismutase (SOD), catalase (CAT) and phospholipid hydroperoxide glutathione peroxidase (GSH-Px) in control rats in study of chronic exposition to electromagnetic radiation (EMR) emitted from a mobile phone for 60 days [32]. Retinal levels of NO and malondialdehyde (MDA), which are markers of lipid peroxidation, increase in EMR exposed rats, while CAPE significantly reduced their levels. CAPE significantly decreased MDA levels and increased the activities of SOD, GSH-Px, and CAT in a model of chronic retinal I/R injury induced by increasing the IOP for 60 min in rat retinas [33]. Additionally, CAPE attenuated the I/R-induced apoptosis of retinal cells in the INL and GCL, possibly by enhancing the antioxidation ability, which suggests that CAPE is potentially useful for treating I/R-induced eye disorders [33]. This data led us to speculate that, due to its similar chemical properties, the neuroprotective effects of CGA and CQA may involve an antioxidative activity that resulted in reduction of the ischemic cell death. Future studies will explore this possibility and the potential that CGA and CQA are more effective than the previously studied compounds.

Another study reported that pretreatment with CGA attenuated cell death in a concentration-dependent manner against retinal degeneration on optic nerve crush in a strain of albino mice from the Institute of Cancer Research in the USA (ICR) and in an *in vitro* retinal ganglion cell culture (RGC) hypoxic stress, possibly by preventing up-regulation of apoptotic proteins such as Bad and cleaved caspase-3 [34], which are potential mechanisms that could also be explored in the context of retinal glaucoma.

A plethora of antioxidants have been examined in models of chronic tissue injury; however, just a few studies have investigated the effects of CGA and CQA in a paradigm of acute ischemia model. In this context, we suggest that pretreatment with CQA and CGA promotes an intravitreal environment that somehow attenuates the cascade of deleterious events triggered by retinal ischemia. Shin et al. reported that both CQA and CGA could act, for example, in the interaction with vascular endothelial growth factor (VEGF) [35]. Inhibition of nuclear factor kappa B (NF-κB) and activating protein-1 (AP-1) may also be targets of action by CGA and CQA, as suggested by Feng et al. They reported for the first time that the effects of CGA may be through its up-regulation of cellular antioxidant enzymes and suppression of ROS-mediated NF-κB, AP-1, and mitogen-activated protein kinase (MAPK) activation [36]. All of these targets are directly or indirectly related to signaling pathways that could increase cell survival.

A recent study shows that CGA prevents oxidative stress and inflammation in liver stellate cells and fibroblast cells during liver fibrosis by inhibiting TGF-β (transforming growth factor-beta) and Nrf2 (Nuclear factor-erythroid 2 related factor 2) signaling pathways [37]. Additionally, Chen et al. reported that CGA administration alleviated spinal cord injury via anti-inflammatory activity mediated by nuclear factor NF-κB and MAPK [38]. A study of the effects of CGA in treating inflammation and alleviating the severity of colitis showed that CGA acts by suppressing phosphorylated extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases 1 and 2 (JNK1/2), Akt, and signal transducer and transcription activator 3 (STAT3) with concomitant up-regulation of phosphatase and tensin homolog (PTEN) expression. Therefore, CGA and CQA are noticeable potential therapeutic agents since new molecules targeting TGF-β (transforming growth factor-beta), MAPK, BDNF (brain-derived neurotrophic factor), or PTEN (phosphatase and tensin homolog) have shown preliminary success in animal models and even human trials, demonstrating that they may eventually be used to preserve retinal neurons and vision [39].

In addition, CGA showed a dose-dependent decrease in p65 subunit expression of NF-κB and suppression of tumor necrosis factor-alpha (TNF-α) expression of pro-inflammatory cytokines. Also, the attenuation of apoptosis and oxidative stress induction was dose-dependent by suppressing the expression of Bcl-2-associated X-protein (Bax), caspase-8, caspase-9, and heme oxygenase-1 (HO-1) proteins in mice administered with CGA. These studies confirm the potential of CGA to suppress the activation of pro-inflammatory and apoptotic signaling pathways [40].

Additionally, the neurotransmitter glutamate plays an important role in the neurodegenerative cascade, predominantly through the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. CGA was shown to be neuroprotective by preventing calcium influx in a model of glutamate-induced neuronal death in mouse primary cortical cultures [41]. A recent study demonstrated that CGA inhibited 4-aminopyridine (4-AP)-induced glutamate release from cortical synaptosomes by suppressing the activation of P/Q-type calcium channels and CaMKII/synapsin I pathways [42] Additionally, CGA reduced glutamate release from microglia

and significantly decreased cell death induced by hydrogen peroxide in primary neuron cultures [43]. Future studies will explore whether the mechanism of neuroprotection of CGA and CQA in our retinal model involves modulation of NMDA receptors and calcium channels.

Our results highlight the potential of these compounds to preserve retinal structure and cells against degeneration caused by increased IOP in models of ischemia and reperfusion. Limitations of this study include lack of clarity of the mechanisms involving CGA and QCA neuroprotective effects. Further studies will aim at identifying possible mechanisms of action, evaluating apoptotic, oxidative stress, and inflammatory signaling pathways in the model of ischemia and ischemia-reperfusion induced by IOP increase.

This study shows that chlorogenic and 4,5-di-*O*-[E]-caffeoylquinic acids exhibit neuroprotective activity in models of acute retinal ischemia and – acute retinal ischemia-reperfusion induced by increased intraocular pressure in Wistar rats. Pretreatments with these compounds preserved the structure of the retinal ganglionic layers and reduced neuronal death in the GCL, INL, and ONL.

Material and Methods

Plants and extracts

Leaves of *Lychnophora ericoides* were collected in Ibiraci, MG, Brazil by Dr. Norberto Peporine Lopes, and a voucher specimen was deposited at the Biology Institute of the State University of Campinas (UNICAMP), Campinas, SP, Brazil (NPL-123; herbarium UEC). *L. ericoides* polar extracts (Asteraceae, Asterales) were obtained from the Plant Extract Processing Laboratory bank from the Laboratory of Organic Chemistry, Physics and Chemistry Department, School of Pharmaceutical Sciences of Ribeirão Preto/University of São Paulo. A methanolic extract of *L. ericoides* was prepared from shade-dried leaves, that potentially increases the probability of finding chlorogenic acid derivatives [44], which was the goal of this study. In summary, initially the leaves were slowly dried in the shade for 10 days. The leaves were then air-dried, powdered (200 g) and extracted with 2.5 L of MeOH for 24 h at room temperature providing 15 g of the methanolic extract. The hydroalcoholic extract was partitioned between *n*-butanol (*n*-BuOH; Merck) and water. The *n*-BuOH-acetic acid phase was concentrated by rota-evaporation (60 °C) and the aqueous phase was concentrated by lyophilization.

Isolation of 4, 5-di-*O*-[E]-caffeoylquinic acid (CQA)

The extract was fractionated as previously described by Santos et al. (2005) [3] and Taleb-Contini et al. (2008) [4]. Briefly, aliquots of the polar fractions of extracts were fractionated on a column Sephadex LH-20 (Sigma-Aldrich) using methanol (Sigma-Aldrich) as eluent. The fractions resulting from this process were monitored in thin layer chromatography on silica plates using *n*-BuOH acetic acid and water (60 : 15 : 25, v/v) as solvent system. The process was monitored with ultraviolet light (254 and 366 nm).

Chemicals

Chlorogenic acid (CGA) was purchased from Sigma-Aldrich.

Animals and ethical statement

Adult male Wistar rats (280–320 g) were obtained from the Central Vivarium of the University of São Paulo, Ribeirão Preto Campus, and housed in the animal facility at the Department of Biology (FFCLRP-USP). The rats were housed in groups of 3 per cage with food and water available ad libitum. The animals were maintained in a controlled environment with 23 ± 2 °C, 60 ± 5 % humidity, and a light-dark cycle of 12 h.

All experiments were performed following the guidelines for animal care from the Committee on Care and Use of Laboratory Animal Resources, from National Research Council (USA), which regulates the procedures for the scientific use of animals, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Ethics Committee for Care and Use of Laboratory Animals from Ribeirão Preto University of São Paulo Campus (CEUA) approved this work at December 07th, 2010, under the protocol number CEUA 2010.1.795.53.6.

Acute retinal ischemia or ischemia-reperfusion injury

Acute retinal ischemia or I/R models were induced in rats according to Louzada-Júnior et al. (1992) [19] and Guizzo et al. (2005) [18]. Briefly, animals were anesthetized with urethane (2.5 g/kg, via intraperitoneal; Acros Organics), and a sterile 27-gauge needle, attached to a manometer/pump, was inserted into the anterior chamber of the left eye to increase the IOP to 120 mm/Hg above systemic systolic blood pressure for 45 min to induce retinal ischemia. The appearance of a pale fundus confirmed the blood flow interruption.

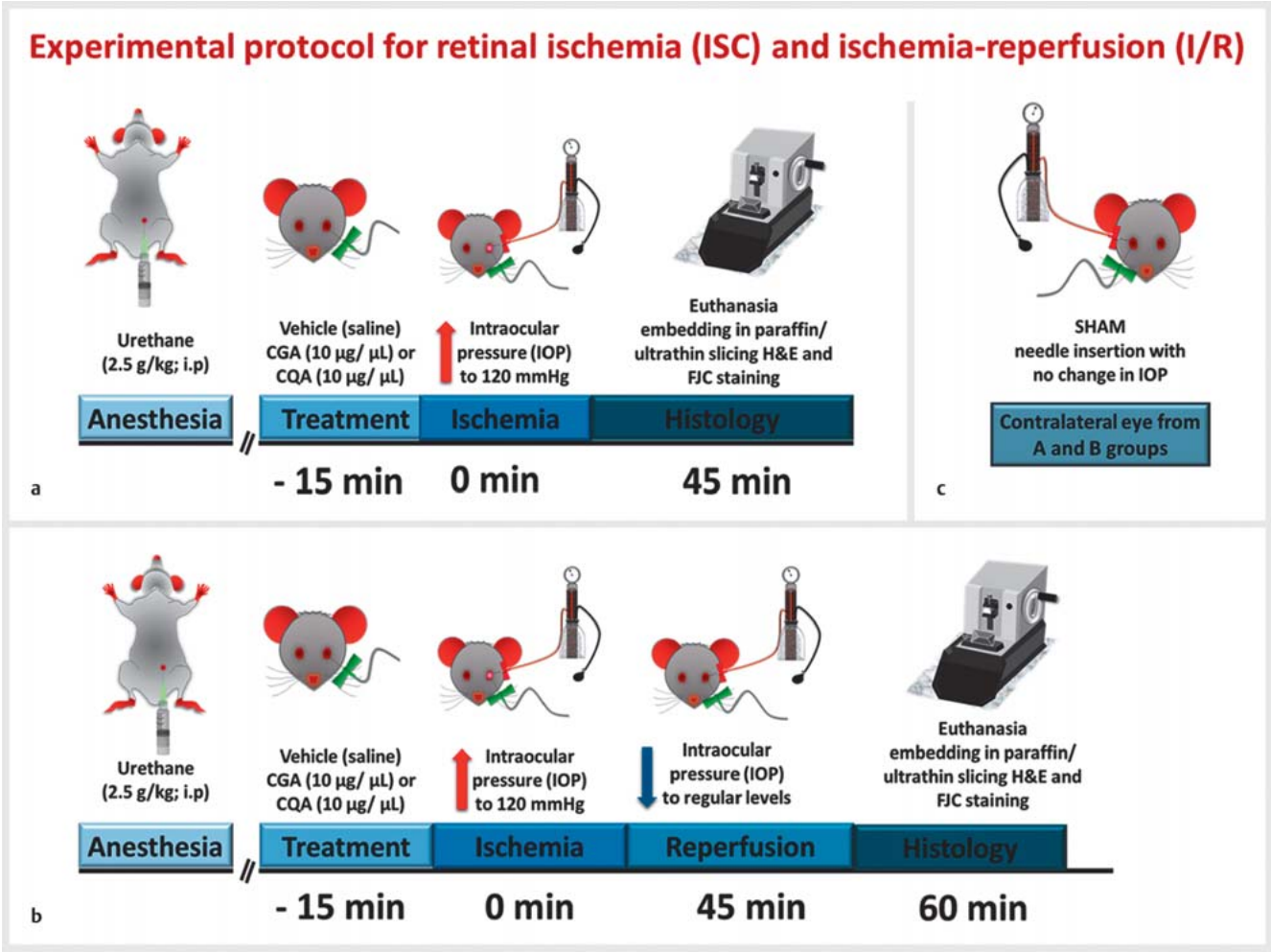
After 45 min, the IOP was reduced to normal levels for 15 min. This is the reperfusion period when the eye fundus color returned to rose.

The contralateral retinas served as sham-controls (SHAM), and for this reason, the cannula was inserted into the anterior chamber of the right eye, but the IOP was not increased. The rats were kept over a heating pad to ensure that the body temperature was maintained above 37 °C during all experimental procedures.

The rats were administered with 1 µL of vehicle (dH_2O), CGA (10 µg/µL; diluted in dH_2O) or CQA (10 µg/µL; diluted in dH_2O), via intravitreal 15 min before the insults. These doses were determined by a prior trial (data not shown) based on the study of Santos et al. (2010) [13], which reported that caffeoylquinic acid derivatives produced inflammatory mediators in cultured cells [13]. Additionally, these compounds were shown to cross the blood-brain barrier [30].

Five rats were used in each group: retinal ischemia group (ISC) treated with vehicle, CGA or CQA (CGA+ISC and CQA+ISQ), and retinal ischemia-reperfusion group (I/R) treated with vehicle, CGA and CQA (CGA+I/R and CQA+I/R). As described above, ipsilateral retinas received the indicated insults, whereas contralateral retinas did not receive insults (SHAM).

Additionally, tree rats per group were administered vehicle, CGA or CQA, without any insult. When compared to the SHAM group, no morphological differences were found between retinas administered with CGA, CQA, or vehicle (not shown), so only five



▶ **Fig. 8** Schematic representation of the experimental protocols used. **a** experimental protocol for induction of retinal ischemia (ISC) and **b** experimental protocol for retinal ischemia-reperfusion (I/R). **c** The contralateral retinas served as a sham-control (SHAM), so the cannula was inserted into the anterior chamber of the right eye, but the IOP was not increased. The figures were created in BioRender.com [rerif].

SHAM retinas, randomly chosen, were analyzed in this study. A total of 39 rats were used in this work. ▶ **Fig. 8** depicts the protocols used in this study.

Histology

Hematoxylin-eosin (H-E)

Animals were euthanatized immediately after retinal ischemia or I/R protocols by an overdose of urethane (3 g/kg; Sigma-Aldrich), and the eyes were rapidly enucleated and fixed in Bouin's solution (75% picric acid, 25% formalin, and 5% acetic acid) for 24 hours. Once fixed, the corneas and lenses were removed, leaving a chalice-shaped structure for the eyecups used for diaphanization and dehydration. The eyecups (structure C shape) were included in paraffin in a standardized position using the muscle tissue located in the supranasal part as reference. After embedding in paraffin, retinas were sectioned in a sagittal plane at 5 µm, stained with H-E, and examined using a light/fluorescent microscope (Leica DM5000B).

Microscopic images (1392 × 1040 pixels) were captured from three retina sections of each eye using the bright field of an epifluorescence microscope (Leica DM5000B) linked to a digital camera (Leica DFC300FX) connected to a computer. The cellular quantification (15 measurements per group) was based upon the number of non-pyknotic nuclei in the nuclear layers of the retina. The condensation of chromatin characterizes pyknotic nuclei. This counting was performed by ImageJ (National Institutes of Health; version 1.53 k) using a blind fashion counting. The areas of the layers were measured by Q-Win software (Leica) in a blind manner. The results were normalized using the Abercrombie correction method, according to the following formula:

$$N\ (\text{per mm}^2) = n\ [T/(T+D)]/A$$

Where *N* is the real number of cells, *n* is the number of cells observed; *T* is the section thickness, *D* is the diameter of the nuclei in each region, and *A* is the measured area (mm^2). Results were

expressed as mean cell density ± standard error of the mean (SEM).

Fluoro-Jade C staining)

Fluoro-Jade C (FJC; Sigma-Aldrich) staining is widely used for the specific detection of degenerating neurons. We used a protocol according to Schmued et al. (2005) [45], with modifications.

Briefly, 6 µm-thick retinal sections were stretched on previously gelatinized slides and kept in an oven at 37 °C for 24 hours. The sections were deparaffinized by three washes of xylene, rehydrated in two washes of absolute ethanol, one of 95%, one of 75%, and one in distilled water. Sections were transferred to 0.06% potassium permanganate for 13 min, washed in distilled water, and incubated in 0.0001% FJC. Slides were washed in distilled water three times for 1 min and mounted with 1 : 1 (v/v) solution of Fluoromount (Sigma-Aldrich) and 0.1% acetic acid. The sections were examined using a light source of a high-pressure mercury lamp and an excitation filter to fluorescein/FITC. Five ganglion cell layer (GCL) microscope fields with the same standard area of the region of interest (1392 × 1040 pixels) were analyzed per group. Results were expressed as mean cells FJC-positive per image photographed ± standard error of the mean (SEM).

Statistical analysis

The treatment effects among the groups were compared using a one-way analysis of variance (ANOVA) followed by the Newman-Keuls post-hoc test. *p*-values below 0.05 were considered statistically significant. Data was analyzed with Graph Prism (version 8.0, GraphPad Software).

Contributors' Statement

J. L. L wrote, reviewed, and edited the manuscript, also contributed to data analysis tools; M. N. R. performed the experiments, collected, and analyzed the data; M. C. R. M. performed the experiments and collected the data; J. L. C. L. conceived and designed the analysis, wrote the manuscript; L. G. N. isolated the CQA compound, collected the data, performed the analysis; A. C. K. F. conceived and designed the analysis, wrote, reviewed and edited the manuscript; N. P. L. conceived and designed the analysis, wrote the paper and acquired funding; W. F. S. conceived and designed the analysis, wrote, reviewed, and edited the manuscript, and acquired funding.

Acknowledgements

This research was supported by São Paulo State Research Foundation (FAPESP, Brazil) grant numbers [2005/60254-0] and [2008/09389-0]; JLL was a recipient of a research scholarship from the National Council for Scientific and Technological Development (CNPq, Brazil); JLL and MNR were also recipients of research scholarships from the Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil).

Conflict of Interest

The authors declare that they have no conflict of interest.

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