

Evaluation of retinal injury in a rat model of transient ischemic stroke

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Summary. Stroke-associated ocular disorders are vision-threatening. This study was designed to evaluate *in vivo* retinal injury induced by transient global cerebral ischemia/reperfusion (I/R). A stroke-induced retinal injury model in Wistar rats was established by electrocoagulation of bilateral vertebral arteries, combined with transient ligation of the bilateral common carotid arteries. Rats were randomly divided into groups based on the time post cerebral perfusion (3 h, 24 h, 48 h, 72 h, and 7 days). Retinal injury was evaluated by histological analysis, examination of eye fundus, and TUNEL staining. The expression of protein kinase C- α (PKC α) and fibrillary acidic protein (GFAP) was determined using qRT-PCR and immunofluorescence analysis. Both retinal neurons and the vasculature underwent significant damage in the cerebral-I/R groups when compared to rats in the sham group. Moreover, when compared to non-stroke rats, TUNEL staining revealed signs of apoptosis in the retina after transient ischemic stroke was induced ($P < 0.001$). In these rats, the expression of PKC α and GFAP in the retinas was enhanced and peaked at 72 h after induction of cerebral-I/R ($P < 0.001$). In this study, we found that retinas are very susceptible to transient global cerebral-I/R injury. The expression of PKC α and GFAP may be implicated in the pathogenesis of ischemic stroke-induced retinal injury.

Key words: Stroke, Ischemia reperfusion, Retinal injury, Apoptosis

Introduction

Stroke has a significant burden on human health (Lawlor et al., 2015). In the clinic, stroke is characterized by a high incidence, multiple complications, and a poor prognosis (Carandang et al., 2006). Reportedly, approximately 87% of strokes are ischemic (Go et al., 2014). The retina is formed as an outpouching of the diencephalon and is part of the central nervous system (CNS) (D'Onofrio and Koeberle, 2013). Therefore, a strong connection exists between stroke and retinal injury (Ho et al., 2012; D'Onofrio and Koeberle, 2013; London et al., 2013). In several large population-based studies, a strong link has been reported between clinical stroke and retinal damage (Wang et al., 2014; Hughes et al., 2016; Kambale et al., 2017). However, the pathogenesis of stroke-induced retinal injury is not well understood.

Several animal models have been developed to study retinal ischemia, including a high intraocular pressure (IOP) model of ischemia (Buchi et al., 1991), a central retinal artery (CRA) ligation model (Magharious et al., 2011), a bilateral common carotid artery ligation model (Stevens et al., 2002), and a retinal intravenous injection of Bengal Rose model (Daugeliene et al., 2000). To date, no model can completely block the retinal blood supply while avoiding mechanical damage to the retinal neurons (Macrae, 2011; D'Onofrio and Koeberle, 2013). Therefore, establishing an ideal method for investigating

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stroke-induced retinal I/R injury is important.

In previous studies, apoptosis has been shown to play a pivotal role in retinal ischemia, and anti-apoptotic treatment alleviated retinal I/R injury (Häcker, 2000; Chao et al., 2014; Wang et al., 2017). The objective of this study was to establish and evaluate retinal injury by using a bilateral vertebral artery electrocoagulation technique, combined with bilateral common carotid artery ligation (four vessels obstruction) in rats. This approach simulated complete ischemia of the retina and prevented damage of retinal neurons during acute ischemic stroke.

Materials and methods

Experimental animals and rat model of cerebral I/R-induced retinal injury

All investigations involving the use of animals were conducted according to the guidelines of the National Institutes of Health for the care and use of laboratory animals, and the experiments were reviewed and approved by the Laboratory Animal Ethics Committee of Jinhua Hospital of Zhejiang University (Zhejiang, China). Fourteen-week-old male Wistar rats (weighing 200-250 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Rats were randomly divided into a sham group (n=8) and a cerebral-I/R group. The latter was subdivided into five subgroups based on the time after cerebral-I/R, which included 3h, 24h, 48h, 72h, and 7d (n=8). In the sham group, bilateral foramen alare of the first cervical vertebrae and common carotid arteries were anatomically exposed, but blood vessels were not blocked. In the cerebral-I/R groups, bilateral vertebral artery electrocoagulation was performed and combined with bilateral common carotid artery ligation as previously described (Pulsinelli and Brieley, 1979). Cerebral blood flow was interrupted for 10 min before reperfusion. Rats were provided with food and water ad libitum, and were kept in an environment at 45-50% humidity. The temperature was 21-25°C, the circadian rhythm involved a 12-h light/dark cycle, and the air change was set at a 12-15 h interval. Rats were sacrificed at different times after cerebral I/R.

Histological procedures

Rats were anesthetized with 1% sodium pentobarbital (40 mg/kg, ip) and a cardiac perfusion was performed using freshly-prepared 4% paraformaldehyde to fix the eyeballs. Then, eyeballs were harvested and continuously fixed in 4% paraformaldehyde for 24 h at 4°C.

Paraffin-embedded retinal sections were cut into 5- μ m thick sections and the morphology was determined by hematoxylin and eosin (HE) staining. Three continuous retinal sections taken from each side of the optic nerve were examined using an optical microscope

(DM4000; Leica, Heidelberg, Germany), and the changes in the overall thickness of the retina (at a distance of 1.5 mm from the center of the optic nerve head) was determined by using a method as previously described (Wang et al., 2017).

Retinas were minced into small pieces using a dissecting microscope, and incubated in 3% trypsin solution at 37°C for 3 h. Then retina pieces were gently rinsed in distilled water several times, and finally a transparent retinal vasculature layer was obtained. Retinal vasculature was smoothly affixed to a slide and dried for future HE staining. Images of the retinal vasculature were taken of the central parts of the retinal vascular tree and the percentage of the vessel area was quantitated using AngioTool software as previously described (Zudaire et al., 2011; Orlova et al., 2014).

Eye fundus examination

Rats were subjected to eye fundus examination at predetermined time points. The retinal vasculature of both eyes of a rat was examined under a slit lamp, and images were taken of the retinal vasculature for analysis.

Analysis of apoptosis

Apoptosis of paraffin-embedded retinal sections was determined by terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) staining, and TUNEL assay (In Situ Cell Death Detection kit, Roche, Mannheim, Germany) was performed in accordance with the instructions provided by the manufacturer. TUNEL-positive cells were counted in ten different, randomly-selected areas (200 \times magnification).

RNA extraction and qRT-PCR

The mRNA levels of protein kinase C alpha (PKC α) and glial fibrillary acidic protein (GFAP) in the retinas were determined using a real-time PCR approach. Total retinal RNA was extracted using TRIZOL reagent (no. 15596026; Ambion, USA) according to the manufacturer's guidelines. Total RNA was reverse transcribed in a final volume of 20 μ l using random primers and standard conditions for the PrimeScript RT reagent Kit (RR037A; TaKaRa, Dalian, China). Quantitative reverse-transcription PCR was performed using SYBR Premix Ex TaqTM II (Tli RNaseH Plus) (RR420A; TaKaRa, Dalian, China) according to manufacturer's guidelines. The specific primers used are presented in Table 1. PCR was initiated by incubation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. The PCR was carried out on a LightCycler[®] 96 real-time PCR system (Roche Diagnostics). Relative quantification was performed using the housekeeping gene β -actin as the internal standard. Relative quantification of the gene expression of PKC α and GFAP was calculated as fold changes

Retinal injury in ischemic stroke

according to the $2^{-\Delta\Delta CT}$ method according to manufacturer's instructions.

Immunofluorescent staining

Frozen retinal sections were thawed at room temperature, then incubated in a wet box for 40 h at 4°C with rabbit anti-PKC α monoclonal antibody (1:200; ab32376; Abcam, Cambridge, UK) or rabbit anti-GFAP monoclonal antibody (1:200; ab68428; Abcam, Cambridge, UK). After washing three times 5 min with PBS, the retinal sections were incubated for 2 h at 37°C with a goat anti-rabbit IgG secondary antibody (green) (1:200; Beyotime Biotechnology, Nanjing, China). Simultaneously, the nuclei of cells were labeled with 4', 6-diamidino-2-phenylindole (DAPI; D9542; 1:1000; Sigma, USA). Next, a fluorescence quenching agent was added and the sections were sealed with a coverslip. Retinal sections were examined using a fluorescent microscope (Leica, Solms, Germany). Composite figures were analyzed using Image-Pro Plus 6.0 software systems (Media Cybernetics, Inc., Maryland, USA).

Statistical analysis

Data were expressed as the mean \pm S.D. (standard deviation) and analyzed using GraphPad software package (Prism 7.0) for Windows (San Diego, CA, USA). One-way analysis of variance (ANOVA) was performed to compare three or more independent groups, and the Tukey's multiple comparison tests were performed for statistical comparison of multiple groups. The unpaired t-test was used for comparison of apoptosis and the percentage of vessel area between the sham group and the 24 h group. $P < 0.05$ was considered statistically significant.

Results

Histological presentations

Histological examination showed that the maximum thickness of the overall retina from the inner limiting membrane to the outer limiting membrane (ILM-OLM) (Fig. 1A) in rats in the sham group was $186.2 \pm 10.05 \mu\text{m}$.

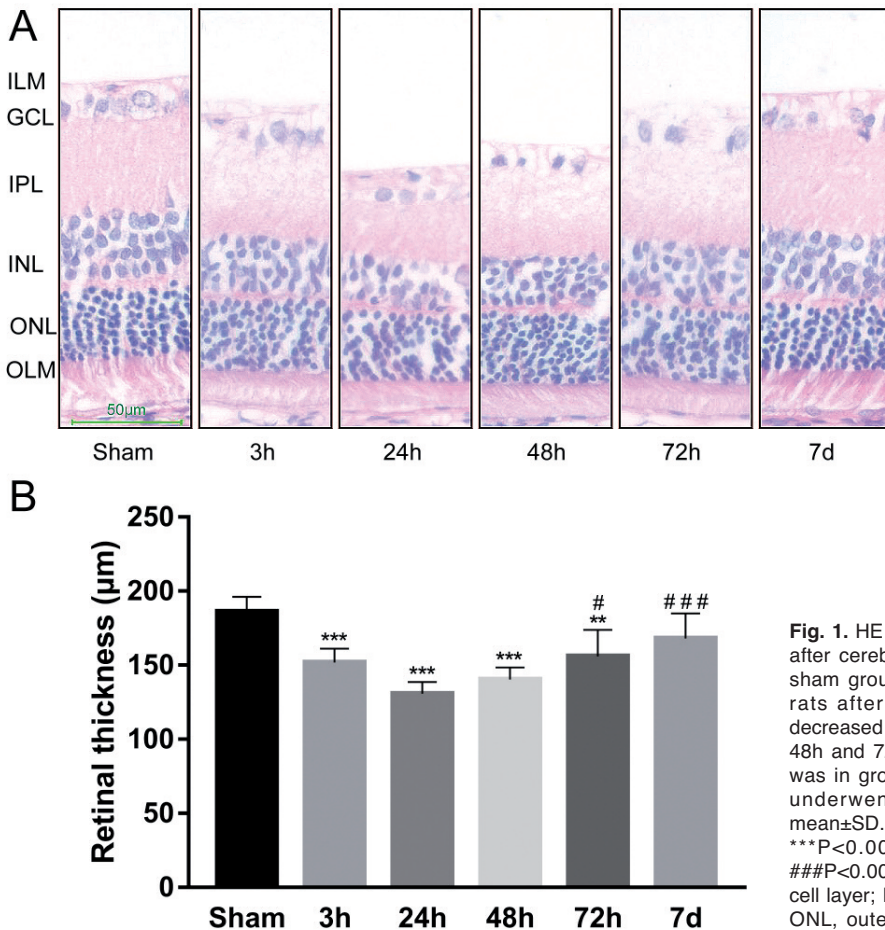


Fig. 1. HE staining demonstrates retinal histological changes after cerebral I/R. **A.** Compared to the retina in rats in the sham group, retinal thickness was reduced significantly in rats after transient cerebral I/R. **B.** Retinal thickness decreased by 18%, 29%, 25% and 16% in groups 3h, 24h, 48h and 72h after transient ischemic stroke, respectively; it was in group 24h after cerebral reperfusion that the retina underwent the most damage. Data are expressed as mean \pm SD. $n=6$. Compared with the sham group, ** $P < 0.01$, *** $P < 0.001$; Compared with the 24h group, # $P < 0.05$, ### $P < 0.001$. ILM, inner limiting membrane; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; OLM, outer limiting membrane. Scale bar: 50 μm .

The retinal thickness of rats in the 24 h cerebral-I/R group decreased to $130.8 \pm 7.86 \mu\text{m}$, which was 29% less when compared to rats in the sham group ($P < 0.001$). The reduction in retinal thickness was more evident in the inner nuclear layer (INL) when compared to the outer nuclear layer (ONL) (Fig. 1A). Nevertheless, the structure of the retina, post cerebral-I/R at day 7 improved. Moreover, the reduction in retinal thickness was statistically significant between the sham group and

the cerebral-I/R group rats at 3 h, 24 h, 48 h, and 72 h (Fig. 1B, $P < 0.01$). However, there was no statistically significant difference between the sham group and rats 7 days after cerebral reperfusion.

Compared with the integrated retinal vasculature in sham-operated rats (Fig. 2A-a,b), a reduced intensity and disarray of the retinal vasculature was observed in cerebral-I/R rats (Fig. 2A-c,d). There were fewer nuclei but more spastic blood vessels were observed in the

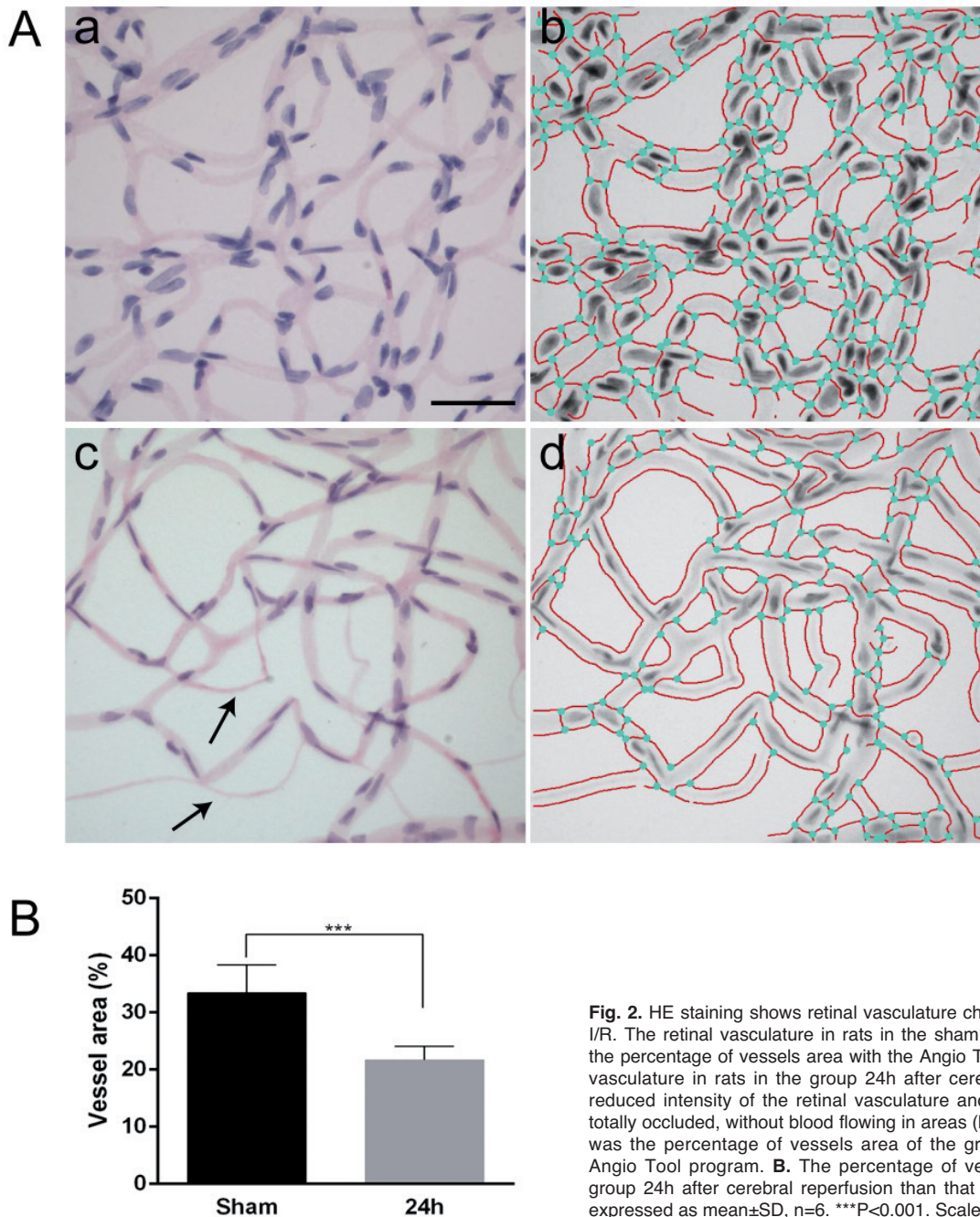


Fig. 2. HE staining shows retinal vasculature changes after transient cerebral I/R. The retinal vasculature in rats in the sham group (**A-a**) and detection of the percentage of vessels area with the Angio Tool program (**A-b**); the retinal vasculature in rats in the group 24h after cerebral reperfusion illustrates a reduced intensity of the retinal vasculature and some vascular lumen were totally occluded, without blood flowing in areas (black arrows) (**A-c**), and (**A-d**) was the percentage of vessels area of the group 24h detection using the Angio Tool program. **B.** The percentage of vessels area was lower in the group 24h after cerebral reperfusion than that in the sham group. Data are expressed as mean \pm SD, $n=6$. *** $P < 0.001$. Scale bar: $50 \mu\text{m}$.

Retinal injury in ischemic stroke

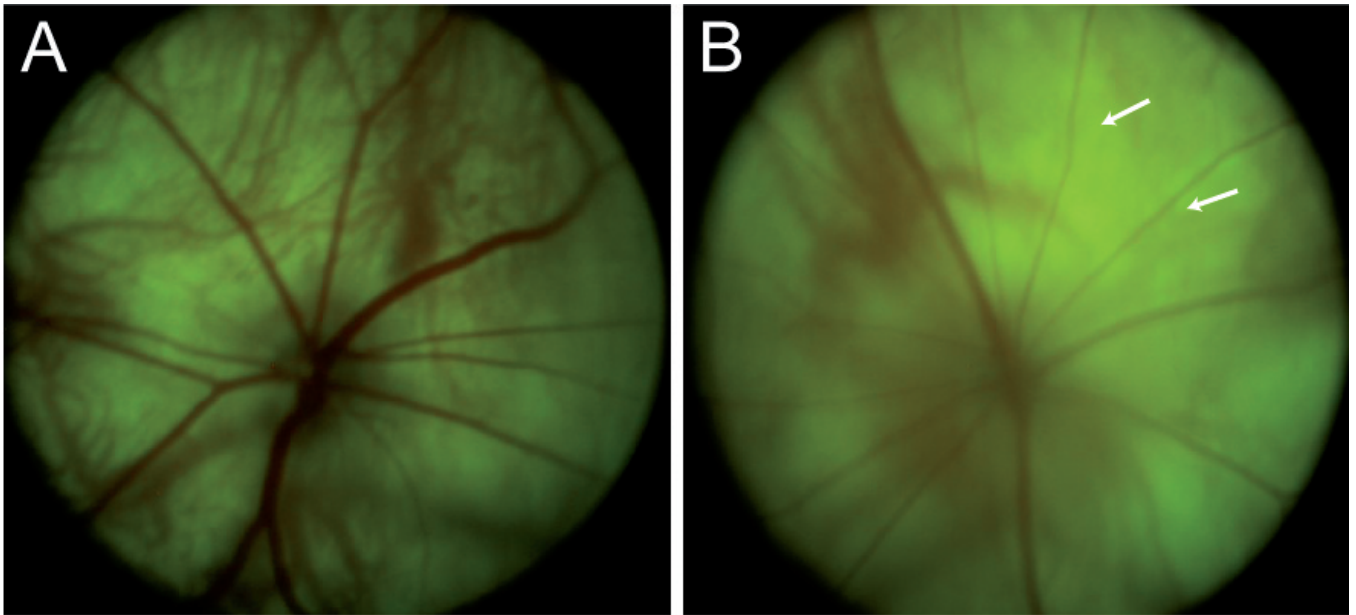


Fig. 3. Eye fundus examination reveals morphological alteration of retinal vasculature. **A.** shows fine networks of blood vessels in rats in the sham group. **B.** shows thinner retinal vessels (white arrows) with intensive light-reflection in rats in the group 24h after cerebral reperfusion.

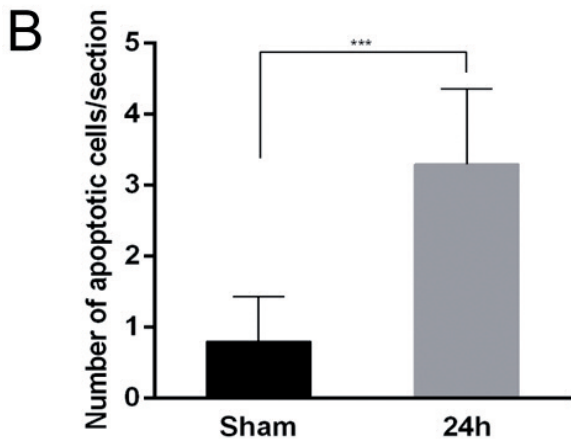
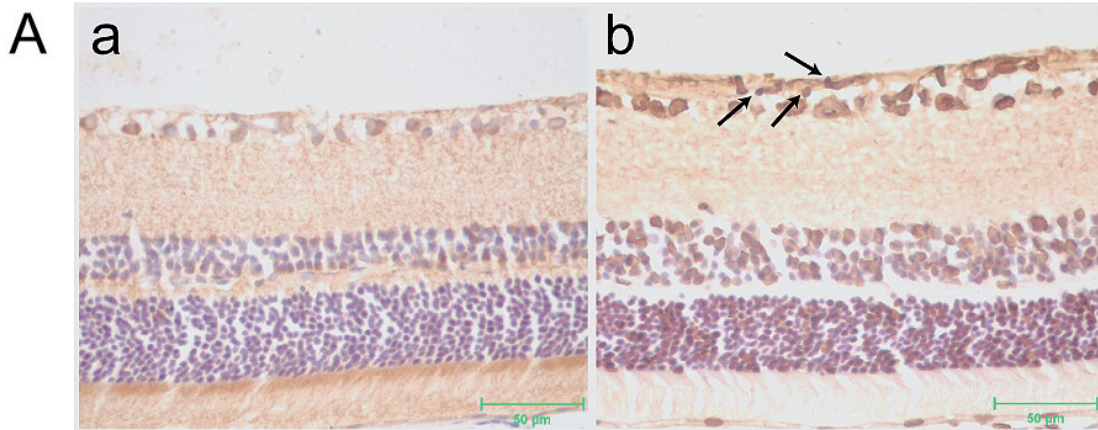


Fig. 4. TUNEL staining analyses of retinal neurons apoptosis in the sham group (**A-a**) and in the group 24h after cerebral reperfusion (**A-b**). TUNEL-positive nuclei, stained brown and distributed unevenly, were mainly detected in the ganglion cell layer (**A-b**, black arrows). **B.** Statistics showing retinal apoptotic cells in the group 24h after cerebral reperfusion was more than that in the sham group. Data are expressed as mean±SD, n=10. ***P<0.001. Scale bar: 50 μm.

retina of rats in the post cerebral I/R groups (Fig. 2A-c,d). These findings suggested the loss of vascular cells in the retinal vasculature. A statistical difference was found in the percentage of vessel area between rats in the sham group and those 24h post cerebral I/R induction (Fig. 2B, $P < 0.001$).

Morphological changes of retinal vasculature were also observed during eye fundus examination. Compared with rats in the sham group (Fig. 3A), a reduction in vessel diameter and intensive light reflection of the vascular wall with limited blood flow was observed in rats after transient stroke (Fig. 3B).

Apoptosis in retina after transient cerebral I/R

The level of apoptosis was biochemically characterized by internucleosomal DNA fragmentation. TUNEL-positive nuclei appeared in a brown color, and showed an uneven distribution and condensation of chromatin. In rats in the sham group, no apparent signs of apoptosis were found in the retina (Fig. 4A-a). However, several TUNEL positive nuclei were observed in the rat retina after induction of cerebral I/R (Fig. 4A-b). TUNEL-positive cells were mainly observed in the retinal ganglion cell layer (GCL). A statistical difference in apoptotic cells was found between rats in the sham group and rats 24 h after cerebral reperfusion (Fig. 4B, $P < 0.001$).

Expression of PKC α and GFAP mRNA

We used a quantitative RT-PCR method to directly assess the changes in PKC α and GFAP mRNA levels in the cerebral I/R-induced retinal tissue. In retinal tissue of rats in the cerebral I/R groups, the mRNA expression of GFAP (Fig. 5A) and PKC α (Fig. 5B) was significantly increased when compared to the control group. Moreover, the mRNA expression of PKC α and GFAP in

the retina peaked at 72 h and 7 d after cerebral-I/R, respectively.

PKC α and GFAP immunofluorescent staining

Positive PKC α expression, as characterized by a green-colored fluorescence in an immunofluorescent assay, was primarily found in the cytoplasm and the plasma membrane of bipolar cells in the INL, and was also increased in the GCL (Fig. 6A). In the rat retina, statistically significant differences in PKC α expression were observed between the control group and the 3 h, 24 h, 48 h, and 72 h cerebral I/R groups (Fig. 6B, $P < 0.001$). Among the cerebral-I/R groups, the intensity of PKC α staining peaked at 72 h (Fig. 6B, $P < 0.001$).

In addition, GFAP-labeled Müller cell processes extended from the end-foot region to the inner plexiform layer, into the INL and ONL (Fig. 6C). Anti-GFAP immunoreactivity gradually increased at 3 h, 24 h, and 48 h after cerebral reperfusion, peaked at 72 h, then decreased on the 7th day after cerebral reperfusion (Fig. 6D, $P < 0.001$). However, no statistically significant difference was observed between rats in the 7 d group and the sham group ($P > 0.05$).

Discussion

In the present study, we demonstrated that both retinal neurons and the retinal vasculature were significantly altered in histology during transient global cerebral I/R *in vivo*. Furthermore, cerebral I/R-induced retinal injury was associated with increased PKC α and GFAP expression. In addition, increased apoptotic changes were observed in RGCs after transient ischemic stroke induction.

In the CNS, ischemia is undoubtedly a complex problem with many facets, among which stroke-related retinal injury is an interesting topic. Since the retina is

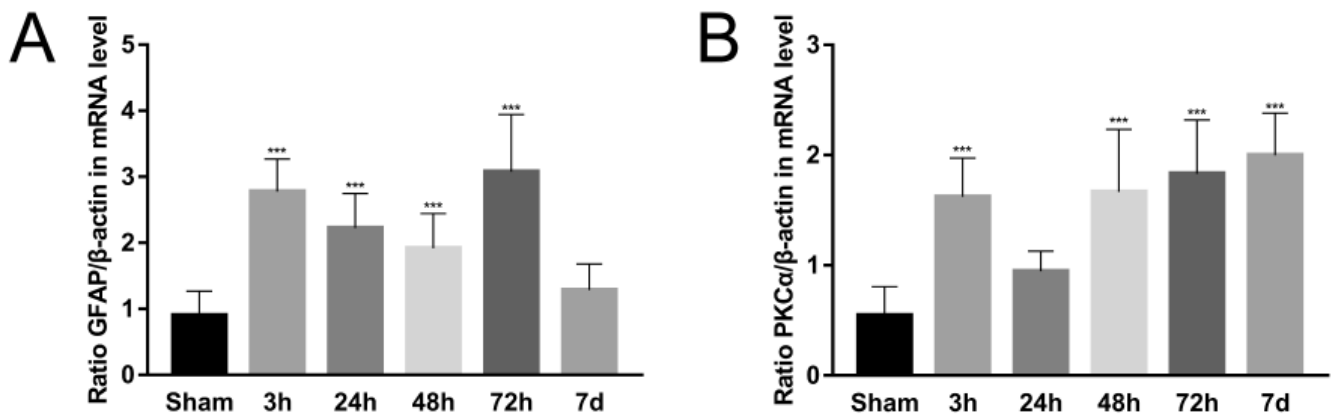


Fig. 5. Quantitative RT-PCR analyses of PKC α and GFAP mRNA expression in the retina. The mRNA expression of GFAP (A) and PKC α (B) were highly up-regulated in the cerebral I/R groups compared to that in the sham group. *** $P < 0.001$.

Retinal injury in ischemic stroke

formed as an outpouching of the diencephalon and is part of the CNS (D'Onofrio and Koeberle, 2013), neurodegenerative processes in the brain may lead to similar changes in the optic nerve (Ho et al., 2012; London et al., 2013). The metabolic demands of the retina are the highest of any tissue within the body (Osborne et al., 2004). Stroke may result in vision damage. Several large population-based studies have reported a strong link between retinal vascular changes and clinical stroke, both in cross-sectional and prospective studies (Wang et al., 2014; Hughes et al., 2016; Kambale et al., 2017).

The retina shares a similar embryological origin, anatomical features, and physiological properties with the brain and therefore offers a unique and accessible “window” to study the correlations and consequences of subclinical pathology in stroke or transient ischemic attack (TIA) (London et al., 2013; Cheung et al., 2014; Nguyen et al., 2017). It has been suggested that the retina can act as a biomarker of the brain (Nguyen et al., 2017). However, our current knowledge of stroke-related retinal injury is incomplete.

In the past, several animal models have been established to study retinal ischemia. The IOP ischemia

model is frequently used as it is relatively simple to perform. Using the IOP method, the retinal blood supply is expelled by introducing sterile fluid into the vitreous chamber of the eye, which results in an increased pressure within the eye and compresses the vasculature passing through the optic disc, thereby supplying the retina (Buchi et al., 1991). However, with such an intervention, it is hard to prevent secondary mechanical damage to the retina in addition to ischemic damage. Another common method of inducing retinal ischemia is the CRA ligation model (Magharious et al., 2011). Because of the close association between the optic nerve and its adjacent vessels, selective compression of the vasculature is usually incomplete, which commonly causes the axons of the optic nerve to be compressed and damaged. The CRA method produces results that are easily inappropriate to retinal ischemia and has confounding effects of optic nerve damage (Gehlbach and Purple, 1994).

In previous studies, the bilateral carotid artery or internal carotid artery ligation model has also been described (Stevens et al., 2002). Retinal damage depends on the number of ligated vessels or collateral circulation. The human retina has a dual blood supply function,

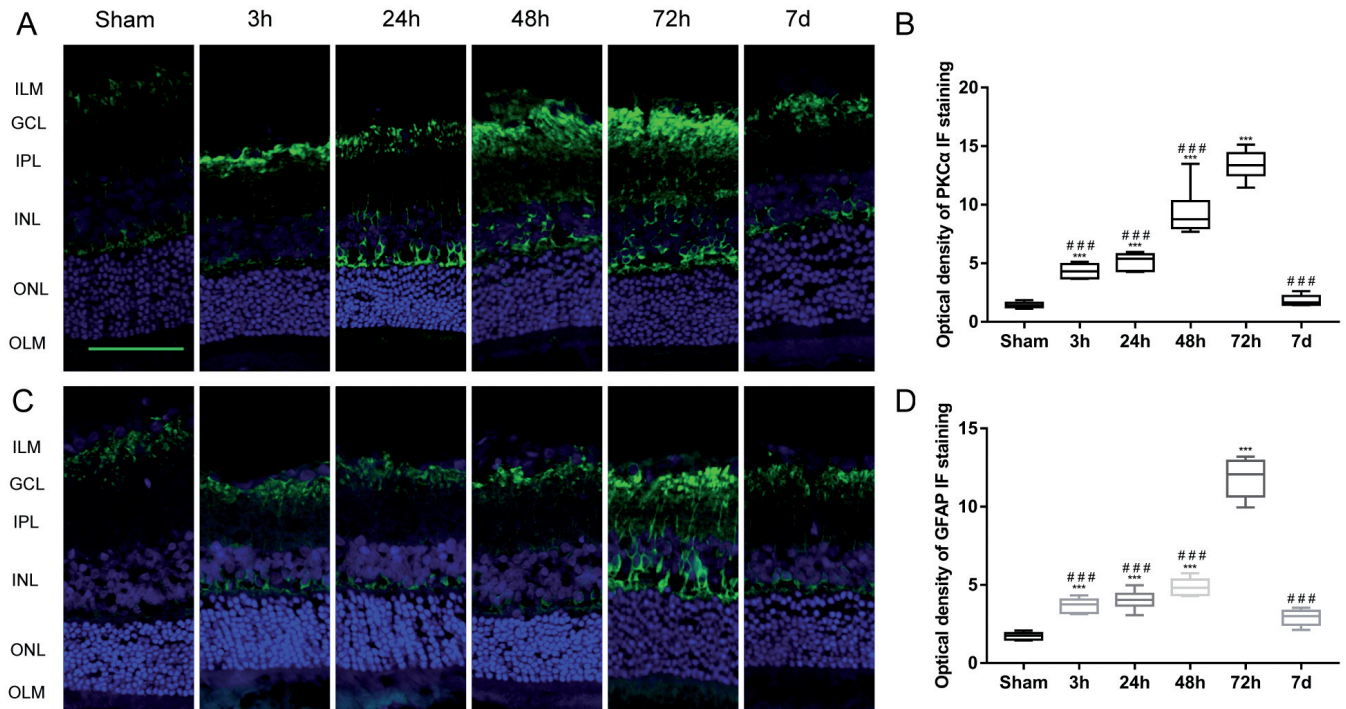


Fig. 6. Immunofluorescent staining analyses of PKC α and GFAP protein expression in the retina. The positive immunofluorescent staining of PKC α characterizes as green, and was mainly expressed in the cytoplasm and the plasma membrane of bipolar cells (A). Anti-PKC α immunoreactivity was enhanced in the retina after cerebral I/R, of which the group 72h was the most obvious (B). The GFAP green fluorescence-labeled Müller cell processes extended from the end-foot region to the IPL, into the INL and ONL (C), and the immunofluorescence optical density values of GFAP were higher in cerebral I/R groups, among which the group 72h was the highest (D). Data are expressed as mean \pm SD, n=6. Compared with the control group, ***P<0.001; in cerebral I/R groups, compared with 72 h group, ###P<0.001. ILM, inner limiting membrane; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; OLM, outer limiting membrane. Scale bar: 50 μ m.

namely the photoreceptors, including their cell bodies in the ONL and most of the outer plexiform layer, are supplied via the choriocapillaries, whereas the inner retinal layers, including the RGC layer, are nourished by CRA (D'Onofrio and Koeberle, 2013). The main drawback of this method is that in experimental animals with an intact circle of Willis in the brain, complete ischemia of the retina cannot be established, because of the continuous blood supply to the retina from the vertebrobasilar arteries.

In addition, retinal intravenous injection of Bengal Rose (Daugeliene et al., 2000) or subconjunctival injection of endothelin-1 has also been reported (Masuzawa et al., 2006; Kim et al., 2012). However, for the study of I/R injury, these two methods are not considered ideal because the created lesion is resistant to the reintroduction of blood flow (Macrae, 2011).

To best extrapolate the data from animal models to a clinical situation, a model that most closely resembles human retinal ischemia is preferred. In our study, the four vessels obstruction model, combined with bilateral common carotid artery ligation was used. Not only was complete ischemia of the retina established without causing mechanical damage to the retina, but ischemic stroke was also successfully simulated *in vivo*. Therefore, this method may be suitable for the study of ischemic stroke-related retinal injury.

It has previously been reported that apoptosis was implicated in the pathogenesis of retinal I/R injury (Häcker, 2000; Chu et al., 2002; D'Onofrio and Koeberle, 2013). In our study, TUNEL staining analysis suggested that after transient ischemic stroke, apoptotic changes mainly occurred in retinal RGCs. In addition, the presence of microvessels in the retina of ischemic stroke rats was reduced compared to that of rats in the sham group. These changes were similar to the retinal vascular lesions that were described in patients with diabetes or TIA (Umemura et al., 2017; Wolz et al., 2017).

In the retina, PKC α and GFAP are mainly expressed in bipolar and Müller cells, respectively (Barmack et al., 2000; Caminos et al., 2000; Chao et al., 2014). The activation of PKC α implied a neurite-promoting effect of injury in the retina (Wu et al., 2003). Since bipolar cells are mainly located in the INL, with synaptic terminals spreading to the GCL (Suzuki and Kaneko, 1990), we found that PKC α was expressed not only in the INL, but also in the GCL. Interestingly, previous studies have described either a temporary increase (Yokota et al., 1992; Dvorianchikova et al., 2014), a decrease (Osborne et al., 1995; Gesslein et al., 2009), or no change (Kumar et al., 1992) of PKC α expression in the retina after I/R. These conflicting results may result from the type of ischemic insult, ischemic duration, as well as the animal model utilized (Gesslein et al., 2009). In the present study, we observed dramatic increases in immunoreactivity for proteins of both PKC α and GFAP in cerebral I/R-conditioned retinal tissue, suggesting that overexpression of PKC α and GFAP was strongly

associated with global cerebral I/R-induced retinal injury. Given this strong connection, the expression of PKC α or GFAP in the retina may portend possible future adverse events, including loss of vision and stroke.

Although the results are promising, the present study has several limitations. First, the time of post-stroke retinal injury remains elusive; we only analyzed several time points within 7 days after induction of stroke. Second, clinical presentations and vision changes may vary with regard to stroke that was induced by different causes. Our study only reflected retinal damage that was caused by transient global ischemic stroke. Third, we are concerned about the retinal damage demonstrated by histological analysis; however, we did not perform any functional studies to evaluate the impact of injury on retinal function. Therefore, future studies are warranted to clarify the relationship between retinal histological injury and changes in vision functionality.

In conclusion, we investigated retinal injury in a rat model of transient cerebral-I/R, which was feasible and appropriate for evaluating ischemic stroke-induced retinal damage. Moreover, this study revealed that retinal injury may be associated with increased expression of GFAP and PKC α during cerebral-I/R. These findings will serve to increase our understanding of ischemic stroke and further characterize the pathophysiological mechanisms underlying retinal injury.

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Retinal injury in ischemic stroke

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