Protective limb remote ischemic post-conditioning against high-intraocular-pressure-induced retinal injury in mice

Qi–Jiang Zhu[1,2], Lei Zhang[2,3], Shuang–Yu Lyu[3], Zhan–Jun Cui[3], En–She Jiang[2,4], Jun Wang[2,3]

[1]School of Clinical Medicine, Henan University, Kaifeng 475001, Henan Province, China
[2]Henan International Joint Laboratory for Nuclear Protein Regulation, Henan University, Kaifeng 475001, Henan Province, China
[3]School of Basic Medical Sciences, Henan University, Kaifeng 475001, Henan Province, China
[4]Institute of Nursing and Health, School of Nursing and Health, Henan University, Kaifeng 475001, Henan Province, China
Correspondence to: Jun Wang. Henan International Joint Laboratory for Nuclear Protein Regulation, Henan University, Kaifeng 475001, Henan Province, China. whb21c@vip.henu.edu.cn
Received: 2021-06-30   Accepted: 2021-11-02

Abstract

● **AIM:** To determine whether limb remote ischemic post-conditioning (LRIC) protects against high-intraocular-pressure (IOP)-induced retinal injury, and to identify underlying molecular mechanisms.

● **METHODS:** In mice, IOP was increased to 110 mm Hg for 50min and LRIC applied to the unilateral leg for three occlusion cycles (5min/release). Three animal groups (control, high IOP, and high IOP+LRIC) were arranged in this study. Plasma was collected from LRIC treated mice. Retinal histology, oxidative stress were determined by histological section staining and chemical kit. C/EBP homologous protein (CHOP), and Iba-1 parameters were evaluated by immunofluorescent staining and Western blot.

● **RESULTS:** The data showed that LRIC treatment alleviated the retinal histological disorganization and ganglion cell loss induced by high IOP. The CHOP, Iba-1 expression and oxidative stress marker also were inhibited by LRIC treatment. To further explore underlying mechanisms, plasma from LRIC treated animals was intravenously transfused into high-IOP animals. The results showed plasma injection decreased caspase 9 expression and DHE staining signals compared with that in high IOP retinas.

● **CONCLUSION:** These data suggest that LRIC treatments exert retinal protective effects against high-IOP injury.

Endogenous humoral factors release into the circulation by LRIC may contribute to homeostatic protection by reducing monocyte infiltration and/or microglia activation.

INTRODUCTION

The retina is an extension of the brain tissue and also is the highest oxygen-consuming organ in the body, with high sensitivity to ischemia[1]. Retinal ischemia can lead to functional and morphological changes culminating in blindness. Several ophthalmic diseases are related to retinal ischemia, including glaucoma, obstructive retinopathy, ischemic optic neuropathy, carotid artery occlusive disease, and diabetic retinopathy[2-4]. Thus far, retinal ischemic injury is still an issue for treatment. Current neuroprotective agents offer incomplete protection, whereas others generate nonspecific effects/risks or toxicity[5]. Therefore, safe and effective alternative therapeutic interventions are required.

Ischemic conditioning was initially discovered by Murry *et al*[6]. Later, it was used to intermittently block remote organs (*e.g.*, limbs) which were relatively ischemia-tolerant. We refer to this as limb remote ischemic post-conditioning (LRIC). Ischemic conditioning may be divided into pre-, per-, and post-conditioning types. Since retinal ischemia onset is usually unpredictable, we used limb ischemic post-conditioning as a clinical strategy. In recent years, extensive research has shown that LRIC has been comprehensively developed from a basic research tool to a complex clinical technique, with its application potential constantly evolving[7]. LRIC as a safe (non-invasive), economical, and effective adjuvant intervention has provided protective roles towards the heart, brain, lung, kidney, liver, and intestine[8]. The retina is the most important tissue in the eye and extends from the central nervous system. In terms of increased oxygen consumption and metabolic

activity, the retina is more sensitive to hypoxia and ischemia. However, LRIC-based treatment studies on retinal ischemic injury are limited. Zhang et al[9] reported that middle cerebral and pterygopalatine artery occlusion induced retinal ischemic injury, and was ameliorated by an LRIC intervention. In our study, we investigated the role of LRIC on high intraocular pressure (IOP)-induced retinal ischemia injury in mice, which is a commonly used animal model without large surgery stress. Also, the pathological process is closer to real ischemia conditions. Furthermore, we assessed if plasma aliquots from LRIC-treated animals could induce protective effects on retinal ischemic injury. We sought to identify possible endogenous humoral molecules released into the circulation to promote LRIC-protective effects. This work provides a research basis for exploring effective molecules induced by LRIC (Figure 1).

MATERIALS AND METHODS

Ethical Approval Procedures relating to animal performance and surgery were approved by the Committee of Medical Ethics and Welfare for Experimental Animals, Henan University School of Medicine (Ref no. MEWEAHUM 2014-0001). Strict efforts were made to reduce animal suffering in accordance with the Association for Research in Vision and Ophthalmology (ARVO) on the use of animals.

Study Animals C57BL6 mice (8-12-week-old and weight range 18-28 g) were purchased from Zhengzhou University (Henan, China) and housed in individually ventilated cages. Environmental conditions were maintained over a 12h/12h light/dark cycle, with humidity at 60%±5% and temperature at 22℃±3℃. Food and water were freely accessible. Animals were initially divided randomly into three or four groups. LRIC in control animals (*n*=18) did not affect retinal histology when compared with sham animals (sham group, *n*=4). Subsequently, three animal groups were used. Animals undergoing high-IOP treatment in the eyeball were designated as the high-IOP group (*n*=18). Animals undergoing high-IOP + LRIC were the high-IOP + LRIC group (*n*=18).

To evaluate the effects of plasma based endogenous factors induced by LRIC, the animals were divided into control (*n*=12), high-IOP + normal saline (NS) group (*n*=12), and high-IOP + plasma (*n*=12) groups. The right eye was typically chosen to perform for high IOP procedures.

Mouse Model of a High-Intraocular Pressure The mice were anaesthetized by using a pentobarbital (100 mg/kg) intraperitoneal injection, and 0.5% proparacaine hydrochloride was used as topical anesthesia in the cornea. Next, 1% tropicamide was applied externally applied onto the cornea to dilate the pupils. Anaesthetized mice were laid down on their left side under a stereo microscope. A 30-gauge needle connected to an NS bag was inserted horizontally into the anterior chamber of the right eye. Then, the bag was raised to
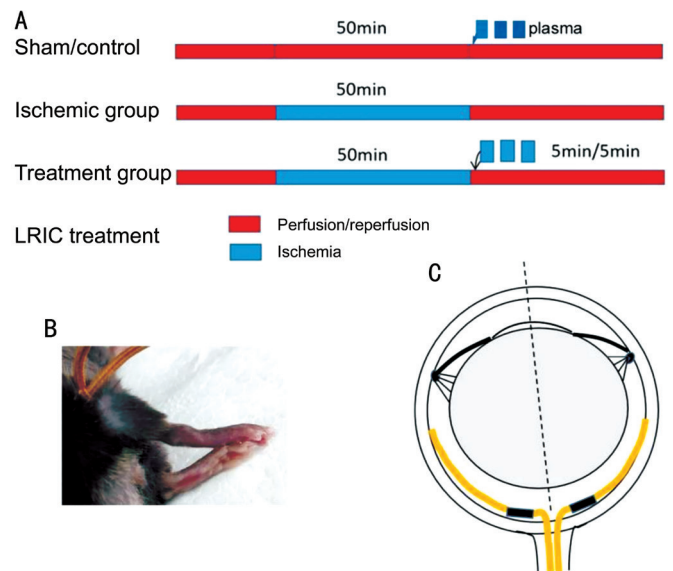

Figure 1 Study schematics A: The ischemic/reperfusion mouse model and limb remote ischemic conditioning (LRIC) treatments across groups; B: Image of the LRIC-treated position; C: Retinal diagram depicting the region of interest around the optic disc.

approximately 150 cm. When the anterior segment of the globe got whitening (vessels were blocked), ischemia conditioning could be complete. The high-IOP was then maintained for 50min and, then returned to normal-IOP by removing the needle. Usually, the whitened anterior eyeball was restored with blood supply. For the sham group, a needle was inserted into the cornea but without elevated IOP. Eyeballs and retinas were collected for analysis at 1, 3, and 7d post high-IOP.

Limb Remote Ischemic Post-Conditioning Treatment LRIC was performed after completing high-IOP procedures. A tourniquet (5 mm) was tightened around the right proximal thigh for three cycles; a cycle comprised of a 5min occlusion phase and a 5min release phase. When the pulse disappeared, skin temperature in the distal limb was decreased and the skin cyanosed, indicating the femoral artery was occluded. LRIC was conducted every day thereafter. Sodium pentobarbital (30 mg/kg) was intraperitoneally applied before LRIC treatment. The same dose of pentobarbital dose was used to treat the sham or control group.

Plasma Collection and Administration Mouse plasma from LRIC (5 min occlusion/5 min release over three cycles) treated animals (30min later) was collected by intracardiac bleeding. Blood collected in anticoagulation tubes was used to prepare plasma by centrifugation at 1000 g. Plasma aliquots were stored at -80℃. Plasma was then systemically intravenously injected (150 μL/injection) into mice *via* the tail.

Immunohistochemical Observations Horizontal retinal paraffin-embedded slides (5 μm in thickness around the optic disc) were deparaffinized in xylene and rehydrated in decreasing ethanol concentrations. Retinal sections

were stained by hematoxylin and eosin. To examine retinal thickness, three measurements were averaged for each retina from the peripheral to the center. For immunofluorescence staining, retinal sections were washed in 0.01 mol/L phosphate buffered saline (PBS, pH 7.4) and incubated with antigen retrieval buffer (Boster, China) according to manufacturer's instructions. After this, sections were blocked in 10% normal goat serum (Boster, China) for 30min and incubated overnight at 4℃ with primary antibodies (anti-NeuN 1:400, Boster, China; anti-Iba-1, 1:1000, Abcam, USA, and anti-CHOP 1:1000, Beyotime, China; and anti-caspase 9 1:100, Boster, China). The following day, after washing with PBS, sections were incubated with relevant secondary antibodies (1:500, Beyotime) for 1h at room temperature. After washing twice in PBS (5min each), section images were observed using an optical or fluorescence microscope. For dihydroethidium (DHE) staining, eyeballs were embedded into compound tissue-tek (SaKura Finetec, USA) and fixed in liquid nitrogen. Retinal cryosections (5 μm) were incubated with DHE (40min at room temperature). Images were observed under a fluorescence microscope and the fluorescence intensity was then calculated.

Western Blotting Retinas were rapidly isolated from eyeballs with pigmentary epithelium discarded. After this, retinal tissues were homogenized in RIPA buffer (Beyotime, China). Protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and gels transferred to nitrocellulose membranes (Millipore, MA, USA). These were blocked in 3% bovine serum albumen and incubated overnight at 4℃ with primary antibodies (CHOP, Beyotime,1:2000; Iba-1, Abcam, 1:5000; caspase 9, Boster, 1:1000). The next day, a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit) was added and incubated for 1h at room temperature on a shaker. Protein band optical densities were semi-quantitatively measured by Image J v2.1. β-actin (1:400, Boster) was used as a loading control.

Malondialdehyde Levels and Super Oxide Dismutase Activity Malondialdehyde (MDA) levels and super oxide dismutase (SOD) enzyme activities were measured by commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical Analysis All data were expressed as the mean± standard error of the mean (SEM). Differences among groups were statistically analyzed using one-way ANOVA. A $P<0.05$ value was considered statistically significant. Statistical analyses were performed using Sigma Stat 3.5.

RESULTS

LRIC Alleviated the Retinal Histological Disorder Induced by High IOP LRIC treatment in the normal animals did not affect retinal histomorphology. Thus, the sham group and

LRIC control group were combined as the control group subsequently. In the high-IOP group, retina cross-sections were disorganized, edema and thickened 1d after application of high-IOP (Figure 2A). In the 7th day after high-IOP, whole retina thickness and inner plexiform layers were significantly decreased since loss of neurons (Figure 2B). These histological changes induced by high-IOP were relieved by the LRIC treatment significantly ($P<0.05$; Figure 2). Immunofluorescence staining for neuN in retinal cross-sections revealed the positive signals were neuron cells (Figure 3). Significant neuronal loss in the ganglion cell layer was observed 7d after high-IOP, however, this loss was remarkably inhibited by LRIC ($P<0.05$). These data suggested LRIC may have protected the retina against high-IOP induced injury.

LRIC Treatment Inhibits CHOP Expression and Oxidative Stress CHOP is an endoplasmic reticulum stress marker. Western blotting and immunohistochemical staining analysis indicated that high-IOP induced increases in CHOP expression could be inhibited by LRIC treatment ($P<0.05$; Figure 4A, 4B). MDA levels and superoxide dismutase (SOD) activities were evaluated as oxidative stress markers. As shown (Figure 4C, 4D), MDA levels were increased, whereas SOD activity was significantly decreased at day 1 after high-IOP ($P<0.05$). In contrast, the increases in MDA levels and the decreased in SOD activities were inhibited significantly by LRIC treatment.

LRIC Treatment Reduces Iba-1 Expression Retinal ischemic injury usually lead to strong microglia activation. Immunohistochemical staining (Figure 5A) and Western blot analyses (Figure 5B) were used to evaluate Iba-1 expression (a microglia and macrophage marker). Our data indicated that low Iba-1 levels were detected in the normal retina. However, at 1d after high-IOP, Iba-1 levels were abundantly expressed in the ganglion cell layer and inner plexiform layer. However, in the high-IOP + LRIC group, increased Iba-1 levels induced by high-IOP were significantly reduced when compared with the high-IOP group ($P<0.05$; Figure 5).

LRIC Plasma Transfusion Reduces Caspase 9 and ROS in High-IOP Retinas To explore whether endogenous protective molecules induced by LRIC exerted remote protective effects against retinal ischemic injury, LRIC-treated plasma was transfused into high-IOP animals. Our results indicated that application of LRIC-treated plasma appeared to inhibit high-IOP-induced increase of caspase 9, a key apoptosis enzyme (Figure 6). Furthermore, we also evaluated reaction oxygen species (ROS) levels in retina tissue, by using a ROS fluorescent DHE probe. The results showed that LRIC treated-plasma inhibited the enhanced ROS fluorescence intensity induced by high-IOP ($P<0.05$; Figure 7). Taken together, LRIC treated-plasma may exert protective effects in high-IOP retinas through anti-apoptosis and anti-oxidative stress. The effective
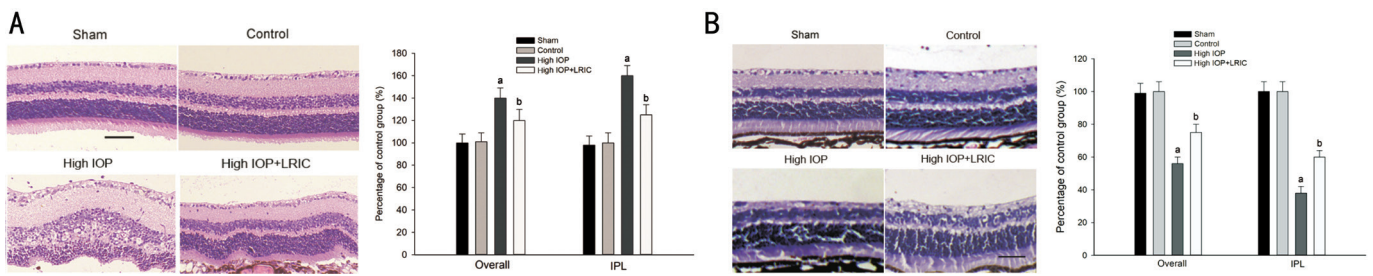
Figure 2 Representative histopathological images showing mouse retina cross-sections in sham, control, high-IOP, and high IOP + LRIC animals Sections were prepared from eyeballs at days 1 (A) and 7 (B) after treatment with high-IOP. Inner plexiform layer (IPL) and whole retinal thickness was analyzed. [a]$P<0.05$ compared with that in control group; [b]$P<0.05$ compared with that in high-IOP group. Scale bar=50 µm.
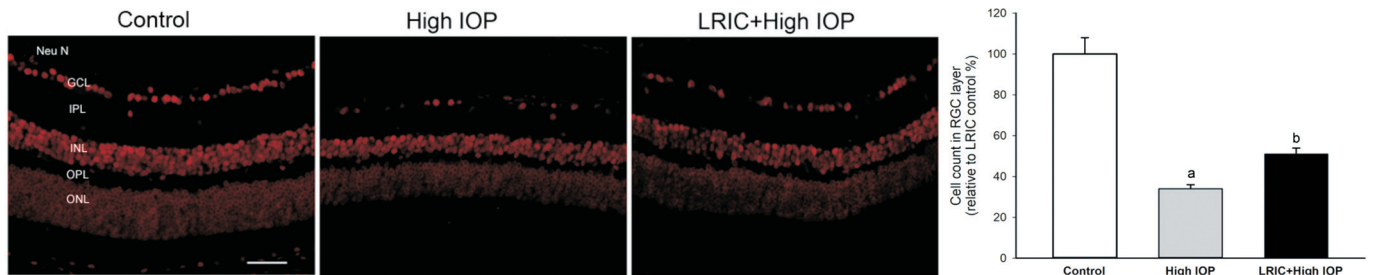

Figure 3 Representative immunohistochemical images showing NeuN positive in the ganglion cell layer at day 7 after high-IOP scale bar=50 µm. [a]$P<0.05$ compared with that in control group; [b]$P<0.05$ compared with that in high-IOP group.
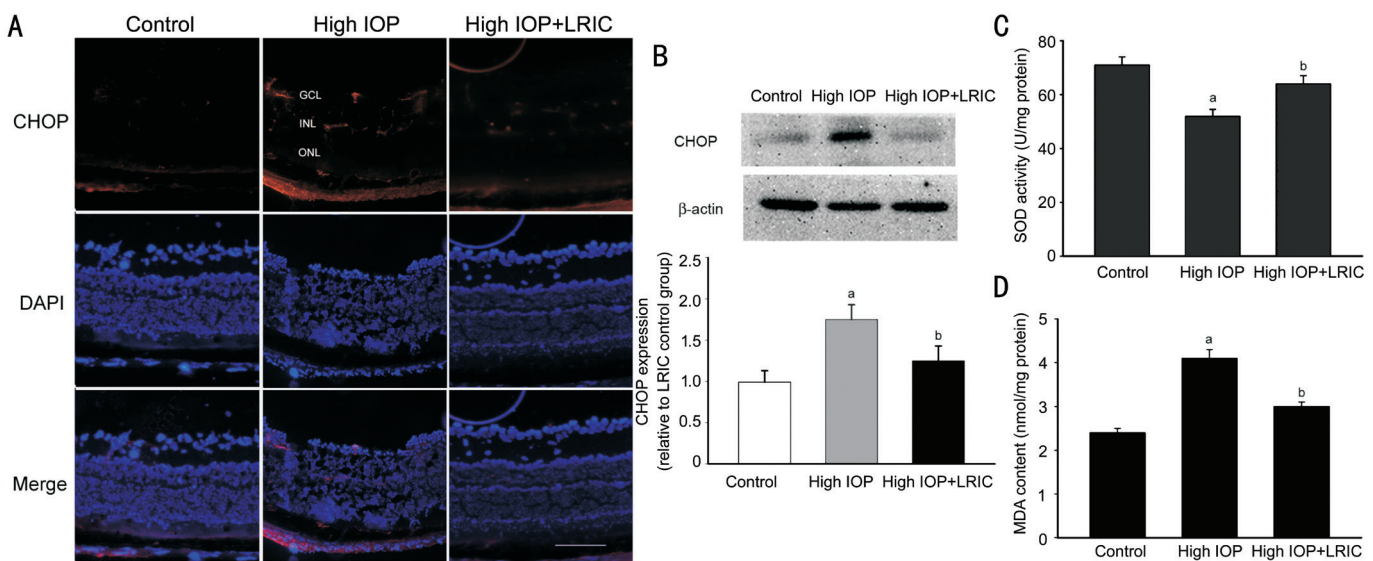

Figure 4 Representative immunohistochemical images (A), Western blot data of CHOP expression (B), SOD activity (C), and MDA levels (D) Retinas were dissected from eyeballs at 1d after high-IOP. Scale bar=100 µm. [a]$P<0.05$ compared with the control group; [b]$P<0.05$ compared with the high-IOP group.

endogenous component maybe came from the endocrine of LRIC treated limb.

DISCUSSION

Our study suggested that LRIC generated retinal protective roles against high-IOP induced injury through the inhibition of CHOP, Iba-1 and oxidative stress levels. In addition, we observed that LRIC-treated plasma decreased caspase-9 levels and ROS formation in high-IOP retinas. Thus, endogenous factors induced by LRIC may release into the circulatory system and exerted remote protective roles.

LRIC is a potent endogenous protection system which potentially triggers a series of endogenously active biological factors to exert protective effects against retinal ischemic injury[10]. It applies intermittent blood flow blocking to an organ (typically the limbs) which in turns exerts protective roles toward the organ experiencing ischemic injury. Typically, three LRIC approaches are available (pre-, per- and post-conditioning) for practical applications[7]. In reality, ischemic events cannot be predicted, therefore, post-conditioning may be considered a therapeutic intervention for multi-organ protection[8]. A
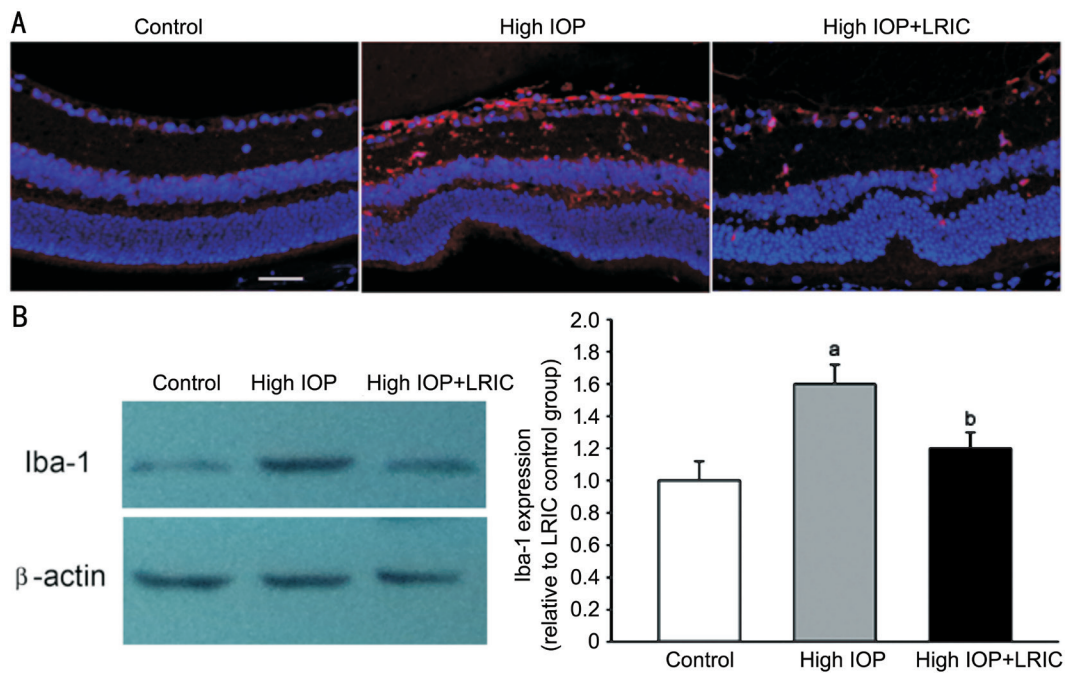
Figure 5 LRIC treatment reduced Iba-1 expression induced by high-IOP Representative immunohistochemical images (A) and Western blotting (B). Retinas were dissected from the eyeball at 1d after high-IOP. Scale bar=50 μm. [a]$P<0.05$ compared with the control group; [b]$P<0.05$ compared with the high-IOP group.
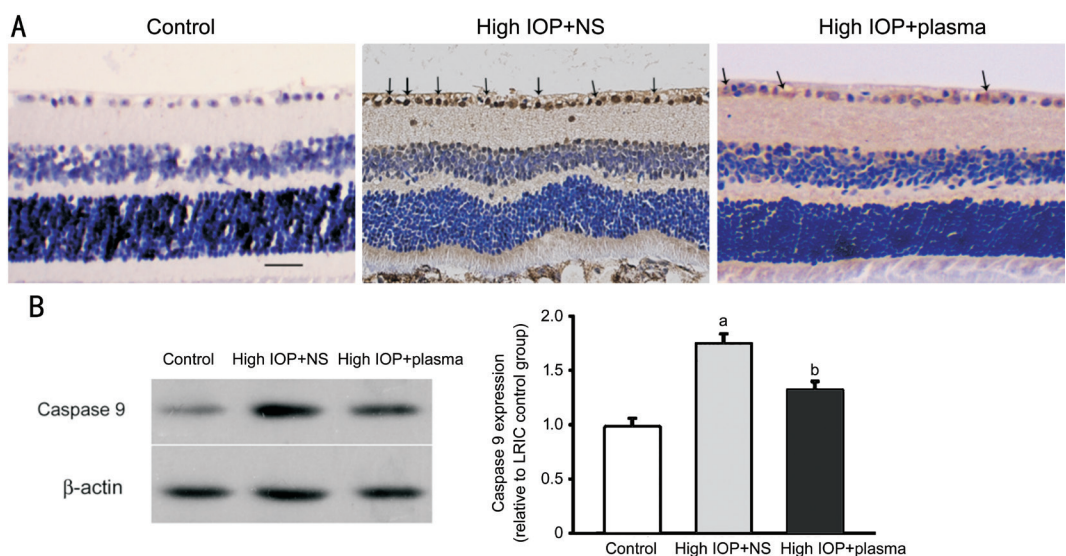

Figure 6 LRIC-treated plasma reduced the high-IOP induced apoptosis signal, caspase 9 Representative immunohistochemical images (A) and Western blotting (B). Eyeballs were excised at day 3 after high-IOP. Scale bar=50 μm. [a]$P<0.05$ compared with the control group; [b]$P<0.05$ compared with the high IOP+NS group.

previous study reported that LRIC exerted protective roles against cerebral artery occlusion induced by retinal ischemia injury[9]. Our study revealed that high-IOP in a mouse model treated with LRIC induced retinal protection. The surgical approach generating high-IOP was less invasive than other vascular occlusion surgery, therefore it may be more practical for exploring underlying LRIC mechanisms. High IOP-induced retinal ischemia is commonly used to generate retinal ischemic reperfusion in mouse models[11]. Because the mouse lens is relatively larger than humans, it is easier to oppress

backwards and induce acute ischemia under acute ocular hypertension conditions. The mouse model reflects the same pathological changes as seen in human conditions, including acute angle-closure glaucoma and retinal vessel occlusion. Similar to previous investigations, high-IOP stress led to changes in retinal histology[11-12]. During the preliminary stages (24h) of retinal ischemic-reperfusion injury, the main pathological changes were vacuolation, edema, and increased thickness of the ganglion cell layer, inner nuclear layer and the whole retina. In later stages (7d), retinal thickness reduced, and
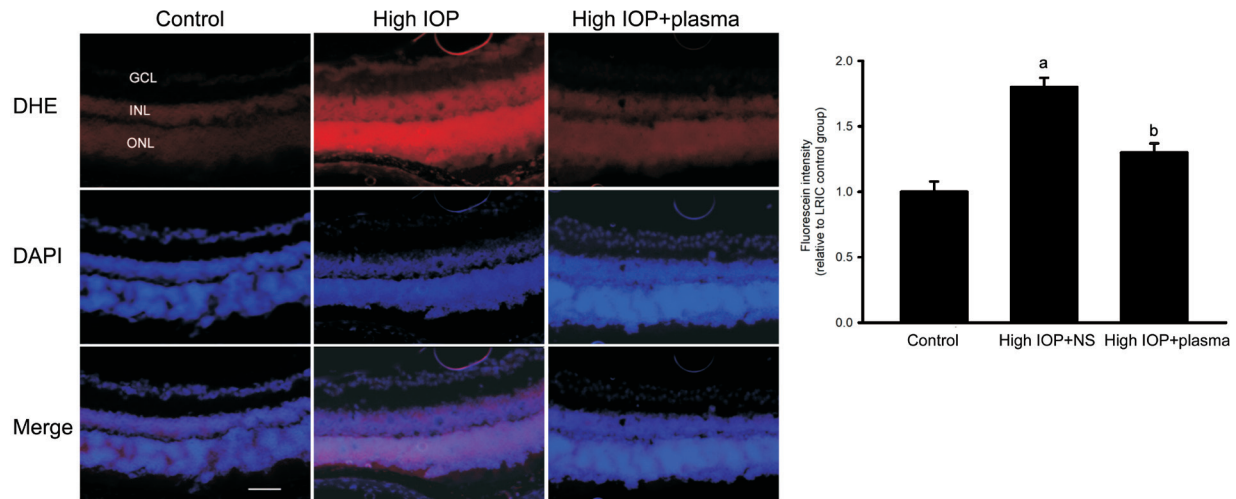
Figure 7 LRIC-treated plasma inhibited high-IOP induced ROS formation Representative DHE fluorescence probe staining. Eyeballs were excised at day 3 after high-IOP. Scale bar=50 μm. [a]$P<0.05$ compared with the control group; [b]$P<0.05$ compared with the high IOP + NS group.

ganglion cell numbers decreased significantly. LRIC at 5min and 3d after reperfusion ameliorated these histological changes induced by ischemic reperfusion injury. These protective roles were consistent with previous post-conditioning mouse models[13].

Ischemic-reperfusion injury is a main source of free radical generation[14], which if in excess, induce oxidative stress damage towards proteins, lipids, and nucleic acids. We showed that LRIC inhibited increased MDA levels and decreased SOD activity. MDA is a product of lipid peroxidation and is an indicator to assess oxidative stress damage and severity. Increasing evidence has identified associations between remote ischemic conditioning and antioxidant activity[15-18]. CHOP is an endoplasmic reticulum (ER) stress-related protein and is used as an ER stress marker. Increased retinal CHOP levels are observed in many conditions, including ischemic reperfusion injury, diabetic retinopathy, and other diseases[4,19-20]. ER homeostasis may be dysregulated by hypoxia, oxidative stress, and inflammation. The ER stress and oxidative stress always interact to generate apoptosis and even cause tissue injury. In our study, LRIC in ischemic reperfusion animals reduced CHOP expression, consistent with the ROS formation.

Ischemia/hypoxia induces direct retinal damage during high-IOP conditions[21-22]. In addition, blood reperfusion induced leukocyte infiltration (including monocytes/macrophage) induces inflammatory response cascades which may induce more subsequent injury[23-24]. Therefore, LRIC could alter these systemic responses and decrease the extent of nervous injury by decreasing leukocyte accumulation and inflammatory factor expression[25-26]. Our results indicated LRIC inhibited high-IOP induced Iba-1 expression in the retina, which was in accord with previous report[27]. Iba-1 is a macrophage/microglia marker. It was not very easy to distinguish Iba-1 positive microglia and infiltrated monocytes *via* our morphology staining. From Ha *et al*'s[20] study, we were aware of the Iba-1 positive staining including both types of the cellular during retinal ischemic reperfusion injury status. Thus, LRIC appeared to reduce infiltrated monocytes and microglia activation, which may contribute to retinal protection against ischemic reperfusion injury.

Plasma transfusion are effective interventional methods and have been demonstrated for several disease conditions including cardioprotection, severe sepsis, Alzheimer's disease, and age related impairment[28-32]. In our study, LRIC plasma was transfused into high-IOP animals, and showed that the apoptotic signal enzyme, caspase 9 and ROS formation in retinas were significantly reduced. Studies have reported that remote ischemic condition protection is generated by the release of multiple humoral factors into the circulation, including heat shock proteins[33-34], extracellular vesicles[35-37], adenosine[38-40], nitric oxide[41], and glutamate[42-43]. Thus, we also preliminarily verified the protective effects of LRIC-treated plasma by intravenous injection. Further research is required to fully clarify the essential humoral factors required for LRIC protection and related neuroregulatory mechanisms.

In conclusion, since remote ischemic condition was proposed in 1986, extensive research and clinical transformational studies have emerged[34,37,43-44]. We demonstrated that LRIC protected high IOP-induced retinal injury *via* antioxidant and ER stress inhibition. Reduced monocyte infiltration and microglia activation may have played an important role in this protection. The present study demonstrated that LRIC-plasma transfusion exerted protective effects from ischemic reperfusion injury by inhibiting caspase 9 and ROS production in high-IOP animals. However, LIRC humoral or/and neural mechanisms require further investigation, therefore, future

studies need to determine and identify key factors released into the circulation. Ultimately, this effective, low cost, low risk therapy could be optimally developed for improved applications in clinical medicine.

REFERENCES

1 Shi SY, Feng XM, Li Y, Li X, Chen XL. Expression of neuroglobin in ocular hypertension induced acute hypoxic-ischemic retinal injury in rats. *Int J Ophthalmol* 2011;4(4):393-395.

2 Osborne NN, Casson RJ, Wood JPM, Chidlow G, Graham M, Melena J. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. *Prog Retin Eye Res* 2004;23(1):91-147.

3 Flammer J, Orgül S, Costa VP, Orzalesi N, Krieglstein GK, Serra LM, Renard JP, Stefánsson E. The impact of ocular blood flow in glaucoma. *Prog Retin Eye Res* 2002;21(4):359-393.

4 Wang HG, Li JM, Zhong PY, Wang SW, Zhang L, Yang RS, Wu DD, Chen ML, Ji AL, Li YZ, Wang J. Blocking CXCR3 with AMG487 ameliorates the blood-retinal barrier disruption in diabetic mice through anti-oxidative. *Life Sci* 2019;228:198-207.

5 Roth S. Endogenous neuroprotection in the retina. *Brain Res Bull* 2004;62(6):461-466.

6 Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;74(5):1124-1136.

7 Chen GL, Thakkar M, Robinson C, Doré S. Limb remote ischemic conditioning: mechanisms, anesthetics, and the potential for expanding therapeutic options. *Front Neurol* 2018;9:40.

8 Zhou D, Ding JY, Ya JY, Pan LQ, Wang Y, Ji XM, Meng R. Remote ischemic conditioning: a promising therapeutic intervention for multi-organ protection. *Aging* 2018;10(8):1825-1855.

9 Zhang XX, Jizhang YN, Xu XY, Kwiecien TD, Li N, Zhang Y, Ji XM, Ren CH, Ding YC. Protective effects of remote ischemic conditioning against ischemia/reperfusion-induced retinal injury in rats. *Vis Neurosci* 2014;31(3):245-252.

10 Mohammad Seyedsaadat S, Kallmes DF, Brinjikji W. Remote ischemic conditioning approach for the treatment of ischemic stroke. *Neural Regen Res* 2020;15(6):1033-1034.

11 Chen H, Deng Y, Gan XL, Li YH, Huang WY, Lu L, Wei L, Su LS, Luo JW, Zou B, Hong YH, Cao YH, Liu YZ, Chi W. NLRP12 collaborates with NLRP3 and NLRC4 to promote pyroptosis inducing ganglion cell death of acute glaucoma. *Mol Neurodegener* 2020;15(1):26.

12 Wan PX, Su WR, Zhang YY, Li ZD, Deng CB, Li JM, Jiang N, Huang SY, Long EP, Zhuo YH. LncRNA H19 initiates microglial pyroptosis and neuronal death in retinal ischemia/reperfusion injury. *Cell Death Differ* 2020;27(1):176-191.

13 Fernandez DC, Chianelli MS, Rosenstein RE. Involvement of glutamate in retinal protection against ischemia/reperfusion damage induced by post-conditioning. *J Neurochem* 2009;111(2):488-498.

14 Fan N, Silverman SM, Liu Y, Wang XZ, Kim BJ, Tang LP, Clark AF, Liu XY, Pang IH. Rapid repeatable *in vivo* detection of retinal reactive oxygen species. *Exp Eye Res* 2017;161:71-81.

15 Chen GL, Ye XY, Zhang JW, Tang TL, Li L, Lu PR, Wu Q, Yu BY, Kou JP. Limb remote ischemic postconditioning reduces ischemia-reperfusion injury by inhibiting NADPH oxidase activation and MyD88-TRAF6-P38MAP-kinase pathway of neutrophils. *Int J Mol Sci* 2016;17(12):1971.

16 Li S, Hu XS, Zhang MX, Zhou FF, Lin N, Xia QJ, Zhou Y, Qi WQ, Zong YH, Yang HJ, Wang TH. Remote ischemic post-conditioning improves neurological function by AQP4 down-regulation in astrocytes. *Behav Brain Res* 2015;289:1-8.

17 Liang JM, Xu HY, Zhang XJ, Li XG, Zhang HB, Ge PF. Role of mitochondrial function in the protective effects of ischaemic postconditioning on ischaemia/reperfusion cerebral damage. *J Int Med Res* 2013;41(3):618-627.

18 Wang JY, Shen J, Gao Q, Ye ZG, Yang SY, Liang HW, Bruce IC, Luo BY, Xia Q. Ischemic postconditioning protects against global cerebral ischemia/reperfusion-induced injury in rats. *Stroke* 2008;39(3):983-990.

19 Li HL, Zhu XH, Fang F, Jiang DY, Tang LS. Down-regulation of GRP78 enhances apoptosis *via* CHOP pathway in retinal ischemia-reperfusion injury. *Neurosci Lett* 2014;575:68-73.

20 Ha Y, Liu H, Xu Z, Yokota H, Narayanan SP, Lemtalsi T, Smith SB, Caldwell RW, Caldwell RB, Zhang W. Endoplasmic reticulum stress-regulated CXCR3 pathway mediates inflammation and neuronal injury in acute glaucoma. *Cell Death Dis* 2015;6(10):e1900.

21 Ulbrich F, Goebel U, Scheid S, Spassov S, Schallner N, Wollborn J, Buerkle H. Argon reduces microglial activation and inflammatory cytokine expression in retinal ischemia/reperfusion injury. *Neural Regen Res* 2021;16(1):192.

22 Luo JY, He T, Yang JY, Yang N, Li ZY, Xing YQ. SIRT1 is required for the neuroprotection of resveratrol on retinal ganglion cells after retinal ischemia-reperfusion injury in mice. *Graefes Arch Clin Exp Ophthalmol* 2020;258(2):335-344.

23 Nakazawa T, Takahashi H, Nishijima K, Shimura M, Fuse N, Tamai M, Hafezi-Moghadam A, Nishida K. Pitavastatin prevents NMDA-induced retinal ganglion cell death by suppressing leukocyte recruitment. *J Neurochem* 2007;100(4):1018-1031.

24 Marcheselli VL, Hong S, Lukiw WJ, Tian XH, Gronert K, Musto A, Hardy M, Gimenez JM, Nan CA, Serhan CN, Bazan NG. Novel

docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. *J Biol Chem* 2003;278(44):43807-43817.

25 Liu ZJ, Chen C, Li XR, Ran YY, Xu T, Zhang Y, Geng XK, Zhang Y, Du HS, Leak RK, Ji XM, Hu XM. Remote ischemic preconditioning-mediated neuroprotection against stroke is associated with significant alterations in peripheral immune responses. *CNS Neurosci Ther* 2016;22(1):43-52.

26 Xing BZ, Chen H, Zhang M, Zhao DM, Jiang R, Liu XH, Zhang SM. Ischemic postconditioning inhibits apoptosis after focal cerebral ischemia/reperfusion injury in the rat. *Stroke* 2008;39(8):2362-2369.

27 Esposito E, Hayakawa K, Ahn BJ, Chan SJ, Xing CH, Liang AC, Kim KW, Arai K, Lo EH. Effects of ischemic post-conditioning on neuronal VEGF regulation and microglial polarization in a rat model of focal cerebral ischemia. *J Neurochem* 2018;146(2):160-172.

28 Villeda SA, Plambeck KE, Middeldorp J, Castellano JM, Mosher KI, Luo J, Smith LK, Bieri G, Lin K, Berdnik D, Wabl R, Udeochu J, Wheatley EG, Zou BD, Simmons DA, Xie XS, Longo FM, Wyss-Coray T. Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice. *Nat Med* 2014;20(6):659-663.

29 Zhang L, Zhang JP, Liu Y, Wang H, Guo JR. Plasma transfusion promoted reprogramming CD4+ T lymphocytes immune response in severe sepsis mice model through modulating the exosome protein Galectin 9. 2020;29:963689720947347.

30 Middeldorp J, Lehallier B, Villeda SA, Miedema SSM, Evans E, Czirr E, Zhang H, Luo J, Stan T, Mosher KI, Masliah E, Wyss-Coray T. Preclinical assessment of young blood plasma for alzheimer disease. *JAMA Neurol* 2016;73(11):1325-1333.

31 Sha SJ, Deutsch GK, Tian L, *et al*. Safety, tolerability, and feasibility of young plasma infusion in the plasma for alzheimer symptom amelioration study: a randomized clinical trial. *JAMA Neurol* 2019;76(1):35-40.

32 Heinen A, Behmenburg F, Aytulun A, Dierkes M, Zerbin L, Kaisers W, Schaefer M, Meyer-Treschan T, Feit S, Bauer I, Hollmann MW, Huhn R. The release of cardioprotective humoral factors after remote ischemic preconditioning in humans is age- and sex-dependent. *J Transl Med* 2018;16(1):112.

33 Kim N, Ullah I, Chung K, Lee D, Cha MJ, Ban H, Choi CS, Kim S, Hwang KC, Kumar P, Lee SK. Targeted delivery of recombinant heat shock protein 27 to cardiomyocytes promotes recovery from myocardial infarction. *Mol Pharmaceutics* 2020;17(6):2034-2043.

34 England TJ, Hedstrom A, O'Sullivan S, Donnelly R, Barrett DA, Sarmad S, Sprigg N, Bath PM. RECAST (remote ischemic conditioning after stroke trial): a pilot randomized placebo controlled phase II trial in acute ischemic stroke. *Stroke* 2017;48(5):1412-1415.

35 Abel F, Murke F, Gaida M, Garnier N, Ochsenfarth C, Theiss C, Thielmann M, Kleinbongard P, Giebel B, Peters J, Frey UH. Extracellular vesicles isolated from patients undergoing remote ischemic preconditioning decrease hypoxia-evoked apoptosis of cardiomyoblasts after isoflurane but not propofol exposure. *PLoS One* 2020;15(2):e0228948.

36 Bartekova M, Jelemensky M, Dhalla NS. Emerging role of non-coding RNAs and extracellular vesicles in cardioprotection by remote ischemic conditioning of the heart. *Rev Cardiovasc Med* 2019;20(2):59-71.

37 Li Y, Ren CH, Li HY, Jiang F, Wang L, Xia CQ, Ji XM. Role of exosomes induced by remote ischemic preconditioning in neuroprotection against cerebral ischemia. *Neuroreport* 2019;30(12):834-841.

38 Randhawa PK, Jaggi AS. Unraveling the role of adenosine in remote ischemic preconditioning-induced cardioprotection. *Life Sci* 2016;155:140-146.

39 Ng MW, Angerosa J, Konstantinov IE, Cheung MM, Pepe S. Remote ischaemic preconditioning modifies serum apolipoprotein D, met-enkephalin, adenosine, and nitric oxide in healthy young adults. *Clin Exp Pharmacol Physiol* 2019;46(11):995-1000.

40 Singh L, Kulshrestha R, Singh N, Jaggi AS. Mechanisms involved in adenosine pharmacological preconditioning-induced cardioprotection. *Korean J Physiol Pharmacol* 2018;22(3):225-234.

41 Totzeck M, Hendgen-Cotta U, Rassaf T. Concepts of hypoxic NO signaling in remote ischemic preconditioning. *World J Cardiol* 2015;7(10):645-651.

42 Bonova P, Burda J, Danielisova V, Nemethova M, Gottlieb M. Delayed post-conditioning reduces post-ischemic glutamate level and improves protein synthesis in brain. *Neurochem Int* 2013;62(6):854-860.

43 You JL, Feng LS, Xin MY, Ma D, Feng JC. Cerebral ischemic postconditioning plays a neuroprotective role through regulation of central and peripheral glutamate. *Biomed Res Int* 2018;2018:6316059.

44 England TJ, Hedstrom A, O'Sullivan SE, Woodhouse L, Jackson B, Sprigg N, Bath PM. Remote ischemic conditioning after stroke trial 2: a phase IIb randomized controlled trial in hyperacute stroke. *J Am Heart Assoc* 2019;8(23):e013572.