Remote ischemic conditioning protected retina injury in mice

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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 50 min and LRIC applied to the unilateral leg for three occlusion cycles (5 min/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 parameters were evaluated by immunofluorescent staining and Western blot.
- **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.

- **KEYWORDS:** retina; ischemic conditioning; high intraocular pressure

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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. 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. MEW2AHUM 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°C±3°C. 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 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°C. 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

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**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.
were stained by hematoxylin and eosin. To examine retinal thickness, three measurements were averaged for each retina from the peripheral to the center. For immuno-fluorescence 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 30 min and incubated overnight at 4°C 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 1 h at room temperature. After washing twice in PBS (5 min 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 (40 min 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°C 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 1 h at room temperature on a shaker. Protein band optical densities were subsequently calculated. The following day, after washing with PBS, sections were incubated with relevant secondary antibodies (1:500, Beyotime) at 4°C for 1 h. After washing twice in PBS (5 min 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 (40 min at room temperature). Images were observed under a fluorescence microscope and the fluorescence intensity was then calculated.

**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 1 d 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 7 d 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 analysis (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 1 d 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
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].

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.
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\(^{11-12}\). 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. 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 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.
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. Ischemic-reperfusion injury is a main source of free radical generation, 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. 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. 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. In addition, blood reperfusion induced leukocyte infiltration (including monocytes/macrophage) induces inflammatory response cascades which may induce more subsequent injury. Therefore, LRIC could alter these systemic responses and decrease the extent of nervous injury by decreasing leukocyte accumulation and inflammatory factor expression. Our results indicated LRIC inhibited high-IOP induced Iba-1 expression in the retina, which was in accord with previous report. 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 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. 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, extracellular vesicles, adenosine, nitric oxide, and glutamate. 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. 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
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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.

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