• Basic Research •

Protection of retinal ganglion cells against optic nerve injury by induction of ischemic preconditioning

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Abstract

• AIM: To explore if ischemic preconditioning (IPC) can enhance the survival of retinal ganglion cells (RGCs) after optic nerve axotomy.

• METHODS: Twenty-four hours prior to retinal ischemia 60min or axotomy, IPC was applied for ten minutes in groups of (*n*=72) animals. The survival of RGCs, the cellular expression of heat shock protein 27 (HSP27) and heat shock protein 70 (HSP70) and the numbers of retinal microglia in the different groups were quantified at 7 and 14d post-injury. The cellular expression of HSP27 and HSP70 and changes in the numbers of retinal microglia were quantified to detect the possible mechanism of the protection of the IPC.

• RESULTS: Ten minutes of IPC promoted RGC survival in both the optic nerve injury (IPC-ONT) and the retinal ischemia 60min (IPC-IR60) groups, examined at 7d and 14d post-injury. Microglial proliferation showed little correlation with the extent of benefit effects of IPC on the rescue of RGCs. The number of HSP27-positive RGCs was significantly higher in the IPC-ONT group than in the sham IPC-ONT group, although the percentage of HSP27-positive RGCs did not significantly differ between groups. For the IPC-IR60 group, neither the number nor the percentage of the HSP27-positive RGCs differed significantly between the IPC and the sham-operated groups. The number of HSP70-positive RGCs was significantly higher for both the IPC-ONT and the IPC-IR60 experimental groups, but the percentages did not differ.

• CONCLUSION: The induction of IPC enhances the survival of RGCs against both axotomy and retinal ischemia.

• KEYWORDS: ischemic preconditioning; retinal ganglion cells; axotomy; retinal ischemia/reperfusion; heat shock protein 27 and 70

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INTRODUCTION

Ischemic preconditioning (IPC), also known as ischemic tolerance or ischemic resistance, refers to the phenomenon that brief nonlethal periods of ischemia can protect local or remote organs from subsequent prolonged periods of critical ischemia. IPC was first described with a canine myocardium model in 1986, which showed that four cycles of short periods of ischemia and reperfusion 40min prior to coronary artery occlusion, reduced by 75%, the size of the ultimate myocardial infarct compared to cases without IPC[1]. Subsequent studies disclosed that IPC provides both histological and psychological protection through eliciting endogenous protective mechanisms, providing a promising strategy for protecting tissues and organ systems with high sensitivity to ischemia, such as the myocardium[2], muscle flaps, the stomach, kidneys, lungs and liver, and the central nervous system[3]. In 1998, one study showed that IPC in the rat retina alleviated functional impairment and cell death and also provided complete protection against retinal ischemia/reperfusion injury[4].

We have earlier shown that application of remote ischemic post-conditioning can promote the survival of retinal ganglion cells (RGCs) after optic nerve axotomy[5]. Induction of ischemic tolerance has also been reported to be a promising strategy to protect RGCs against diabetic retinopathy[6], increasing survival and function of retinal neurons in a model of glaucomatous retinopathy[7]. Given this background we tested the hypothesis that induction of ischemic tolerance would

rescue ganglion cells from degeneration after subsequent optic nerve axotomy and compare the protective effects of IPC after retinal ischemia/reperfusion injury. Retinal microglia and the expression of heat shock protein 27 (HSP27) and heat shock protein 70 (HSP70) were quantitated to investigate the possible mechanism of IPC in surviving RGCs.

MATERIALS AND METHODS

The experiments were performed in adult 8 to 12-week-old Syrian golden hamsters (*Mesocricetus auratus*). Surgical manipulations were performed after induction of general anesthesia by intraperitoneal (i.p.) injection of ketamine/xylazine (200 mg/20 mg per kg body weight). All hamsters were randomly assigned to one of two different groups: optic nerve injury (optic nerve transection, ONT) and retinal ischemia/reperfusion (IP) groups. In the ONT group, the RGCs of one eye were damaged by a complete transection of the optic nerve proximal to the orbit, whereas in the IP group, retinal ischemia was induced by ligature of the ophthalmic vessels (LOV) lasting 60min after the induction of a 10min IPC performed 24h previously. The survival of RGCs was quantified at 7 and 14d post-injury, as was the cellular expression of HSP27 and HSP70 and changes in the numbers of retinal microglia in the different groups. The experimental protocols have been approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

Ligature of the Ophthalmic Vessels to Induce Ischemic Preconditioning Twenty-four hours prior to retinal ischemia or axotomy, IPC was applied for 10min in groups of animals (n=72). Following the method described by Lafuente *et al*[8] with slight modifications, LOV was induced to produce transient retinal ischemia, followed by retinal reperfusion upon releasing the suture. In this procedure, the optic nerve was first exposed, and the superior dural sheath was opened longitudinally. A 10/0 nylon suture was inserted between the dural sheath and the optic nerve, the two ends of the suture were tied with a loose knot around the dura, and the vessels were also ligated within the dura. During the surgery, great care was taken to avoid damage to the optic nerve. Ten minutes later, the suture was released and removed with great care in order to ensure resumption of retinal perfusion. For each group, a corresponding sham IPC procedure group was prepared in similar manner, *i.e.* with exposure and loose suturing of the ophthalmic vessels, but without ligation of the suture.

Transection of the Optic Nerve to Induce Ganglion Cell Axonal Injury Twenty-four hours after the 10min of IPC, the optic nerve of the right eye was cut both in the IPC-ONT and sham IPC-ONT groups. In this procedure, the animals were re-anesthetized as above. The optic nerve of the right eye was exposed in the orbit and transected with microsurgical scissors 2 mm behind the eyeball, taking care not to injure the ophthalmic artery running along the inferior aspect of the dura.

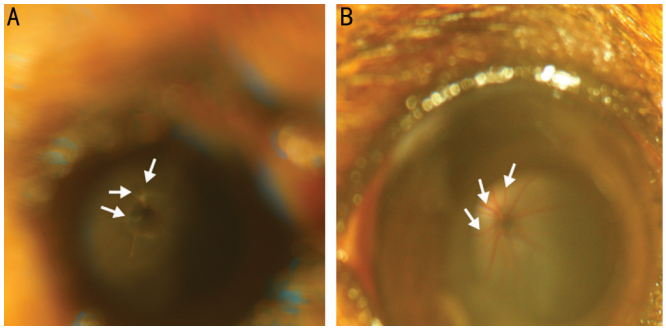

Figure 1 The conditions of the blood flow in the Syrian golden hamster retina Images (A) and (B) are both from the operated eye; the funduscopic examination pictures were taken during the process of LOV (A) and after reperfusion (B). A: The entire fundus looked pale with clear white vessels branching from the optic disc initially, and the arrows point to the whitening branches; B: The blood flow of the retina was resumed after reperfusion, here the arrows point to the red branches. Magnification: 4×.

Ligature of the Ophthalmic Vessels to Induce Transient Retinal Ischemia/Reperfusion The LOV method to induce retinal ischemia/reperfusion was performed as described above. The interruption of the retinal blood flow was assessed by ophthalmoscopy of the eye fundus through an operating microscope (Figure 1). Animals not showing a complete interruption of the retinal blood flow underwent a second LOV operation until complete interruption was observed. The duration of the ischemic period lasted 60min, whereupon the suture was released for subsequent reperfusion. The sham IPC-IR60 group was processed in the same manner, but without LOV. Before release of the suture, observation of the eye fundus through the operating microscope corroborated and confirmed the interruption throughout the targeted time. After the suture was released and the skin was closed, resumption of the retinal blood flow was investigated every five minutes, and the total time to complete reperfusion time was recorded. Animals not exhibiting a complete recovery of retinal blood flow within the first 10min after release were excluded from the study. Transient cloudiness of the lens was occasionally observed during the LOV process, which might have hampered the observation of reperfusion time. However, the cloudiness reversed spontaneously within a few minutes after the onset of reperfusion. The observation of the fundus was easy and non-invasive, providing a reliable and feasible measure to confirm the cessation and recovery of blood flow for each experimental animal.

Quantification of Ganglion Cell Survival Survival of ganglion cell at 7 or 14d post-optic nerve injury or post-retinal ischemia/reperfusion was assessed by immunostaining with anti-βIII-tubulin (clone TuJ1, CovanceInc., USA). The immunohistochemical staining and quantification of the surviving RGCs followed the methods described in previous paper[5,9]. In the ONT groups, retrograde labeling was also applied to confirm the

results at 14d post-optic nerve injury. In this procedure, several crystals of the fluorescent dye 4-[4-(didecylamino)styryl]-N-methylpyridinium iodide (4-Di-10ASP; Molecular Probes), were placed on the cut surface of the proximal stump upon surgical re-exposure of the right optic nerve with truncation to 0.5 mm from the orbit. The wound was closed and the animals recovered for a period of 2d after dye application, namely at 14d post-optic nerve injury. At this time, the animals were killed and the orbit resected. The entire retina fixed as a whole mount in glycerol and observed under epifluorescence to quantify the number of surviving retrogradely labelled RGCs, as in TuJ1 staining.

Changes in Microglia Number in the Optic Nerve Injury Groups Results of previous studies suggest that the number and activity of microglia increase after neuronal injury, and influence neuronal survival. To test the relationship between microglial activation and beneficial effects of IPC on RGC survival, we quantified retinal microglial numbers in the IPC-ONT and sham IPC-ONT groups at 7d post-optic nerve section. Whole mount retinas were incubated in a medium containing anti-βIII-tubulin and anti-Iba-1 (1:1000 rabbit polyclonal, Wako). Anti-Iba-1 is a pan-microglia marker, which is visualized with anti-rabbit-biotin plus streptavidin-Cy2. The number of stained microglia per retina was quantified with Neurolucida as described previously[5].

Expression of Heat Shock Protein 27 and Heat Shock Protein 70 After Optic Nerve Transection or Ischemia/Reperfusion 60min The expression of HSP27 by RGCs was examined in the IPC-ONT group, the IPC-IR60 group, and the corresponding sham groups. At 7d post-injury, the whole mount retina was processed for double immunofluorescence with antibodies against TuJ1 and HSP27 (rabbit polyclonal, StressGen; 1:1000) according to procedures described previously[5]. The numbers of TuJ1- and HSP27-stained ganglion cells were quantified and the percentage of HSP27-expressing ganglion cells in the surviving population (as identified by double labeling with TuJ1) were calculated[4].

For the expression of HSP70 by RGCs in the IPC-ONT or IPC-IR60 groups, paraffin-embedded sagittal sections across the optic disc were selected and processed. At 7d post-injury, the retinal sections were processed for double immunofluorescence with antibodies against TuJ1 and HSP70 (rabbit polyclonal, Chemicals USA, Inc.; 1:100). TuJ1 staining was visualized by anti-mouse-Cy3, whereas HSP70 staining was visualized by anti-rabbit-biotin, followed by application of streptavidin-Cy2 (Jackson; 1:500).

The mean densities of TuJ1- and HSP70-positive RGCs in the retinal sections were obtained from the average of 8 counted grids; 6 sections were counted for every sample, and mean was obtained. The number of HSP70-positive RGCs in the sections was counted in four grids (200×200 μm^2) for each the superior and inferior portions of the retina. Furthermore, the percentage of HSP70-positive RGCs was calculated for every section. In addition, the retinal thickness at the center and the periphery was measured, *i.e.* in the first and the last grids in the superior and inferior portions. To reduce sampling errors, six sections were measured, and the mean thickness of the center and periphery of the retina were obtained for every sample.

Statistical Analysis For the quantification of the survival of TuJ1-positive RGCs, and also for quantification of HSP27- or HSP70-positive ganglion cells, each experimental group consisted of four or five animals. All quantitative data were presented as the means±SEM. For statistical comparison of the outcomes between two groups, the two-tailed Student's *t*-test was used with the level of statistical significance set at $P<0.05$.

RESULTS

Ischemic Preconditioning Promoted Survival of Retinal Ganglion Cells After Optic Nerve Transection Ten minutes of IPC at 24h prior to axotomy had a protective effect on the survival of RGCs at 7 and 14d post-axotomy (Figure 2A, 2C). In the IPC-ONT group (40 229±1205), the number of TuJ1-positive RGCs was significantly higher than that in the sham IPC-ONT group (35 298±847; *t*-test, $P=0.02$; Figure 2A *vs* 2B) at 7d post-axotomy. The number of surviving RGCs was approximately 5000 to 7000 RGCs higher in the IPC-ONT treatment group at 7d post-axotomy than in the sham IPC-ONT group. In the case of the retinas examined at 14d post-axotomy, the number of TuJ1-positive RGCs was 22% higher in the IPC-ONT group (25 358±1326) compared with the sham group (20 788±1435; *t*-test, $P=0.001$; Figure 2C *vs* 2D). RGCs survival as accessed by retrograde labelling with dye at 14d post-optic nerve injury also confirmed the beneficial effects of the IPC pre-treatment. The number of surviving ganglion cells was 14.7% higher in the treatment group compared to the sham group (10 749±287 *vs* 9165± 463; $P=0.02$) (Figure 2E *vs* 2F).

Increased Ganglion Cell Survival did not Correlate with Changes in Microglia Number Microglia are located in different laminae of the healthy retina, mainly in: 1) the ganglion cell layer (GCL); 2) inner plexiform layer (IPL); 3) outer plexiform layer (OPL). In our analysis we focused on microglia counts in the GCL, due to the intimate association of microglia in those regions with RGCs and their axons (Figure 3A). Morphologically, normal retinal microglia has small cell bodies and short fine branches, which are extended without overlapping (Figure 3A). The total number of microglia in the GCL of normal retina amounted to 4394±200 ($n=4$) per retina (data not shown). According to our results, the number of microglia increased to about 100 000 at 7d post-optic nerve sectioning (Figure 3B). Microglia counts also increased to a similar extent in both the IPL and OPL but to similar extents in the IPC and sham-pretreated groups (data not shown).

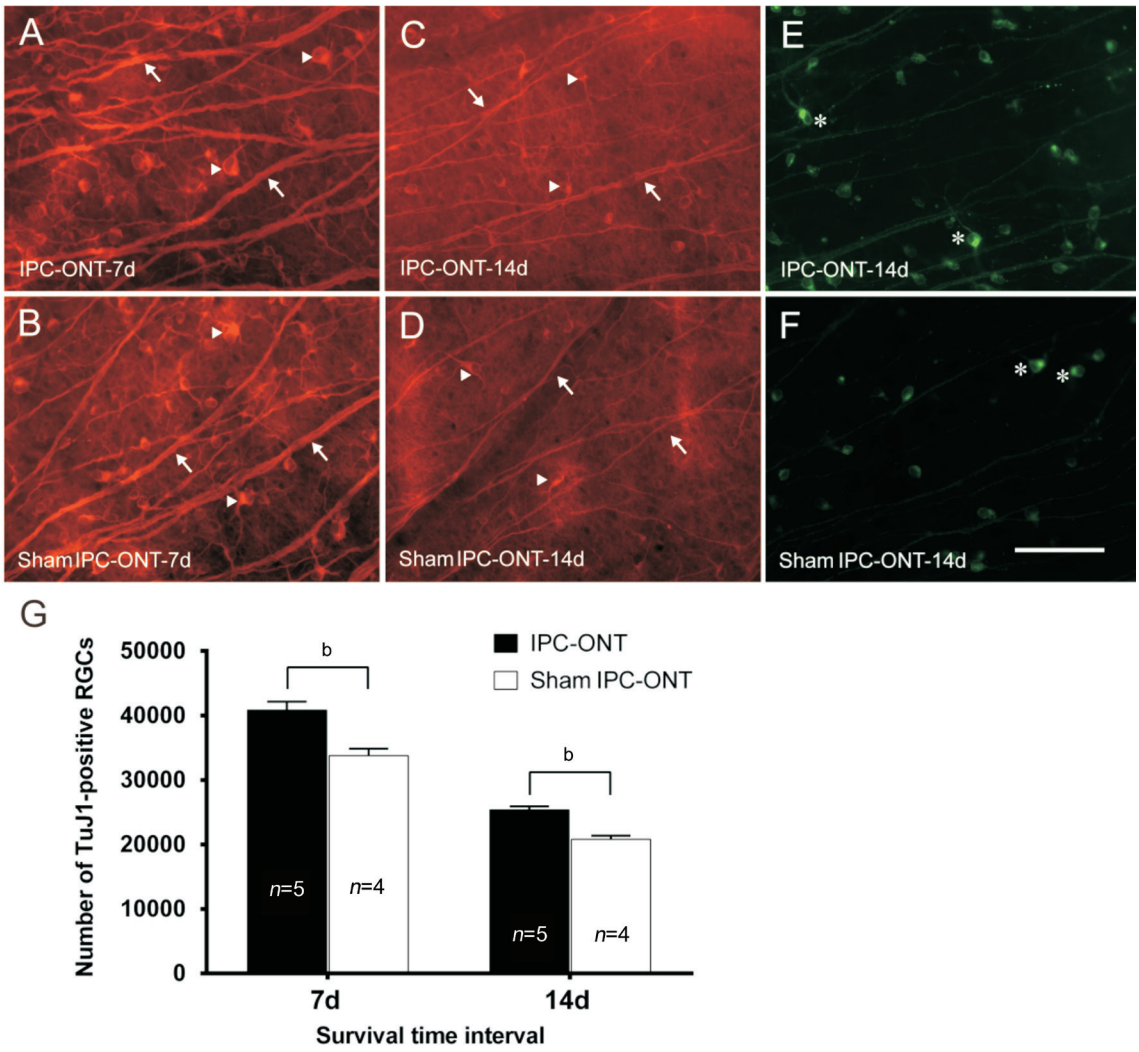

Figure 2 TuJ1-positive RGCs IPC-ONT group (A) and sham IPC-ONT group (B) at 7d post-optic nerve section; IPC or sham IPC was induced for 10min and 24h later, ONT was conducted; there were more surviving RGCs in IPC-ONT group than in the sham conditioning group. IPC-ONT group (C) and shamIPC-ONT group (D) at 14d post-optic nerve section; IPC or sham IPC was applied for 10min and 24h later, ONT was conducted; there were significantly more surviving RGCs in IPC-ONT group than in sham conditioning group. IPC-ONT group (E) and sham IPC-ONT group (F) at 14d post-optic nerve cut labeled by 4-Di-10ASP in a retina; there were more surviving ganglion cells (some marked by asterisk) in the retina of animals pretreated with IPC. The number of TuJ1-positive RGCs (G) in the different groups at 7 and 14d post-optic nerve cut; significantly more RGCs were observed in the IPC treatment group than in the sham group at both 7 and 14d post-axotomy (*t*-test, $^{b}P<0.01$). The arrows indicate axons extending from the RGCs; the arrowheads indicate the bodies of RGCs labeled by TuJ1; the asterisks indicate the surviving ganglion cells labeled by 4-Di-10ASP in a retina. Error bar=SEM. Scale bar=100 μm (A-F).

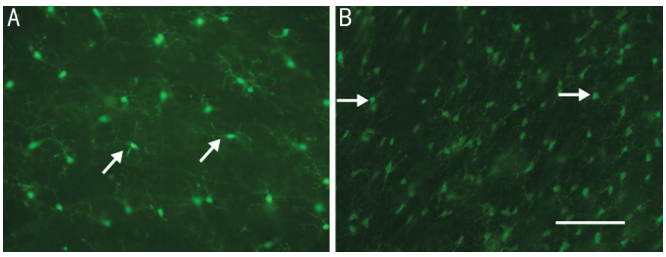

Figure 3 The activation of retinal microglia at 7d after optic nerve cut compared to the normal group A: GCL microglia from the normal group; B: GCL microglia from the retina at 7d post-optic nerve cut. Arrows indicate microglia in GCL. Scale bar=100 μm.

Thus, the extent of microglial proliferation was unaltered by the induction of IPC, and seeming without relation to RGC survival after axotomy.

Different Expression of HSP27 Induced by IPC Plus ONT or IR60 Under normal conditions, retinal blood vessels and astrocytes are labeled by HSP27, but no HSP27-positive RGCs are seen in the whole-mount retina. Seven days after injury, in both the axotomy and 60min of retinal ischemia groups, the expression of HSP27 in the RGCs was significantly increased. We could distinguish RGCs labeled with HSP27 from astrocytes because HSP27-positive RGCs are morphologically distinct, having a larger soma size with several processes (Figure 4B and 4E). Furthermore, in combination with the TuJ1 staining, some of HSP27-positive cells were confirmed as being RGCs.

Significantly more HSP27-positive RGCs were observed in the IPC-ONT group (1018±34) than in the sham IPC-ONT group

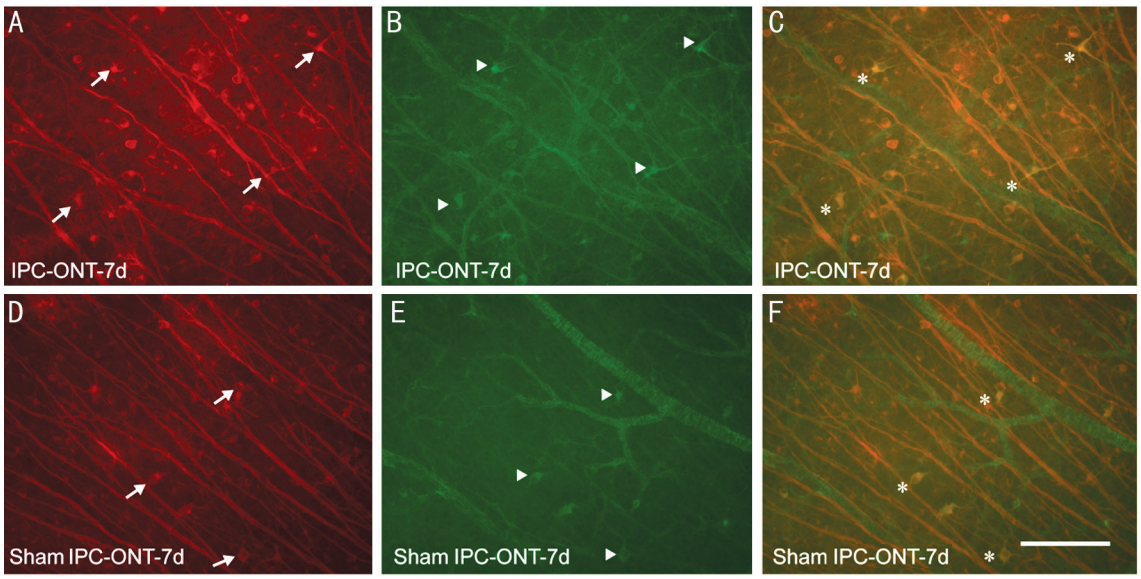

Figure 4 TuJ1-positive surviving RGCs (A, D), HSP27-positive RGCs in the same field of view (B, E) and merged images (C, F) Arrows indicate RGCs labeled by TUJ-1 (red), arrowheads indicate RGCs labeled by HSP27 (green), asterisks indicate RGCs double-labeled by TuJ1 and HSP27 (yellow), respectively. Note that more HSP27-positive RGCs are observed in the IPC group (B) compared to the sham IPC group (E). A, B and C show surviving TuJ1-positive and HSP27-positive RGCs at 7d after optic nerve axotomy in the IPC group and D, E and F show corresponding results them in the sham IPC group. Scale bar=100 μm.

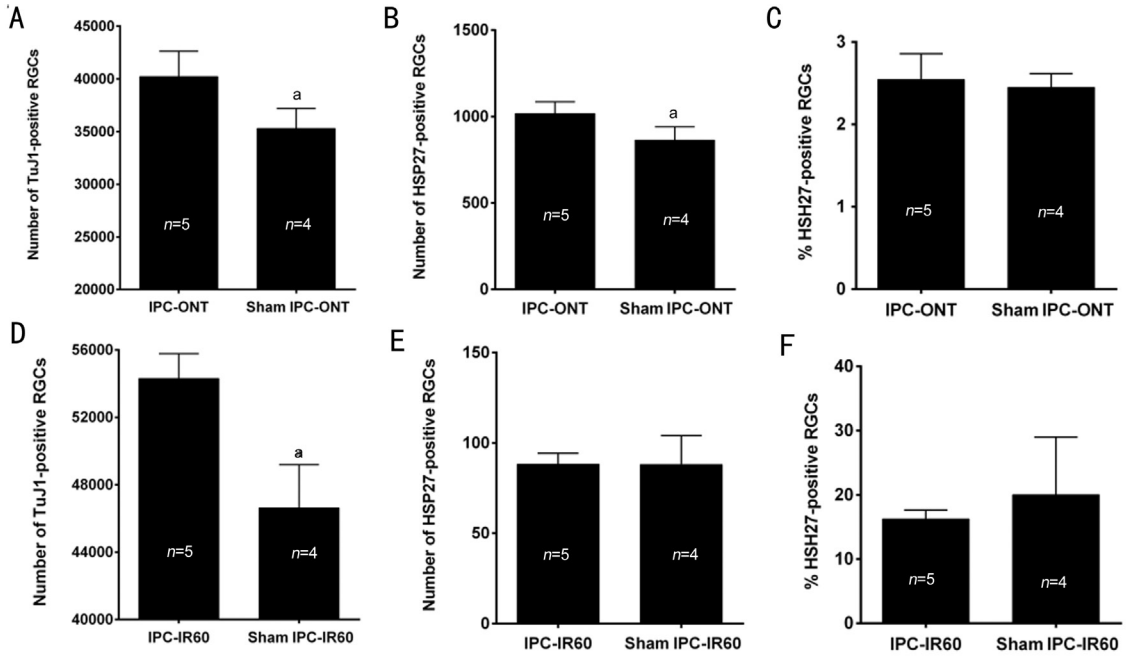

Figure 5 The numbers of TuJ1-positive RGCs (A, D) and HSP27-positive RGCs (B, E) in the IPC-ONT, IPC-IR60 and their corresponding sham groups at 7d post-ischemia Significantly more TuJ1-positive RGCs were found in the IPC-ONT group than in the sham IPC-ONT group at 7d post-ischemia (A), and a significant difference in number of HSP27-positive RGCs was observed between IPC-ONT and sham IPC-ONT groups (B), while no difference in percentage of HSP27-positive RGCs between IPC-ONT and sham IPC-ONT groups (C). Meanwhile, significantly more surviving RGCs were found in the IPC-IR60 group than in the sham IPC-IR60 group at 7d post-ischemia (D), but no difference in number and percentage of HSP27-positive RGCs was observed between IPC-IR60 and sham IPC-IR60 groups (E, F). *t*-test, [a]$P<0.05$; Error bar=SEM.

(865±35) (Figure 5B). Although significantly more surviving TuJ1-positive RGCs were also consistently observed in the IPC-ONT group, the percentage of HSP27-positive RGCs (double-stained with TuJ1) did not differ between the two groups (2.5% in the IPC-ONT group and 2.4% in the sham IPC-ONT group) (Figure 5C).

Although the number of TuJ1-positive RGCs was significantly higher in the IPC-IR60 group (54 304±1474) than in the sham IPC-IR60 group (46 633±2565) at 7d post-ischemia (P=0.03) (Figure 5D), the numbers of HSP27-positive RGCs were not different between the two groups (88±6 in the IPC-IR60 group and 88±16 in the sham IPC-IR60 group (Figure 5E). In

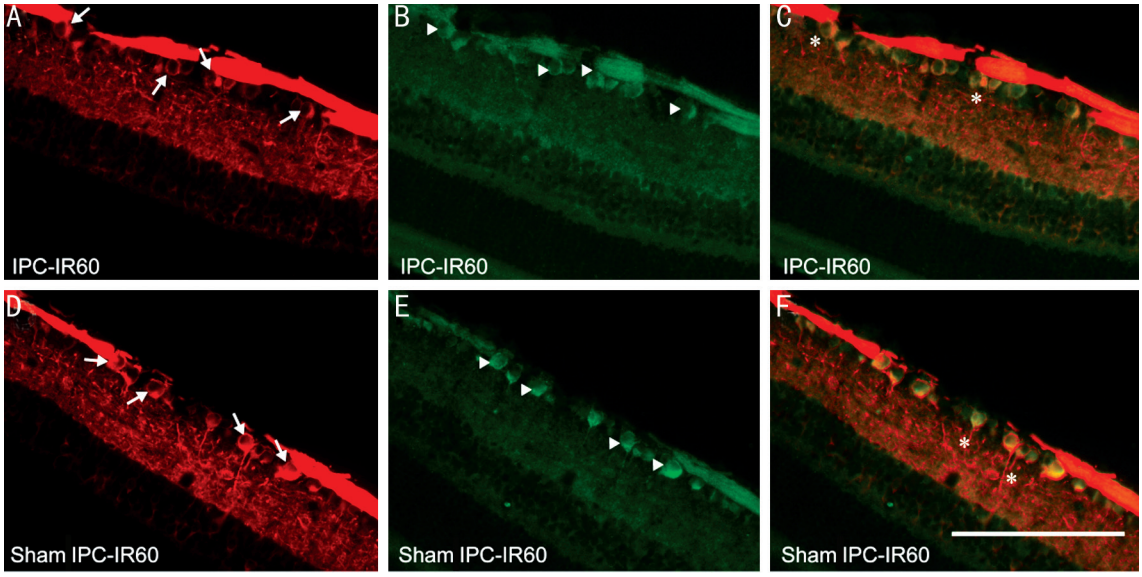

Figure 6 Micrographs showing surviving TuJ1-positive RGCs (A, D), HSP70-positive cells (B, E) and the merged images (C, F) in the central retinal section 7d after 60min of retinal ischemia A, B and C are from the IPC-IR60 group, which received 10min of IPC 1d before 60min of retinal ischemia and survived 7d. D, E and F are from the sham IPC-IR60 group, which received 10min of sham IPC 1d before 60min of retinal ischemia and survived 7d. Arrows indicate RGCs positive for TuJ1 (red); arrowheads indicate RGCs positive for HSP70; asterisks indicate RGCs double-labeled by antibodies for both TuJ1 and HSP70. Scale bar=100 μm.

addition, much fewer HSP27-positive RGCs were found in the IPC-IR60 group than in the IPC-ONT group, although the numbers of TuJ1-positive RGCs were slightly higher in the IPC-IR60 group than in the IPC-ONT group; fewer than 100 HSP27-positive RGCs were found in the IPC-IR60 and sham IPC-IR60 groups, whereas more than 800 HSP27-positive RGCs were found in the ONT groups.

Similar Expression of HSP70 Induced by IPC Plus ONT or IR60 HSP70-positive cells were RGCs and astrocytes in the GCL; cells that were double-stained with both TuJ1 and HSP70 were recognized as RGCs and counted (Figure 6).

For the axotomy groups, the mean density of the HSP70-positive RGCs in the IPC-ONT group was significantly higher than in the sham IPC-ONT group at 7d post-axotomy. However, the percentage of HSP70-positive RGCs did not differ significantly with IPC treatment when compared with the sham or control groups 89.1% and 83.8% of TuJ1-positive RGC population were labeled with HSP70 in the IPC and the sham IPC treatment groups, respectively (Figure 6).

For the IPC-IR60 group, a mean of 7.15 (±0.50) HSP70-positive RGCs were found on the retinal sagittal sections, versus only 4.92 (±0.46) HSP70-positive RGCs in the sham IPC-IR60 group at 7d post-ischemia (Figure 7A). Consistent with the results of the TuJ1-positive RGCs, significantly more TuJ1-positive RGCs were found in the IPC-IR60 group than in the shamIPC-IR60 group. Similar to findings in the axotomy group, the percentage of HSP70-positive RGCs did not differ between the two groups; 85.2% and 86.2% of TuJ1-positive RGCs were labeled with HSP70 in the IPC-IR60 or the sham IPC-IR60 groups, respectively (Figure 7B).

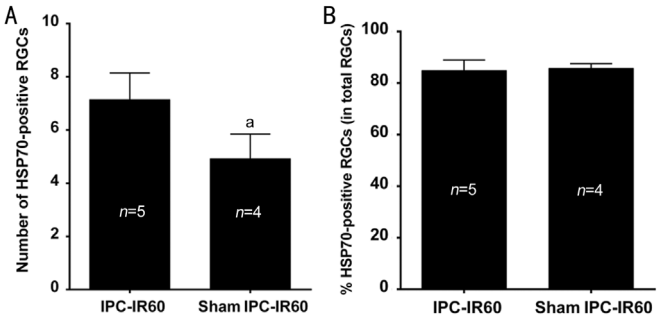

Figure 7 The number and percentage of HSP70-positive RGCs in the IPC-IR60 and the sham IPC-IR60 groups 7d post-ischemia respectively Significantly more HSP70-positive RGCs were found in the IPC-IR60 group than in the sham IPC-IR60 group at 7d post-ischemia (A), while percentage of HSP70-positive RGCs did not show significant difference between two groups (B). *t*-test, $^{a}P<0.05$; Error bar=SEM.

DISCUSSION

We chose to use LOV in order to induce an IPC stimulus, and subsequently evoked lethal ischemia/reperfusion injury of retinal neurons in our experiment because this model scenario is most similar to central retinal artery occlusion, a clinical disease leading to ischemia. The advantage of our selected method is that reversible ligature can induce pure retinal ischemia without an obvious mechanical effect on the retina. In contrast, high intraocular pressure (HIOP) is sometimes used to induce IPC, but at the risk of generating pressure-related mechanical injury to the retina[10]. Furthermore, the duration of ischemia can be readily adjusted with timely removal of the suture. When performed with precision and skill, the LOV surgical operation should not lead to optic nerve damage. LOV

has not previously been used as an IPC stimulus, although it has commonly been used as a model of retinal ischemia per second.

Results of our study indicate that the loss of RGCs induced by optic nerve section was efficiently rescued with the prior application of IPC. As far as we know, this is the first report that has investigated the effect of IPC on RGCs after axotomy, although IPC-induced protection against subsequent ischemic injury has been previously reported[1-4]. A multitude of strategies have proven to promote the survival and regeneration of RGCs after axotomy, such as application of peripheral nerve, intravitreal injection of various neurotrophic factors, and stem cell replacement therapy. Among the possible therapeutic mechanisms of IPC, it has been supposed that induction of IPC could activate endogenous protective mechanisms, thus potentially rescuing RGCs from a number of types of injuries and it is assumed that IPC may also be effective on other types of injuries. Thus, ischemic tolerance induced by six weekly rounds of retinal ischemic stimulus prior to the onset of experimental diabetes has been examined to protect the retina from diabetic retinopathy by preserving retinal function (as measured by electroretinogram) and also the local integrity of the blood-retinal barrier and by decreasing the circulating levels of vascular endothelial growth factor[10]. The same research group found that a brief ischemic pulse applied for 6 successive weeks prior to experimental glaucoma protected the rat retina from glaucomatous damage through both functional and morphological preservation[7]. These findings indicated that the induction of ischemic tolerance is a promising therapy for treating different types of retinal injuries.

Microglia have been considered as a "sensor" for pathological events in the central nervous system, given their rapid proliferation in response to minor injury of diverse etiologies[3]. As such, the extent of microglial proliferation might reflect the efficacy of protective interventions. In the present study we saw similar retinal microgliosis not only in the GCL but also in the IPL and OPL. However, the loss of RGCs and the increase of microglia in the GCL region exhibited a significant positive correlation at 7d after the different injuries (data not shown). There was no significant difference between the IPC treatment and sham-operated groups with respect of activated microglia in response to the IPC and axotomy. There has been no previous quantitative study of microglia proliferation after IPC and subsequent severe insult, nor has the relationship between microglia counts in the GCL or elsewhere in the retina and the number of surviving RGCs after axotomy been reported previously. Our results suggest that the effect of IPC played a minor role in the activation of microglia, or, in other words, the protective effects of IPC on RGCs survival are not importantly mediated by microglia.

A great number of cellular and molecular mechanisms have been associated with the benefits of preconditioning, and many key molecules may be involved as candidate protective mechanisms and pathways in IPC. The inhibition of cyclooxygenase (COX) enzymes[11], HSP27 up-regulation[12-13], hypoxia-inclucible factor-1 transcription factor[14], б-opioid receptor activation[15], and inducible nitric oxide synthase[16] have all been implicated in IPC. Ganglion cells in uninjured retinas do not normally express HSP27, but after axotomy, a small population of surviving ganglion cells start to express this marker, with a significant positive correlation existing between HSP27 expression and axonal regeneration[17]. In our study, the number of HSP27-positive RGCs showed dramatically different responses to different injuries, namely optic nerve cut and retinal ischemia 60min. In particular, there were nearly 10 times more HSP27-positive RGCs in optic nerve cut groups (865±35 in the sham IPC-ONT group) than in the retinal ischemia groups (88±16 in the sham IPC-IR60 group), although the number of surviving RGCs was slightly greater in the retinal ischemia 60min group. More importantly, given the correlation of HSP27 expression with the ability of neurons to regenerate their axons[18], its expression in ganglion cells after ischemic or optic nerve injury may be relevant to their different regenerative propensities. This observation suggests a greater capacity for axonal regeneration after axotomy, when more HSP27 positive RGCs are present. On the other hand, HSPs may be a marker for damage rather than protective mechanism[19], since axotomy is a more severe insult to the retina and leads to greater RGC loss than did 60min of retinal ischemia, such that more HSP27-positive RGCs were found in both the IPC- and sham IPC-ONT groups.

We observed that the number of HSP70-positive cells was markedly higher in the IPC-IR60 group, although the percentage of the HSP70-positive RGCs did not show a significant difference between the IPC-IR60 and sham IPC-IR60. This result implies that, the expression of HSP70 may play only a small role in IPC-induced neuroprotection of RGCs.

This study has several limitations that should be addressed in future work. The activation of HSP27 and HSP70 were investigated only in the groups that received axotomy or retinal ischemia for 60min, but effects of longer duration of retinal ischemia were not explored. Thus, the expression of HSP27 and HSP70 after retinal ischemia lasting 120min or longer is unknown. Furthermore, the neuronal expression of HSP27 exhibited dramatic differences between the groups with optic nerve injury and retinal ischemia, a phenomenon that merits further examination.

In conclusion, the neuroprotection provided by IPC can protect RGCs against not only subsequent retinal ischemia, but also optic nerve axotomy. Endogenous protective mechanisms activated by IPC maintained the thickness of the retina and the

GCL almost within the normal range. Although direct or local IPC can protect vulnerable tissues against ischemia/reperfusion injury, its application necessarily entails direct stress to the target organ and mechanical trauma to major vascular structures, which has hitherto limited its clinical application. Recently, the concept of remote ischemic preconditioning (RIPC) has emerged, in which brief ischemia of one tissue confers protection to important distant organs without direct stress to those organs, presumably through the release of blood-born factors. Encouragingly, brief IPC of a hind limb provided remote protection to the heart in children who underwent cardiopulmonary bypass surgery for congenital heart disease[18]. Furthermore, the induction of RIPC protected the retina against ischemia/reperfusion injury in rats[20-21]. We suppose that the induction of RIPC should provide a promising strategy to protect organs against subsequent injury.

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REFERENCES

1 Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: underlying mechanisms and clinical application. *Atherosclerosis* 2009;204(2):334-341.

2 Wever KE, Hooijmans CR, Riksen NP, Sterenborg TB, Sena ES, Ritskes-Hoitinga M, Warlé MC. Determinants of the efficacy of cardiac ischemic preconditioning: a systematic review and Meta-analysis of animal studies. *PLoS One* 2015;10(11):e0142021.

3 Kanoria S, Jalan R, Seifalian AM, Williams R, Davidson BR. Protocols and mechanisms for remote ischemic preconditioning: a novel method for reducing ischemia reperfusion injury. *Transplantation* 2007;84(4):445-458.

4 Roth S, Li B, Rosenbaum PS, Gupta H, Goldstein IM, Maxwell KM, Gidday JM. Preconditioning provides complete protection against retinal ischemic injury in rats. *Invest Ophthalmol Vis Sci* 1998;39(5):777-785.

5 Liu X, Sha O, Cho EY. Remote ischemic postconditioning promotes the survival of retinal ganglion cells after optic nerve injury. *J Mol Neurosci* 2013;51(3):639-646.

6 Rosenstein RE, Fernandez DC. Induction of ischemic tolerance as a promising treatment against diabetic retinopathy. *Neural Regen Res* 2014;9(17):1581-1584.

7 Belforte N, Sande PH, de Zavalía N, Fernandez DC, Silberman DM, Chianelli MS, Rosenstein RE. Ischemic tolerance protects the rat retina from glaucomatous damage. *PLoS One* 2011;6(8):e23763.

8 Lafuente MP, Villegas-Pérez MP, Sellés-Navarro I, Mayor-Torroglosa S, Miralles de Imperial J, Vidal-Sanz M. Retinal ganglion cell death after acute retinal ischemia is an ongoing process whose severity and duration depends on the duration of the insult. *Neuroscience* 2002;109(1):157-168.

9 Wong WK, Cheung AW, Yu SW, Sha O, Cho EY. Hepatocyte growth factor promotes long-term survival and axonal regeneration of retinal ganglion cells after optic nerve injury: comparison with CNTF and BDNF. *CNS Neurosci Ther* 2014;20(10):916-929.

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

11 Fleischman A, Oron Y, Geyer O. COX-2 inhibition improves retinal function in rats' ischemic eyes. *J Ocul Pharmacol Ther* 2014;30(8):634-641.

12 Li Y, Roth S, Laser M, Ma JX, Crosson CE. Retinal preconditioning and the induction of heat-shock protein 27. *Invest Ophthalmol Vis Sci* 2003;44(3):1299-1304.

13 Whitlock NA, Lindsey K, Agarwal N, Crosson CE, Ma JX. Heat shock protein 27 delays Ca2+-induced cell death in a caspase-dependent and -independent manner in rat retinal ganglion cells. *Invest Ophthalmol Vis Sci* 2005;46(3):1085-1091.

14 Whitlock NA, Agarwal N, Ma JX, Crosson CE. Hsp27 upregulation by HIF-1 signaling offers protection against retinal ischemia in rats. *Invest Ophthalmol Vis Sci* 2005;46(3):1092-1098.

15 Husain S, Potter DE, Crosson CE. Opioid receptor-activation: retina protected from ischemic injury. *Invest Ophthalmol Vis Sci* 2009;50(8):3853-3859.

16 Sakamoto K, Yonoki Y, Kubota Y, Kuwagata M, Saito M, Nakahara T, Ishii K. Inducible nitric oxide synthase inhibitors abolished histological protection by late ischemic preconditioning in rat retina. *Exp Eye Res* 2006;82(3):512-518.

17 Hebb MO, Myers TL, Clarke DB. Enhanced expression of heat shock protein 27 is correlated with axonal regeneration in mature retinal ganglion cells. *Brain Res* 2006;1073-1074:146-150.

18 Cho EYP, Wong WK. Transient induction of heat shock protein 27 in axotomized retinal ganglion cells, and their sustained up-regulation in regenerating ganglion cells. *Investigative Ophthalmology & Visual Science* 2005;46(13):189.

19 Windisch BK, LeVatte TL, Archibald ML, Chauhan BC. Induction of heat shock proteins 27 and 72 in retinal ganglion cells after acute pressure-induced ischaemia. *Clin Exp Ophthalmol* 2009;37(3):299-307.

20 Brandli A, Johnstone DM, Remote ischemic preconditioning protects retinal photoreceptors: evidence from a rat model of light-induced photoreceptor degeneration. *Invest Ophthalmol Vis Sci* 2016;57(13):5302-5313.

21 Brandli A. Remote limb ischemic preconditioning: a neuroprotective technique in rodents. *J Vis Exp* 2015;(100):e52213.