

# Fasudil, a Rho-associated protein kinase inhibitor, attenuates retinal ischemia and reperfusion injury in rats

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**Abstract.** Abnormal activation of Rho kinase (ROCK) plays a vital role in the pathogenesis of ischemia/reperfusion (I/R)-induced retinal injury. The aim of this study was to investigate whether fasudil, a potent inhibitor of ROCK, has a protective effect on retinal I/R injury in rats and to explore the possible underlying mechanisms. Forty adult Sprague-Dawley rats were randomly assigned into sham, I/R injury model (I/R), model plus normal saline (control), and model plus fasudil (fasudil) groups. Rats in the control and fasudil groups were intravitreally injected with normal saline and fasudil, respectively, 5 min prior to the induction of ischemia. Retinal ischemia was induced by increasing the intraocular pressure to 100 mmHg for 60 min. Overall retinal thickness and retinal cell apoptosis was evaluated by histological analysis and the TUNEL assay, respectively. The protein expression of caspase-3 and the Bax/Bcl-2 mRNA ratio were also examined. Moreover, the retinal expression of inducible nitric oxide synthase (iNOS) was determined by immunohistochemical staining, quantitative real-time RT-PCR and Western blot analysis. Fasudil attenuated the I/R-induced apoptosis of retinal cells in the inner nuclear and ganglion cells of the rat retina. Fasudil significantly decreased the Bax/Bcl-2 mRNA ratio and the expression of caspase-3 and iNOS compared to the control group ( $P<0.05$ ). Seven days after I/R, the overall retinal thickness in the fasudil group was significantly greater compared to that in the control group ( $P<0.05$ ). In conclusion, fasudil can protect the rat retina from I/R injury by inhibiting apoptosis and iNOS expression, suggesting that fasudil may have a therapeutic potential for the prevention of retinal diseases associated with I/R.

## Introduction

Ischemia/reperfusion (I/R) injury is a major complicating feature of many pathological conditions, such as myocardial infarction, cerebral ischemic events, and hepatic, renal, and intestinal ischemia, as well as of vascular surgery, trauma, and transplantation (1,2). In general, I/R injury represents an acute inflammatory response after an ischemic event and subsequent restoration of blood flow (2). I/R injury to the retina is a major cause of visual impairment and blindness that occurs in a number of ocular diseases, such as retinal vascular occlusion, acute glaucoma, diabetic retinopathy, and retinopathy of prematurity (3-5). Despite pharmacological efforts that have been made to discover effective drugs, this complication still cannot be completely prevented.

Rho kinase (ROCK) belongs to a family of serine/threonine kinases that are stimulated by G protein coupled receptor activation of the small plasma membrane bound GTP-binding proteins (6). Based on the ubiquitous expression pattern across human, mouse and rat tissues, ROCK has been shown to be involved in a variety of fundamental cellular functions, such as contraction, adhesion, migration and proliferation (7). Recently, evidence has accumulated suggesting that abnormal activation of ROCK plays an important role in various pathological conditions, including cerebral and coronary vasospasm, hypertension, vascular inflammation and remodeling, and arteriosclerosis (8). Studies with fasudil and the structurally unrelated inhibitor Y-27632 have indicated beneficial effects of selective ROCK inhibition in experimental models of ischemic diseases, including cerebral vasospasm, and end-organ (renal, liver, colon, brain, intestine) I/R injury (9-14). Regarding retinal I/R injury, Hirata *et al* have recently reported that administration of Y-27632 attenuates neuronal cell death and dramatically inhibits leukocyte infiltration and endothelial disarrangement after transient retinal ischemia in rats (15). However, the possibility that fasudil may have a protective effect on retinal I/R injury has not yet been examined.

The aim of this study, therefore, was to investigate whether fasudil has a protective effect against I/R-induced retinal cell damage in a rat retinal I/R model. Furthermore, we also examined the apoptosis of retinal cells and the expression of inducible nitric oxide synthase (iNOS) in retinas in an effort to elucidate the mechanisms by which fasudil protects retinal cells.

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## Materials and methods

**Animals.** Forty adult Sprague-Dawley rats weighing 200–250 g were obtained from the Experimental Animal Centre of China Medical University. They were housed in plastic cages containing wood shaving and maintained in a room with a 12-h light cycle with free access to food and water. All experiments were performed in accordance with the Statement on the Use of Animals of the Association for Research in Vision and Ophthalmology.

**Experimental design.** Animals were randomly divided into four experimental groups (10 rats each): (i) the sham group, in which needle cannulation was performed into the anterior chamber of the rat's right eye without causing elevation of the intraocular pressure (IOP); (ii) the retinal I/R group, in which 60 min retinal ischemia was induced without any pre-treatment; (iii) the control group, in which rats were intravitreally injected with normal saline 5 min prior to the 60 min retinal ischemia; and (iv) the fasudil group, in which rats were intravitreally injected with fasudil (Asahi Kasei, Osaka, Japan) at a final concentration of 10  $\mu$ M 5 min prior to the 60 min retinal ischemia.

**Induction of ischemia and reperfusion.** Rats were anesthetized with intraperitoneal injection of chloral hydrate (40 mg/kg). Corneal analgesia was achieved using 1 or 2 drops of oxybuprocaine hydrochloride (Santen, Osaka, Japan). Dilatation of the pupil was maintained with tropicamide (Santen, Osaka, Japan). Retinal ischemia was induced only in the right eye of each rat by increasing the IOP to 100 mmHg for 60 min. After dilating the pupil, the anterior chamber of the right eye was cannulated with a 30-gauge needle connected to a bottle containing normal saline, and the IOP was raised to 100 mmHg by lifting the bottle. Retinal ischemia was confirmed by fundus examination. After 60 min of ocular hypertension, the cannulating needle was removed and the IOP was normalized. The operating microscope was used to verify reperfusion of the vessels. On day 1 and day 7 after the induction of I/R, five rats from each group were sacrificed by an intraperitoneal overdose injection of chloral hydrate. The right eyes were rapidly enucleated, the eyeball was cut through the pars plana, and the anterior segment was discarded, the vitreous was removed, and the retina was harvested and immersed in liquid nitrogen prior to analysis. The remaining retinal tissues were fixed in 4% paraformaldehyde for 24 h at 4°C and later used for preparation of flat mounted retinas. This animal study protocol was approved by the Ethics Committee for Animal Experiments of the China Medical University.

**Histological examination.** For histological examination, the retinas were fixed in 4% paraformaldehyde and embedded in paraffin. Transverse, 4- $\mu$ m sections were obtained through the optic disc, stained with hematoxylin and eosin, and examined using light microscopy. The degree of hypertension-induced neuronal damage in the retina was quantified by measuring the thickness of the retina, 1.5 mm from the optic disc. Three sections for each eye were randomly selected, and the thickness is expressed as the mean of the three measurements obtained.

**Immunohistochemical staining.** Following deparaffinization of the sections, immunohistochemical staining was performed with streptavidin-biotinylated peroxidase to detect the expression of iNOS, as described elsewhere. A rabbit polyclonal antibody against rat iNOS (1:100, Boster Bio-Engineering Co. Ltd., Wuhan, China) was used as a primary antibody.

**Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay.** The TUNEL assay was performed to detect internucleosomal DNA fragmentation. The staining was performed as described elsewhere. The number of TUNEL-positive cells per 0.2 mm length of each area were counted by two authors in a blinded fashion. A confocal microscope was used for the measurements. The apoptotic index was expressed as the percentage of TUNEL-positive cells out of the total counted cells.

**Quantitative real-time RT-PCR.** Total RNA was isolated from retinas using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration and purity of the RNA in each sample was determined using a spectrophotometer at 260 and 280 nm. cDNA was synthesized from 1  $\mu$ g of total RNA using a PrimeScript RT reagent kit (Takara, Dalian, China). Quantitative real-time RT-PCR was performed using SYBR-Green (Takara) on a Real-Time quantitative thermal block (Biomtra, Göttingen, Germany). The PCR primer sequences were designed according to the rat Bcl-2, Bax, iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequences reported in GenBank and were chemically synthesized: Bcl-2, forward 5'-TGAACCGGCATCTGCACAC-3' and reverse 5'-CGTCTTCAGAGACAGCCAGGAG-3'; Bax, forward 5'-GTTACAGGGTTTCATCCAGG-3' and reverse 5'-CGTGTCCACGTCAGCAAT-3'; iNOS, forward 5'-CTCACTGTGGCTGTGGTCACCTA-3' and reverse 5'-GGGTCTTCGGGCTTCAGGTTA-3'; GAPDH, forward 5'-GCAAGTTCAACGGCACCA-3' and reverse 5'-CATTTGATGTTAGCGGGAT-3'. The specificity of the amplified products was analyzed through dissociation curves generated by the equipment yielding single peaks. GAPDH was used as an internal control to normalize samples. PCR reactions of each sample were conducted in triplicate. Data were analyzed through the comparative threshold cycle ( $C_T$ ) method (16).

**Western blot analysis.** The frozen retinas were homogenized in ice-cold lysis buffer with a protease inhibitor cocktail followed by centrifugation at 13,000  $\times$  g for 10 min. The supernatant was solubilized in Laemmli's buffer and the protein concentrations were determined by the Lowry method. Equal amounts of protein were separated by 10% SDS-PAGE and then electrotransferred to PVDF membranes (Millipore, Billerica, MA). The blotted membranes were blocked with 5% skim milk at 4°C overnight, and then incubated with rabbit anti-caspase-3, anti-iNOS and anti- $\beta$ -actin antibodies (1:400 dilution; all from Boster Bio-Engineering Co. Ltd.) at room temperature for 2 h. After incubation of the membranes with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, visualization of the bands was performed by an enhanced chemiluminescence kit. Immunoblotting with anti- $\beta$ -actin antibody was used as an internal control to confirm equivalent protein loading. The relative intensity of each

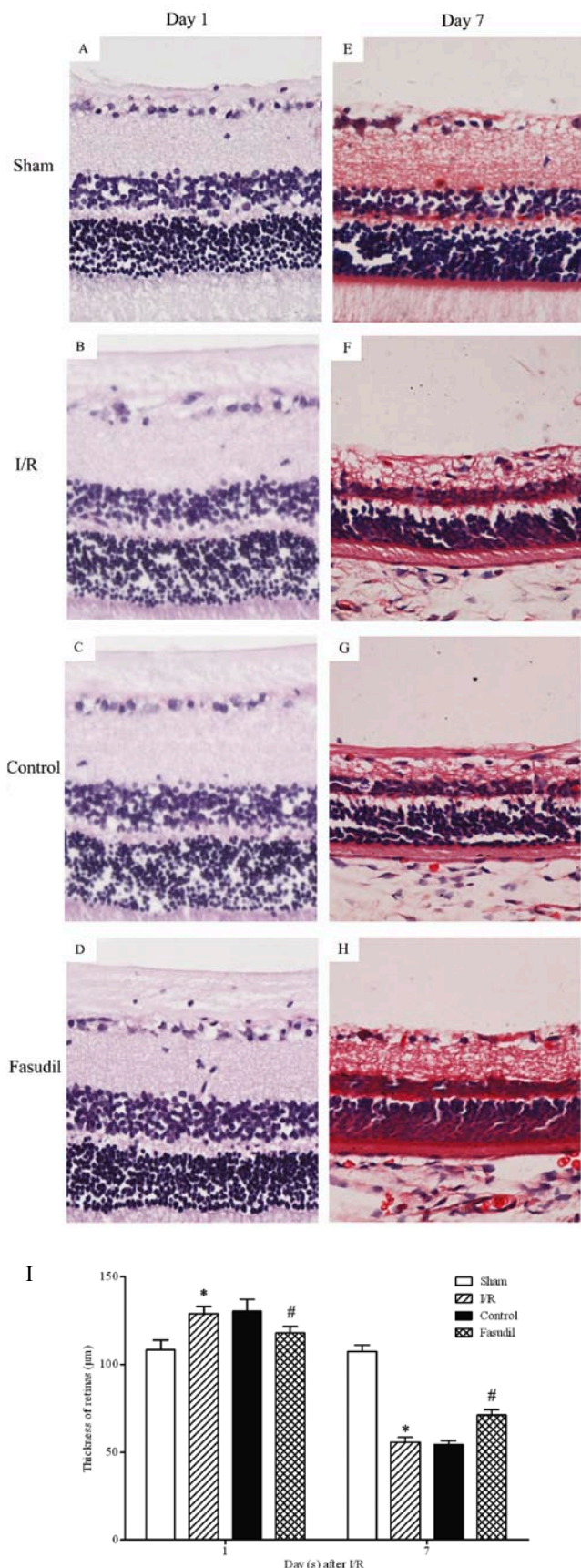


Figure 1. Effect of intravitreal pre-treatment with fasudil on the histological damage induced by retinal I/R. Representative photomicrographs showing the histological appearance of retinas in the sham group (A and E), and in I/R retinas 1 day (B-D) and 7 days (F-H) after 60 min of ischemia. Fasudil was intravitreally injected 5 min before 60 min of ischemia. (I) Morphometry of the overall retinal thickness. Each column represents the mean  $\pm$  SD. (n=5, \*P<0.05 vs. the sham group, #P<0.01 vs. the control group).

protein band was scanned by Chemi-Imager™ 5500 (Alpha Innotech Inc, Santa Clara, CA, USA).

**Statistical analysis.** All experiments were performed in triplicate and all data were expressed as means  $\pm$  SD. Statistical differences were determined by the Student's t-test using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). A P-value <0.05 was considered to be statistically significant.

## Results

**Histological changes in the retinas.** We first investigated the effects of fasudil against retinal I/R injury induced by 60 min of ocular hypertension. Typical photomicrographs of the retina taken 1 day and 7 days after reperfusion are shown in Fig. 1. One day after I/R, appearance of pyknotic nuclei, vacuolated spaces, and degenerative changes in the ganglion cell layer (GCL) and inner nuclear layer (INL) were noticed in the I/R group (Fig. 1B) and the control group (Fig. 1C), but such changes were not seen in the sham group (Fig. 1A). The overall retinal thickness in the I/R group was significantly greater than that in the sham group (P<0.05, Fig. 1I). In the fasudil group, administration of fasudil before the induction of I/R clearly protected against retinal damage; the retinas appeared much more normal, with a reduced overall thickness of retinas compared to that in the control group (P<0.05, Fig. 1D and I). Seven days after I/R, the overall retinal thickness in the I/R group was significantly decreased compared to that of the retinas in the sham group, whereas no histopathological difference was observed between the I/R and control groups (Fig. 1E-G and I). Compared to retinas of the normal saline-treated group with I/R, fasudil pre-treatment resulted in a significant protection against ischemic damage, as shown by the rescue of the overall retinal thickness (P<0.05, Fig. 1H and I). These data suggest that fasudil attenuates retinal damage induced by retinal I/R.

The apoptotic index, as assessed by the TUNEL method, was evaluated on day 1 after I/R. For the rats in the sham group, the retina cell nuclei were almost negative for TUNEL staining (Fig. 2A). Abundant TUNEL-positive cells were found within the inner nuclear and ganglion cells in the I/R and control groups (Fig. 2B and C). In contrast, in rats pre-treated with fasudil, only a few TUNEL-positive cells were found in the retinas (Fig. 2D). As shown in Fig. 2E, the apoptotic index of retinas in the fasudil group was significantly lower than that in control group (P<0.05), suggesting that fasudil is able to reduce cell apoptosis.

**Effects of fasudil on the expression of caspase-3, and on the Bax/Bcl-2 ratio.** In order to provide more evidence for the fasudil-induced apoptosis reduction, we examined the protein expression of caspase-3 on day 1 after I/R by Western blot analysis. In comparison to retinas in the sham group, the expression levels of caspase-3 protein significantly increased in the I/R and control groups (P<0.05). Fasudil pre-treatment markedly decreased these levels in comparison to the control group (P<0.05, Fig. 3).

The ratio of the pro-apoptotic Bax to the anti-apoptotic Bcl-2 is a crucial determinant of cellular susceptibility to apoptosis. We measured the ratio of Bax/Bcl-2 mRNA levels



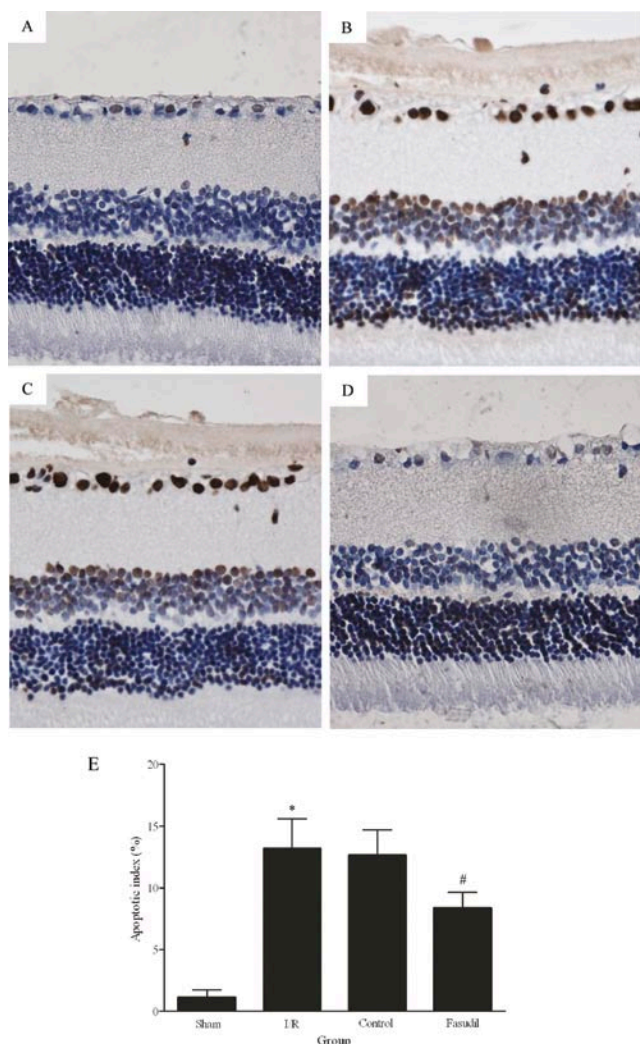


Figure 2. Analysis of the morphology and the apoptotic index in retinal cells 1 day after I/R. Representative photomicrographs of retinas stained for DNA breaks by the TUNEL assay. (A) sham group; (B) I/R group; (C) control group; (D) fasudil group. Abundant TUNEL-positive cells were found in the I/R and control groups. (E) The apoptotic index of retinal cells in the different groups. Each column represents the mean  $\pm$  SD. (n=5, \*P<0.05 vs. the sham group, #P<0.01 vs. the control group).

by quantitative real-time RT-PCR. In comparison to retinas in the sham group, the Bax/Bcl-2 mRNA ratio significantly increased in the I/R and control groups (P<0.05). After fasudil pre-treatment, the Bax/Bcl-2 mRNA ratio markedly decreased in comparison to the control group (P<0.05, Fig. 4).

**Effect of fasudil on the expression of iNOS.** A significant body of evidence has indicated an involvement of nitric oxide (NO) in the pathogenesis of ischemia damage in the retina (2). We first examined the expression of iNOS on day 1 after I/R by immunohistochemical staining. The photomicrographs of the immunohistochemical localization of iNOS in the ischemic retinas are shown in Fig. 5. The rats in the I/R and control groups exhibited a remarkable increase in iNOS reactivity. However, fasudil pre-treatment attenuated these changes in retinas.

In order to further determine the expression of iNOS in I/R retinas, iNOS mRNA and protein levels were measured by quantitative real-time RT-PCR and Western blot analysis,

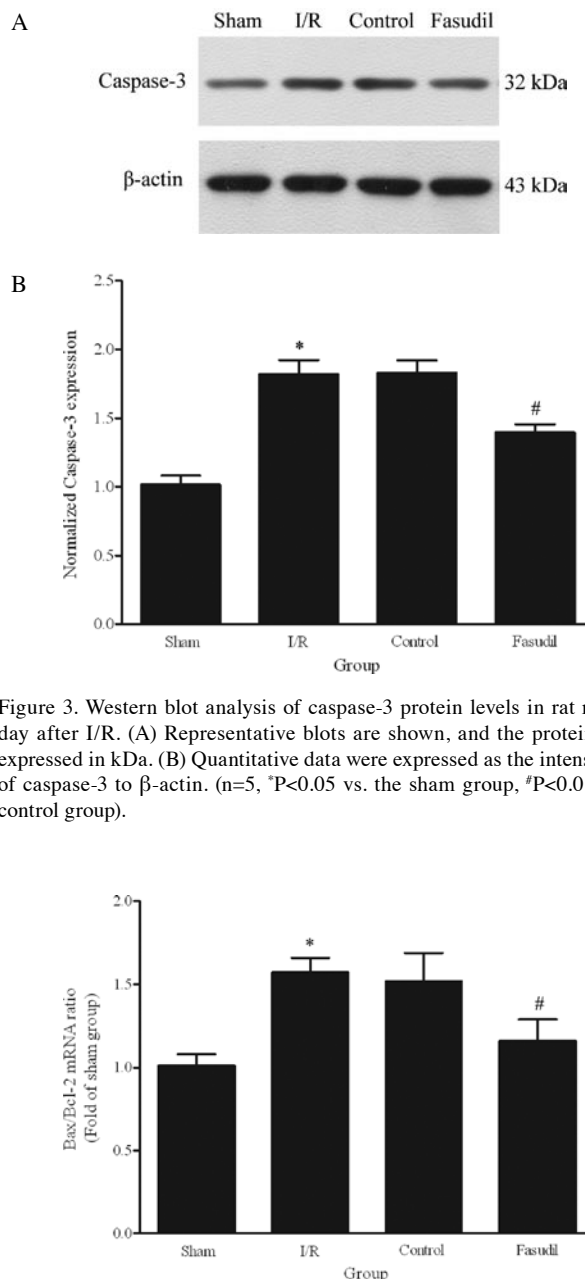


Figure 3. Western blot analysis of caspase-3 protein levels in rat retinas 1 day after I/R. (A) Representative blots are shown, and the protein size is expressed in kDa. (B) Quantitative data were expressed as the intensity ratio of caspase-3 to  $\beta$ -actin. (n=5, \*P<0.05 vs. the sham group, #P<0.01 vs. the control group).

Figure 4. Quantitative real-time RT-PCR analysis of the Bax/Bcl-2 mRNA ratio in rat retinas 1 day after I/R. Quantification of the ratio of Bax/Bcl-2 mRNA levels compared to the sham and control groups. (n=5, \*P<0.05 vs. the sham group, #P<0.01 vs. the control group).

respectively. Compared with the sham group, there was a marked increase in iNOS mRNA levels in the I/R and control groups (P<0.05). Fasudil pre-treatment significantly attenuated the increase of iNOS mRNA in retinas of rats with I/R (P<0.05, Fig. 6A). Meanwhile, changes observed by Western blot analysis were in accordance with the findings in the quantitative real-time RT-PCR study (Fig. 6B and C). The data confirm that fasudil pre-treatment largely reverses the changes in the expression of iNOS caused by retinal I/R.

## Discussion

The present study investigated the pre-conditioning effects of fasudil on the retinal damage caused by I/R in a rat retinal

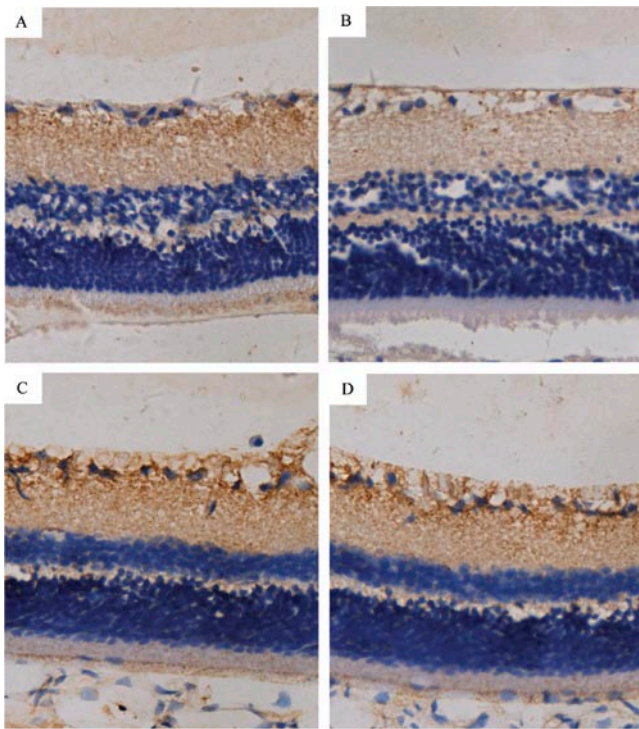


Figure 5. Immunohistochemical staining of iNOS in rat retinas at 1 day after I/R. (A) sham group; (B) I/R group; (C) control group; (D) fasudil group. The rats in I/R group and control group exhibited a remarkable increase in iNOS reactivity (n=5).

I/R model. We showed that fasudil pre-treatment markedly reduced retinal cell injury following I/R, as demonstrated by a reduction in both the histological damage and the function changes in comparison to the I/R and control groups. In addition, we also found that fasudil inhibited the apoptosis of retinal cells and reduced the expression of iNOS in a retinal I/R model in rats, suggesting the possible mechanisms by which fasudil exerts protective effects on retinal I/R injury.

In our animal model, appearance of pyknotic nuclei, vacuolated spaces, and degenerative changes in the GCL and INL were observed 1 day after retinal I/R, as described in previous reports (17,18). Moreover, numerous studies have indicated a significant decrease in retinal thickness at 7 days after retinal I/R (17,19,20). Consistent with these studies, our ocular hypertension model, in which I/R in the retinal vessels has been confirmed microscopically, also markedly decreases the retinal thickness at 7 days after retinal I/R, reflecting the destruction of the inner retinal elements. However, these morphological changes were significantly improved by fasudil pre-treatment, implying the protective effect of fasudil on retinal I/R injury.

Recent studies have revealed that both apoptosis and necrosis are involved in I/R-induced cell damage (21-23). In the current study, a significant number of TUNEL-positive cells were found within the inner nuclear and ganglion cells 1 day after I/R. Caspase-3, a key enzyme required for execution of apoptosis, was also up-regulated in retinas after 1 day of retinal I/R. The Bcl-2 family is thought to be the critical factor in the apoptotic signaling pathways, and the relative ratio of anti- and pro-apoptotic proteins is important to determine cell survival or death (24). In the current study, we observed that the retinas

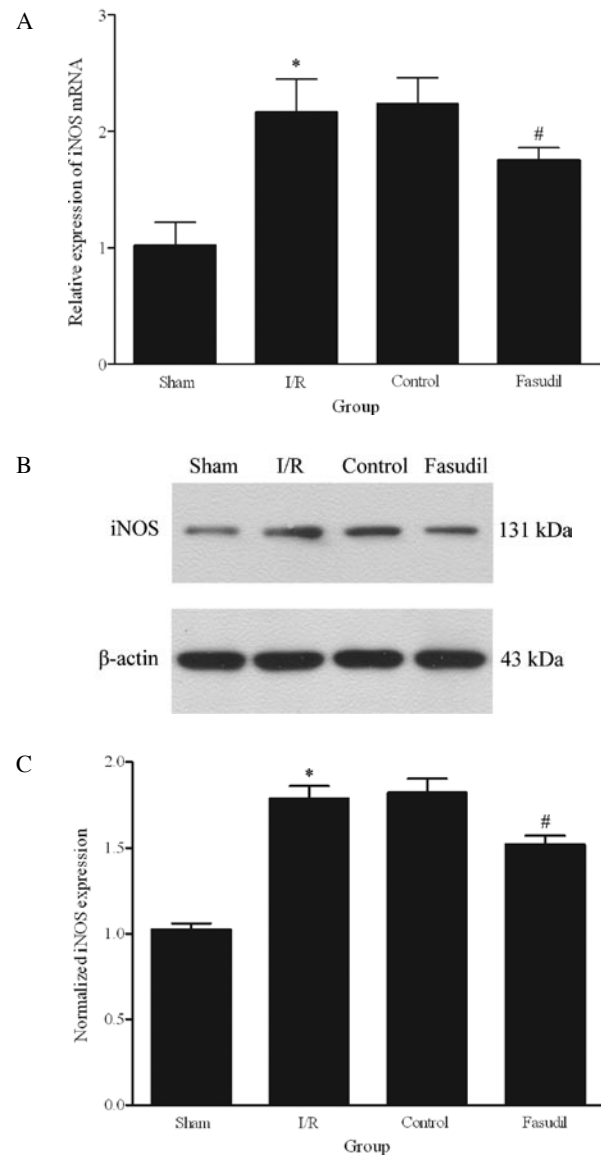


Figure 6. iNOS mRNA and protein levels in rat retinas at 1 day after I/R were measured by quantitative real-time RT-PCR (A) and Western blot analysis (B), respectively. (A) Compared to the sham group, there was a marked increase in iNOS mRNA in the I/R group and the control group. Fasudil pre-treatment significantly attenuated the increase of iNOS mRNA in retinas of rats with I/R. (B) iNOS protein levels in the above-mentioned groups were consistent with the mRNA expression by Western blotting. Representative blots are shown, and the protein size is expressed in kDa. (n=5, \*P<0.05 vs. the sham group, #P<0.01 vs. the control group).

in the I/R group exhibited an increased ratio of Bax/Bcl-2 in mRNA level. Nonetheless, these changes were significantly reversed by fasudil pre-treatment. Our findings are consistent with a recent report from Li *et al* showing that restoration of the levels of some apoptotic markers, such as Bcl-2, and Bax play an important role in the neuroprotective effects of fasudil mesylate (25). Taken together, our data not only confirmed previous reports that apoptosis produces retinal cell death after I/R injury, but also demonstrated that fasudil could prevent the apoptosis of retinal cells.

NO is an important signaling molecule that plays a variety of roles in the pathophysiology of many diseases. It is synthesized by three isoforms of NO synthase (NOS): neuronal (nNOS), endothelial (eNOS), and iNOS (26). Hangai *et al*

found that the mRNA level of iNOS increased during retinal ischemia in rats and iNOS mRNA was mainly expressed in the heterophil granulocytes infiltrated into the retina as detected by *in situ* hybridization, suggesting that NO induction by iNOS in leukocytes could be one of the factors involved in retinal I/R injury (27). Recently, Zheng *et al* demonstrated that aminoguanidine, a non-specific inhibitor of iNOS, inhibited I/R-induced degeneration of both neuronal and vascular cells of the retina (28). In the current study, we consistently found a substantial increase of iNOS expression both in mRNA and protein level after 1 day retinal I/R. Fasudil significantly decreased iNOS mRNA and protein expression. This result provides evidence that the protective effect of fasudil on retinal I/R injury may be mediated, at least in part, by inhibition of the inflammatory immune reaction injury through down-regulation of iNOS. However, the precise mechanisms by which fasudil leads to retinal protection need to be further elucidated.

In summary, our results demonstrate that pre-ischemic administration of fasudil may effectively protect retinal cells during retinal I/R and improve I/R-induced retinal dysfunction, possibly and at least partially by inhibiting apoptosis and iNOS expression. Fasudil may have a therapeutic potential for the prevention of retinal diseases associated with I/R. However, pharmacological treatment sometimes leads to undesirable side effects *in vivo*. Further *in vivo* examination, therefore, is necessary to evaluate the therapeutic potential and safety of fasudil for clinical use in ocular diseases.

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