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Fasudil protects retinal ganglion cells and promotes axonal regeneration

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Abstract: This study was aimed to investigate whether fasudil can protect retinal ganglion cells and promote axonal regeneration by inhibiting RhoA/Rock pathway. Long Evans rats were used to establish an optic nerve injury model. Apoptosis was detected by TUNEL, and surviving RGCs was detected by Fluoro-Gold retrograde label and hematoxylin-eosin (HE) staining was used to evaluate pathological changes and western blot was used to measure the expression of protein. After 10 days of optic nerve injury rat model, increased cell apoptosis and decreased FG-positive RGCs in rat eye, but fasudil could reverse these changes. *In vitro*, fasudil could not only increase the number of RGCs with protuberances, but also increase the length of protuberances. Moreover, fasudil could not only reduce the expression of total-cohoin, Rock, total-cofilin and total-MLC protein induced by optic nerve injury, but also reduce the relative expression of GTP-RhoA, p-cofilin and p-MLC protein. Fasudil protects retinal ganglion cells and promotes axonal regeneration by inhibiting RhoA / Rock pathway.

Keywords: Fasudil, retinal ganglion cells, axonal regeneration, RhoA / Rock pathway.

INTRODUCTION

Optic nerve injury is a common blinding ophthalmologic disease in the clinic, and it is mainly characterized by sharp decline in visual acuity, abnormal visual field and light reflex disorder. Statistics showed that there were nearly half of the patients were permanently blinded after optic nerve injury, which had become the main blinding factor in modern society (Haring *et al*., 2016, Vidalsanz *et al*., 2017). Previous studies have found that secondary apoptosis of retinal ganglion cells (RGCs) after optic nerve injury is one of the reasons why the optic nerve is difficult to regenerate (Isenmann *et al*., 2003, Leaver *et al*., 2010). However, simply protecting the survival of RGCs did not cause effective regeneration of the optic nerve, as the inhibitory substance in the local environment was another key factor that hindered optic nerve regeneration (Thomas *et al*., 2017).

Studies had shown that many nerve regeneration inhibitors blocked the central nervous system regeneration by activating RhoA/Rock (Rho-associated kinase) and causing growth cone atrophy through signal transduction (Fujita and Yamashita, 2014), while C3 transferase that was a RhoA activation inhibitor could successfully promote optic nerve regeneration in incomplete injury (Bertrand *et al*., 2007) and could promote the growth of RGCs axons on glial scars (Fu *et al*., 2007). Similarly, many growth cone-directing materials such as Ephexin (Margolis *et al*., 2016) and Semaphorin/Plexin (Lin *et al*., 2007) could inhibit axonal regeneration by activating RhoA/Rock pathway. In addition, RhoA gene was highly expressed after spinal cord injury in rats and the application of RhoA/Rock inhibitor could significantly promote the recovery of motor function in rats (Forgione and Fehlings, 2014, Fehlings *et al*., 2018). These indicated that the expression and activity changes of RhoA/Rock had a direct effect on neuronal axon regeneration and were the common point of signal transduction of axon regeneration inhibitors.

Fasudil is a selective RhoA/Rock inhibitor (Nagumo *et al*., 2000) . Since it was discovered, it has been used for the treatment of cerebral vasospasm, which is often due to subarachnoid hemorrhage (Liu *et al*., 2012, Ishihara *et al*., 2012), as well as to improve the cognitive decline seen in stroke patients (Chan and Cipolla, 2017). Moreover, Fasudil has been found to not only affect the optic nerve blood flow in rabbits (Tetsuya *et al*., 2011), but also to restore normal motor nerve conduction velocity in diabetic rats (Kanazawa *et al*., 2013) and to be effective for the treatment of nonarteritic anterior ischemic optic neuropathy (Sanjari *et al*., 2016). However, the effect of fasudil in the survival and axonal regeneration of RGCs after optic nerve injury remains unclear. In this study, we treated the rats with optic nerve injury by intraperitoneal injection of fasudil and found that fasudil could protect retinal ganglion cells and promote axonal regeneration by inhibiting RhoA / Rock pathway. This means that fasudil may be used as a drug for the treatment of optic nerve damage.

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MATERIALS AND METHODS

Experimental animals and grouping

In this study, Long Evans rats (all were females, 5 weeks old, 250-280 g) were used to establish animal model and fed by the animal center (free-feeding, 12h each day and night, 22±2°C). One week after adaptive feeding, all rats were randomly divided into Sham group, Control group, and Fasudil group according to the random number table method. All animal care and experiments were approved by the Ethics Committee of Shijiazhuang Third Hospital by reference No.2017 Ethics Approval No. 002.

Optic nerve injury rat model and Fasudil treatment

For Control and Fasudil group, rats were fixed after anesthesia, and cut off the hair around the eyes and disinfected. The temporal conjunctiva was cut 5mm along the limbus and the optic nerve was exposed to the peribulbar tissue. The optic nerve was compressed for 2 seconds with a constant pressure reverse forceps (1mm wide, pressure 148g) and clamped for 20 seconds. Clamp and observe the blood supply to the fundus of the rat and if the pupil dilated, the light reflection disappeared and the blood supply to the retina indicated successful modeling. After successful modeling, a 7-needle suture was used to suture the ball and conjunctiva in the conjunctival sac. Waiting for the rat to wake up and return to the cage. For Sham group, only anesthesia, disinfection and suture, but no optic nerve damage and were given normal basal diet.

In addition, rats in fasudil group were intraperitoneally injected with 15 mg/kg/day fasudil hydrochloride injection (H20123204, Chengdu Huayu Pharmaceutical Co., Ltd., Chengdu, China) and rats in Control group were intraperitoneally injected with equal amount of saline.

Apoptotic cell assay

After 10 days of establishment, the eyeballs of each group were taken out and made into paraffin sections. The TUNEL kit (11684817910, Roche, IN, USA) was used to detect apoptotic cells. Counting the number of cells with brown-yellow particles in the nucleus in six high-definition fields were randomly selected around the injured area and recorded the average.

Fluoro-Gold retrograde mark

The rat model of optic nerve injury was completed for 7 days, and each group of rats was anesthetized and fixed to the head. The head skin was cut longitudinally in the middle of the head, the subcutaneous tissue was separated and the skull was exposed. The former skull was marked with a hand-held electric bone drill at the corresponding position on the surface of the skull (6.0mm behind the anterior cranium, 1.4mm apart, depth 4.0mm) Drill the skull and expose the dura mater, inject 3% of Fluoro-Gold (FG) (Fluorochorome, CO, USA) 3μL at each point.

The rat model of optic nerve injury was completed for 10 days, each group of rats were sacrificed, the eyeball was removed, and the retina was removed. The retina was placed on a slide (glass face up) and naturally dry. The retina was covered with a slide and the fluorescent enhancer was added for sealing. Using a fluorescence microscope and 2 mm from the center of the optic nipple, taken a picture of each of the top, bottom, left and right, and represented the RGCs of the four quadrants.

Culture of retinal ganglion cells

Isolation and culture of Long Evans rat retinal ganglion cells as previously described (Wang *et al*., 2004). The first and fifth days of culture were observed under a phase contrast microscope and photographed. The number of cells with protrusions and the length of the longest protrusion (Randomly select 5 fields of view) were analyzed using the Leica QWin image analysis system, and recorded the average.

Western blot

Tissue lysates were separated by SDS-page and then transferred to PVDF membrane. Primary antibody: Bcl-2 (GTX100064, gene tex, USA), Fas (GTX116024, gene tex, USA), FasL (GTX66619, gene tex, USA) and active-caspase 3 (ab2302, abcam, UK), Anti-RhoA (ab187027, 1:2000), anti-ROCK (ab45171, 1:1000), anti-cofilin (ab42824, 1:5000), anti-cofilin (phospho S3) (ab12866, 1:2000), anti-Myosin Light Chain (MLC) (ab92721, 1:5000), anti-Myosin light chain (phospho S20) (ab2480, 1:3000).Second antibody: goat anti-rabbit (ab150077, 1:1000), or goat anti-rat (ab150117, 1:1000). Primary antibody was incubated overnight at 4^{o}C and second antibody was incubated for 1 hour at roomtemperature. All antibodies were purchased from ABCAM (Cambs, UK) unless otherwise stated. The detection of GTP-RhoA was first treated by RhoA Activation Assay Kit (ab211164, abcam), and followed by Western blot.

STATISTICAL ANALYSIS

SPSS 20.0 was used for the statistical analysis of the data in this study. Data were expressed as (mean ± standard deviation). Student's t-test was used to compare differences between two groups. One-way ANOVA with Tukey-Kramer post hoc test was used to compare differences between Multiple groups. $P<0.05$ indicated that difference was statistically significant.

RESULTS

Fasudil protected RGCs in rat model of optic nerve injury

After 10 days of Optic nerve injury rat model, 6 rats were sacrificed in each group, the eyeballs were isolated, paraffin sections were made, and apoptosis was detected

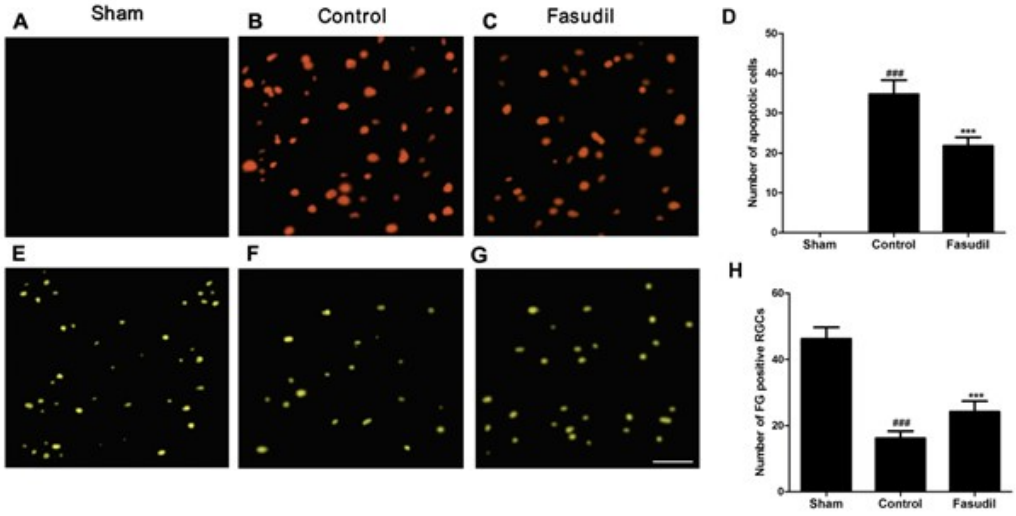

Fig. 1: Apoptosis and survival statistics of RGCs in rats 10 days after optic nerve injury. A-D, Tunel was used to detect apoptosis in the damaged area and statistics (D); E-H, FG-positive RGCs in the injured area were stained by retrograde FG label, statistics (H). 6 rats per group and one-way ANOVA with Tukey-Kramer post hoc test. ### was P<0.001 versus Sham group and *** was P<0.001 versus Control group.

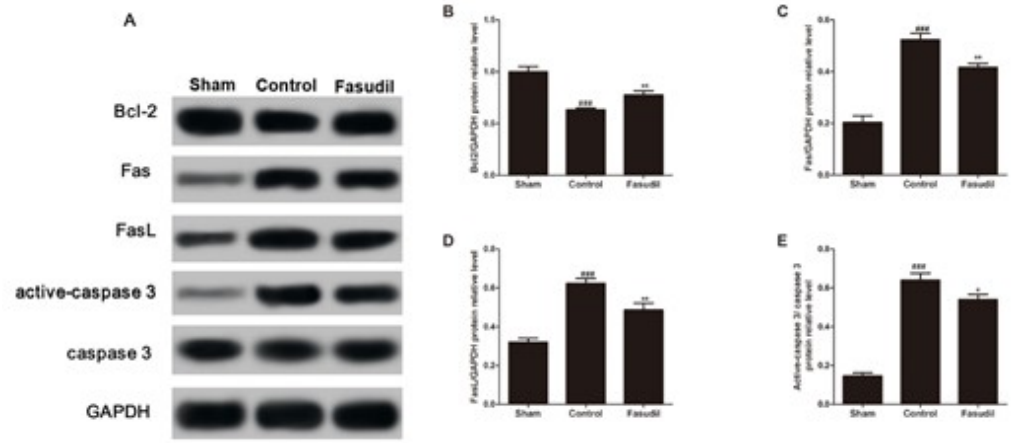

Fig. 2: Expression of apoptosis-related proteins in the injured area of rats 10 days after optic nerve injury. A-E, The expression of Bcl-2, Fas, FasL and active-caspase 3 / caspase 9 in the eyeballs tissues of different group after 10 days of Optic nerve injury rat model. 6 rats per group. * was P<0.05, ** was P<0.01 and *** was P<0.001 vs Control group; ### was P<0.001 vs Sham group.

by Tunel. As shown in fig. 1A-D, there was no apoptotic cells in Sham group, but many in Control group and Fasudil group and the number of apoptotic cells in the Fasudil group was significantly lower than that in the Control group. Moreover, FG was used to label RGCs and as shown in fig. 1E-H that the number of FG positive RGCs in Sham group was the most and Control group was the least.

We also used western blot to detect the apoptosis-related proteins expression of retina in different groups of rats. And we found that (fig. 2) compared with the Sham group, Bcl2, a protein that inhibits apoptosis, had a significantly reduced expression in Control group; Fas, FasL and active-caspase 3/ caspase 3 protein, proteins that promotes apoptosis had a significantly increased expression in the Control group. However, compared with the Control group, Bcl2, a protein that inhibits apoptosis, had a significantly increased expression in Fasudil group; Fas, FasL and active-caspase 3/ caspase 3 protein, proteins that promotes apoptosis, had a significantly reduced = expression in the Fasudil group.

Fasudil promoted axonal regeneration in RGCs in vitro

Rat RGCs were isolated and cultured. After 1 day of culture, a small number of cells in the WT group grew short protuberances (fig. 3A), while the Fasudil group (fig. 3B) had significantly more protuberances than the WT group (fig. 3E) and the prominence was significantly longer than the WT group (fig, 3E-F). After 5 day of culture, the number of cells with protrusions and the length of protrusions increased significantly in both groups (fig. 3C-D) and the Fasudil group was also significantly more than the WT group (fig. 3E-F).

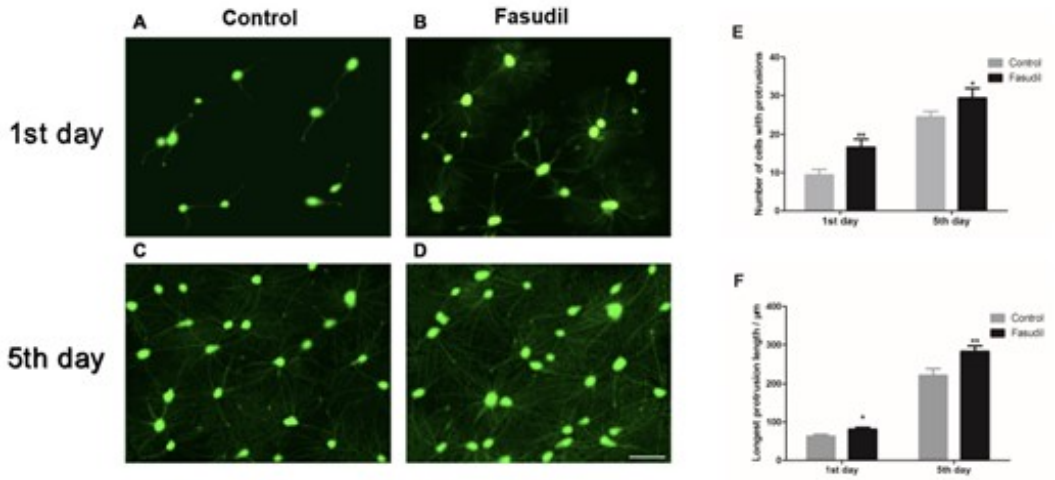

Fig. 3: Effect of Fasudil on the number of protruding RGCs and the length of the longest protrusion in vitro. A-D, Morphology of different groups of RGCs cultured at different times, and representative image displayed; E, The number of cells with protrusions in different group at different time; F, The longest protuberance length of RGCs cells in different group at different time. All experiments were performed in three independent replicates. One-way ANOVA with Tukey-Kramer post hoc test. * was P<0.05 and ** was P<0.001 versus Control group.

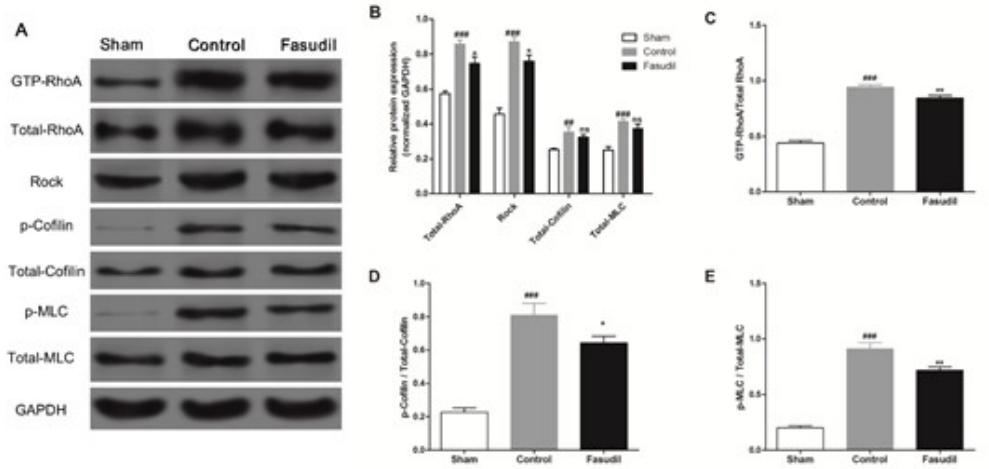

Fig. 4: The change of protein expression in the retina in mice 10 days after optic nerve injury. A-E, Western blot was used to measure the expression of protein in the retina in vivo (A) and compare gray values. 6 rats per group and all experiments were performed in three independent replicates. One-way ANOVA with Tukey-Kramer post hoc test. ### was P<0.001 versus Sham group and * was P<0.05, ** was P<0.01 and ns was P>0.05 versus Control group.

Fasudil inhibited activation of the RhoA / Rock pathway in vitro

RhoA / Rock pathway was a pathway closely related to RGCs survival and axonal regeneration, so we isolated rat retina to detect the expression of key proteins on RhoA / Rock pathway 10 days after optic nerve injury. 10 days after optic nerve injury, the expression of total-RhoA, Rock, total-cofilin and total-MLC protein in Control group was significantly increased which was compared with sham group (fig. 4A and 4B), and fasudil could significantly reduce the expression of total-RhoA and Rock protein. More importantly was the relative expression of GTP-RhoA, p-cofilin and p-MLC protein in Control group was significantly increased which was compared with sham group (fig. 4A and 4C-E), and fasudil could significantly reduce them.

DISCUSSION

The reason why the optic nerve is difficult to regenerate after injury is consistent with the problem that the central nervous system is difficult to regenerate, that was (Li *et al*., 2017, You *et al*., 2013): (1) secondary death of neurons after injury; (2) losing the nutrients provided by the target cells; (3) the formation of localized glial scars; (4) the hindrance of damage to the local inhibitory microenvironment. Therefore, researchers have explored various methods to promote neuronal axon regeneration.

Supplementation of exogenous neurotrophic substances could promote the survival of RGCs, but this protection was only transient and did not prevent RGCs from eventually leading to death (Hertz *et al*., 2013, Li *et al*., 2015). It could reduce the apoptosis of RGCs by interfering with apoptotic signaling pathways, but it did not enhance the ability of axonal regeneration (Wang *et al*., 2013). Peripheral nerve transplantation at the injury site could promote optic nerve regeneration, but failed to achieve functional connection between the regenerated fiber and the visual center (Wu *et al*., 2018, Jonathan *et al*., 2014). Thus, it can be seen that none of these methods can achieve long-term, effective, and functional regeneration of central nervous system axons.

In this study, we treated the optic nerve injury in rats by intraperitoneal injection of fasudil, and found that fasudil could not only promote the survival of RGCs after optic nerve injury in vivo, but also promote axonal regeneration of RGCs in vitro. Fasudil is currently the only clinically available RhoA/Rock pathway inhibitor and can dilate brain blood vessels, protect neuronal cells, and promote axonal regeneration (Takekazu *et al*., 2008). Sung JK *et al* (Sung *et al*., 2003) found that intraperitoneal injection of 10mg / kg of fasudil for 4 weeks ccould improve the coordination of hindlimb reflex and exercise in rats with spinal cord injury, and also been found it played a neuroprotective, reduced tissue damage, promoted nerves Fiber growth and the role of reducing void formation (Nishio *et al*., 2006, Furuya *et al*., 2009). For other neurological diseases, such as Alzheimer's disease, fasudil had also been found to have certain therapeutic potential (Couch *et al*., 2010, Hou *et al*., 2012). Couch *et al* (Couch *et al*., 2010) found that fasudil can significantly increase the dendritic length of CA1 vertebral cells in the hippocampus of APP/PS1 transgenic rats. At the same time, intraperitoneal injection of fasudil could reverse the decline of learning and memory function in rats induced by STZ (Hou *et al*., 2012).

In addition, also found that fasudil significantly inhibited the activation and conduction of RhoA/ROCK signaling pathway in the retina of rats with optic nerve injury. RhoA is a member of the Rho GTPases family and is a small molecule GTPase whose primary function is to mediate aggregation of actin filaments and contraction of cell bodies (Sadok and Marshall, 2014, Ridley *et al*., 2012). Rock, also known as Rho kinase, is a serine/threonine protein kinase that is a downstream effector of RhoA. And activated RhoA directly activates the Rock to further activate downstream substances to mediate signal transduction (Shimokawa *et al*., 2016). Previous studies have demonstrated that RhoA/Rock is an important substance that inhibits axonal regeneration in central neurons and aslo plays an important role in inhibiting optic nerve regeneration (Fujita and Yamashita, 2014). We found that fasudil could reduce the expression of RhoA and ROCK proteins in the retina of rats with optic nerve injury. More importantly, fasudil could aslo significantly reduce the expression of GTP-RhoA.

RhoA has two forms of presence in cells, one is the GTP-bound form and the other is the GDP-bound form. GTPase activating proteins (GAPs) catalyzes the hydrolysis and dephosphorylation of GTP, converting RhoA into a GDP-bound form without GTPase activity (Ming *et al*., 2010, Yamashita *et al*., 2007); but exchange factors (GEFs) catalyzes the binding of RhoA to GTP dissociation to GTP-RhoA, which has GTPase activity (Ming *et al*., 2010, Yamashita *et al*., 2007). Only when RhoA is activated, that is, in the form of GTP-RhoA, it has the effect of inhibiting central nervous system regeneration (Fujita and Yamashita, 2014). The mechanism by which many axon regeneration inhibitors block nerve regeneration is through the activation of RhoA, which is transformed into GTP-RhoA with GTPase activity, causing the activation of Rock, a downstream substance (Ridley *et al*., 2012, Shi and Wei, 2007). And activated Rock inhibits the atrophy of the axon terminal growth cone by enhancing the cofilin that regulates actin aggregation in the growth cone and the phosphorylation of the myosin light chain (MLC) that mediates myosin filament contraction, and thereby inhibiting the regeneration of central neuron axons (Ridley *et al*., 2012, Shi and Wei, 2007).

CONCLUSION

Fasudil protects retinal ganglion cells and promotes axonal regeneration by inhibiting RhoA / Rock pathway, and fasudil may be used as a drug for the treatment of optic nerve damage.

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