Int J Clin Exp Pathol 2015;8(11):14717-14724
www.ijcep.com /ISSN:1936-2625/IJCEP0015517

Original Article

Impacts of Rho kinase inhibitor Fasudil on Rho/ROCK signaling pathway in rabbits with optic nerve injury

Jianglong Yu[1,2*], Lin Lin[3*], Xinping Luan[1], Xiepan Jing[2], Maierab[1]

[1]Department of Neurosurgery, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi 830063, Xinjiang, P.R. China; [2]Department of Neurosurgery, People's Hospital of Xinjiang Bazhou Region, Korla 841000, Xinjiang, P.R. China ; [3]Department of Neurosurgery, The Fourth Affiliated Hospital of Xinjiang Medical University, Urumqi, 830000, P.R. China. [*]Equal contributors.

Received September 2, 2015; Accepted October 20, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Objective: The aim of this study was to study the impacts of Rho kinase inhibitor Fasudil on expressions of Rho/ROCK signaling pathway associated genes in rabbits with optic nerve injury (ONI), and to explore the therapeutic mechanisms towards ONI. Methods: The rabbit ONI model was established, then the rabbits were divided into model group (treated with saline), control group (treated with dexamethasone, Dex), and intervention group (treated with Fasudil, Fas). The eyeball and optic nerve were sampled at 3, 7, 14 and 21 days after injury. The morphological changes of retina and optic nerve were observed. The expressions of RhoA, Caspase-3, Rock 2 and Nogo-A gene were determined by immunohistochemistry and real-time polymerase chain reaction (RT-PCR) methods. Results: At different time after injury, there were significant differences of RhoA, Caspase-3, Rock 2 and Nogo-A gene expression among three groups ($P < 0.05$). Conclusions: After ONI, Fas can decrease the expression of Caspase-3 gene, and down-regulate the expressions of Nogo-A and Rock 2 gene. Therefore, it can treat ONI through affecting the Rho/ROCK signaling pathway.

Keywords: Fasudil, optic nerve injury, Nogo-A, Rock 2

Introduction

Acute optic nerve injury (ONI) was a common surgical traumatic disorder, with high blinding rate and difficult regeneration. The apoptosis of retinal ganglion cells (RGCs) was the main pathological change in ONI, therefore, maintaining the RGCs' activities was the basis to ensure the repairing and regeneration of optic nerves' structures and functions [1, 2]. Therefore, studying the post-ONI protection and restoration of RGCs had become a hot topic in recent years [3-5]. We applied the Rho kinase inhibitor after ONI, aiming to study the number and morphological changes of RGCs, as well as the expression changes of intracellular molecules, thus helping to determine the apoptosis of RGCs, and the mechanisms that how the inhibitor could impact the post-ONI recovery through the Rho/ROCK signaling pathway.

The Caspase family was the important genes of cellular apoptosis. The activation of Caspase-3 was key to the cellular apoptosis. It was found that [6], the Caspase-3 expression of RGCs in animal diabetic model was increased, while the RGCs apoptotic rate was decreased after injected the Caspase-3 inhibitor. Certain study found that in the animal glaucomatous model, when applied the inhibitor therapy, the apoptosis of RGCs was reduced, and the Caspase-3 expression was reduced [7]. This study detected the Caspase-3 expressions in rabbit ONI model at different time points, aiming to determine the apoptotic situations of RGCs, and make clear the initial factors that could induce the secondary degeneration of RGCs. The Rho/ROCK signaling pathway was widespread inside biological bodies, mainly involved in the physiological processes of cytoskeleton and cell contraction [8], and it was also the main signal pathway towards the repairing and regeneration of central nervous system, it could explain, from the molecular level, the mechanisms of ultrastructural reconstruction and functional recovery after the nerve tissues were injured. Rock 2 was a Rho kinase, which expression was detected in many researches to determine the activa-

tion status of Rho/ROCK signaling pathway [9, 10]. So we detected the expression of Rock 2 in rabbit ONI model to determine how this signaling pathway could affect ONI. Nogo-A was the typical nerve growth inhibitor, expressed on the membrane of neural glial cells, as well as in the endoplasmic reticulum and Golgi body, and it could inhibit the nerve regeneration. Nogo-A bound with the receptors on the cell membrane, then transferred the signals into the cells, caused the signaling cascade, activated the Rho/ROCK signaling pathway, thus affecting the actions of actin-myosin, causing the collapse of cell growth cone, and inhibiting the growth of nerve axons [11]. The existing study [12] more focused on the injury repairing and regeneration of brain tissues and spinal cord tissues, this study focused on using the Rho kinase inhibitor to investigate the expression changes of Rho/ROCK signaling pathway associated proteins inside the optic nerve tissues of rabbit ONI model, as well as the morphological changes of optic nerves, and the number changes of the related cells, aiming to investigate the relationships between the Rho kinase inhibitor and the repairing and regeneration of ONI, and to explore its inhibitory mechanisms.

Materials and methods

Animals and grouping

108 New Zealand white rabbits, 2-3 months old, male/female, weighed 2.0 to 2.5 kg, were provided by the Experimental Animal Center of Xinjiang Medical University. The management of laboratory animals followed the "Management Rules of Medical Laboratory Animals", and this study was approved by the Ethics Committee of Medical Experimental Animal, Xinjiang Medical University. The rabbits were divided into three groups (the control group, the model group and the intervention group) according to random number table method, with 36 rabbits (72 eyes) in each group.

Establishment of rabbit ONI model

Before prepared the traumatic ONI model, the rabbits were numbered, weighed and recorded; then anesthetized with 3% sodium pentobarbital, 30 mg/kg, slowly injected through the rabbit ear vein; incised the right eyelid, then curt open the fornix bulbar conjunctiva along 10-14:00 direction under binocular microscope; drew the superior and lateral rectus muscles towards the lateral bilateral directions with suture, while drew the eyeball towards the nose bottom; exposed the bilateral vortex veins, and bluntly dissected towards the posterior direction of eyeball; exposed the optic nerve, carefully peeled the fascia on the optic nerve surface; separated the optic nerve at the bone tube (or ring), extended REF 65263T type Yasargil Aneurgsm clip (clamping force: 110 g ± 7.5%; Rebstock Instruments GmbH, Germany) into the bone tube (or ring); clamped the optic nerve 3-5 mm above and below the nerve root behind the eyeball, continued 30 seconds before removed the aneurysm clip to prepare the ONI model. After the rabbit recovered from anesthesia, observed the pupil size and light reflection, as well as whether the eyeball obviously bulged, whether the eyelid could be closed completely, such phenomena as mydriasis in operated eye, direct light reflection delayed or disappeared, no retinal hemorrhage and vascular obstruction found in the fundus examination could be seen as the successful modeling, and the rabbits were then included into the experiment. Tobramycin Eyedrops (Alcon) was then used to wash the surgical field, and then sutured the bulbar conjunctiva. Tobramycin Eyedrops was used for three consecutive days after surgery, twice a day. 108 rabbit ONI models were successfully prepared for the experiment.

Reagent injection

Reagents were injected 12 hours after modeling. The intervention group was injected with 6 mg/kg Fas (Shanxi Pude Pharm. Co., Chinese) every 12 hours through the ear vein for the longest 12 days. The control group was injected with the same amount of Dex at the same time, 1.0 mg/kg for former 6 days, decreased dose for the later days. The longest continuous injection was 14 days. The model group was injected with the same amount of saline at the same time points.

Sampling time points

The samples were taken at 4 best time points when the pathological changes were the most obvious [13]: T1: 3 days after injury, at this time point exhibited the peaks of bleeding, oozing, vacuolar degeneration and telangiectasia; T2: 7 days after injury, at which the oedematous

Table 1. Sequence of primers

Primer	Sequence (5' to 3')	Length (bp)
GAPDH_F	ACCATCTTCCAGGAGCGAGA	187
GAPDH_R	GGTTCACGCCCATCACAAAC	
ROCK2_F	CTGGTGGTCTGTGGGTGTTT	209
ROCK2_R	CTACCCCATTTCGCCCAAGT	
RhoA_F	CCGTGCATCTTGCAGTACATCT	195
RhoA_R	CCAGCTCTACCTGTTTCCCATC	
RTN4-F	GCCTGTGATACACTCCTCTGC	124
RTN4-R	AGAAGGAAGAGAAGCAGCAGTT	
Caspase-3-F	CTGGACAGTGGCATCGAGAC	120
Caspase-3-R	TCGCCAGGAATAGTAACCAGG	

axons exhibited infarction, thrombosis and vacuolar degeneration seriously; T3: 14 days after surgery, at which the edema faded and gliocytes proliferated obviously, and it was the best time to observe the arrangement and structure of reproduced nerve fibers; T4: 21 days after surgery, at which the structures were repaired, the vascular circulation was reopened, and the fibers began adhesion. At each time point, 3 rabbits were sampled for HE as well as immunohistochemical staining, 6 rabbits were sampled for RT-PCR and 3 of whom were also for Western blot analysis.

Sampling

After been anesthetized, the rabbits were chest exposed to perfuse the fixation solution (4% paraformaldehyde, Wuhan Boster, China) by deep venous puncture in left ventricle, then the retina and optic nerve (cross section) were collected to prepare slices (6 for each eye) for examination.

Immunohistochemical staining

The paraffin slices were dewaxed with xylene, rehydrated with gradient alcohol, rinsed with PBS 3 min × 3 times; added 0.01 M pH 6.0 citrate buffer solution then kept in microwave for 5-min low fire treatment, rinsed with PBS 3 min × 3 times; added goat serum for the closure at room temperature for 10 min, rinsed with PBS 3 min × 5 times, added primary antibody (rabbit anti-mouse Caspase-3 antibody and rabbit anti-mouse Nogo-A antibody; dilution: 1:100, Beijing Boosen Biological Company, Beijing, China), incubated at 37°C for 1 hour, rinsed with PBS 3 min × 3 times; added secondary antibody (biotinylated goat anti-rabbit IgG; dilution: depends on the introduction; Beijing Zhongshan Jinqiao Biological Company, Beijing, China), incubated at 37°C for 30 min, rinsed with PBS 3 min × 3 times; performed DAB staining, rinsed with distilled water to terminate the reaction 5 min later after the positive and negative results were obvious, then observed and recorded the numbers of positive Nogo-A cells to calculate the Caspase-3 immunohistochemical positive area percentage by gray density analysis.

RT-PCR

The quantitative real-time polymerase chain reaction (RT-PCR) was conducted on CFX96 Touch PCR detection system (Bio-Rad Laboratories, CA, USA). The total RNA was extracted from the tumor tissue. The 20 μL PCR-reaction mixture containing total RNA was prepared for PCR. GAPDH was used as internal reference. The primer sequences were shown in **Table 1**. All primers were synthesized by Shanghai Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The PCR steps were as follows: initial denaturation, 2 min at 50°C; denaturation, 2 min at 95°C; annealing, 15 sec at 95°C; elongation, 1 min at 60°C. Above steps were performed for 40 cycles. The relative expression level was determined using the $2^{-\Delta\Delta Ct}$ analysis method [14].

Statistical methods

SPSS17.0 statistical software was used for ANOVA. The data were expressed as mean ± SD, and tested with t test. $P < 0.05$ was considered as statistically significant.

Results

Expression of Rock 2 mRNA in three groups

The Rock 2 mRNA expression in intervention group at each time point was lower than the model group ($P < 0.05$) and control group. The Rock 2 mRNA expression in control group at T2 and T3 was significantly lower than the model group ($P < 0.05$), while at T1 and T4, the Rock 2 mRNA expression in control group was higher than model group although there was no significance (**Table 2**).

Table 2. Expressions of Rock 2 mRNA in three groups ($\bar{x} \pm s$, n = 18)

Group	T1	T2	T3	T4
Model	1.018±0.220	1.038±0.308	1.033±0.281	1.035±0.312
Control	1.340±0.886	0.548±0.056*	0.501±0.280*	1.088±0.435
Intervention	0.604±0.272*,#	0.442±0.112*	0.477±0.164*	0.431±0.247*,#

Note: *P < 0.05 compared with model group; #P < 0.05 compared with control group. T1, 3 days; T2, 7 days; T3, 14 days; T4, 21 days.

Table 3. Number of Nogo-A positive oligodendrocytes in three groups ($\bar{x} \pm s$, n = 9)

Group	T1	T2	T3	T4
Model	136.2±2.0	169.7±1.8	162.7±2.8	161.9±1.5
Control	127.4±2.7*	147.3±1.5*	144.3±2.5*	129.5±2.9*
Intervention	126.6±2.2*	146.9±2.1*	143.4±2.3*	124.7±2.7*,#

Note: *P < 0.05 compared with model group; #P < 0.05 compared with control group. T1, 3 days; T2, 7 days; T3, 14 days; T4, 21 days.

Immunohistochemical staining of Nogo-A in three groups

After ONI, the number of Nogo-A positive oligodendrocytes in three groups increased, and reached the maximum at T2. The number of Nogo-A positive oligodendrocytes in control group at each time point was significantly lower than model group ($P < 0.05$), and that in intervention group at each time point was also significantly lower than model group ($P < 0.05$). At T4, the number of Nogo-A positive oligodendrocytes in intervention group was significantly lower than control group ($P < 0.05$) (**Table 3**; **Figure 1**).

Immunohistochemical staining of Caspase-3 in the groups

The positive slice exhibited brown nuclei and cytoplasm. After injury, the model group exhibited a large amount of Caspase-3 expression, mainly in the ganglion cell layer and inner nuclear layer, while no expression was found in the retinal outer nuclear layer (**Figure 2**). The positive rate of Caspase-3 in intervention group or control group was significantly lower than model group ($P < 0.05$). There was no significant difference between intervention group and control group ($P > 0.05$) (**Table 4**).

Expression of Caspase-3 in the groups

The Caspase-3 expressed extremely high in the model group than that of control and intervention group with significant difference ($P < 0.05$), at the same time, the relative transcript level of Caspase-3 in the intervention group was the lowest at each time point, while only at T1 there was statistical difference ($P < 0.05$, **Table 4**).

Expression of optic nerve Nogo-A mRNA in three groups

The expression of Nogo-A mRNA was extremely high in model group, almost twice the level of other 2 group (with significance at T1 and T2), and the expression increased along with the time; and the expression was lowest in the intervention group, which only be found a increase at T4; in control group, the expression was higher at T1 than T2/T3/T4 (**Table 5**).

Expression of retinal RhoA mRNA in three groups

The expression of retinal RhoA mRNA was higher in model group than other 2 groups with significant difference ($P < 0.05$, except for control group at T4); the expression in intervention group was almost the lowest (except for control group at T3, **Table 6**).

Retinal pathological changes in three groups

After HE staining, the optical microscopy revealed that the normal adult rabbit retina exhibited clear levels, the cells in RGCs layer exhibited monolayer arrangement. While at T1, the nuclei of RGCs of the model group degenerated, the numbers were reduced, but the retinal structures were still neat and legible; the numbers of RGCs on the intervention group and the control group were more than the model group, and the nuclear degeneration was not that severe. At T2, T3 and T4, with time extending, the nuclei of the three groups exhibited more and more serious degeneration, the numbers of RGCs were reduced, the retinal thickness became thinner and thinner, and the cells began to show more and more serious random arrangement. However, the state of the intervention group at each time point was better than the model group and the control group (**Figure 3**).

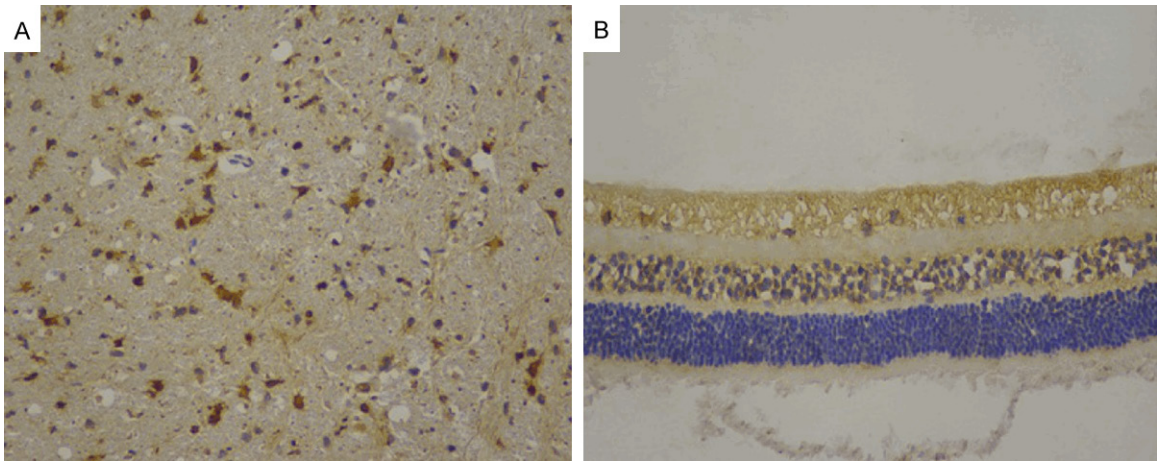

Figure 1. Immunohistochemical staining of optic nerve (A) and retinal (B) Nogo-A in injury side in intervention group (14 days, × 400).

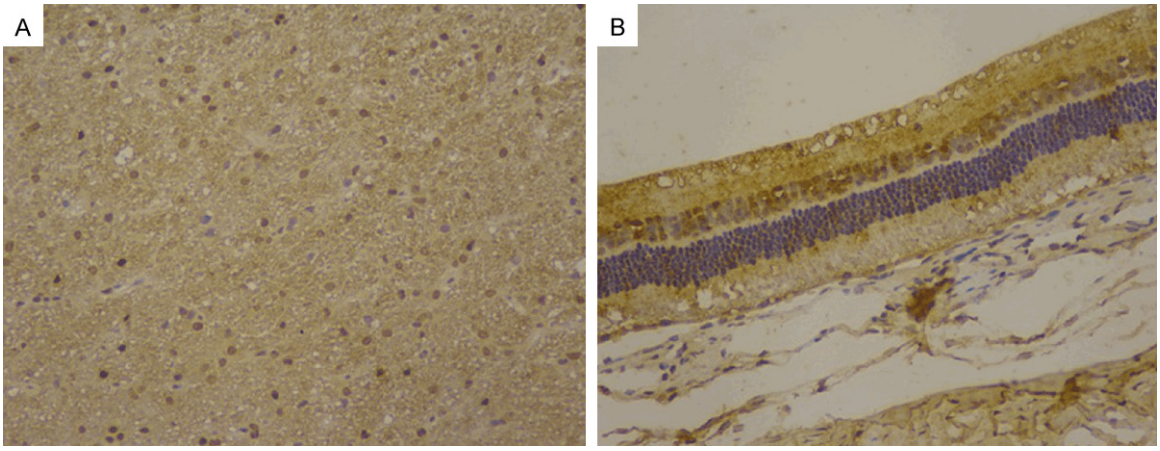

Figure 2. Immunohistochemical staining of optic nerve (A) and retinal (B) Caspase-3 in injury side in intervention group (14 days, × 400).

Table 4. Relative transcript level of Caspase-3 ($\bar{x} \pm s$, n = 18)

Group	T1	T2	T3	T4
Model	1.005±0.112	1.009±0.145	1.016±0.186	1.013±0.180
Intervention	0.461±0.122$^{\Delta}$	0.438±0.122$^{\Delta}$	0.592±0.129$^{\Delta}$	0.510±0.087$^{\Delta}$
Control	0.665±0.106$^{\Delta,\blacktriangle}$	0.590±0.145$^{\Delta}$	0.793±0.171$^{\Delta}$	0.680±0.114$^{\Delta}$

Note: $^{\Delta}P < 0.05$ compared with model group. $^{\blacktriangle}P < 0.05$ compared with intervention group; T1, 3 days; T2, 7 days; T3, 14 days; T4, 21 days.

Discussion

In adult mammals, ONI only exhibited the regeneration responses instead of the regeneration abilities. The apoptosis of RGCs was the main pathological change after ONI, while no effective treatment had been found. Higher RGCs survival rate would promote the growth and extension of axons. Currently, the researches about RCGs were mainly focused on describing the deformation process, but little associated with ONI. The initial factors that could induce RGCs' secondary degeneration were not studied clearly, thus further studies were still needed. Inhibiting the secondary degeneration of RGCs after ONI was the basis for the effective treatment of ONI.

We found that after modeling, the Rock 2 expression was gradually increased, after administrated Fas, the intervention group exhibited decreased expression of Rock 2 than the model group and the control group. The

Table 5. Relative transcript level of Nogo-A mRNA ($\bar{x} \pm s$, n = 18)

Group	T1	T2	T3	T4
Model	1.012±0.165	1.027±0.270	1.044±0.333	1.050±0.354
Intervention	0.545±0.192$^{\Delta}$	0.588±0.152$^{\Delta}$	0.578±0.174	0.632±0.163$^{\Delta}$
Control	0.897±0.179$^{\blacktriangle}$	0.733±0.125$^{\Delta}$	0.722±0.147	0.791±0.269

Note: $^{\Delta}P < 0.05$ compared with model group; $^{\blacktriangle}P < 0.05$ compared with intervention group.

Table 6. Relative tanscript level of retinal RhoA mRNA ($\bar{x} \pm s$, n = 18)

Group	T1	T2	T3	T4
Model	1.012±0.172	1.009±0.151	1.013±0.180	1.007±0.130
Intervention	0.525±0.128$^{\Delta}$	0.662±0.128$^{\Delta}$	0.751±0.207$^{\Delta}$	0.720±0.136$^{\Delta}$
Control	0.740±0.172$^{\Delta,\blacktriangle}$	0.752±0.139$^{\Delta}$	0.738±0.227$^{\Delta}$	0.903±0.250

Note: $^{\Delta}P < 0.05$ compared with model group; $^{\blacktriangle}P < 0.05$ compared with intervention group.

Rho/ROCK signaling pathway was widespread inside biological bodies, and it was the main signal pathway towards the repairing and regeneration of central nervous system. As a GTP enzyme of molecular switch [15, 16], Rho could be activated into Rho-GTP by G protein-coupled receptors. Rock 2 was a Rho kinase present in the central nervous system, belonging to the Ser/Thr protein kinase, and it was an important molecular target of Rho/ROCK signaling pathway. After Rock 2 and activated Rho-GTP bound, they could be activated, and then induced the directional translocation, thus prompting the phosphorylation of myosin light chain (MLC). The phosphorylated MLC would then promote interactions of actin and myosin; therefore, it could regulate the cytoskeletal reorganization, resulting in the growth cone depression and neurite retraction [17]. In addition, study found that inhibiting the Rho/ROCK signaling pathway would promote the regeneration and functional recovery of damaged central nervous system, increase the blood flow, inhibit the neutrophil-mediated inflammatory injuries, and inhibit the ischemia-induced death of neurons [18]. Some study showed that [19], the activated RhoA was substantially increased in the processes of nerve cells, Rock 2 was thus activated, and the Rho/ROCK signaling pathway was then activated, blocking RhoA and ROCK could promote the axonal growth. The common ROCK inhibitor was Y-27632. Y-27632 might combine with the catalytic site of ROCK, thus inhibited the activities of ROCK. Fasudil was the isoquinolinesulfonyl drug, could act as a selective ROCK inhibitor, like Y-27632, to restore the regeneration of neurons to some extent [20], in the current clinical applications, it was not found any adverse reactions. Fas could inhibit the Rho/ROCK signaling pathway, thus reducing the neuronal apoptosis in the ischemic region [21], and adjusting the polymerization of neuronal actin cytoskeleton [22]. So we used Fas in this study to reduce the in vivo expression of Rock 2 in rabbit ONI model, it could thus possibly inhibit the Rho/ROCK signaling pathway, and play the recovery roles towards the damaged retina.

Nogo-A was a myelin-associated glycoprotein, involved in the pathogenesis of a variety of central nervous system diseases, and it was also the key factor in suppressing the regeneration of central nervous system [23]. We found that, after ONI, the Nogo-A expression was increased, when administrated Dex and Fas, the control group and the intervention group exhibited decreased Nogo-A expression than the model group. Nogo-A could bind with the neuronal membrane receptors than activate the Rho/ROCK signaling pathway, causing the growth cone collapse and inhibiting the axonal regeneration [24]. Inhibiting the abnormal activation of Rho kinase played an important roles towards ONI. Dex was a glucocorticoid, as the early medication after ONI, it could promote the nerve regeneration, protect the retina and the optic nerve. The study found that after ONI, RGCs would induce apoptosis; the application of glucocorticoid could reduce the apoptosis. Dex could also inhibit the expression of Nogo-A, so this study used Dex as the control, the results showed that Dex could inhibit the expression of Nogo-A, and increase the number of RGCs, so it could be used as the control group of Fas. This experiment showed that in rabbits with ONI, the Nogo-A expression continued to increase, reached the highest at T2 and maintained the high expression, consistent to other researches. The results showed that after ONI, the inhibitory factors were activated, and

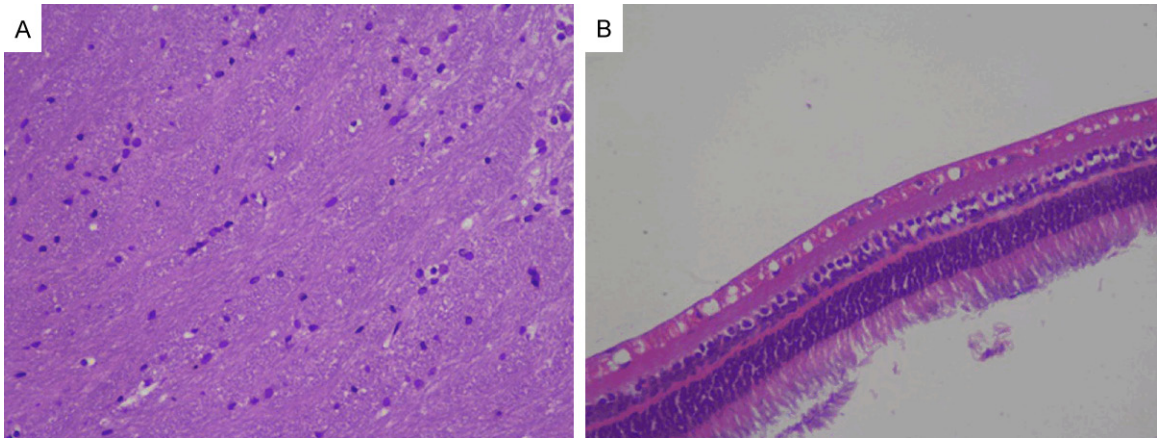

Figure 3. HE staining of optic nerve (A) and retinal (B) Caspase-3 in injury side in intervention group (14 days, × 400).

rapidly increased, which reached the peak 7 days later, then declined, although the expression was decreased to some extent, it was still maintained at a high status. After administrated Fas, the amount of Nogo-A was significantly lower than the model group, indicating that Fas could inhibit the activation of axonal regeneration inhibitors, thus promoting the regeneration and functional recovery of optic nerve, and this provided theoretical basis for the clinical application research of Fas.

In summary, the optic nerve would be repaired through Rho/ROCK signaling pathway by fasudil, which could down-regulate the expression of Caspase-3 and lead to Nogo-A/Rock 2 reduced.

Acknowledgements

This work was supported by Xinjiang Medical University and the national natural science Foundation of China (No. 81160153).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xinping Luan, Department of Neurosurgery, The Second Affiliated Hospital of Xinjiang Medical University, 38 Nanhu East Road, Urumqi 830063, Xinjiang, P.R. China. Tel: +86 991 4609099; 15299158818; Fax: +86 991 4609099; E-mail: xinpingluancn@126.com

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