Original Article
Fasudil alleviates traumatic optic neuropathy by inhibiting Rho signaling pathway

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Abstract: Objectives: The present study is to investigate the pathological changes in rabbits with traumatic optic neuropathy (TON), as well as the effect of fasudil on the lesions. Methods: A total of 144 New Zealand rabbits were successfully established as TON models. Twelve hours after surgery, the rabbits in control, dexamethasone, and fasudil groups were administrated with saline, dexamethasone, and fasudil via ear veins, respectively. Then, retinas of the rabbits were obtained at 72 h and on days 7, 14 and 21 after surgery. The pathological changes in retina and optic nerves were observed by hematoxylin and eosin staining and transmission electron microscopy. The expression levels of Rho-associated genes were measured using quantitative real-time polymerase chain reaction. Results: In control group, the axons were swelling, and mitochondria showed vacuolation after optic nerve crush. Mitochondria were swelled slightly in dexamethasone group. By contrast, nerves in fasudil group were repaired. Retinal ganglion cells in control group were reduced significantly due to optic nerve crush. The loss of retinal ganglion cells was alleviated in fasudil group. Quantitative real-time polymerase chain reaction showed that the expression of Rho-associated genes were down-regulated. Conclusions: The present study demonstrates that fasudil inhibits the apoptosis of retinal ganglion cells and ameliorates damages of optic nerves in traumatic optic neuropathy.

Keywords: Traumatic optic neuropathy, Rho kinase, pathology

Introduction

Traumatic optic neuropathy (TON) is indirect lesion in optic nerves caused by external force via skeleton or the movement of eye balls. The clinical manifestation of TON is progressive decline of visual function [1]. TON is the main reason for traumatic blindness, with 40-50% patients losing eye sight after the occurrence of TON [2, 3]. Because it is difficult to perform prospective clinical trials for TON, little progress is made in the clinical and animal researches of TON. Clinically, sustained systemic administration of steroids [4], surgical decompression [5], the combination of hormones and surgery [6], and conservative observation are usually adopted to suppress secondary pathological lesions, but little significant progress is made. In optic nerve system, Rho family plays important roles in the growth of axons [7-11]. The activation of Rho-ROCK signaling pathway damages myosin light chain phosphorylation, and inhibits the regeneration of optic nerve axons [12]. In the present study, we investigate the effect of fasudil, a kind of Rho kinase inhibitor, on the pathological changes caused by TON.

Materials and methods

Animals

A total of 144 New Zealand rabbits were divided into control, fasudil and dexamethasone groups of 48 rabbits. Twelve hours after TON rabbit models were built [13], rabbits in fasudil group were injected with fasudil hydrochloride (6 mg/kg body weight; Pude Pharma, Datong, China) via ear veins every 12 hours. The maximal administration time was 12 days. Dexamethasone group of rabbits were injected via ear veins with dexamethasone (1 mg/kg; Pude Pharma, Datong, China) twice a day for a maximal duration of 14 days. Rabbits in control group received the same volumes of saline. All animal experiments were conducted according to the ethical guidelines of Xinjiang Medical University.
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**Tissues**

The 48 rabbits in each group were randomly divided into four subgroups of 12 rabbits that were sacrificed at four different time points (72 h, day 7, day 14, and day 21). Among the 12 rabbits, optical nerve tissues from 3 rabbits were used for hematoxylin and eosin (HE) staining (Beyotime Biotechnology, Shanghai, China), tissues from another 3 rabbits were processed for transmission electron microscopy (TEM), and tissues from the last 6 rabbits were subject to RNA extraction.

**HE staining**

Tissue samples were fixed with 10% formaldehyde for 24 h, followed by dehydration with 70% ethanol for 4 h, 80% ethanol for 4 h, and 90% ethanol overnight. Then, the samples were treated with 95% ethanol for 4 h twice, 100% ethanol for 1.5 h twice, and then xylene for 50 min before transparency with xylene. The tissues were then soaked in wax at 58-60°C for 1.5 h twice, and sliced into 4-6 μm. Then the slices were soaked in water at 45-47°C, before being baked at 65°C for 8 h. The sections were stained with HE (Richard-Allan Scientific, Kalamazoo, MI, USA) and examined under a magnification of ×100.

**TEM**

The samples were cut into pieces of 1 mm³, and fixed by 4% glutaraldehyde for 1 or 2 hours, followed by washing with phosphate-buffered saline for 3 times of 10-15 min. Then, the samples were fixed again with 1% osmium tetroxide for an hour, followed by washing with phosphate-buffered saline for 3 times of 15 min. The samples were then dehydrated using acetone (50%, 70%, 80%, 90% and 100%) for 3 times of 10-15 min. Subsequently, the samples were soaked with EPON812 and acetone (1:1) for 1 hour, EPON812 and acetone (3:1) for 3 hour, and EPON812 for 12 hour. The samples were embedded and labeled before aggregation at 37°C for 12 hour, 45°C for 12 hour, and 60°C for 24 hour. After slicing, the slices were stained with lead nitrate for 10-20 min, and uranyl acetate for 20-30 min, before observation of the integrity of the nerve fibers under transmission electron microscope (JEM-1230, JEOL, Tokyo, Japan).

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extract from 100 mg tissues using TRizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). RNA template (1 μg) was denaturated at 65°C for 5 min, followed by immediate cooling on ice. cDNA was obtained by reverse transcriptions using TIANGen RT Kit (Tiangen, Beijing, China) under 77°C for 15 min, followed by enzyme inactivation reaction at 98°C for 5 min. qRT-PCR was performed using SYBR Select Master Mix PCR kit (Thermo Fisher Scientific, Waltham, MA, USA) on ABI 7500 FAST Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The PCR system (20.0 μl) was composed of 10.0 μl SYBR Select Master Mix (2X), 0.4 μl forward primer (10 μM), 0.4 μl reverse primer (10 μM), 1.0 μl cDNA template, and 8.2 μl ddH₂O. PCR protocol was as follows: UDG Activation AmpliTaq DNA at 50°C for 2 min and Polymerase, UP Activation at 95°C for 2 min; 40 cycles of 95°C for 15 s and 60°C for 60 s. The sequences of primers were as follows. RhoA: forward primer, CCGTGACTTGGCAGTTACT; reverse primer, CAGCCTTCACTGTTCCCATC. ROCK1: forward primer, CATGCAAGCGCAAATTGGTAGA; reverse primer, CGAAGCTTAGCAGGACT. ROCK2: forward primer, CTGGTGCTGTGGTGGTTT; reverse primer, CTACCACATTTCGCCCAAGT. GAPDH: forward primer, ACCATCTTCCAGGAGCGAGA; reverse primer, GGTTCACGCCCATCACACAAAC.

**Statistical analysis**

All results were analyzed using SPSS 17.0 (IBM, Armonk, NY, USA). Measurement data were expressed as means ± standard deviation. Comparisons between multiple groups were performed using one-way analysis of variance. Differences were considered significant if P < 0.05.

**Results**

_Fasudil ameliorates pathological lesions in retina induced by TON_

To examine pathological changes in retina, HE staining was performed. In control group, blood capillaries in ganglion cell layer (GCL) were broken or filled with blood, and cells in GCL had reduced size of nucleus. In addition, the number of cells in inner nuclear layer (INL) and outer nuclear layer (ONL) was slightly reduced. On
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72 h  Day 7  Day 14  Day 21
Control

Dexamethasone

Fasudil

Figure 1. Pathological changes in retina of rabbits with traumatic optic neuropathy. Hematoxylin and eosin staining of retina in (A) control, (B) dexamethasone, and (C) fasudil on day 14 after the occurrence of traumatic optic neuropathy. Magnification, 400×.

Figure 2. Lesions in optic nerves of rabbits with traumatic optic neuropathy. Transmission electron microscopy was performed (scale bar = 2 μm; magnification, 1200×).

day 14 after TON, the nucleuses of cells in GCL became sparse, and the number of cells in INL and ONL was reduced. In addition, a large number of empty cells were observed in GCL, and retina became thinner. In dexamethasone group, fewer empty cells were observed in GCL, and the arrangement of cells in photoreceptor cell layer (PCL) was more ordered than control. Moreover, the number of apoptotic retinal ganglion cells (RGCs) in fasudil group was fewer than that in control group, and the number of cells in INL and ONL was more than that in control group. In addition, the number of vacuoles in fasudil group was greater than that in control group and dexamethasone group, and the thickness of retina in fasudil group was thicker than that in control group and dexamethasone group (Figure 1). These results suggest that fasudil ameliorates pathological lesions in retina induced by TON.
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To observe the structural changes in optic nerves induced by TON, TEM was employed. TEM images showed that mitochondria in control group was reduced in number, became swelled, and exhibited vacuole-like degeneration. On day 14 after the occurrence of TON, axon vacuolar structures were observed, microfilaments and microtubules became swelled and twisted, and lamellar separation appeared. On day 21, the number of myelin was reduced, microfilaments were dissolved, and bundles of myelin were observed. In dexamethasone group, the swelling of mitochondria was alleviated compared with control group. In fasudil group, little lamellar separation was observed, and more axonal buds appeared (Figure 2). These results indicate that fasudil treatment repairs damaged structures in optic nerves induced by TON.

**Fasudil treatment repairs damaged structures in optic nerves induced by TON**

Fasudil inhibits Rho signaling pathway by reducing the expression of RhoA, ROCK1 and ROCK2

To measure the expression of Rho-associated genes in optic nerves, qRT-PCR was used. The data showed that the levels of RhoA, ROCK1 and ROCK2 at 72 h and on day 7 were high, while those on days 14 and 21 were reduced compared with earlier time points. Furthermore, the levels of RhoA, ROCK1 and ROCK2 in fasudil group were significantly lower than control group and dexamethasone group at all time points (P < 0.05) (Figure 3). These results suggest that fasudil inhibits Rho signaling pathway by reducing the expression of RhoA, ROCK1 and ROCK2.

**Discussion**

TON is a kind of acute lesions in optic nerves that can lead to visual function decline or even blindness [14]. Hormones, neurotrophic drugs or vasodilators are the main drugs for the treatment of TON. However, their effects are not good enough, and hormones sometimes show severe adverse effects during treatment [15, 16]. The pathologic mechanism of TON is thought to mainly include primary injury, secondary injury and the apoptosis of RGCs. Primary injury is usually caused by factors that directly break optic nerves; secondary injury is mainly caused by tissue edema, inflammation, lipid peroxidation, or Ca\(^{2+}\) influx [17]; the apoptosis of RGCs is the result of axonal damages in...
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In optic nerves [18, 19]. Inhibition of apoptosis of RGCs after damages in optic nerves is the basis for the treatment of optic neuropathy and improvement of visual function. Compared with surrounding nerves, the regeneration ability of axons in central nervous system is weaker. A study indicates that RhoA/ROCK signaling pathway induces the inhibition of axonal regeneration and repair after central nervous system is damaged [20]. Rho is a member of the Ras superfamily in GTPase family, including RhoA, RhoB, and RhoC [21]. Rho kinase, the downstream effector molecule of RhoA, has two subtypes, ROCK1 and ROCK2 [22]. In central nervous system, ROCK is a downstream effector molecule of Rho that enhances myosin light chain (MLC) phosphorylation by directly phosphorylating MLC and indirectly inhibiting MLC phosphatase. Rho/ROCK signaling pathway not only regulates actin reorganization, but also regulates microtubules and intermediate filaments [23-25]. Because of the importance of Rho signaling pathway in optic nerves [26], Rho inhibitors are used in several optic nerve diseases [27, 28]. Fasudil is the most widely used ROCK inhibitor that expands brain blood vessels, promotes axonal regeneration, and protects neurons [29, 30].

Our results show that fasudil inhibits the apoptosis of RGCs in rabbit optic nerves, and protects the repair and regeneration of axons. Results by HE staining show that the repaired function of optic nerves in fasudil group is better than that in control. TEM results further demonstrate that the repair of microfilaments and microtubules in optic nerves of fasudil group is better than control. Subsequent determination of expression of RhoA, ROCK1 and ROCK2 genes shows that Rho/ROCK signaling pathway in fasudil group is inhibited. In the present study, the repair effect of dexamethasone is not as good as fasudil. Other Rho inhibitors such as Y-39983 and Y-27632 are also reported to promote the regeneration of axons [31-33]. However, they are not suitable for clinical treatments because they are chemical drugs. Fasudil also provides a good microenvironment for axonal repair after TON by alleviating inflammation by the inhibition of Rho signaling pathway. In conclusion, the present study demonstrates that treatment with fasudil promotes the repair and regeneration of damaged axons induced by TON, and inhibits secondary degeneration of RGCs. This provides a novel way for the clinical treatment of TON.

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Disclosure of conflict of interest

None.

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