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# Combination of fasudil and celecoxib promotes the recovery of injured spinal cord in rats better than celecoxib or fasudil alone

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## Abstract

Resistance mechanisms of rho-associated kinase (ROCK) inhibitors are associated with the enhanced expression of cyclooxygenase-2 (COX-2). The therapeutic effects of ROCK on nervous system diseases might be enhanced by COX-2 inhibitors. This study investigated the synergistic effect of the combined use of the ROCK inhibitor fasudil and a COX-2 inhibitor celecoxib on spinal cord injury in a rat model established by transecting the right half of the spinal cord at $T_{11}$. Rat models were orally administrated with celecoxib (20 mg/kg) and/or intramuscularly with fasudil (10 mg/kg) for 2 weeks. Results demonstrated that the combined use of celecoxib and fasudil significantly decreased COX-2 and Rho kinase II expression surrounding the lesion site in rats with spinal cord injury, improved the pathomorphology of the injured spinal cord, and promoted the recovery of motor function. Moreover, the effects of the drug combination were better than celecoxib or fasudil alone. This study demonstrated that the combined use of fasudil and celecoxib synergistically enhanced the functional recovery of injured spinal cord in rats.

***Key Words:*** *nerve regeneration; Rho kinase; fasudil; cyclooxygenase-2; celecoxib; spinal cord injury; NSFC grant; neural regeneration*

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## Introduction

The most important step in the recovery of central nervous system injury is to promote neurite growth and to re-establish the physical neural network. In recent years, rho-associated kinase (ROCK) has been recognized as a promising new target for central nervous system injury (Cui et al., 2013), as well as being involved in neural development and neural precursor cell migration (Mueller et al., 2005; Leong et al., 2011; Phillips et al., 2012; Chen et al., 2013; Cui et al., 2013). ROCK is an important regulator of cytoskeleton control, cell adhesion, and gene expression (Leung et al., 1996; Mueller et al., 2005; Groysman et al., 2008; Leong et al., 2011; Feng et al., 2012). There are two isoforms of ROCK, which are encoded by two different genes named ROCK I (ROK α or p160 ROCK) and ROCK II (ROK α) (Mueller et al., 2005). ROCK II is preferentially expressed in the nervous system and plays a pivotal role in neurite outgrowth. After binding to its rho-binding domain and unmasking its catalytic domain, active rho proteins (mainly rho A-GTP), which are activated by membrane signals such as adrenergic receptor activation and prostaglandin E receptor activation (Katoh et al., 1998; Sakurada et al., 2003; Broggini et al., 2010), activate ROCK II. These events lead to substrate phosphorylation (mainly myosin light chain) and trigger cytoskeleton contraction (Mueller et al., 2005) that eventually causes neurite collapse and damage to neural functions (Mueller et al., 2005).

It is believed that neurite outgrowth is the physical foundation for neural network construction and remodeling. Many reports have confirmed that ROCK II over-expression and/or over-activation inhibited neurite outgrowth induced by nerve growth factor and other stimuli (Lambrechts et al., 2006; Racchetti et al., 2010). In contrast, the low expression of ROCK II and/or ROCK II inhibition triggered or promoted neurite outgrowth (Katoh et al., 1998; Chan et al., 2008). ROCK up-regulation and/or activation have been observed in inflammation and in many neural disorders, and in animal models ROCK inhibition promoted neural function recovery after nerve damage or brain ischemia/reperfusion injuries (Fournier et al., 2003; Hiraga et al., 2006; Ding et al., 2010).

Our previous study in PC12 cells found that ROCK inhibitor Y27632 decreased noradrenalin synthesis and release whereas nerve growth factor increased these processes (Duan

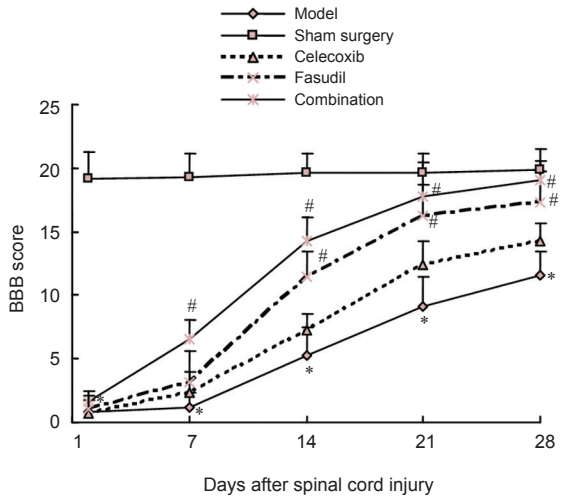


**Figure 1 Effects of fasudil and celecoxib on locomotor behavior in rats with spinal cord injury.**
Rats were evaluated using the BBB rating scale at 1, 7, 14, 21, and 28 days postoperatively. High BBB scores indicate poor motor ability. Data are expressed as the mean ± SD, with eight rats in each group. One-way analysis of variance followed by the least significant difference *post hoc* test was performed to compare the difference between groups. *$P < 0.05$, *vs.* sham surgery group; #$P < 0.05$, *vs.* model group. BBB: Basso-Beattie-Bresnahan locomotor rating scale.

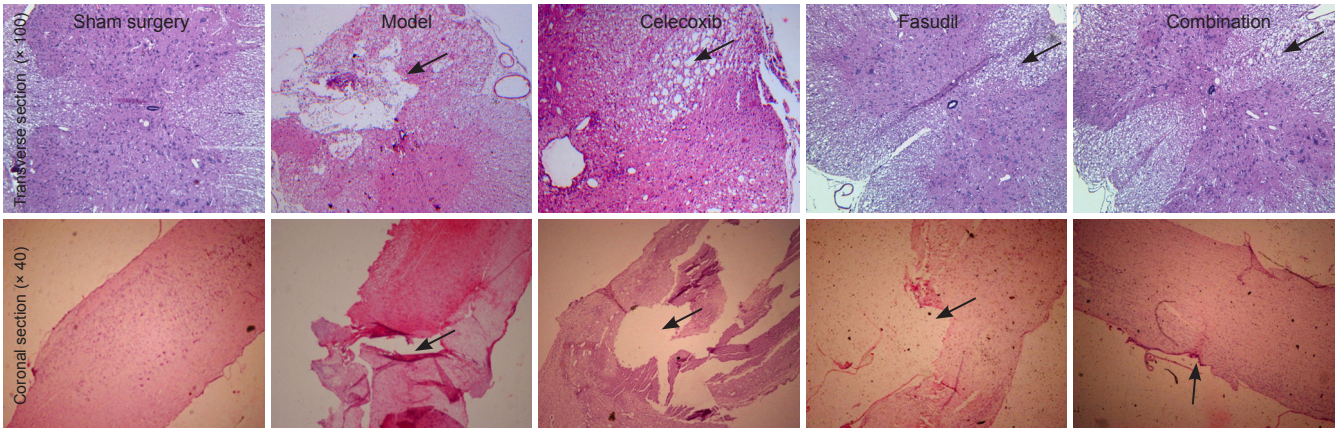


**Figure 2 Effects of fasudil and celecoxib on pathological changes in rat spinal cords at 4 weeks after spinal cord injury (hematoxylin-eosin staining).**
No apparent pathological injuries were detected in the sham surgery group. Cavities were visible in the model group. The cavities were smaller in the celecoxib, fasudil and combination groups compared with the model group. Morphology in the fasudil and combination groups was similar to that in the sham surgery group. Arrows show the injury sites.

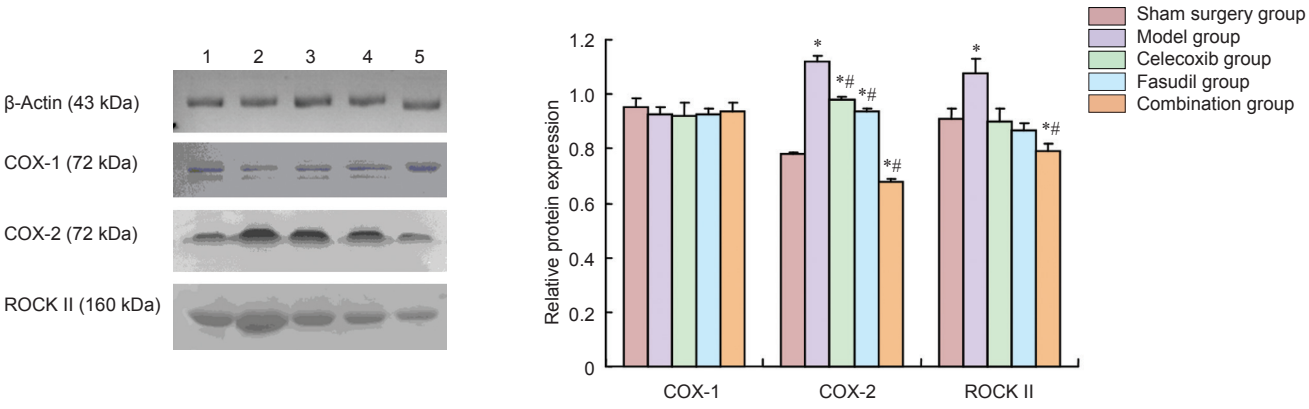


**Figure 3 Effects of fasudil and celecoxib on COX-2, ROCK II and COX-1 expression in injured spinal tissues of rats at 4 weeks after surgery.**
Relative expression was determined as the optical density ratio of the target protein to β-actin. Data are expressed as the mean ± SD ($n = 8$). One-way analysis of variance followed by the least significant difference *post hoc* test was performed to compare the difference between groups. *$P < 0.05$, *vs.* sham surgery group; #$P < 0.05$, *vs.* model group. COX: Cyclooxygenase; ROCK: rho-associated kinase.

et al., 2009). Furthermore, a resistant effect of ROCK inhibitors on neurite outgrowth was observed recently when neural PC12 Adh cells and Neuro-2a cells were exposed to ROCK inhibitors for 3 or more days (Que et al., 2011; Duan et al., 2012). In addition, our previous study indicated the mechanism of resistance was associated with the up-regulation of inflammatory pathways, especially the cyclooxygenase-2 (COX-2) pathway (Duan et al., 2012; Yin et al., 2015), because in cell cultures, the resistant effect was partly improved by a COX-2 selective inhibitor, NS-398 (Duan et al., 2012). This previous evidence suggests that the effect of ROCK inhibitors in treating neurological disorders might be enhanced by COX-2 inhibitors. Therefore, this study investigated the synergistic effect of a ROCK inhibitor fasudil and a COX-2 inhibitor celecoxib to enhance neural function recovery in spinal cord injury of rats.

## Materials and Methods

### Spinal cord hemi-transection

Forty adult, clean, female, Sprague-Dawley rats aged 3 months and weighing 280–330 g, were obtained from Jianyang Shuoda Science and Technology Ltd., China (Certification No. SCXK (Chuan) 2008-24). Rats were housed in temperature-controlled (22°C) and humidity-controlled (45–55%) conditions, under natural light. This project was approved by the Experimental Animal Committee of Yunnan University of Traditional Chinese Medicine in China. Forty rats were randomized to five groups as follows: sham surgery, model, celecoxib, fasudil and combination groups, with eight rats in each group. All rats were anesthetized with pentobarbital sodium 40 mg/kg. The crest of $T_{11}$ was excised with a pair of nipper pliers, and a hole with a diameter of 2 mm was drilled with the cusp of scissors at the center where the crest was located. Then, a syringe needle was gently placed into the spinal cord *via* the hole and the right part of the spinal cord was cut by moving the needle to the right in rats. In the sham surgery group, the needle was placed into spinal cord without movement.

### Drug administration

When the operation was finished, rats in the sham surgery and model groups were treated normally. Rats in the celecoxib group were intragastrically administrated with a suspension of celecoxib (20 mg/kg; Pfizer Inc., Dalian, China), and a suspension of celecoxib containing 0.5% sodium carboxymethylcellulose was made from the capsules. Rats in the fasudil group were intramuscularly administrated with fasudil hydrochloride injection (10 mg/kg; Tianjin Chase Sun Pharmaceutical Co., Ltd., Tianjin, China) *via* the dorsal muscle. Rats in the combination group were administrated with both a suspension of celecoxib (20 mg/kg) and fasudil hydrochloride (10 mg/kg). The fasudil and celecoxib doses were based on doses administered to adults and these were adjusted in a pre-study. Administration was once every day for 2 weeks. Subsequently, all rats were treated normally for another 2 weeks, and then sacrificed either for histological examination or for western blot assay.

### Behavioral analysis

All rats were subjected to behavioral examination preoperatively, and at 1, 7, 14, 21, and 28 days after surgery. The Basso-Beattie-Bresnahan (BBB) locomotor rating scale (Basso et al., 1995) was used to analyze individual components of limb movement, weight support, plantar and dorsal stepping, forelimb-hindlimb coordination, paw rotation, toe clearance, trunk stability, and tail placement. Scores from 0 to 21 were given based on these observations. The BBB scores of normal rats were 21.

### Histological examination

Four rats in each group at 4 weeks after surgery were perfused with 4% paraformaldehyde (pH 7.2) *via* the left ventricle after euthanasia. The vertebral column including the injury site and the surrounding area (2 mm) was harvested, and immersed in 4% paraformaldehyde until a routine histological operation was conducted. Paraffin sections of the spinal cord through the lesion were cut parasagittally or paracoronally (10 μm). Transverse sections were collected from the spinal cord rostral and caudal to the injury site, and coronal sections were also collected from the spinal cord proster and posterior to the injury site. The sections were stained with a hematoxylin-eosin staining kit. Images were obtained with a light microscope (Nikon, Tokyo, Japan).

### Western blot assay

The remaining four rats in each group at 4 weeks after surgery were sacrificed and their spinal cords were carefully removed. Spinal cord at 2.5 mm from the lesion site was discarded. The remaining spinal cord of 5 mm was homogenized with PBS in ice-cold water. The homogenate was centrifuged at 6,000 × *g*, 4°C for 10 minutes and the supernatant was collected as a protein sample for western blot assay as previously described (Fukuda et al., 2005) with some modifications. Briefly, protein (50 μg, 5 μg/μL × 10 μL) of different samples was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein was transferred to a nitrocellulose filter at 10 V for 60 minutes in a Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). The nitrocellulose filter was blocked with 3% bovine serum albumin at room temperature for 2 hours, incubated in a solution containing rabbit anti-rat ROCK II monoclonal antibody (1:400; Merck Millipore Ltd., Darmstadt, Germany), rabbit anti-rat COX-1 monoclonal antibody (1:400; Boster Company, Wuhan, Hubei Province, China), rabbit anti-rat COX-2 monoclonal antibody (1:400; Boster Company) and rabbit anti-rat β-actin monoclonal antibody (1:400; Boster Company) at 4°C overnight. The filter was rinsed three times with Tris-HCl buffer with Tween-20 solution (20 mM Tris-HCl, pH 7.5, 0.05% Tween-20) each for 10 minutes, and incubated in a solution containing goat anti-rabbit antibody linked with horseradish peroxidase (1:800; Boster Company) for an additional 2 hours. The nitrocellulose filter was rinsed with Tris-HCl buffer and Tween-20 solution for 10 minutes three times and developed on X-ray films by enhanced chemiluminescence detection kits (Pierce Biotechnology Inc.,

Rockford, IL, USA). The X-ray films were scanned by a gel imager (Bio-Rad, Hercules, CA, USA). The results were expressed as the optical density ratio to β-actin.

**Statistical analysis**

Data are expressed as the mean ± SD. One-way analysis of variance followed by the least significant difference *post hoc* test was performed to compare the difference between groups. A value of $P < 0.05$ was considered statistically significant. The statistical analysis was conducted with SPSS for Windows 16.0 (SPSS, Chicago, IL, USA).

## Results

**Combined administration of fasudil and celecoxib improved locomotor behavior of rats with spinal cord injury**

The body weight of rats after surgery increased slowly. Rat locomotor activities in the celecoxib, fasudil and combination groups were similar to that of the model group immediately after injury. However, the recovery of rats in the celecoxib, fasudil or combination groups was increased compared to controls ($P < 0.05$). The recovery of rat locomotor activity was enhanced in the combination group compared with the model, celecoxib and fasudil groups (**Figure 1**).

**Effects of fasudil and celecoxib on pathological changes in rats with spinal cord injury**

Hematoxylin-eosin staining in transverse sections showed that no micropathological injury was visible in the sham surgery group rats. However, abundant cell death was observed in the damaged spinal tissue in rats from the model group. The damaged spinal tissue was recovered to a certain extent in the celecoxib, fasudil, and combination groups. The recovered tissues in rats of the fasudil and combination groups were similar to those in the sham surgery group, but with the presence of slight cellular edema and inflammation (**Figure 2**).

**Effects of fasudil and celecoxib on protein expression of COX-2, ROCK II, and COX-1 in injured spinal cord tissues of rats**

The results of the western blot assay showed that at 28 days after spinal cord injury, the expression of COX-2 and ROCK II in injured spinal tissues was up-regulated in the model group, followed by the celecoxib group, fasudil group and combination group (**Figure 3**). The expression profiling of COX-2 in injured spinal tissues was similar to that of ROCK II. COX-1 is a constitutively expressed molecule and its expression in injured spinal tissues was maintained at a stable level in all the different groups.

## Discussion

That ROCK inhibitors are promising agents for neurological disorders, and have been widely accepted by pharmacologists (Mueller et al., 2005; Chen et al., 2013). However, their therapeutic effect was further enhanced in a spinal cord injury model (Hiraga et al., 2006) and in our previous studies (Que et al., 2011; Duan et al., 2012).

In the present study, fasudil was intramuscularly administrated and celecoxib was orally administrated. The only ROCK inhibitor licensed for clinical use is fasudil and this is administered *via* injection (fasudil is not stable enough for oral administration). Most COX-2 inhibitors are prepared orally. Ideally, the delivery route of both drugs should have been the same. However, as the main aim of our study was to indicate the clinical application of the drugs, we consider the effects of different delivery routes to be negligible. The different ways of drug delivery could, however, introduce some interference in the therapeutic effect. However, we consider these effects to be negligible as the celecoxib group and fasudil group are control groups for the combined group. The beneficial synergistic effects can be found by comparing the combined group with the fasudil group and celecoxib group.

Other studies demonstrated that spinal cord injury causes inflammatory reactions that prevent spinal cord recovery and the up-regulation of COX-2 and ROCK II (Schäfers et al., 2004; Conrad et al., 2005). These evidences were confirmed by the present study. Based on these findings, it could be deduced that either COX-2 or ROCK inhibition might promote the recovery of spinal cord injury, and this was confirmed by Schafers's group (Schäfers et al., 2004) and Conrad's group (Conrad et al., 2005), respectively. Furthermore, Schwab et al. (2004) suggested that COX inhibitors might be useful for the treatment of spinal cord injury, and are candidates for a safe, synergistic, adjuvant treatment option in combination with cell-specific approaches of rho inactivation. This would minimize the pool of $rhoA^+$ (whose effector protein is ROCK) cells at the lesion site following spinal cord injury (Schwab et al., 2004). However, the synergestic effect of a COX inhibitor and ROCK inhibitor in improving spinal cord injury has not been confirmed directly. In the present study, the combined application of a ROCK inhibitor (fasudil) and a COX-2 inhibitor (celecoxib) confirmed the synergestic effect, because behavioral scores of the combination group were higher than those of the other groups except the sham surgery group. The results of histological examination also supported the synergestic effect. The effect of the ROCK inhibitor in enhancing spinal cord injury (Hiraga et al., 2006) was confirmed in the present study. However, slightly different from a previous report (Schwab et al., 2004), there was a trend in the beneficial effect of celecoxib, but it was not statistically significant.

Our previous study (Duan et al., 2012) suggested that: (1) inflammatory reactions might cause the over-expression of COX-2 and other bio-macromolecules; (2) COX (especially COX-2) would catalyze arachidonic acid from membrane phospholipids to prostaglandins (PGs), including thromboxane A2, PGF2α, and PGE2; (3) these PGs might diffuse out of the cell to act on neural cells and glial cells; (4) PGs activated their membrane receptors whose effector protein was ROCK II; (5) receptor activation would result in the contraction of the cytoskeleton; and (6) that eventually, cytoskeleton contraction would cause neurite collapse. In the present animal model, two factors, inflammation and the

prolonged use of a ROCK inhibitor, might have contributed to COX-2 over-expression (Schäfers et al., 2004; Duan et al., 2012; Yin et al., 2015). However, it was difficult for us to distinguish between these mechanisms in the animal model. Nevertheless, a beneficial synergistic effect was observed after the combined use of ROCK inhibitor fasudil and COX-2 inhibitor celecoxib.

In summary, the combined use of ROCK inhibitor fasudil and celecoxib synergistically enhanced the functional recovery in spinal cord injury in rats.

**Author contributions:** *WGD conceived and designed the study as well as analyzed and interpreted the data. YC carried out histological examination. HY performed western blot assay. WGD wrote the paper. All authors performed animal experiments and approved the final version of the paper.*
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