해면체 신경 손상 발기부전 백서 모델에서 LIMK2 억제제의 음경해면체 섬유화 차단 효과를 통한 발기력의 호전

Role of LIM-kinase2 inhibition in Improvement of erectile function through suppression of corporal fibrosis in a rat model of cavernous nerve injury

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ABSTRACT

Role of LIM-kinase2 inhibition in Improvement of erectile function through suppression of corporal fibrosis in a rat model of cavernous nerve injury

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Purpose: To determine whether LIM-kinase2 inhibitors (LIMK2i) could improve erectile function by suppression of corporal fibrosis through normalization of ROCK1/LIMK2/Cofilin pathway in a rat model of CN crush injury (CNCI).

Materials and Methods: Sixty 10-week-old male Sprague-Dawley rats were divided equally into five groups: sham surgery (S), CNCI (I), and CNCI treated with low-dose (L), medium-dose (M) and high-dose (H) LIMK2i. The L, M and H groups was treated with daily intraperitoneal injection of LIMK2i (2.5, 5.0 and 10.0 mg/kg, respectively) for 1-week from the following day after surgery. Erectile response was assessed using electrostimulation at 1-week postoperatively. Penile tissue was processed for Masson's trichrome staining, double immunofluorescence and Western blotting.

Results: The I group showed significantly lower intracavernous pressure (ICP)/mean arterial pressure (MAP) and lower area under the curve (AUC)/MAP than the S group. The erectile responses in the H group improved compared to the I group, while the M group showed only

partial improvements. Significantly decreased smooth muscle/collagen ratio and increased content of fibroblasts positive for phospho-LIMK2 were noted in the I group. The I group showed the increase in ROCK1 protein expression, LIMK2 phosphorylation and Cofilin phosphorylation. The M and H groups showed significant improvements in the histological alterations and the dysregulated LIMK2/Cofilin pathway, except for LIMK2 phosphorylation in the M group. The LIMK2 inhibition did not affect the ROCK1 protein expression. The content of fibroblasts positive for phospho-LIMK2 in the H group returned to the level found in the S group, while it did not in the M group. However, the L group did not show their improvements.

Conclusions: Our data suggest that inhibition of LIMK2, particularly with administration of 10.0 mg/kg LIMK2i, can improve corporal fibrosis and erectile function by normalizing LIMK2/Cofilin pathway.

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Introduction

Corporal fibrosis after cavernous nerve injury during radical prostatectomy

Despite the continuous optimization of nerve-sparing techniques during radical prostatectomy (RP), the prevalence of post-RP erectile dysfunction (ED) has been reported to range from 20% to 90%.[1-4] A previous study showed that even visualization of the cavernous nerve (CN) without direct injury to CN resulted in rats' impaired erectile responses, indicating that even the most minimal damage to CNs might lead to at least short-term erectile impairment.[5] Thus, neuropraxia caused by traction, compression and minimal manipulation during RP can have adverse effects on recovery of erectile function in a short-term period after RP.

A temporary block of neurotransmission due to CN injuries leads to persistence of a flaccid penis and, thereby, low oxygen supply to the penis during the early postoperative period after RP, which results in structural alterations of the corpus cavernosum such as corporal fibrosis through the TGF-β-driven fibrotic pathway.[6-9] Thus, CN injury is a detrimental factor in the process of corporal fibrosis[10], where the latter plays a critical role in the development of corporal veno-occlusive dysfunction (CVOD) which is a main pathophysiological factor of ED after RP. However little is known about the molecular mechanisms leading to corporal fibrosis.

ROCK1/LIMK2/Cofilin pathway in corporal fibrosis

Recently, we demonstrated that the RhoA/ROCK1/LIM-kinase 2 (LIMK2)/Cofilin pathway was involved in corporal fibrosis with a loss of smooth muscle (SM) through coordination with TGF-β/sphingosine-1-phosphate signaling after CN injuries (Fig.1).[11,12] Furthermore, early inhibition of Rho-kinase, an upstream molecule of LIMK2 in the ROCK1/LIMK2/Cofilin pathway, after CN crush injury (CNCI) could prevent corporal apoptosis and fibrosis by suppressing the Akt-driven and ROCK1/LIMK2/Cofilin pathways, preventing both CVOD and ED.[13] However, given

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the risk for significant adverse effects of the ROCK inhibitor, inhibition of LIMK2, a down-stream target of Rho-kinase, could be a reasonable strategy for the treatment of corporal fibrosis after CN injury. Selectively inhibiting a downstream pathway of ROCK such as LIMK2/Cofilin would be better than targeting ROCK itself, analogous to a surgeon's scalpel being better than a chisel.[14] Fig.1)

Thus, the aim of this study was to determine whether inhibition of LIMK2 could improve erectile functions through normalizing the dysregulated ROCK1/LIMK2/Cofilin pathway related to corporal fibrosis after RP in a rat model of CNCI.

Materials and methods

Study Design

The experiments were performed in accordance with the Institutional Animal Care and Use Committee of the Clinical Research Institute at our hospital, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. All rats were cared for in accordance with the National Research Council guidelines for the care and use of laboratory animals.

To determine treatment effects of LIMK2 inhibitor (LIMKi) on CNCI-induced corporal fibrosis and ED, sixty 11-week-old male Sprague Dawley rats, weighing 300 - 350 g, were categorized into five equal experimental groups (n = 12 each): sham surgery, bilateral CNCI, and bilateral CNCI treated with intraperitoneal injection of low-dose (2.5 mg/kg), medium-dose (5.0 mg/kg) and high-dose (10.0 mg/kg) LIMK2 inhibitor (LIMK2i: LX-7101, Cellagen Technology, USA), respectively.[15,16] The Low-dose, Medium-dose, and High-dose LIMK2i groups were treated with once-daily intraperitoneal injections of LIMK2i for one week beginning on the day following CNCI. The Sham and Crush Injury groups were treated with intraperitoneal administration of vehicle only (saline).

The rats were anesthetized with an intraperitoneal injection of zoletil (10 mg/kg) and an isoflurane inhalation. In the Crush Injury group, which mimicked a clinical context of nerve-sparing RP, the CNs were crushed by applying a microsurgical vascular clamp at a location 2 - 3 mm distal to the major pelvic ganglion for 70 seconds, and then re-applied additional 70 seconds after a 35-second rest periods.[13] Both CNs were just exposed by an identical procedure of pelvic dissection in the Sham group, without any direct nerve damage.[13] The CNCIs were produced by the same trained surgeon. Six rats in each group were evaluated for erectile function. The remaining six rats in each group were sacrificed without nerve stimulation or intracavernous cannulation to avoid inadvertent changes in histological staining or western blotting.

Assessment of Erectile Functions

After a 48-hour washout period, in vivo erectile function was assessed in anesthetized rats using erectile responses to electrical stimulation of the CNs at one week after surgery, as described previously.[13] In brief, the carotid artery was cannulated using a 24-gauge angiocatheter for continuous monitoring of the mean arterial pressure (MAP). A 26-gauge needle connected to a pressure transducer was introduced into the corpus cavernosum for continuous monitoring of intracavernous pressure (ICP). After identification of the CN, it was stimulated using a platinum bipolar electrode, and the erectile responses were evaluated by increasing voltage settings. The stimulation parameters included 1.0, 2.5, and 4.0 V at 15 Hz with a square wave duration of 0.2 ms for 30 seconds. Evaluation parameters were ICP and the area under the curve (AUC) corresponding to the duration of electrical stimulation, normalized with MAP.

After completion of the functional studies in six rats in each group, the whole penis of the remaining six rats in each group was harvested for histological studies and western blotting. The middle part of the skin-denuded penile shaft was maintained overnight in a 10% formaldehyde solution and wax-embedded paraffin. The remaining penile tissues were rapidly frozen in liquid nitrogen and stored at -80°C until further processing.

Masson's Trichrome Staining

To calculate the SM/collagen ratio, the specimens were stained for Masson's trichrome according to a standard protocol, as described previously.[13] For each stained slide, a ×40 magnification image of the penis comprising the corpora cavernosum half was analyzed for SM cells (stained in red) and collagen (blue), using the Image Pro Plus 4.5 software (Medica Cybernetics, Silver Spring, MD, USA). Six rats from each group were evaluated, and two tissue sections per animal were reviewed. The slides were evaluated by an independent observer who was blinded to the group allocation.

Double Immunofluorescence for Confocal Laser Microscopy

To assess the content of fibroblasts positive for phosphorylated LIMK2 (co-localization of vimentin with phosphorylated LIMK2), to identify whether LIMK2 phosphorylation in the fibroblasts could contribute to corporal fibrosis and to evaluate the efficacy of LIMK2 inhibition in the cavernous tissue, double immunofluorescence microscopy was performed using paraffin embedded sections (2.5μm) of penile tissues, as described before.[13] They were incubated overnight with primary antibodies to phospho-LIMK2 (phospho T505) (1:50, AbCam) and vimentin (1:100, Dako), a fibroblast marker. After PBS-washing, the sections were incubated with two secondary antibodies (goats' anti-rabbit IgG 594 and anti-mouse IgG 488) in 1% BSA for 1 hour at room temperature. Digital images were obtained using a confocal microscope (Leica TCS SP8, Leica Microsystems, Germany). Six rats from each group (two sections per animal) were analyzed. The slides were evaluated by an independent observer who was blinded to the group allocation.

Western Blot Analysis

Western blot analyses were performed as described previously.[11-13] Primary antibodies included anti-ROCK1 (1:2,000, Cell-Signaling Technology, USA), anti-phospho-LIMK2 (phospho T505) (1:1,000, Abcam), anti-LIMK2 (1:2,000, Abcam), anti-phospho-Cofilin (1:500, Cell-Signaling Technology) and anti-Cofilin (1:1,000, Cell-Signaling Technology). Densitometry results were normalized to β-actin expressions.

Statistical Analysis

All data are presented as means ± standard error of the mean (SEM). Statistical comparisons among groups were performed using the Mann-Whitney U-test or the Kruskal-Wallis test. The p-values were two-sided with $p < 0.05$ considered to be statistically significant. The Statistical Package for the Social Sciences, Version 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp.) was used for analysis.

Results

Effect of bilateral CNCI on erectile response, corporal fibrosis, and the ROCK/LIMK2/Cofilin pathway

There was no significant difference in baseline MAP and changes of body weight among the experimental groups. Compared with the Sham group, the Crush Injury group showed both lower ICP/MAP (1.0 V; p = 0.004, 2.5 V; p = 0.004 4.0 V; p = 0.004) and AUC/MAP (1.0 V; p = 0.002, 2.5 V; p = 0.004 4.0 V; p = 0.002) at all stimulation voltages one week after surgery (Fig. 2), which was also the case for the SM/collagen ratio compared with the Sham group (p = 0.008), according to Masson's trichrome staining (Fig. 3). The densitometry revealed that ROCK1 protein expression (p = 0.004), LIMK2 phosphorylation (p < 0.001) and cofilin phosphorylation (p < 0.001) in the Crush Injury group were significantly increased compared with that of the Sham group (Fig. 4-6). The double immunofluorescent staining of cavernous tissue with antibodies to phospho-LIMK2 and vimentin demonstrated that the content of fibroblasts positive for phospho-LIMK2 in the Crush Injury group was also significantly higher than that in the Sham group (p < 0.001) (Fig. 7).

Daily administration of LIMK2 inhibitors improves the erectile response in a rat model of bilateral CNCI

To verify the physiological relevance of administration of LIMK2i, in vivo erectile responses to electrical stimulation of the CNs were determined. In the Low-dose LIMK2i group, daily intraperitoneal administration of 2.5 mg/kg LIMK2i did not improve the erectile responses, which were not different from those in the Crush Injury group at any stimulation voltage. In the Medium-dose LIMK2i group, ICP/MAP obtained with 4.0 V stimulation (p = 0.017) and AUC/MAP with electrical stimulation (1.0 V; p = 0.009, 4.0 V; p = 0.009) were significantly increased compared with the Crush Injury group, but AUC/MAP with 1.0 or 4.0 V stimulation did not recover to the

level determined in the Sham group. Additionally, ICP/MAP obtained with 1.0 or 2.5 V stimulation and AUC/MAP with 2.5 V stimulation in the Medium-dose LIMK2i group were not significantly different from than those in the Crush Injury group. In the High-dose LIMK2i group, daily intraperitoneal administration of 10.0 mg/kg LIMK2i significantly increased the ICP/MAP (1.0 V; p = 0.004, 2.5 V; p = 0.015 4.0 V; p = 0.004) and AUC/MAP (1.0 V; p = 0.004, 2.5 V; p = 0.041 4.0 V; p = 0.004) at all stimulation voltages compared with the Crush Injury group. However, the values of AUC/MAP at all stimulation voltages in the High-dose LIMK2i group did not completely return to those found in the Sham group, the age-matched control.

Inhibition of LIMK2 alleviates corporal fibrosis through normalization of ROCK1/LIMK2/Cofilin pathway

At one week after surgery, the SM/collagen ratios in the Medium-dose (p = 0.016) and High-dose LIMK2i groups (p = 0.008) were significantly higher than that in the Crush Injury group, but lower than that in the Sham group (Medium-dose LIMK2i group; p = 0.008, High-dose LIMK2i group; p = 0.016) (Fig. 3). Additionally, the content of fibroblasts positive for phospho-LIMK2 in the Medium-dose (p = 0.026) and High-dose LIMK2i groups (p = 0.001) were significantly reduced as compared with the Crush Injury group (Fig. 7). On the contrary, daily administration of 2.5 mg/kg LIMK2i in the Low-dose LIMK2i group did not improve the SM/collagen ratio or the extent of LIMK2 phosphorylation in fibroblasts.

According to the densitometry, LIMK2 phosphorylation in the High-dose LIMK2i group was significantly reduced compared with that in the Crush Injury group (p = 0.035), while Cofilin phosphorylation was lowered in both the Medium-dose (p = 0.027) and High-dose LIMK2i groups (p = 0.043). However, LIMK2 and Cofilin phosphorylation in the Low-dose LIMK2i group did not significantly differ from those in the Crush Injury group. Inhibition of LIMK2 in the Low-dose, Medium-dose, and High-dose LIMK2i groups did not reduce the protein expression of ROCK1 compared with the Crush Injury group (Fig. 4-6).

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Discussion

The main findings of current study

To our knowledge, the present study is the first to demonstrate that the inhibition of LIMK2, particularly with administration of 10.0 mg/kg LIMK2i, could improve erectile function by suppression of corporal fibrosis via normalization of the LIMK2/Cofilin pathway in a rat model of CNCI. The main findings of this study are summarized as follows: 1) It was confirmed that the ROCK1/LIMK2/Cofilin signaling pathway could contribute to corporal fibrosis after CNCI. 2) Inhibition of LIMK2 with 10.0 mg/kg LIMK2i from the immediate post-injury period significantly alleviated corporal fibrosis by normalizing the ROCK1/LIMK2/Cofilin signaling pathway and the content of fibroblasts positive for phosphorylated LIMK2. The inhibition of LIMK2 with 5.0 mg/kg LIMK2i partially improved the dysregulated LIMK2/Cofilin pathway and the increased content of fibroblasts positive for phosphorylated LIMK2. 3) Daily administration of 10.0 mg/kg LIMK2i significantly mended erectile functions compared with the vehicle treatment, while the administration of 5.0 mg/kg LIMK2i partially rectified erectile functions.Additionally, the present study demonstrated, to some degree, dose-escalation effects of early treatment with LIMK2i on corporal fibrosis and erectile dysfunctions caused by CNCI.

ROCK1/LIMK2/Cofilin pathway in corporal fibrosis

Although corporal fibrosis caused by CN injury plays a critical role in the development of post-RP ED, there has been a scarcity of studies about specific pathways responsible for the fibrosis of cavernosal tissues after CN injury or whether the restoration of specific dysregulated pathways can improve erectile functions by suppression of corporal fibrosis. Thus, there is still room for improving the alleviation of post-RP ED through suppression of specific target molecules that contribute to corporal fibrosis after CN injury. The ROCK pathway is important in the progression of TGF-β-induced vascular fibrosis in cardiovascular disease.[17] Activation of LIMK2, a down-stream effector of ROCK, induces cytoskeletal rearrangements through Cofilin phosphorylation,

which results in a fibroblast-to-myofibroblast differentiation, a pathophysiological feature of fibrosis.[18] In this context, we considered the ROCK1/LIMK2/Cofilin pathway as a potential candidate that can cause corporal fibrosis. Recently, we also revealed that fibroblasts positive for ROCK1 or phosphorylated LIMK2 significantly increased in cavernosal tissue from the early post-CNCI period.[12] Furthermore, the present study confirmed that the ROCK1/LIMK2/Cofilin pathways might play a critical role in corporal fibrosis caused by CNCI, which corroborated our previous findings.[11,12]

The role of LIMK2 inhibitor

A fibrogenic response may have adaptive features during a very short period.[19] However, when it progresses over a prolonged period of time, parenchymal scarring and, ultimately, cellular dysfunction or organ failure can ensue.[19] Previous reports using rat models on bilateral CN injuries showed that corporal fibrosis developed from early post-injury period and progressed over time.[11,12,20] Therefore, after structural alterations such as the progressive fibrosis of cavernosal tissues are developed, erectile functions are unlikely to recover despite an improvement from neuropraxia, a finding possibly supported by previous research showing that corporal fibrosis was not alleviated by a single sildenafil administration at various time points after CN injury.[21] One of our previous studies demonstrated that an inhibition of the Rho-kinase/LIMK2/Cofilin pathway with daily administration of Rho-kinase inhibitors at an early post-CNCI period improved corporal fibrosis and CVOD.[13] However, for improvement in erectile function through alleviation of corporal fibrosis, the specific inhibition by a downstream effector of a fibrosis-related pathway such as LIMK2 may have benefits in terms of both efficacy and safety. Thus, we hypothesized that early intervention with LIMK2 inhibitors beginning from the immediate post-CNCI period would increase the chance of improvement in erectile functions by corporal fibrosis suppression. In accordance with our hypothesis, the present study noted that early LIMK2 inhibition could improve erectile functions through the alleviation of corporal fibrosis. Similarly, a prior in vitro

investigation revealed that down-regulation of LIMK by direct application of LIMK siRNA in corneal fibroblasts suppressed TGF-beta mediated ocular fibrosis.[22] Additionally, a previous report by Morin et al. showed that selective inhibition of LIMK effectively blocked TGF-ß-induced cell motility or invasion and indicated that LIMK represented an attractive therapeutic target in TGF-ß-induced organ fibrosis.[14] Preclinical research utilizing a mouse model of glaucoma revealed that LIMK2i (LX7101) significantly reduced intraocular pressure by inducing depolymerization of actin filaments in the trabecular meshwork, resulting in tissue relaxation.[23]

Rho Kinase inhibitors vs. LIMK2 inhibitors

Rho kinase inhibitors are a series of compounds that interfere with the RhoA/ROCK pathway.[24] Fasudil, a potent Rho-kinase inhibitor, is used for improvement and prevention of cerebral vasospasm and cerebral ischemia after subarachnoid hemorrhage surgery in China and Japan.[25] However, it is not approved by the USA and Europe. Ripasudil, a Rho-kianse inhibitor eye drop, is used for the treatment of glaucoma and ocular hypertension in Japan.[26] However, the Rho-kinase inhibitors have not yet been approved by the USA and Europe. The Rho-kinase inhibitors have potential adverse effects such as systemic vasodilation or hypotension.[27-29] Thus, selective inhibition of LIMK, a downstream effector of ROCK, would be better than targeting ROCK itself in terms of both efficacy and safety.[14] LIMK is a serine protein kinase that plays a critical role in the regulation of the actin cytoskeleton by phosphorylating and deactivating cofilin.[30] The LX7101, a pyrrolopyrimidine-based inhibitor of LIMK2, has been recently developed.[23] A randomized, double-blind, placebo-controlled Phase 1/2a trial of topically administered LX7101 for patients with open angle glaucoma or ocular hypertension is ongoing. [31]

LX7101, the novel class of LIMK2 inhibitor

Interestingly, the LX7101 is reported to be a dual LIMK2 and ROCK inhibitor.[23] According to the densitometry of our study, the administered dose of 10 mg LX-7101 in our study tended to

suppress the increase of ROCK1 expression. However, there was no statistically significant suppression of the increased ROCK1 expression. Meanwhile, 10 mg LX7101 significantly improved the dysregulated LIMK2 phosphorylation. There are some plausible explanations for it. First, the LX7101 is thought to be a potent inhibitor of LIMK2, although we cannot rule out the possibility that some of the observed activity is due to weak inhibition of ROCK.[23] This is supported by a recent finding showing that the LX7101 has proved significantly selective for LIMK2 (300-fold vs. ROCK1).[23] Second, there is a possibility that the administered dose of 10 mg LX7101 in our study is not sufficient for significant suppression of ROCK1 expression.

Suggestions of future research

According to our results, the inhibition of LIMK2 by administering LIMK2i improved erectile function, but could not completely restore it to normal values. Additionally, the SM/collagen ratio was significantly corrected; however it did not recover to the point of control values despite the normalization of fibroblasts' content positive for phosphorylated LIMK2 by administration of 10.0 mg/kg LIMK2i. On the basis of the incomplete erectile functions' recovery observed in our study, this indicates that other structural alterations including corporal apoptosis may also contribute to the development of post-RP ED. Moreover, a possible explanation for the SM/collagen ratio's incomplete recovery in the cavernosum is that other molecular pathways such as the Smad pathway might play a role in corporal fibrosis after CN injury.[32] Thus, further research is needed to explore whether a combination of LIMK2i with anti- apoptotic agents or anti-fibrotic agents targeting other fibrotic pathways can restore erectile function to control values. Furthermore, additional time-course studies with long-term follow-ups using dynamic infusion cavernosometry might be necessary to determine the effect of chronic treatment with LIMK2i on CVOD caused by CN injury.

Limitations of this study

A limitation of our study is that although both ROCK1 and ROCK2 are expressed in the penis, expression of cavernosal ROCK2 was not evaluated. Several studies have suggested a potential role for RhoA/ROCK1 signaling pathway in the development of fibrosis.[33,34] We also noticed that the activation of RhoA is followed by the activation of downstream target kinases, such as ROCK1, and subsequently accompanied by phosphorylation of the downstream effectors LIMK2 and cofilin, resulting in fibroblast-to-myofibroblast differentiation, a pathophysiologic feature of fibrosis.[11,12] However, previous studies reported that ROCK2 was increased after CN injury.[35,36] Therefore, expression of ROCK2 needs be evaluated in future studies.

Another limitation is that vimentin can be stained in macrophages and SM cells of mesenchymal origin as well as fibroblasts. Thus, although previous studies have used vimentin as a fibroblast marker, the subsequent studies need to use a more specific marker of fibroblasts or additional protocols such as flow cytometry for sorting vimentin-positive cells into fibroblasts.

Additionally, although the inhibition of LIMK2 could improve the maximal ICP/MAP in a similar level to sham control, it did not normalize the AUC/MAP (maintenance of erection) to the level observed in sham control. Thus, the maximal ICP/MAP was increased, but the animals were unable to maintain an erection.

Lastly, it was also thought as a limitation that LIMK2 inhibitor which had been developed as a local agent, was systemically used as intraperitoneal injection. However, the examples of systemic LIMK inhibition were found in the cancer related researches, despite the systemic use, there was no side effect such as hypotention.[37,38] When studying with large animals or people in the future, using the LIMK2 inhibitor as local injection, it is possible to reduce the systemic side effects and increase the drug efficacy.

Nevertheless, using a rat model of CNCI that could approximate a clinical situation in human males undergoing nerve-sparing RP, this study's results suggest the inhibition of LMK2 as an early treatment strategy, at least as a part of combination therapy, for improving post-RP ED through suppressing the corporal fibrosis caused by CN injury.

Conclusions

Our data suggest that the inhibition of LIMK2 beginning from the early postoperative period, particularly with administration of 10.0 mg/kg LIMK2i can improve corporal fibrosis and erectile functions by normalizing the LIMK2/Cofilin pathway in a rat model of CNCI, although the subjects did not completely recover to normal values. Thus, an early therapeutic strategy targeting the LIMK2/Cofilin pathway may alleviate corporal fibrosis caused by CN injury, resulting in improvement of post-RP ED. Future time-course studies with long-term follow-ups for determining treatment effects of LIMK2 inhibition combined with anti-apoptotic agents or anti-fibrotic agents targeting other fibrotic pathways are needed to render our results more clinically useful.

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Figure 1. The role of RhoA/ROCK1/LIMK2/Cofilin signaling pathway in the corporal fibrosis after CN injury. CN = cavernous nerve.
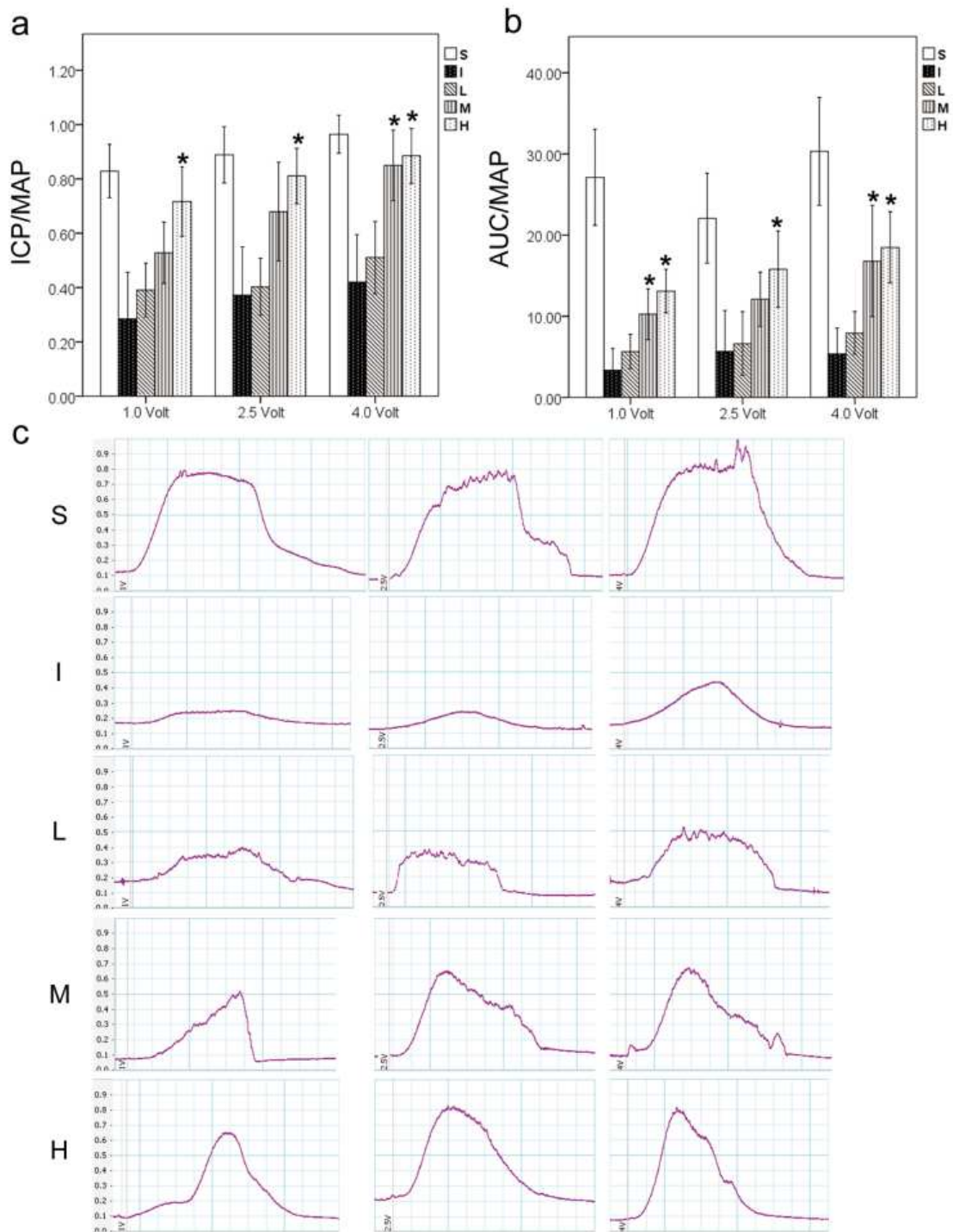
Figure 2. Bar graphs showing the comparison in erectile responses to electrostimulation at 1 week after bilateral CNCI among the five groups (S, I, L, M and H). (a) ICP/MAP (b) AUC/MAP (c) Representative trace of ICP/MAP. Six rats in each group were evaluated for erectile function. CNCI = cavernous nerve crush injury, ICP/MAP = intracavernous pressure/mean arterial pressure,

AUC/MAP = area under the curve corresponding to the duration of electrical stimulation/mean arterial pressure, S = sham surgery group, I = bilateral CNCI group, L = bilateral CNCI group treated with low-dose LIMK2 inhibitors, M = bilateral CNCI group treated with medium-dose LIMK2 inhibitors, H = bilateral CNCI group treated with high-dose LIMK2 inhibitors. * $p < 0.05$ vs. bilateral CNCI (I) group.
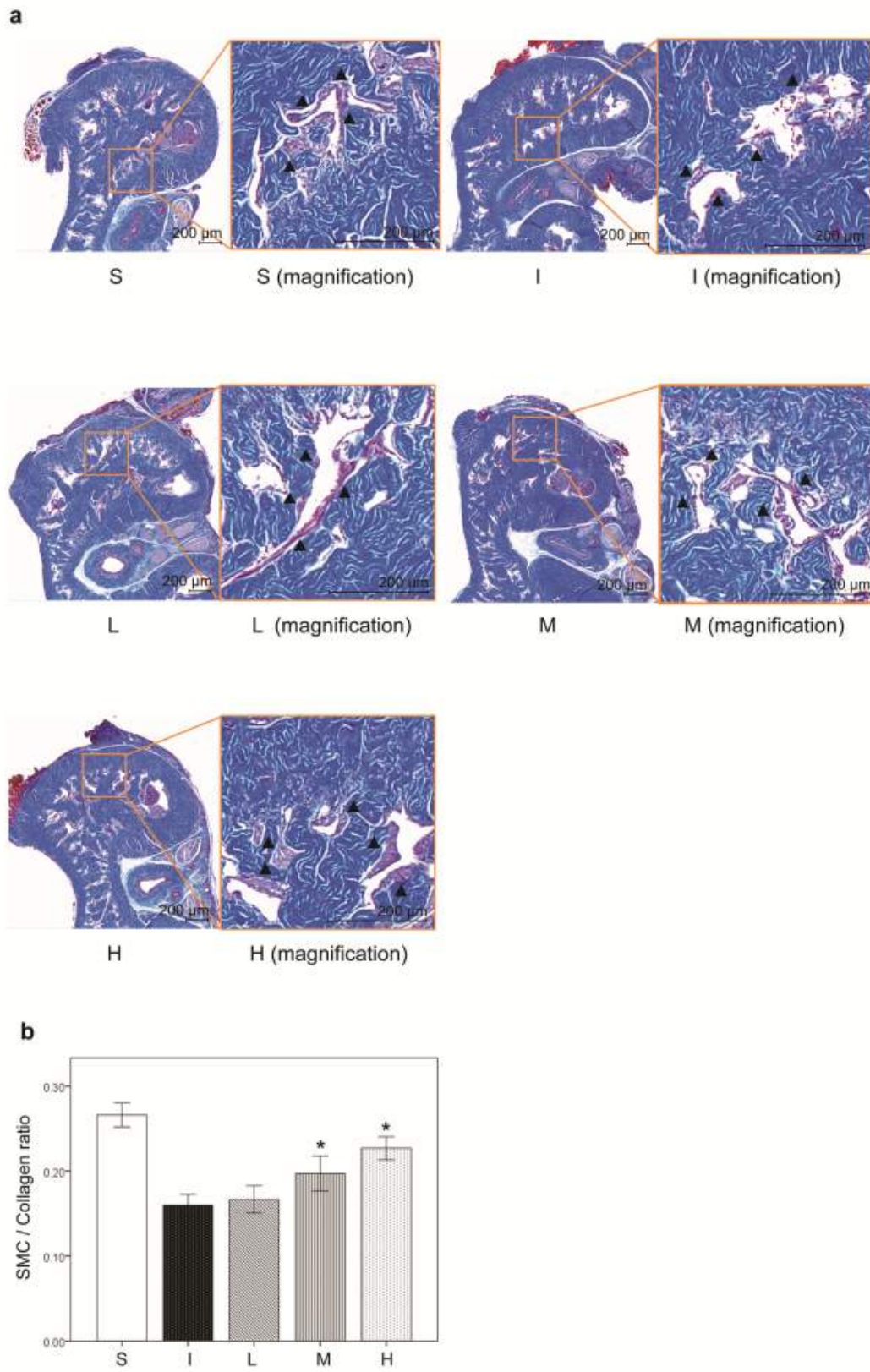
Figure 3. Effect of bilateral CNCI and LIMK2 inhibition on corporal fibrosis after 1 week of

surgery, as evaluated by smooth muscle cell/collagen ratio. (a) Representative images for Masson's trichrome staining and (b) bar graphs showing the smooth muscle cell/collagen ratio (mean ± SEM) among the five groups at 1 weeks after surgery. Smooth muscle and collagen fibers were stained in red and blue, respectively. Magnification images and black short arrows indicated the smooth muscle contents. Six rats in each group were analyzed for smooth muscle cell/collagen ratio. CNCI = cavernous nerve crush injury, S = sham surgery group, I = bilateral CNCI group, L = bilateral CNCI group treated with low-dose LIMK2 inhibitors, M = bilateral CNCI group treated with medium-dose LIMK2 inhibitors, H = bilateral CNCI group treated with high-dose LIMK2 inhibitors. * $p < 0.05$ vs. bilateral CNCI (I) group.
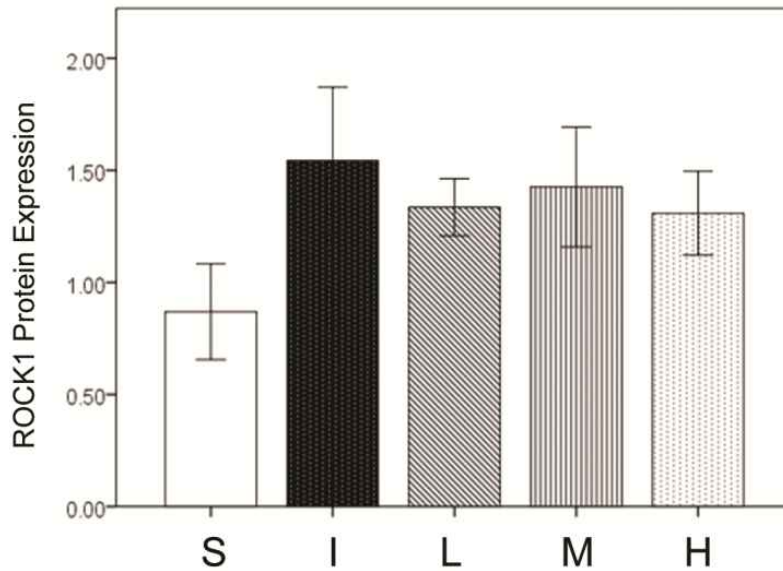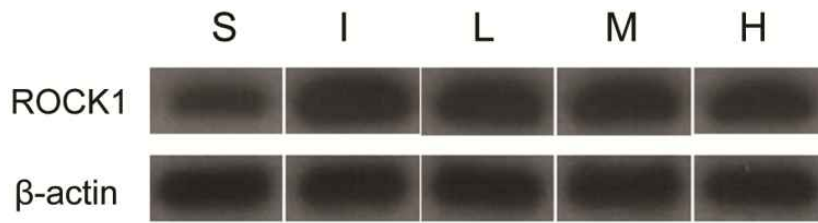
Figure 4. Representative immunoblot images and bar graphs (mean ± SEM) showing the comparison in the protein expression of ROCK from the cavernosal tissues among the five groups using densitometry. The results were normalized by β-actin expression and presented as fold changes over controls. Six rats in each group were analyzed by densitometry. S = sham surgery group, I = bilateral CNCI group, L = bilateral CNCI group treated with low-dose LIMK2 inhibitors, M = bilateral CNCI group treated with medium-dose LIMK2 inhibitors, H = bilateral CNCI group treated with high-dose LIMK2 inhibitors, CNCI = cavernous nerve crush injury. * p < 0.05 vs. bilateral CNCI (I) group.
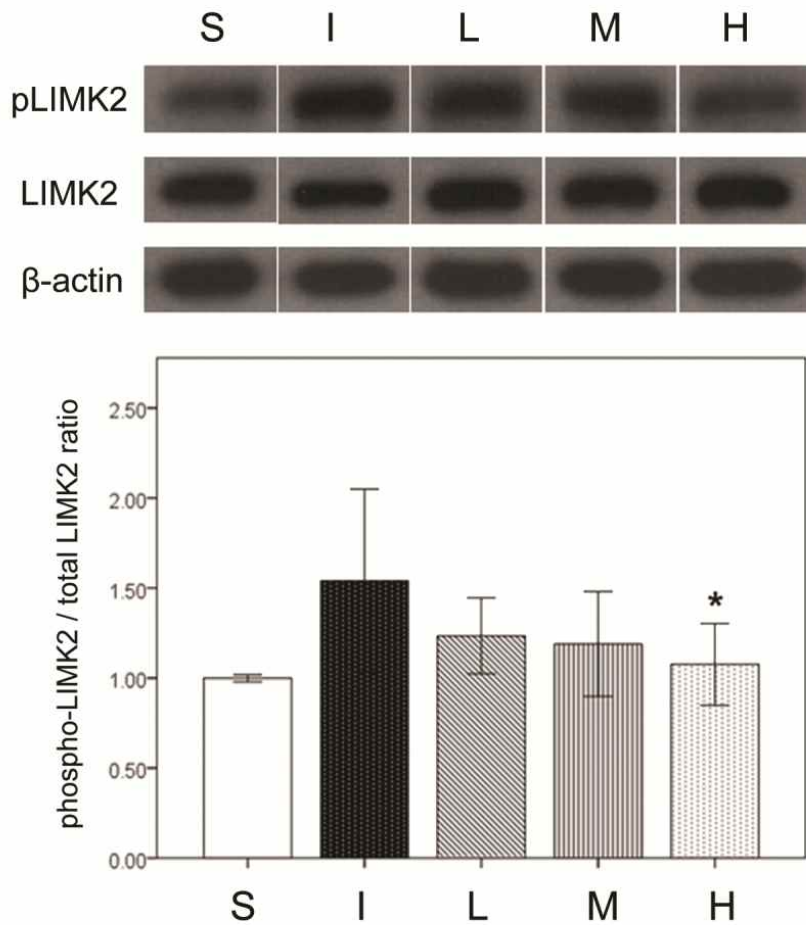
Figure 5. Representative immunoblot images and bar graphs (mean ± SEM) showing the comparison in the protein expression of phosphorylated LIMK2/total LIMK2 from the cavernosal tissues among the five groups using densitometry. The results were normalized by β-actin expression and presented as fold changes over controls. Six rats in each group were analyzed by densitometry. S = sham surgery group, I = bilateral CNCI group, L = bilateral CNCI group treated with low-dose LIMK2 inhibitors, M = bilateral CNCI group treated with medium-dose LIMK2 inhibitors, H = bilateral CNCI group treated with high-dose LIMK2 inhibitors, CNCI = cavernous nerve crush injury. * $p < 0.05$ vs. bilateral CNCI (I) group.
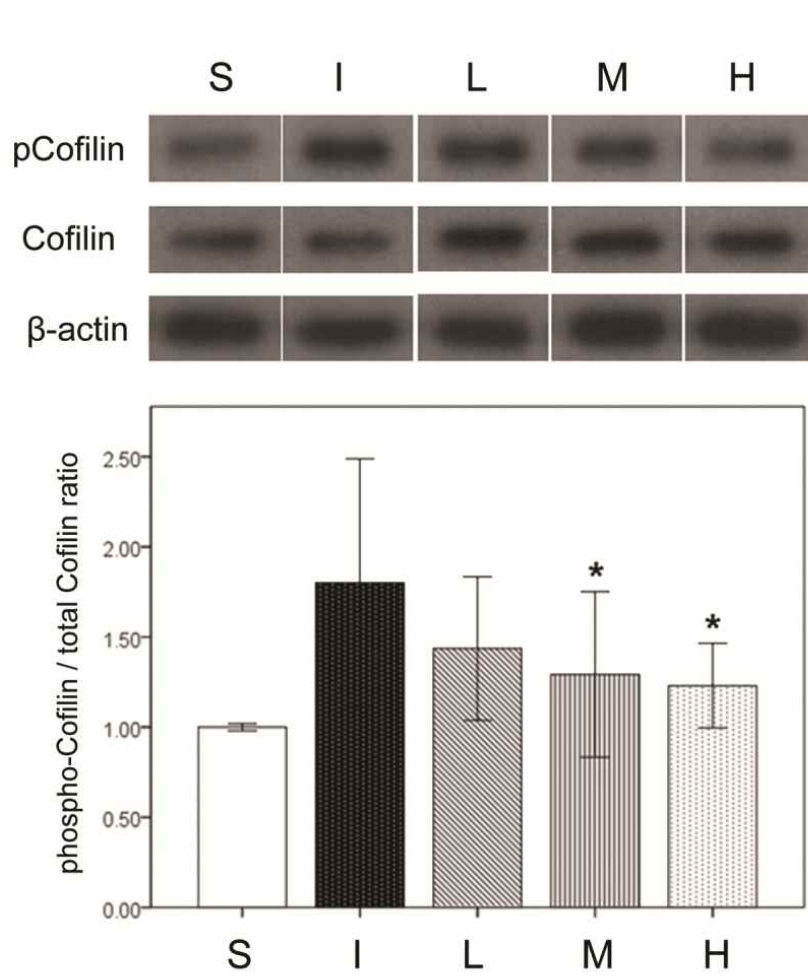
Figure 6. Representative immunoblot images and bar graphs (mean ± SEM) showing the comparison in the protein expression of phosphorylated Cofilin/total Cofilin from the cavernosal tissues among the five groups using densitometry. The results were normalized by β-actin expression and presented as fold changes over controls. Six rats in each group were analyzed by densitometry. S = sham surgery group, I = bilateral CNCI group, L = bilateral CNCI group treated with low-dose LIMK2 inhibitors, M = bilateral CNCI group treated with medium-dose LIMK2 inhibitors, H = bilateral CNCI group treated with high-dose LIMK2 inhibitors, CNCI = cavernous nerve crush injury. * $p < 0.05$ vs. bilateral CNCI (I) group.
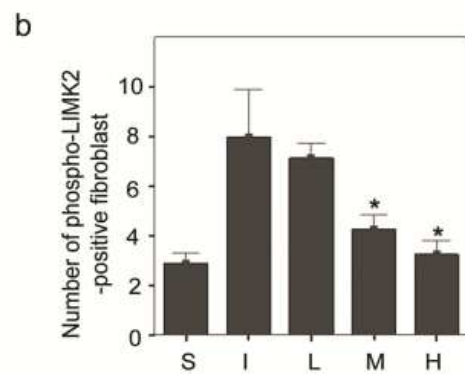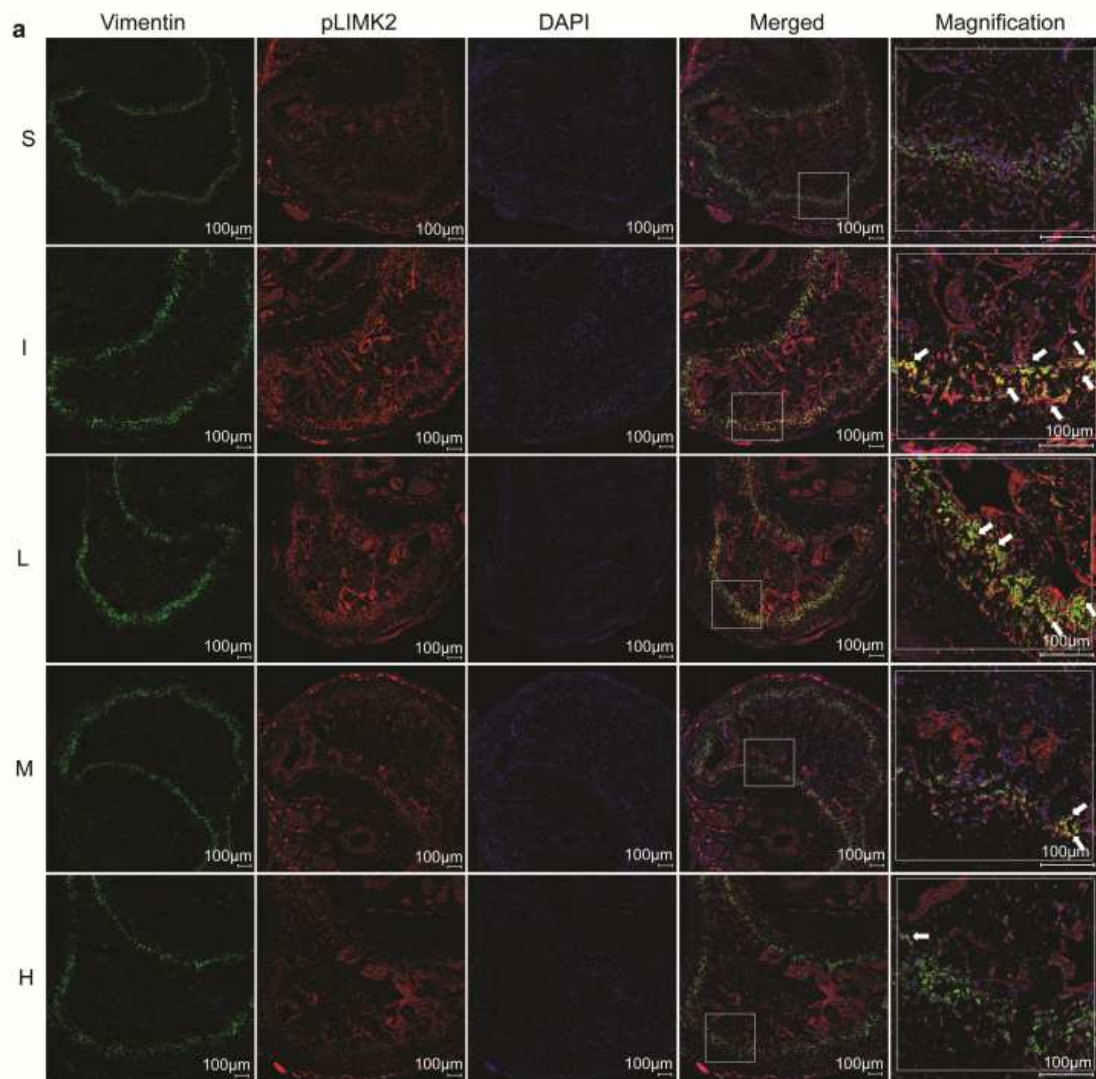
Figure 7. Effect of bilateral CNCI and LIMK2 inhibition on the content of fibroblasts positive

for phosphorylated LIMK2 after 1 week of surgery. Six rats in each group were analyzed by

confocal laser scanning. (a) Representative images for double immunofluorescent staining of cavernosal tissue with anti-vimentin and anti-phospho-LIMK2 in the five groups using confocal microscope. White arrow indicates significant expression of phosphorylated LIMK2 in cavernosal fibroblasts (yellow color in merged or magnified image). Scale bar = 100μm. (b) Bar graphs showing the comparison in fibroblasts positive for phosphorylated LIMK2 (mean ± SEM) among the five groups. S = sham surgery group, I = bilateral CNCI group, L = bilateral CNCI group treated with low-dose LIMK2 inhibitors, M = bilateral CNCI group treated with medium-dose LIMK2 inhibitors, H = bilateral CNCI group treated with high-dose LIMK2 inhibitors. * $p < 0.05$ vs. bilateral CNCI (I) group.

국문초록

해면체 신경 손상 발기부전 백서 모델에서 LIMK2 억제제의 음경해면체 섬유화 차단 효과를 통한 발기력의 호전

박주현

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서론: 연구자들은 해면체 신경 손상 발기부전 백서모델에서 LIM-kinase2 (LIMK2) 억제제 투여 시 Rho-kinase1 (ROCK1)/LIMK2/Cofilin 경로 차단을 통해 음경해면체 섬유화 억제 및 발기력을 호전시키는지 알아보고자 하였다.

대상 및 방법: 10 주령의 웅성 Sprague-Dawley 백서 60 마리를 Sham 대조군 (S), 양측 해면체신경 으깸손상군 (I), 양측 해면체신경 으깸손상 직후부터 LIMK2 억제제를 각각 2.5mg/kg 일일 1 회 복강내 투여 군 (L), 양측 해면체신경 으깸손상 직후 LIMK2 억제제를 각각 5mg/kg 일일 1 회 복강 내 투여 군 (M), 양측 해면체신경 으깸손상 직후 LIMK2 억제제를 각각 10mg/kg 일일 1 회 복강 내 투여 군 (H)의

다섯 군 (각 군당 12 마리)으로 구분하였다. 술후 1 주째 각 군당 6 마리의 백서에서 발기능을 해면체내압측정 (ICP)으로 평가하였고, ICP 를 측정하지 않은 각 군당 6 마리의 백서에서 조직학적 염색 및 Western blot 의 시행을 위해 음경조직을 적출하였다. 파라핀 포매된 조직절편에 대해 Masson's trichrome 염색을 시행하였고, phospho-LIMK2 (p-LIMK2)와 섬유아세포 (fibroblast)의 표지자인 vimentin 에 대해 더블 면역형광염색을 시행하였다. 단백을 추출하여 ROCK1, LIMK2, p-LIMK2, Cofilin, p-Cofilin 에 대한 Western blot 을 시행하였다.

결과: 술 후 1 주 째 I 군의 평균동맥압에 대한 최대해면체내압의 비 (ICP/MAP)와 해면체내압의 곡선 (AUC/MAP)은 S 군에 비해 유의하게 저하되었다. L 군에서 ICP/MAP 및 AUC/MAP 는 I 군과 유의한 차이가 없었다. M 군의 경우 일부 전기자극 조건에서 I 군에 비해 ICP/MAP 와 AUC/MAP 이 호전되었고, H 군에서는 모든 전기자극 조건에서 호전되었다. I 군에서 S 군에 비해 평활근/콜라겐 비율은 감소하였고, p-LIMK2 발현 양성 섬유아세포 수는 증가하였다. L 군에서 평활근/콜라겐 비율 및 p-LIMK2 발현 양성 섬유아세포 수는 I군과 차이가 없었으나, M 군과 H 군에서는 I 군에 비해 호전되었다. Western blot 에서 ROCK1 단백발현, LIMK 에 대한 p-LIMK2 의 단백발현 비율 및 Cofilin 에 대한 p-Cofilin 의 단백발현 비율은 S 군에 비해 I 군에서 유의하게 증가하였다. H 군에서는 I 군에 비해 상기 단백발현이 감소되었지만, L 군 및 M 군에서는 I군과 차이가 없었다.

결론: 본 연구결과 해면체신경 손상 후 초기부터 LIMK2 억제제 10mg/kg 의 지속 투여는 LIMK2/Cofilin 경로 정상화를 통해 음경해면체 섬유화 및 발기력을 호전시켰다.

주요어: 발기부전, 섬유화, LIM kinase, 음경, 전립선절제술