Combination of LIM-kinase 2 and Jun Amino-terminal Kinase Inhibitors Improves Erectile Function in a Rat Model of Cavernous Nerve Injury

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**OBJECTIVE**
To determine if combined administration of LIMK2 and JNK inhibitors in a rat model of erectile dysfunction induced by cavernosal nerve (CN) injury could restore erectile function by suppressing both cavernosal apoptosis and fibrosis via rectification of molecular pathways related to the structural alterations.

**METHODS**
Sixty 12-week-old male Sprague-Dawley rats were categorized into 4 groups: (1) Sham-surgery (Sham) group, (2) CN-crush-injury (CNCI), (3) CNCI group (CNCI+L+1.0J) treated with a combination of 10.0 mg/kg LIMK2-inhibitors and low-dose (1.0 mg/kg) JNK-inhibitors, and (4) CNCI group (CNCI+L+10.0J) treated with a combination of 10.0 mg/kg LIMK2-inhibitors and a high dose (10.0 mg/kg) of JNK-inhibitors. Ten days after surgery, erectile response, histological-studies, and Western-blot was investigated.

**RESULTS**
The CNCI group showed a reduced maximal ICP/MAP or AUC/MAP, decreased immunohistochemical-staining of α-SMA, decreased SM/collagen ratio, increased phospho-cJun-positive apoptotic cells, increased phospho-LIMK2-positive fibroblasts, increased cJun-phosphorylation, increased LIMK2/Cofilin-phosphorylation, decreased Bcl-2/Bax ratio, and increased protein-expression of fibronectin, compared to the Sham group. Both the CNCI+L+1.0J and CNCI+L+10.0J groups showed improvements in erectile-responses, content of cavernosal α-SMA, number of phospho-cJun-positive apoptotic cells, Bcl-2/Bax ratio and cJun phosphorylation. Their improvements in the CNCI+L+10.0J group showed a tendency to be greater than those in the CNCI+L+1.0J group. Also, in the 2 treatment groups, rectification of SM/collagen ratio, number of phospho-LIMK2-positive fibroblasts, LIMK2/Cofilin-phosphorylation, and protein-expression of fibronectin was observed.

**CONCLUSION**
This study suggests that combined inhibition of JNK and LIMK2 may improve erectile function by suppressing cavernosal apoptosis and fibrosis via restoration of cJun/Bcl-2/Bax and LIMK2/Cofilin pathways at 10 days after CN injury. UROLOGY 131: 136–143, 2019. © 2019 Elsevier Inc.

Erectile dysfunction (ED) after radical prostatectomy (RP) is a major complication that has a significant adverse impact on the quality of life of patients with prostate cancer. Despite many advances in our knowledge of the surgical anatomy and the surgical techniques, a recent analysis showed that the incidence rate of post-RP ED was in the range 75%-80% in many studies. Also, these rates have not substantially improved or changed over the past 17 years. However, the exact pathophysiology of post-RP ED still remains to be elucidated.
The causes of post-RP ED are regarded as neurogenic (nerve injury), arteriogenic (vascular injury), venogenic, or a combinations thereof. Reduced bioavailability of neuronal nitric oxide due to damage to the cavernosal nerve (CN) may be a causative factor in post-RP ED. A mechanism responsible for arteriogenic ED after RP can be damage to the aberrant or accessory pudendal arteries. The vascular injury causes both blockage of arterial inflow into the penis and penile hypoxia independent of hypoxia as a result of denervation after CN injury. Furthermore, even with the bilateral nerve-sparing and minimally invasive approaches, neuapraxia is inevitably developed by CN injury during RP, leading to the lack of penile erection, at least during the early postoperative period. The subsequent ischemia of the corpus cavernosum induces structural alterations in penile tissues including cavernosal apoptosis and fibrosis, resulting in cavernosal veno-occlusive dysfunction (CVOD) and, thereby, ED. Thus, cavernosal apoptosis and fibrosis are important pathophysiology of post-RP ED.

Because of significant pathophysiology such as the CVOD developed by the 2 structural alterations, the response to phosphodiesterase-type 5 inhibitors (PDE5Is) in men with post-RP ED is poorer than that in those with ED of other etiologies. In addition, a recent study showed that down-regulation of PDE5 protein expression in the erectile tissue after RP could be a cause of poor response to phosphodiesterase-type 5 inhibitors (PDE5Is). The clinical role of penile rehabilitation using the existing protocols could be a meaningful strategy to recover from the ED induced by CN injury. The objective of the current study was to find out if the combined administration of a LIMK2 inhibitor (LIMK2i) and a JNK inhibitor (JNKi) in a rat model of ED induced by CN injury could restore erectile function by suppressing both apoptosis and fibrosis of the corpus cavernosum via rectification of molecular pathways related to the structural alterations.

MATERIALS AND METHODS

Animals and Study Design

Twelve-week-old male Sprague-Dawley rats (n = 60) (Orient Bio Co., Ltd., Seongnam, Korea), weighing 350-380 g, were randomly categorized into the 4 experimental groups, as follows: (1) Sham surgery (Sham) group treated with daily intraperitoneal injection of vehicle (25% dimethylsulfoxide in saline), (2) CN crush injury (CNI) group treated with daily intraperitoneal injection of vehicle (25% dimethylsulfoxide in saline), (3) CNI group (CNI+L+1.0J) treated with a combination of 10.0 mg/kg LIMK2i (LX-7101, Cellagen Technology, CA) and 1.0 mg/kg JNKi (SP600125, Abcam, MO), and (4) CNI group (CNI+L+10.0J) treated with a combination of 10 mg/kg LIMK2i and 10.0 mg/kg JNKi. The drugs were dissolved in 1 mL of 25% dimethylsulfoxide in saline. The treatments were started on the day after surgery and were stopped 2 days before in vivo evaluation of erectile response (washout) at postoperative day 10.

All surgical procedures were performed by the same trained surgeon. The sham surgery was performed in a routine manner, as previously described. The rats were anesthetized with an intraperitoneal injection of zoletil (10 mg/kg; Vibac Laboratories, Carros, France) and an isoflurane inhalation (Abbott Laboratories, North Chicago, IL). Also, bilateral crush injury of the CNs was induced by two 80-second applications of pressure on the CNs 4-5 mm below the major pelvic ganglion (MPG), using a microsurgical vascular clamp. 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 reviewed and approved the experimental study. According to the National Research Council guidelines for the care and use of laboratory animals, we cared for the experimental rats.

Penile Erection Studies and Tissue collection

At 10 days after surgery, the erectile response of intracavernosal pressure (ICP) to electrostimulation (stimulation parameters: 1.0 and 4.0 V at 16 Hz with a square-wave duration of 0.3 milliseconds for 30 seconds and a 5-minute interval before subsequent stimulation) of the CNs was used to assess the erectile function of each rat in a routine manner. The erectile responses were presented as the ratios of ICP to mean arterial pressure (MAP) and the area under the curve (AUC) to MAP during the entire erectile response. We measured in vivo erectile response in 8 rats per group. In the remaining 7 rats of each group, the whole penis was harvested without performing in vivo electrostimulation, to avoid inadvertent changes in western blotting and histological staining. The middle parts of the corpus cavernosum were paraffin-embedded, and the remaining tissues were rapidly frozen in liquid nitrogen and stored at −80°C.

Quantitative Image Analysis

For stained slides of 7 animals in each group (2 tissue sections per rat), quantitative image analyses were performed using Image Pro Plus 4.5 software (Medica Cybernetics, Silver Spring, MD). The slides were analyzed using the same standard by an independent investigator blinded to the random group allocation.

For Masson's trichrome staining to evaluate the ratio of smooth muscle (SM)/collagen in the corpus cavernosum, the staining was carried out in a routine manner. At 40× magnification images of the penis composed of half the cavernosum, we quantified the content of collagen (stained in blue) and SM (stained in red).
For immunohistochemical staining of α-SM actin (α-SMA) to evaluate cavernosal SM content, a primary antibody against α-SMA (1:100, monoclonal mouse, Dako, Glostrup, Denmark) was used as described previously. The image of each slide was analyzed in the 40× magnification pictures.

**Dual Immunofluorescence Confocal Laser Scanning Microscopy**

Using paraffin-embedded sections of cavernosal tissues (2.5 μm), dual immunofluorescent labeling for Vimentin (a fibroblast marker) and phosphorylated LIMK2 was carried out to evaluate the amount of phosphorylated LIMK2-positive fibroblasts in the corpus cavernosum. Also, dual immunofluorescent labeling for terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine-5'-triphosphate nick end labeling (TUNEL) and phosphorylated-e-Jun was done to measure the amount of phosphorylated-e-Jun-positive apoptotic cells, as described previously. Primary antibodies included anti-Vimentin (1:100, monoclonal mouse, Dako, Glostrup, Denmark), antiphospho-LIMK2 (phospho-T505) (1:50, polyclonal rabbit, AbCam, Cambridge, UK), and phospho-e-Jun (1:20, polyclonal rabbit, Cell-Signaling Technology, Danvers, MA). Digital images were obtained using a confocal microscope (Leica TCS SP8, Leica Microsystems, Germany). On each slide, 4 high-power (×400) zones were randomly selected and analyzed by using confocal laser scanning. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Among cells positive for DAPI (stained in blue or purple in merged/magnified images) in the given area, the phosphorylated LIMK2-positive fibroblasts (stained in yellow) were counted by an independent observer blinded to the treatment group. Also, using the same standards in all of the groups, we counted the phosphorylated-e-Jun-positive apoptotic cells (stained in yellow) among the apoptotic cells (stained in pink) observed in the cavernosal sinuses under confocal microscopy.

**Western Blot Analysis**

To measure the protein expression levels of LIMK2 or JNK signaling molecules, Western blot analyses were done routinely. Primary antibodies included anti-e-Jun (1:1,000, monoclonal rabbit, Cell-Signaling Technology, Danvers, MA), antiphospho-e-Jun (1:1000, polyclonal rabbit, Cell-Signaling Technology, Danvers, MA), anti-Bcl-2 (1:2,000, monoclonal rabbit, Cell-Signaling Technology, Danvers, MA), anti-Bax (1:2,000, polyclonal rabbit, Cell-Signaling Technology, Danvers, MA), antiphospho-LIMK2 (phospho-T505) (1:50, polyclonal rabbit, AbCam, Cambridge, UK), and phospho-e-Jun (1:20, polyclonal rabbit, Cell-Signaling Technology, Danvers, MA).

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**Figure 1.** (A) Comparison of erectile function (ICP/MAP & AUC/MAP & representative ICP/MAP trace) at 10 days after surgery among the 4 experimental groups (8 rats from each group). (B) Comparison of smooth muscle/collagen ratio according to Masson’s trichrome staining and (C) comparison in amount of smooth muscle according to immunohistochemical staining of α-smooth muscle actin, at 10 days after surgery among the 4 experimental groups (7 rats from each group). Black asterisks indicate collagen deposition in the interstitium of the corpus cavernosum. Black arrowheads indicate the trabecular smooth muscle. ICP/MAP, intracavernous pressure/mean arterial pressure; AUC/MAP, area under the curve corresponding to the duration of electrical stimulation/mean arterial pressure; Sham surgery, sham surgery (Sham) group; Injury, bilateral cavernosal nerve crush injury (CNCI) group; Injury + LIMKi 10.0 mg + JNKi 1.0 mg, bilateral cavernosal nerve crush injury group (CNCI+L+1.0J) treated with daily combined administration of 10.0 mg/kg LIMK2 inhibitors and 1.0 mg/kg JNK inhibitors; Injury + LIMKi 10.0 mg + JNKi 10.0 mg, bilateral cavernosal nerve crush injury group (CNCI+L+10.0J) treated with daily combined administration of 10.0 mg/kg LIMK2 inhibitors and 10.0 mg/kg JNK inhibitors. *P < .05 compared to the CNCI group. †P < .05 compared to the Sham group. ‡P < .05 comparison between to the CNCI+L+1.0J and CNCI+L+10.0J groups. (Color version available online.)
(phospho-T505) (1:1,000, polyclonal rabbit, Abcam, Cambridge, UK), anti-Cofilin (1:1,000, monoclonal rabbit, Cell-Signaling Technology, Danvers, MA), antiphospho-Cofilin (1:1,000, monoclonal rabbit, Cell-Signaling Technology, Danvers, MA), and anti-fibronectin (1:2,000, polyclonal rabbit, Abcam, Cambridge, UK). Densitometric results were analyzed using ImageJ software to measure the integrated density of the protein band and were normalized by β-actin expression.

Statistical Analysis
Variables were presented as means ± standard errors of the mean (SEM). Statistical comparison between the groups was done using Mann-Whitney U test or Kruskal-Wallis test or one-way analysis of variance test or modified T-test, as indicated. The level of statistical significance was taken as a P value less than 5%, and the reported P values were two-sided. All calculations were performed using SPSS Version 20.0 (SPSS Inc., Chicago).

RESULTS

Combined Treatment With JNKi (1.0 mg/kg or 10.0 mg/kg) and LIMK2i Significantly Improves Erectile Response
The CNCI group showed a significant reduction of maximal ICP/MAP and AUC/MAP at all stimulation parameters (1.0 and 4.0 V) compared to the Sham group (Fig. 1A). The maximal ICP/MAP and AUC/MAP at stimulation at 1.0 V in the CNCI+L+10.0J group were significantly greater compared to those in the CNCI+L+1.0J group. No significant difference in baseline MAP or change of body weight or mortality was observed among the 4 groups.

Combined Treatment With JNKi and LIMK2i Significantly Improves Apoptosis and Fibrosis of the Cavernosal Tissue by Restoring cJun/Bcl-2/Bax and LIMK2/Cofilin Pathways
The CNCI group showed significantly decreased SM/collagen ratio compared with the Sham group (Fig. 1B). The SM/collagen ratios in the CNCI+L+1.0J and the CNCI+L+10.0J groups significantly improved compared to that in the CNCI group. The SM/collagen ratio in the CNCI+L+10.0J group recovered to the

Figure 2. Comparison of the amount of apoptotic cells positive for phosphorylated c-Jun at 10 days after surgery among the 4 experimental groups. (A) Representative immunofluorescence pictures for double labeling of cavernosal tissue with TUNEL and antiphospho-c-Jun in the 4 experimental groups under confocal microscopic scanning. Nuclei were labeled with DAPI (stained in blue). White arrowheads point to apoptotic cells positive for phosphorylated c-Jun in the cavernosal sinusoids (stained in yellow). Scale bar = 100 μm. (B) Quantification of the amount of apoptotic cells positive for phosphorylated c-Jun in the 4 experimental groups (high-power field). *P < .05 compared to the CNCI group. †P < .05 compared to the Sham group. ‡P < .05 comparison between to the CNCI+L+1.0J and CNCI+L+10.0J groups. (Color version available online.)
level observed in the Sham group, whereas that in the CNCI+L+1.0J group did not. The SM/collagen ratio in the CNCI+L+1.0J group was greater than that in the CNCI+L+1.0J group.

The α-SMA content in the CNCI group was significantly reduced compared to that in the Sham group (Fig. 1C). The CNCI+L+1.0J and the CNCI+L+10.0J groups showed significant improvements in the α-SMA content compared with the CNCI group. The α-SMA content in the CNCI+L+1.0J or the CNCI+L+10.0J group was not restored to the level observed in the Sham group. Also, there was no significant difference in the α-SMA content between the CNCI+L+1.0J and the CNCI+L+10.0J groups.

The CNCI group showed a significantly increased number of apoptotic cells positive for phospho-c-Jun compared with the Sham group (Fig. 2). Both the CNCI+L+1.0J and the CNCI+L+10.0J groups showed significant improvements in the number of apoptotic cells positive for phospho-c-Jun compared with the CNCI group. The number of apoptotic cells positive for phospho-c-Jun in the CNCI+L+10.0J group recovered to values like those observed in the Sham group. Also, there was no significant difference between the CNCI+L+1.0J and the CNCI+L+10.0J groups.

According to the densitometry, the CNCI group showed increased c-Jun phosphorylation, decreased Bcl-2/Bax ratio, increased LIMK2/Cofilin phosphorylation and increased protein expression of fibronectin compared with the Sham group (Fig. 4). In the CNCI+L+1.0J and the CNCI+L+10.0J groups, the c-Jun phosphorylation and the Bcl-2/Bax ratio significantly improved compared to the CNCI group. In the CNCI+L+10.0J group, the c-Jun phosphorylation and the Bcl-2/Bax ratio recovered to the level observed in the Sham group, whereas those in the CNCI+L+1.0J group did not. Also, there were significant differences in the c-Jun phosphorylation and the Bcl-2/Bax ratio between the CNCI+L+1.0J and the CNCI+L+10.0J groups. Meanwhile, the LIMK2/Cofilin phosphorylation and the protein expression of fibronectin in the CNCI+L+1.0J and the CNCI+L+10.0J groups were restored to values like those observed in the Sham group.

**COMMENT**

This study aimed to identify the therapeutic effect of combined inhibition of JNK and LIMK2 on improving erectile function by suppressing both cavernosal apoptosis and fibrosis after CN injury and, ultimately, to suggest a potential
strategy for recovery from post-RP ED, a difficult-to-treat condition. A few main findings of this study are as follows:

(1) Dual inhibition of JNK and LIMK2 significantly improved erectile function by suppression of cavernosal apoptosis and fibrosis via restoration of JNK and LIMK2/Cofilin pathways at 10 days after CN injury.

(2) Dual inhibition of JNK and LIMK2 rectified erectile function, cavernosal apoptosis, cavernosal Bcl-2/Bax ratio, and c-Jun phosphorylation in a dose-dependent manner of a JNKi, together with restoration of the cavernosal fibrosis and LIMK2/Cofilin pathways.

Cavernosal apoptosis and fibrosis are 2 important structural alterations of the corpus cavernosum induced by CN injury that lead to development of post-RP ED. However, there have been few studies of mechanism-specific targeted therapies for suppression of both cavernosal apoptosis and fibrosis. Although previous studies showed that chronic administration of PDE5Is improved ED by the suppression of both cavernosal apoptosis and fibrosis in rat models of CN injury, they did not elucidate the molecular pathways specifically related to the improvement of cavernosal apoptosis or fibrosis. Also, 2 previous studies showed an improvement in cavernosal apoptosis or fibrosis by intracavernosal administration of sonic hedgehog in a rat model of CN resection, but they did not evaluate the therapeutic effect of combined suppression of the 2 structural alterations on improvement of erection function. Also, according to a few previous studies, the inhibition of Rho-kinase from the early postinjury period after CN injury has been suggested as a therapeutic strategy for the suppression of both cavernosal apoptosis and fibrosis. In this context, this study showed promising results in rectifying ED by means of the mechanism-specific targeted therapy for the suppression of both cavernosal apoptosis and fibrosis. Therefore, this study might raise a possibility of targeting specific molecular pathways of cavernosal apoptosis and fibrosis to recover from ED induced by CN injury.

The JNK/c-Jun/Bcl-2/Bax pathway appears to be a downstream effector of Rho-kinase in apoptosis of various cells, including cardiomyocytes, endothelial cells, and neuronal cells. A previous study showed that increased JNK

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Figure 4. (A) Representative Western blot pictures for protein expression of phosphorylated c-Jun/total c-Jun, Bcl-2, Bax, phosphorylated LIMK2/total LIMK2, and phosphorylated Cofilin/total Cofilin and fibronectin in the cavernosal tissues of the 4 experimental groups. (B) Quantification of their protein expression in the cavernosal tissues of the 4 experimental groups using densitometry. Each bar graph depicts the mean ± SEM. *P < .05 compared to the CNCI group. †P < .05 compared to the Sham group. ‡P < .05 comparison between to the CNCI+L+1.0J and CNCI+L+10.0J groups.
phosphorylation was involved in cavernosal apoptosis starting from 2 weeks after CN injury. In accordance with these, our recent study demonstrated that the daily administration of JNKi for 2 weeks right after the CNCI partially improved erectile function by improving α-SMA content, the amount of phospho-c-Jun-positive apoptotic cells, and the dysregulated c-Jun/Bcl-2/Bax pathway. Meanwhile, a LIMK2/Cofilin pathway has been reported to be a downstream effector of ROCK1 in development of fibroblast-to-myofibroblast differentiation, a hallmark of fibrotic disease. Recently, we demonstrated that the daily administration of LIMK2i (particularly, 10.0 mg/kg) for a week right after the CNCI partially improved erectile function by improving the SM/collagen ratio, the amount of phospho-LIMK2-positive fibroblasts, and the dysregulated LIMK2/Cofilin pathway. In line with these, Cui et al showed that the ROCK1/LIMK2/Cofilin pathway was involved in the ED and cavernosal fibrosis caused by advanced age. Therefore, a combination of selective inhibition of the downstream molecule of the Rho-kinase-driven apoptotic pathway and selective inhibition of the downstream molecule of the Rho-kinase-driven fibrotic pathway may be a reasonable strategy to recover from post-RP ED. In this sense, the current study indicates that selectively inhibiting the downstream effectors of Rho-kinase (LIMK2 and JNK) may efficiently improve ED induced by CN injury through suppressing both cavernosal apoptosis and fibrosis.

One of the limitations of this study was that we did not evaluate an effect of JNK or LIMK2 inhibition on the MPG, although CN injury can induce the apoptotic pathways in the MPG. And, we did not evaluate the inflammatory cytokines or reactive oxygen species in our study. Although this study suggests the selective inhibition of LIMK2 and JNK pathways may be a mechanism-specific targeted therapy for an ED induced by CN injury, a future study is needed to identify whether the combined inhibition of the 2 pathways improves CVOD, a main cause of ED induced by CN injury, at a subacute or chronic phase after CN injury. Also, to make this therapeutic strategy more meaningful, a subsequent study may be necessary for finding out if the combined inhibition of the 2 pathways improves the already-developed alterations of penile integrity when the selective inhibitors are administrated starting from the subacute or chronic phase after CN injury.

CONCLUSION

Combined inhibition of JNK and LIMK2 may improve erectile function by suppression of cavernosal apoptosis and fibrosis via restoration of c-Jun/Bcl-2/Bax and LIMK2/Cofilin pathways at 10 days after CN injury, according to this study. Currently, given the limitations of penile rehabilitation after RP, specific inhibition of JNK and LIMK2 may be a potential mechanism-specific targeted therapy for post-RP ED induced by CN injury. Future studies evaluating the combined JNKi and LIMK2i treatments given at different time points following CN injury may determine the maximum protection on cavernosal apoptosis and fibrosis in animal model of post-RP ED.

References

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