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Restoration of Cavernous Veno-Occlusive Function through Chronic Administration of a Jun-Amino Terminal Kinase Inhibitor and a LIM-Kinase 2 Inhibitor by Suppressing Cavernous Apoptosis and Fibrosis in a Rat Model of Cavernous Nerve Injury: A Comparison with a Phosphodiesterase Type 5 Inhibitor

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Purpose: To determine if chronic administration of Jun-amino terminal kinase (JNK)-inhibitors and LIM-kinase 2 (LIMK2)-inhibitors from the immediate post-injury period in a rat model of cavernous-nerve-crush-injury could normalize cavernous-veno-occlusive-function, and to compare it with phosphodiesterase type 5 (PDE5)-inhibitors.

Materials and Methods: A total of 75 12-week-old male Sprague–Dawley-rats were randomized into five groups: sham-surgery (S), cavernous-nerve-crush-injury (I), cavernous-nerve-crush-injury treated with 10.0 mg/kg LIMK2-inhibitor (L) or 10.0 mg/kg JNK-inhibitor and 10.0 mg/kg LIMK2-inhibitor (J+L) or 20.0 mg/kg udenafil (P) for five-weeks. Five-weeks after surgery, dynamic-infusion-cavernosometry, histological-studies, caspase-3-activity-assay, and Western-blot were investigated.

Results: Group-I had lower papaverine-response, higher maintenance-rate and higher drop-rate, compared to Group-S. Group-L, Group-J+L and Group-P showed improvement in the three dynamic-infusion-cavernosometry parameters. The papaverine-response and drop-rate in Group-J+L and Group-P recovered to sham-control level, but those in Group-L did not. Regarding apoptosis, Group-I had decreased content of α-smooth-muscle-actin, increased caspase-3 activity and increased cJun-phosphorylation. The cJun-phosphorylation improved only in Group-J+L. The α-smooth-muscle-actin content and caspase-3-activity in Group-J+L and Group-P improved, but those in Group-L were not. Regarding fibrosis, Group-I had decreased smooth muscle (SM)/collagen-ratio, increased protein-expression of fibronectin, and increased Cofilin-phosphorylation. Cofilin-phosphorylation was normalized in Group-L and Group-J+L, but not in Group-P. SM/collagen-ratio and protein-expression of fibronectin in Group-L, Group-J+L and Group-P improved.

Conclusions: Our data indicate that chronic inhibition of JNK and LIMK2 can restore cavernous-veno-occlusive-function by suppressing cavernous-apoptosis and cavernous-fibrosis, comparable to the results by PDE5-inhibitors. Chronic inhibition of JNK and LIMK2 might be a potential mechanism-specific targeted therapy for cavernous-veno-occlusive-dysfunction induced

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by cavernous nerve-injury.

Keywords: Apoptosis; Erectile dysfunction; Fibrosis; Prostatectomy

INTRODUCTION

Prostate cancer is the second common cancer in males globally with an increasing incidence [1]. Despite the efficacy of radical prostatectomy (RP) in treating prostate cancer, RP has a pernicious effect on the patient's erectile function, and hence, the patient's quality of life and general well-being [2,3]. Erectile dysfunction (ED) at one year after robot-assisted RP persists in up to 50% of the patients who underwent the surgery [4].

The response to phosphodiesterase type 5 inhibitors (PDE5Is) in males with post-RP ED is poor [5]. This is mainly attributed to development of cavernous veno-occlusive dysfunction (CVOD) induced by cavernous nerve (CN) injury during RP, although the pathogenesis of post-RP ED is multifactorial [6]. The major pathogenesis related to development of CVOD induced by CN injury includes cavernous apoptosis and fibrosis [6,7]. Under this background, several therapeutic strategies for cavernous fibrosis or apoptosis including PDE5Is, intracavernous stem cell injection, angiotensin-II antagonists, Rho-kinase inhibitors, intracavernous delivery of sonic hedgehog, and histone deacetylase inhibitors have shown positive results in terms of improving cavernosal apoptosis, fibrosis and erectile function in animal models of ED induced by CN injury [8-14]. However, their routine use is limited because of insufficient clinical efficacy of PDE5Is, potential adverse effects of Rho-kinase inhibitors, pending safety issues of stem cell therapy, and insufficient data on sonic hedgehog or angiotensin-II antagonists or histone deacetylase inhibitors [15-18].

Several previous studies reported that Jun-amino terminal kinase (JNK) pathway plays a role in development of cavernous apoptosis in animal models of ED induced by CN injury [8,19]. Also, a recent study showed that JNK inhibition resulted in partial improvement of erectile function through the suppression of cavernous apoptosis [20]. Regarding cavernous fibrosis, previous studies have demonstrated that inhibition of LIM-kinase 2 (LIMK2) pathway leads to partial improvement of erectile response or cavernous veno-occlusive function (CVOF) through suppression of cavernous fibrosis in a rat model of ED induced by CN injury [21,22]. A recent study showed that short-term administration of both a JNK inhibitor and an LIMK2 inhibitor from the immediate post-injury period significantly improved erectile response to electrostimulation in a rat model of CN injury [23].

Thus, we hypothesized that inhibition of JNK and LIMK2 would restore CVOF, a key pathophysiology of ED induced by CN injury, through suppression of both cavernosal apoptosis and fibrosis. We investigated if the mechanism-specific therapy targeted for JNK and LIMK2 would restore CVOF through suppression of the key structural alterations in a rat model of CN injury. Also, we compared the treatment outcomes of the mechanism-specific therapy with those of a PDE5I (the positive control).

MATERIALS AND METHODS

1. Experimental groups and treatments

A total of 75 12-week-old male Sprague–Dawley rats were randomized into five groups: (1) sham surgery (S) group, (2) CN crush injury (CNCI) (I) group, (3) CNCI treated with daily administration of 10.0 mg/kg LIMK2 inhibitor (L) group (LX-7101; Cellagen Technology, San Diego, CA, USA) [21,22], (4) CNCI treated with daily administration of 10.0 mg/kg JNK inhibitor (J+L) group (SP600125; Abcam, Cambridge, MA, USA) and 10.0 mg/kg LIMK2 inhibitor [20-22], and (5) CNCI treated with daily administration of 20.0 mg/kg udenafil (P) group (PDE5I; Dong-A, Seoul, Korea). All surgical procedures were performed by one surgeon who was blinded to the group allocation, as a routine manner [12-14]. In the S group, only exploration of the pelvis was performed without direct damage to the CNs. In the I group, the

crush injuries were induced by two 80-second applications of pressure on the bilateral CNs 4–5 mm below the major pelvic ganglion. The S group and I group received daily intraperitoneal injection of vehicle (25% dimethylsulfoxide in saline) for five weeks from the day following the surgery. The three treatment groups (L or J+L or P group) received the respective treatments for five weeks from the day following the CNCI. The treatments were interrupted two days (washout period) before dynamic infusion cavernosometry (DIC) at postoperative five weeks. The CVOF was assessed using DIC in eight rats of each group, and the remaining seven rats of each group received total penectomy for histological staining and western blot analyses. The middle part of each cavernosum was embedded in paraffin, and the remaining cavernous tissues were stored at -80℃.

2. Ethics statements

These experiments were approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute at Seoul National University Hospital (No. 18-0035-S1A0), an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. And, we followed the National Research Council guidelines for the care and use of laboratory animals regarding caring for the rats.

3. Dynamic infusion cavernosometry

For evaluation of CVOF of each rat, DIC was performed at five weeks after surgery, as previously described [22]. Briefly, after each rat was anesthetized, a 26-gauge angiocatheter was introduced into the cavernosum to assess intracavernous pressure (ICP) and the carotid artery was cannulated by a 24-gauge angiocatheter to monitor arterial pressure. Also, for infusion of papaverine or normal saline, another 26-gauge angiocatheter was introduced into the contralateral cavernosum. The ICP during the tumescence (approximately five minutes) after infusion of 800 μg papaverine was recorded as "ICP after papaverine". And then, the infusion of normal saline was started at a rate of 0.05 mL/min. Until the ICP reached a plateau of 80 mmHg, the infusion rate was increased by 0.05 mL/min every 10 seconds. After the ICP was on a plateau of 80 mmHg for 30 seconds, the saline infusion was stopped and the fall in ICP was recorded for one minute after the infusion had been stopped. The "maintenance rate" was defined as the saline infusion rate required for a plateau of 80 mmHg. Also, the "drop rate" was defined as the degree of reduction in ICP for one minute.

4. Histological staining

The ratio of smooth muscle (SM)/collagen in the corpus cavernosum of each rat was evaluated using Masson's trichrome staining, in a routine manner [21,22]. For stained slides, the area of SM (stained in red) and collagen (stained in blue) were analyzed quantitatively at 40× magnification images of the penis composed of half the cavernosum.

For determining the SM content, immunohistochemical staining of α-SM actin (α-SMA) was performed using a primary antibody against α-SMA (1:100, monoclonal mouse; Dako, Glostrup, Denmark) in a routine manner [19,20].

The quantitative image analyses were conducted at 40× magnification images of stained slides of seven rats from each group (two tissue sections per animal), using Image Pro Plus 4.5 software (Medica Cybernetics, Silver Spring, MD, USA). An independent investigator blinded to the experimental group allocation performed the analyses using the same standard.

5. Caspase-3 activity assay

Caspase-3 activity of the cavernosal tissue was quantitatively measured using caspase-3 colorimetric assay kit (Abcam) in a routine manner [24]. The results are presented as fold over the control.

6. Western blot analysis

Western blot analyses were performed to quantify protein expression in a routine manner [19-22]. Primary antibodies were as follow: anti-cJun (1:1,000, monoclonal rabbit; Cell-Signaling Technology, Danvers, MA, USA), anti-phospho-cJun (1:1,000, polyclonal rabbit; Cell-Signaling Technology), anti-Cofilin (1:1,000, monoclonal rabbit; Cell-Signaling Technology), anti-phospho-Cofilin (1:1,000, monoclonal rabbit; Cell-Signaling Technology), and anti-fibronectin (1:2,000, polyclonal rabbit; Abcam, Cambridge, UK). Densitometry was conducted using ImageJ software to analyze the results, reported as relative density of each protein compared to that of β-actin.

7. Statistical analysis

Variables were expressed as means±standard errors

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of the mean. We compared between the groups using the Mann–Whitney U-test or Kruskal–Wallis test, as indicated. The statistical significance was a p-value less than 0.05. All statistical analyses were conducted using IBM SPSS version 20.0 (IBM Corp., Armonk, NY, USA).

RESULTS

1. Restoration of cavernous veno-occlusive function to the level observed in sham control by chronic administration of both a Jun-amino terminal kinase inhibitor and an LIM-kinase 2 inhibitor

At five weeks after surgery, Group I had significantly lower papaverine response, higher maintenance rate and higher drop rate, compared to Group S ($p<0.001$, $p=0.001$, and $p=0.005$, respectively) (Fig. 1). Group L, Group J+L and Group P showed significant improvement in the three DIC parameters, compared to Group I. However, none of the three DIC parameters in Group L recovered to the level observed in Group S. The degree of improvement of the three DIC parameters in Group L was lower than that in Group J+L or Group P. The papaverine response and drop rate in Group J+L were restored to the level observed in Group S, identical to the results in Group P.

2. Rectification of cavernous apoptosis and fibrosis by chronic administration of a Jun-amino terminal kinase inhibitor and an LIM-kinase 2 inhibitor *via* normalization of Jun-amino terminal kinase/cJun and LIM-kinase 2/Cofilin pathways

Regarding apoptosis, Group I had significantly lower content of α-SMA, increased caspase-3 activity and increased phosphorylation of cJun, compared to Group S (Fig. 1-3). Group L did not show improvement in the α-SMA content or caspase-3 activity compared to Group I. The α-SMA content and caspase-3 activity in Group J+L and Group P improved compared to Group I. The improvement of α-SMA in Group J+L was comparable to that in Group P. The caspase-3 activity in Group J+L was restored to the level observed in Group S, but that in Group P was not. Also, the cJun phosphorylation improved in Group J+L, but not in Group L or Group P.

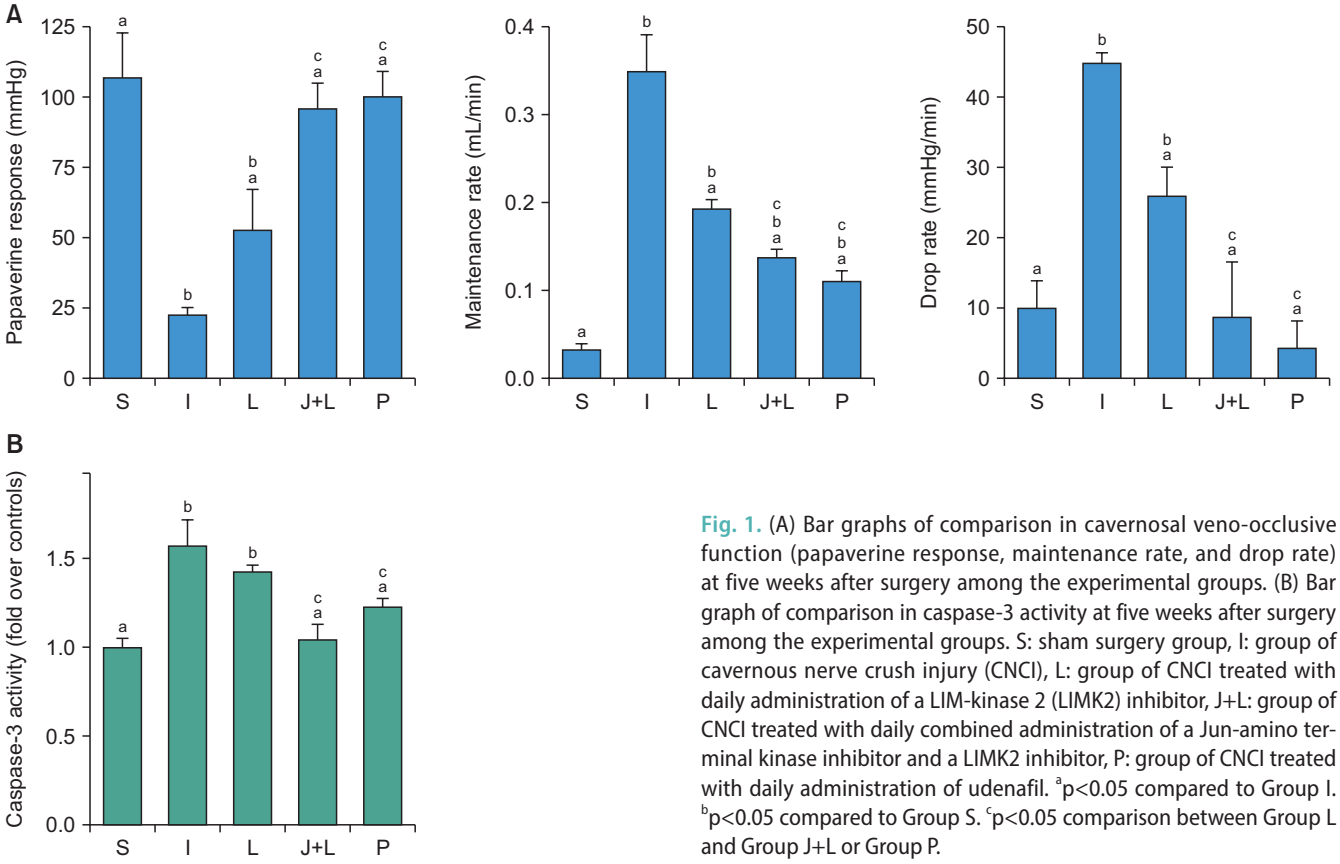

Fig. 1. (A) Bar graphs of comparison in cavernosal veno-occlusive function (papaverine response, maintenance rate, and drop rate) at five weeks after surgery among the experimental groups. (B) Bar graph of comparison in caspase-3 activity at five weeks after surgery among the experimental groups. S: sham surgery group, I: group of cavernous nerve crush injury (CNCI), L: group of CNCI treated with daily administration of a LIM-kinase 2 (LIMK2) inhibitor, J+L: group of CNCI treated with daily combined administration of a Jun-amino terminal kinase inhibitor and a LIMK2 inhibitor, P: group of CNCI treated with daily administration of udenafil. [a]$p<0.05$ compared to Group I. [b]$p<0.05$ compared to Group S. [c]$p<0.05$ comparison between Group L and Group J+L or Group P.

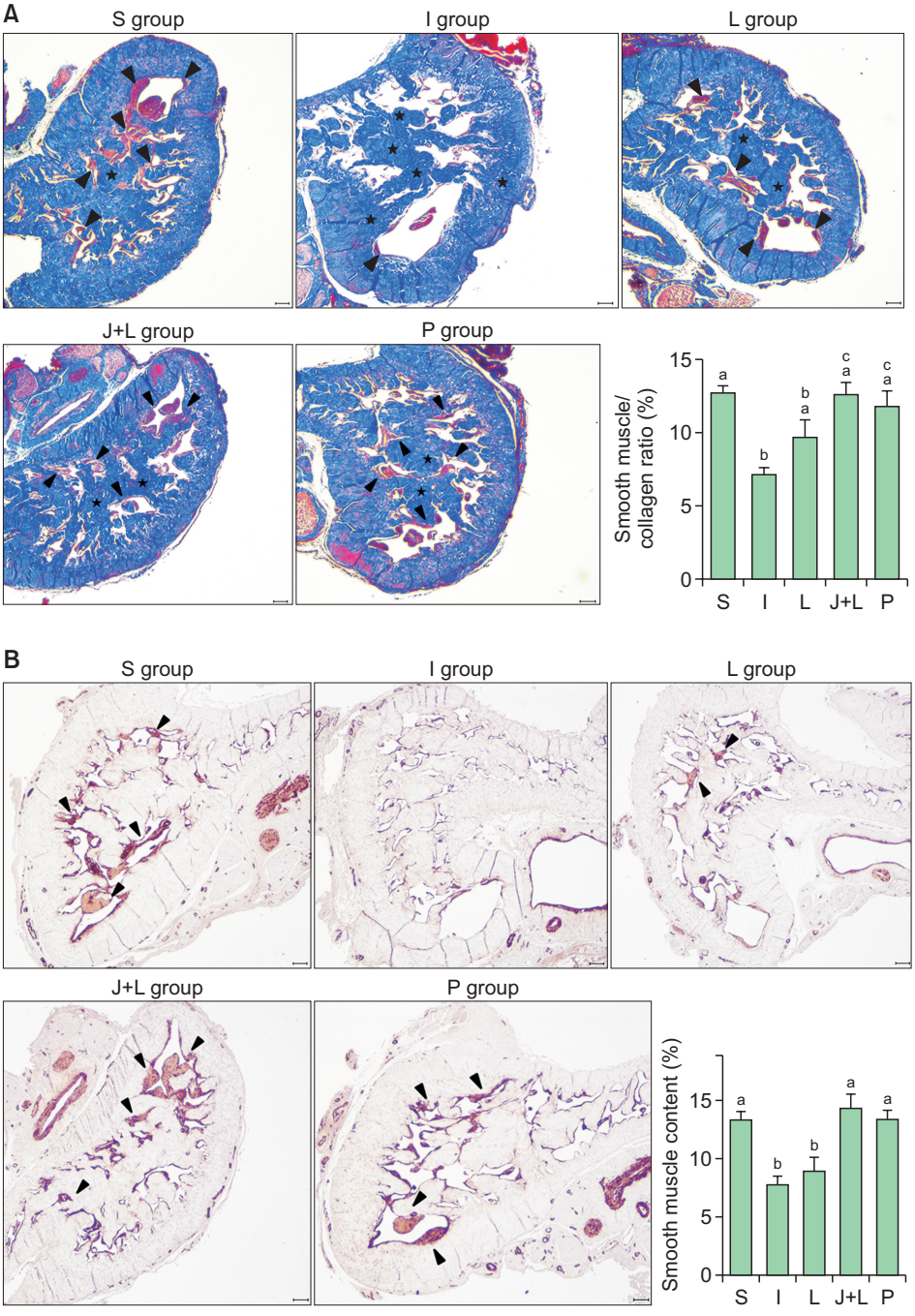

Fig. 2. (A) Representative figures and a bar graph of Masson's trichrome staining at five weeks after surgery. The results are presented as smooth muscle/collagen ratio. Collagenous fibrils in the interstitial tissue of the corpus cavernosum were indicated by black asterisks. The smooth muscle of the corpus cavernosum was indicated by black arrowheads. Magnification ×40, scale bars=100 μm. (B) Representative figures and a bar graph of immunohistochemical staining of α-smooth muscle actin. The smooth muscle components are shown as brown areas. The results are presented as the percentage of smooth muscle fibers in a given area. The smooth muscle of the corpus cavernosum is indicated by black arrowheads. Magnification ×40, scale bars=100 μm. S: sham surgery group, I: group of cavernous nerve crush injury (CNCI), L: group of CNCI treated with daily administration of a LIM-kinase 2 (LIMK2 inhibitor), J+L: group of CNCI treated with daily combined administration of a Jun-amino terminal kinase inhibitor and a LIMK2 inhibitor, P: group of CNCI treated with daily administration of udenafil. $^{a}p<0.05$ compared to Group I. $^{b}p<0.05$ compared to Group S. $^{c}p < 0.05$ comparison between Group L and Group J+L or Group P.

Regarding fibrosis, Group I had lower SM/collagen ratio, increased protein expression of fibronectin, and increased phosphorylation of Cofilin, compared to Group S (Fig. 2, 3). The SM/collagen ratio and the protein expression of fibronectin in Group L, Group J+L and Group P significantly improved compared to Group I. The SM/collagen ratio in Group L improved but not restored to the sham control level. The protein expression of fibronectin and Cofilin phosphorylation in Group L recovered to the control level. Group J+L showed recovery of the SM/collagen ratio and the fibronectin protein expression to the sham control level, comparable to the result in Group P. The Cofilin phosphorylation was normalized in Group J+L, but not in Group P.

DISCUSSION

To date, there has been no irrefutable method for treatment of post-RP ED or penile rehabilitation after

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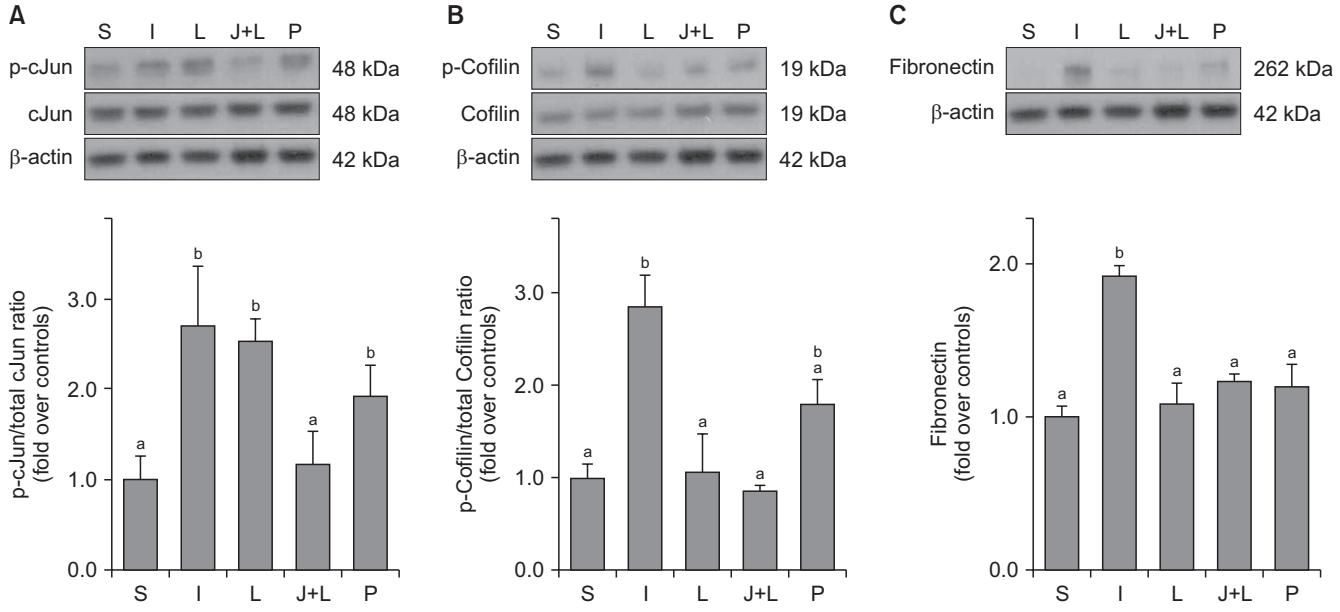

Fig. 3. Comparison in protein expression of (A) phosphorylated cJun (p-cJun)/total cJun, (B) phosphorylated Cofilin (p-Cofilin)/total Cofilin, and (C) Fibronectin of the corpus cavernosum among the experimental groups at five weeks after surgery. Each bar graph depicts the mean±standard error of mean. The data were normalized by β-actin expression and presented as a fold change over controls. S: sham surgery group, I: group of cavernous nerve crush injury (CNCI), L: group of CNCI treated with daily administration of a LIM-kinase 2 (LIMK2) inhibitor, J+L: group of CNCI treated with daily combined administration of a Jun-amino terminal kinase inhibitor and a LIMK2 inhibitor, P: group of CNCI treated with daily administration of udenafil. [a]p<0.05 compared to Group I. [b]p<0.05 compared to Group S.

surgery [16,25]. This is likely attributed to the development of post-RP CVOD, a difficult-to-treat condition, resulting in poor response to PDE5Is [6]. Although penile rehabilitation using several agents such as PDE5Is showed fantastic results in animal models of ED induced by CN injury, current evidence is inadequate to support specific regimens as optimal for penile rehabilitation in clinical practice [16,25]. Also, they are not the mechanism-specific therapy targeted for apoptosis and fibrosis of the corpus cavernosum, the two main structural alterations after CN injury. In this context, the purpose of this study was to determine if the combined administration of a JNK inhibitor and a LIMK2 inhibitor immediately after CN injury would be potential treatment strategy for restoring CVOF to normal control level by suppression of cavernous apoptosis and fibrosis. Our data demonstrates that the combined inhibition of JNK and LIMK2 pathways restored CVOF by suppressing the two structural alterations *via* normalization of apoptotic and fibrotic pathways. Also, these promising results by the combination therapy were comparable to those by a PDE5I (the positive control). Thus, the results of this study suggest that the early therapeutic strategy targeting the JNK and the LIMK2 pathways after CN injury may be a potential mechanism-specific therapy for preservation of penile integrity, and thereby CVOF.

A previous study by Ferrini et al [26] showed that cavernous apoptosis and fibrosis developed from the early post-injury period after CN injury and the fibrosis progressed with time, thereby resulting in CVOD starting from four weeks after injury. Also, previous studies showed that cavernous apoptosis developed immediately after CN injury and persisted up to four or five weeks after CN injury [12,27]. A recent study reported that daily administration of a JNK inhibitor for two weeks partially improved erectile response to electrostimulation by suppressing cavernous apoptosis in a rat model of CN injury [20]. Too, Park et al [22] demonstrated that inhibition of LIMK2 for one month resulted in partial recovery from CVOD through suppression of cavernous fibrosis in a rat model of CN injury. Thus, on the basis of previous studies, this study demonstrates that the combined inhibition of JNK and LIMK2 for five weeks restored CVOF to normal control level through suppressing apoptosis and fibrosis of the corpus cavernosum in a rat model of CN injury. Meanwhile, in this study, the treatment results for the two structural alterations of the combination therapies were comparable to those of a PDE5I. Similar to

our data, several previous studies showed that daily administration of PDE5Is for 45 days after CN injury rectified CVOD by suppressing apoptosis and fibrosis of corpus cavernosum in rat models of ED induced by CN injury [28,29]. However, except for transforming growth factor-β1 (TGF-β1), no molecular mechanisms related to the improvement of cavernous apoptosis or fibrosis were elucidated in the previous studies using PDE5Is. To the contrary, this study tested the combined inhibition of JNK and LIMK2 for a mechanism-specific therapy in a rat model of CVOD induced by CN injury. According to the results of our study, the combination therapy restored CVOF to normal control level, comparable to the results by a PDE5I. Thus, our data may be used as a basis for subsequent mechanism-specific therapies of post-RP CVOD in future studies.

This study made a comparison in CVOF, cavernous apoptosis, cavernous fibrosis and protein expression of some molecules related to LIMK2 or JNK between the combination therapy and the treatment with a PDE5I (udenafil). The SM content or caspase-3 activity improved by daily administration of a PDE5I but not normalized, whereas the two parameters were restored to normal control level by combined inhibition of JNK and LIMK2. Interestingly, however, there were no differences in the DIC parameters or SM content between the two treatment groups. The findings may be attributed to an increase in cell proliferation of SM by PDE5Is. This could be supported by several previous studies showing that daily administration of a tadalafil or a sildenafil improved cell proliferation of SM in a rat model of CN injury [28,29]. To the contrary, the combined inhibition of JNK and LIMK2 was unlikely to affect the proliferation of SM cell. Regarding the apoptosis of corpus cavernosum, the daily administration of PDE5Is improved the caspase-3 activity or SM content without improving cJun phosphorylation. A possible explanation for this finding is that daily administration may have a favorable influence on survival-related kinases such Akt or Erk 1/2, as suggested by a previous study showing that daily administration of tadalafil up-regulated the phosphorylation of Akt and Erk 1/2 [8]. Meanwhile, in terms of cavernous fibrosis, the combination therapy and the daily administration of a PDE5I restored the SM/collagen ratio and the protein expression of fibronectin in the corpus cavernosum of the CN-injured rats to the normal control level. The combined inhibition of JNK and LIMK2 normalized the LIMK2/Cofilin pathway, while the daily administration of a PDE5I partially improved the Cofilin phosphorylation. Previous studies showed that daily administration of a sildenafil or a udenafil down-regulated TGF-β1 expression in a rat model of CN injury [28,30]. Taken together, daily administration of a PDE5I may improve other TGF-β1-driven pathways with a partial improvement in LIMK2/Cofilin pathway, although the molecular mechanisms related to improvement in fibrosis by a PDE5I remain to be further elucidated.

This study may be limited by the absence of evaluation of cell proliferation in corpus cavernosum. However, the previous studies showed that JNK inhibition and LIMK2 inhibition in CN-injured rats improved erectile function by suppressing cavernous apoptosis and fibrosis, respectively. Thus, our study focused on restoration of CVOF through alleviation of the two main structural alterations by combined inhibition of JNK and LIMK2 rather than cell proliferation in corpus cavernosum. Also, because there was no significant difference in improvement of DIC parameters between the treatment with a PDE5I alone and that with combined inhibition of JNK and LIMK2, the advantage of the use of both JNK inhibitors and LIMK inhibitors over the use of a PDE5 inhibitor alone may be questionable in practical aspects. Nevertheless, this study may provide base data for subsequent mechanism-specific therapies of post-RP ED in future studies, although further time-course studies are needed to validate our results.

CONCLUSIONS

Our data indicate that chronic inhibition of both JNK and LIMK2 from the immediate post-injury period after CNCI can restore CVOF by suppressing both cavernous apoptosis and fibrosis *via* normalization of JNK and LIMK2 pathways. In that CVOD developed by cavernous apoptosis and fibrosis is an important pathophysiology of difficult-to-treat ED induced by CN injury during RP, inhibition of both JNK and LIMK2 may be a potential mechanism-specific therapeutic strategy for CVOD induced by CN injury. In futures, further studies are needed to validate our findings.

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Conflict of Interest

The authors have nothing to disclose.

Author Contribution

Conceptualization: MCC, JL, JP, SWK. Data curation: MCC, JL, JP. Formal analysis: MCC. Funding acquisition: MCC. Investigation: MCC, JL, JP, SWK. Methodology: MCC, JL, JP, SWK. Project administration: MCC, SWK. Resources: MCC, JL, JP, SWK. Software: JL, JP. Supervision: MCC, SWK. Validation: MCC, JL, JP, SWK. Visualization: MCC, JL, JP, SWK. Writing – original draft: MCC. Writing – review & editing: MCC, JL, JP, SWK.

Data Sharing Statement

The data analyzed for this study have been deposited in HARVARD Dataverse and are available at https://doi.org/10.7910/DVN/WPFAQN.

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