Asian Journal of Andrology (2019) 21, 493–500
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ORIGINAL ARTICLE

Erectile Dysfunction

The effects of single versus combined therapy using LIM-kinase 2 inhibitor and type 5 phosphodiesterase inhibitor on erectile function in a rat model of cavernous nerve injury-induced erectile dysfunction

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We aimed to determine whether combination of LIM-kinase 2 inhibitor (LIMK2i) and phosphodiesterase type-5 inhibitor (PDE5i) could restore erectile function through suppressing cavernous fibrosis and improving cavernous apoptosis in a rat model of cavernous nerve crush injury (CNCI). Seventy 12-week-old Sprague–Dawley rats were equally distributed into five groups as follows: (1) sham surgery (Group S), (2) CNCI (Group I), (3) CNCI treated with daily intraperitoneal administration of 10.0 mg kg^{-1} LIMK2i (Group I + L), (4) daily oral administration of 20.0 mg kg^{-1} udenafil, PDE5i (Group I + U), and (5) combined administration of 10.0 mg kg^{-1} LIMK2i and 20.0 mg kg^{-1} udenafil (Group I + L + U). Rats in Groups I + L, I + U, and I + L + U were treated with respective regimens for 2 weeks after CNCI. At 2 weeks after surgery, erectile response was assessed using electrostimulation. Penile tissues were processed for histological studies and western blot. Group I showed lower intracavernous pressure (ICP)/mean arterial pressure (MAP), lower area under the curve (AUC)/MAP, decreased immunohistochemical staining for alpha-smooth muscle (SM) actin, higher apoptotic index, lower SM/collagen ratio, increased phospho-LIMK2-positive fibroblasts, decreased protein kinase B/endothelial nitric oxide synthase (Akt/eNOS) phosphorylation, increased LIMK2/cofilin phosphorylation, and increased protein expression of fibronectin, compared to Group S. In all three treatment groups, erectile responses, protein expression of fibronectin, and SM/collagen ratio were improved. Group I + L + U showed greater improvement in erectile response than Group I + L. SM content and apoptotic index in Groups I + U and I + L + U were improved compared to those in Group I. However, Group I + L did not show a significant improvement in SM content or apoptotic index. The number of phospho-LIMK2-positive fibroblasts was normalized in Groups I + L and I + L + U, but not in Group I + U. Akt/eNOS phosphorylation was improved in Groups I + U and I + L + U, but not in Group I + L. LIMK2/cofilin phosphorylation was improved in Groups I + L and I + L + U, but not in Group I + U. Our data indicate that combined treatment of LIMK2i and PDE5i immediate after CN injury could improve erectile function by improving cavernous apoptosis or eNOS phosphorylation and suppressing cavernous fibrosis. Rectification of Akt/eNOS and LIMK2/cofilin pathways appears to be involved in their improvement.

Asian Journal of Andrology (2019) **21**, 493–500; doi: 10.4103/aja.aja_114_18; published online: 22 February 2019

Keywords: apoptosis; erectile dysfunction; fibrosis; LIM kinase; phosphodiesterase

INTRODUCTION

Cavernous nerve (CN) injury during radical prostatectomy (RP) causes structural alterations of penis including apoptosis of smooth muscle (SM) or endothelium and cavernous fibrosis, resulting in erectile dysfunction (ED).[1–6] Thus, cavernous apoptosis and fibrosis are important contributors to post-RP ED. A few potential therapies for cavernous apoptosis including phosphodiesterase type 5 inhibitors (PDE5is), Rho-kinase inhibitors, intracavernous delivery of sonic hedgehog, intracavernous stem cell injection, and Jun amino-terminal kinase (JNK) inhibitors have been tested in rat models of ED induced by CN injury.[5,7–10] Although these studies have shown positive results in terms of improving cavernous apoptosis or ED,[5,7–10] their routine use is limited due to 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 JNK inhibitors.[11–14]

Several therapeutic strategies that can improve CN-associated ED through inhibiting cavernous fibrosis have been reported.[15–18] However, studies devoted specifically to the alleviation of cavernous fibrosis in animal models of ED induced by CN injury are limited. Mechanism-specific targeted therapies for cavernous fibrosis after CN injury are insufficient. Our recent studies have shown that Rho-associated, coiled-coil-containing protein kinase 1 (ROCK1)/LIM-kinase 2 (LIMK2)/cofilin

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Received: 03 June 2018; Accepted: 12 November 2018

pathway plays a role in cavernous fibrosis through coordination with transforming growth factor (TGF)-β1 after CN injury.[19–22] However, LIMK2 inhibition does not normalize erectile function or cavernous fibrosis completely,[21,22] suggesting that a combination therapy of LIMK2 inhibitor (LIMK2i) and other agents might be needed to restore erectile function in animal models of ED after CN injury. Therefore, we noted that treatment with PDE5i in a rat model of ED induced by CN injury alleviated ED through the anti-apoptotic effect or positive effect on endothelial nitric oxide synthase (eNOS).[4,23]

In this context, we hypothesized that erectile function in a rat model of ED after CN injury could be further improved by combined therapy of LIMK2i and PDE5i with known anti-apoptotic effect or positive effect on eNOS phosphorylation, compared to administration of LIMK2i alone or PDE5i alone.[4,23] Thus, the objective of this study was to determine whether combined administration of LIMK2i and PDE5i could further improve erectile function through anti-fibrotic effect and improvement of cavernous apoptosis or positive effect on eNOS.

MATERIALS AND METHODS

Experimental design

Seventy 12-week-old Sprague–Dawley rats were equally divided into the following five groups (n = 14 per group): (1) sham surgery (Group S), rats were treated with daily intraperitoneal administration of saline vehicle and daily oral administration of saline vehicle, (2) bilateral CN crush injury (Group I), rats were treated with daily intraperitoneal administration of saline vehicle and daily oral administration of saline vehicle, (3) rats with bilateral CN crush injury were treated with daily intraperitoneal administration of 10.0 mg kg^{-1} LIMK2i (LX-7101, Cellagen Technology, San Diego, CA, USA)[21,22] and daily oral administration of saline vehicle (Group I + L), (4) rats with bilateral CN crush injury were treated with daily intraperitoneal administration of saline vehicle and daily oral administration of 20.0 mg kg^{-1} udenafil (PDE5i, Dong-A, Seoul, Korea) (Group I + U), and (5) rats with bilateral CN crush injury were treated with combined administration of 10.0 mg kg^{-1} LIMK2i[21,22] and 20.0 mg kg^{-1} udenafil (Group I + L + U). After anesthetizing rats with intraperitoneal injection of zoletil (10.0 mg kg^{-1}; Vibac Laboratories, Carros, France) and isoflurane (Abbott Laboratories, North Chicago, IL, USA) inhalation, a lower abdominal midline incision and pelvic dissection were made by the same trained surgeon. For rats in Group S, bilateral CNs were dissected without any direct injury to CNs. Crush injury was induced by mechanical compression of bilateral CNs at a location 3–4 mm distal to the major pelvic ganglion using a microsurgical vascular clamp (Solco, Pyeongtaek, Korea). The microsurgical vascular clamp was held to the closure twice for 70 s each. Treatment was started from the next day after surgery. It was interrupted 2 days before *in vivo* assessment of erectile function (a 48-h washout period). Our animal studies were approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute at the Seoul National University Hospital (Seoul, Korea), an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. Care for these animals was conducted in accordance with the National Research Council guidelines for the care and use of laboratory animals.

Evaluation of in vivo erectile function

Erectile function was determined using a standardized model by electrical stimulation of the CNs at 2 weeks after surgery to generate erectile responses as described previously.[10,20–22] After a lower midline incision, major pelvic ganglions and the CNs were isolated. Then, a platinum bipolar electrode (Grass Instrument Company, Quincy, MA, USA) was placed around the CN distal to the site of nerve injury. Stimulation parameters were as follows: 1.0 V, 2.5 V, and 4.0 V at 16 Hz with a square wave duration of 0.3 ms for 30 s. Erectile responses were expressed as intracavernous pressure (ICP) and the area under the curve (AUC) during the entire erectile response normalized with mean arterial pressure (MAP). *In vivo* assessment of erectile function was performed for eight rats in each group. Total penectomy was performed for the remaining six rats in each group for western blot and histological analyses. The middle part of the skin-denuded penile shaft undamaged by a needle was harvested, fixed in 10% formaldehyde solution (Sigma-Aldrich, St. Louis, MO, USA) overnight, and embedded in paraffin (Sigma-Aldrich) for histological staining. The remaining cavernous tissues were stored at −80°C until processing.

Histological examinations

To determine the ratio of SM/collagen fibril in the cavernous tissue, Masson's trichrome staining (Abcam, Cambridge, UK) was performed as described previously.[19,20,22] For each stained slide, areas of SM (stained in red) and collagen fibril (stained in blue) were analyzed. To determine SM content in the cavernous tissue, immunohistochemical staining was carried out using a primary antibody against α-SM actin (α-SMA) (1:100, Dako, Glostrup, Denmark) as described previously.[10,19,20]

For histological studies, each stained slide was reviewed through quantitative analysis for the image of the penis comprising the corpora cavernosum half at ×40 using ImagePro Plus 4.5 software (Medica Cybernetics, Silver Spring, MD, USA). We analyzed stained slides of six rats from each group (two tissue sections per animal). An independent examiner reviewed these slides in a blinded fashion using the same standard.

Evaluation of cavernous apoptosis

To evaluate apoptosis in the cavernous tissue, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using ApopTag® Red *In Situ* Apoptosis Detection Kit (Merck Millipore, Darmstadt, Germany) as described previously.[20] Cell nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI, stained in blue). Six rats from each group were evaluated, and two tissue sections per animal were analyzed. For each slide, four nonoverlapping zones were randomly selected at ×400 and examined using a confocal laser microscope (Leica TCS SP8; Leica Microsystems, Mannheim, Germany). The apoptotic index was presented as a ratio of apoptotic nuclei (stained in pink) to the total number of nuclei counted in the given area.

Double immunofluorescent microscopy

To determine the content of fibroblasts positive for phosphorylated LIMK2 in the cavernous tissue, we performed double immunofluorescent labeling for cavernous tissue with antibodies to vimentin and phosphorylated LIMK2 as described previously.[20,22] Paraffin-embedded tissue sections (2.5 μm) were incubated with primary antibodies to vimentin (a fibroblast marker, 1:100, Dako) and phospho-LIMK2 (phospho T505, 1:50; Abcam). After several washings with phosphate-buffered saline, sections were incubated with two secondary antibodies (anti-mouse IgG 488 and goats' anti-rabbit IgG 594, Abcam) in 1% bovine serum albumin at room temperature for 1 h. We acquired images through confocal laser scanning using a confocal microscope. Nuclear staining was performed with DAPI. For each slide, four nonoverlapping zones were randomly selected at ×400. Among DAPI-positive (blue or purple color in merged or magnified images, respectively) cells in the cavernous tissue, the number of fibroblasts positive for phosphorylated LIMK2 (yellow color) was determined by an independent examiner who was blinded to group allocation.

Western blot assay

As described previously,[10,19–22] western blot assay was carried out to determine protein expression levels. Briefly, after cavernous tissue samples were homogenized, equal amounts of protein (50 μg in each well) were electrophoresed on Mini Protean TGX gels (7.5% or 12.0%; Bio-Rad, Hercules, CA, USA). They were run at 200 V for 30 min and transferred to polyvinylidene fluoride (PDVF) membrane (Merck Millipore) at 100 V for 60 min. After adding primary antibodies, they were incubated overnight at 4°C. After adding secondary antibody, they were incubated for 60 min. Primary antibodies were as follows: anti-Akt (1:5000, Cell-Signaling Technology, Danvers, MA, USA), anti-phospho-Akt (Ser473, 1:1000, Cell-Signaling Technology), anti-eNOS (1:3000, BD Biosciences, San Jose, CA, USA), anti-phospho-eNOS (Ser1177, 1:1000, Cell-Signaling Technology), anti-nNOS (1:2000, Cell-Signaling Technology), anti-phospho-nNOS (Ser1417, 1:1000, Thermo Fisher Scientific, Waltham, MA, USA), anti-phospho-LIMK2 (phospho T505; Thr505, 1:1000, Abcam), anti-LIMK2 (1:2000, Abcam), anti-phospho-cofilin (Ser3, 1:1000, Cell-Signaling Technology), anti-cofilin (1:1000, Cell-Signaling Technology), and anti-fibronectin (1:1000, Abcam). Results were quantified by densitometry and presented as relative density of each protein compared to that of β-actin.

Statistical analyses

Results in bar graphs of figures are reported as a median (interquartile range). We analyzed statistical differences among groups using Mann–Whitney U test or Kruskal–Wallis test. $P < 0.05$ was considered statistically significant. All statistical tests were two-sided. SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) was used for all data analyses.

RESULTS

Effect of combined administration of LIMK2i and udenafil on erectile response

Rats in Group I showed lower ICP/MAP and lower AUC/MAP after stimulation at 1.0 V, 2.5 V, and 4.0 V, compared to those in Group S (ICP/MAP: $P = 0.001$; AUC/MAP: $P = 0.001$; **Figure 1**). In all treatment groups, ICP/MAP and AUC/MAP after stimulation at 1.0 V, 2.5 V, and 4.0 V were improved compared to those in Group I (ICP/MAP: $P < 0.05$; AUC/MAP: $P < 0.05$). However, they did not recover to values observed in Group S (**Figure 1**). After stimulation at 4.0 V, rats in both I + U and I + L + U groups showed higher ICP/MAP and AUC/MAP compared to those in Group I + L (ICP/MAP: $P = 0.018$ in Group I + U and $P = 0.001$ in Group I + L + U; AUC/MAP: $P = 0.022$ in Group I + L + U). However, there were no differences in ICP/MAP or AUC/MAP among Groups I + L, I + U, and I + L + U after stimulation at 1.0 V. There were no statistically significant differences in ICP/MAP or AUC/MAP at any stimulation parameter between Groups I + U and I + L + U.

Effect of daily administration of udenafil alone, LIMK2i alone, or combined administration of the two drugs on cavernous apoptosis and eNOS phosphorylation

Rats in Group I showed decreased immunohistochemical staining of α-SMA ($P < 0.001$), higher apoptotic index ($P <$

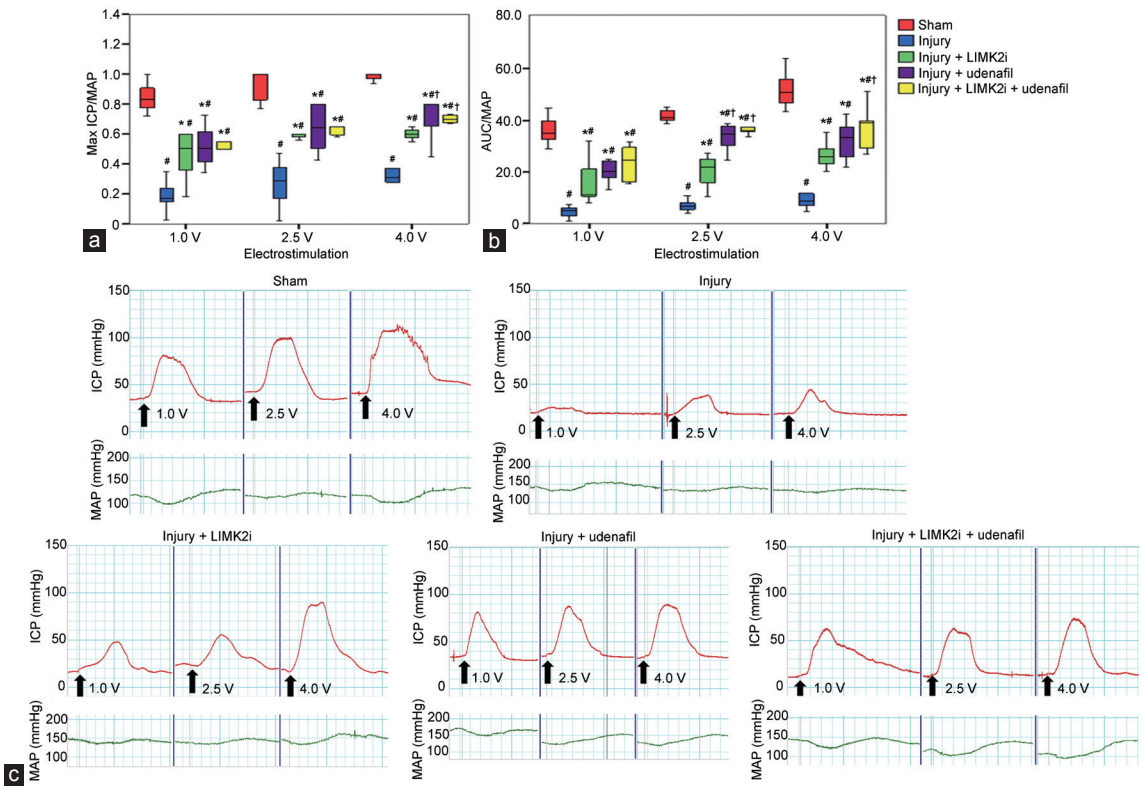

Figure 1: Comparison in erectile responses (**a**: ICP/MAP, **b**: AUC/MAP) to electrostimulation at 2 weeks after bilateral CNCI among the five groups (sham, injury, injury + LIMK2i, injury + udenafil, and injury + LIMK2i + udenafil). (**c**) Representative ICP and MAP for the five groups. Black arrow indicates the time point of electrical stimulation of the cavernous nerve. Eight rats from each group were evaluated. $^{*}P < 0.05$, the indicated group compared to injury group; $^{\#}P < 0.05$, the indicated group compared to sham group; and $^{\dagger}P < 0.05$, the indicated group compared to injury + LIMK2i group. CNCI: cavernous nerve crush injury; ICP: intracavernous pressure; MAP: mean arterial pressure; AUC: area under the curve corresponding to the duration of electrical stimulation; LIMK2i: LIM-kinase 2 inhibitor; sham: sham surgery group; injury: bilateral CNCI group; injury + LIMK2i: bilateral CNCI group treated with daily administration of 10.0 mg kg^{-1} LIMK2i; injury + udenafil: bilateral CNCI group treated with daily administration of 20.0 mg kg^{-1} udenafil; injury + LIMK2i + udenafil: bilateral CNCI group treated with combined administration with 10.0 mg kg^{-1} LIMK2i and 20.0 mg kg^{-1} udenafil.

0.001), decreased Akt and eNOS phosphorylation ($P < 0.001$ and $P = 0.002$, respectively), decreased protein expression of total Akt, total eNOS and total nNOS ($P = 0.048$, $P = 0.033$, and $P < 0.001$, respectively), and increased nNOS phosphorylation ($P = 0.025$) compared to those in Group S (**Figure 2a, 2b** and **3**). Regarding immunohistochemical staining of α-SMA, SM contents in Groups I + U and I + L + U were improved compared to those in Group I ($P < 0.001$ in Group I + U and $P < 0.001$ in Group I + L + U; **Figure 2a**). However, Group I + L did not show improvement in the SM content compared to Group I. Results of TUNEL analysis showed that apoptotic indices in Groups I + U and I + L + U were restored to sham control level, whereas Group I + L did not show such improvement (**Figure 2b**). According to densitometry, protein expression of total Akt, Akt phosphorylation level, protein expression of total eNOS, and eNOS phosphorylation level in Groups I + U (all $P < 0.05$) and I + L + U (all $P < 0.05$) were improved compared to those in Group I. However, those in Group I + L did not show such improvement (**Figure 3**). Protein expression level of total nNOS or NOS phosphorylation level did not change in any of these three treatment groups.

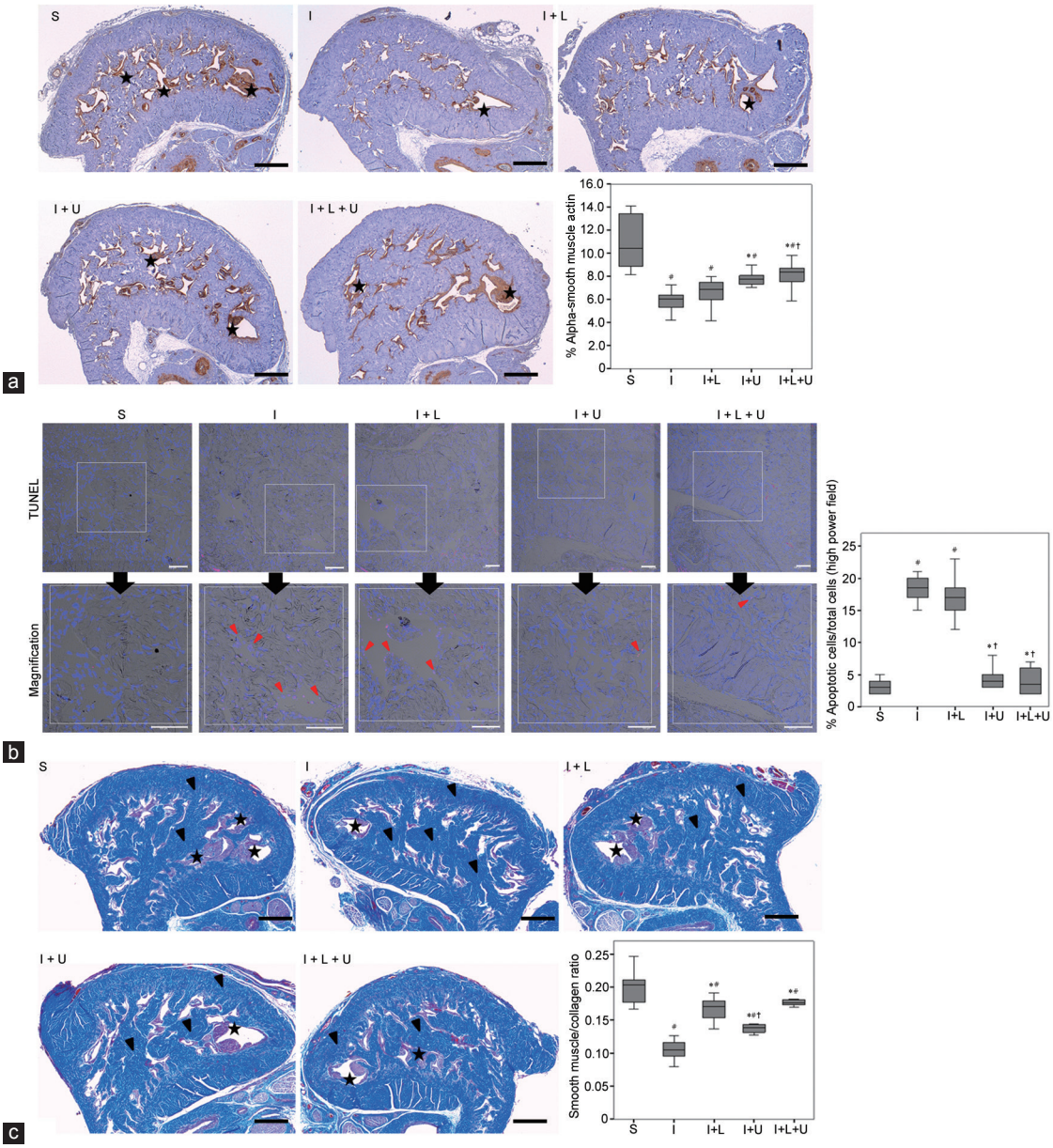

Figure 2: Comparison in (**a**) smooth muscle content (immunohistochemical staining of α-smooth muscle actin, scale bars = 200 μm), (**b**) cavernous apoptosis (immunofluorescent staining with TUNEL, scale bars = 100 μm), and (**c**) smooth muscle/collagen ratio (Masson's trichrome staining, scale bars = 200 μm) at 2 weeks after bilateral CNCI among the five groups (S, I, I + L, I + U and I + L + U). Six rats in each group were analyzed for the histological studies. Black asterisks indicate the cavernous sinusoids and the smooth muscle trabeculae. Red arrow heads indicate the apoptotic cells. Black arrowheads indicate the interstitium with collagen deposition. $^{*}P < 0.05$, the indicated group compared to I group; $^{\#}P < 0.05$, the indicated group compared to S group; and $^{\dagger}P < 0.05$, the indicated group compared to I + L group. α-SMA: α-smooth muscle actin; TUNEL: terminal deoxynucleotidyl transferase-mediated 2' deoxyuridine 5'-triphosphate (dUTP) nick-end labeling; CNCI: cavernous nerve crush injury; S: sham surgery group; I: bilateral CNCI group; I + L: bilateral CNCI group treated with daily administration of 10.0 mg kg^{-1} LIMK2i; I + U: bilateral CNCI group treated with daily administration of 20.0 mg kg^{-1} udenafil; I + L + U: bilateral CNCI group treated with combined administration with 10.0 mg kg^{-1} LIMK2i and 20.0 mg kg^{-1} udenafil.

Daily administration of udenafil alone, LIMK2i alone, or combined administration of the two drugs can improve cavernous fibrosis

Rats in Group I showed lower SM/collagen ratio ($P < 0.001$), increased protein expression of fibronectin ($P = 0.001$), increased amounts of fibroblasts positive for phosphorylated LIMK2 ($P < 0.001$), and increased LIMK2 and cofilin phosphorylation ($P = 0.001$ and $P < 0.001$, respectively) compared to those in Group S (**Figure 2c, 3** and **4**). Regarding SM/collagen ratios and protein expression of fibronectin, all three treatment groups showed improvement compared to Group I ($P < 0.001$ and $P = 0.002$ in Group I + L; $P = 0.021$ and $P = 0.024$ in Group I + U; $P < 0.001$ and $P = 0.004$ in Group I + L + U, respectively). However, they did not recover to levels observed in Group S (**Figure 2c**). Both Groups I + L and I + L + U had greater improvement in SM/collagen ratio ($P = 0.014$ in Group I + L; $P < 0.001$ in Group I + L + U) and protein expression of fibronectin ($P = 0.033$ in Group I + L; $P = 0.029$ in Group I + L + U), compared to Group I + U. After double immunofluorescent staining of cavernous tissue with antibodies to phospho-LIMK2 and vimentin, amounts of fibroblasts positive for phosphorylated LIMK2 in Groups I + L and I + L + U were normalized, but not in Group I + U (**Figure 4**). LIMK2/cofilin phosphorylation levels in Groups I + L and I + L + U, but those in Group I + U, were restored to sham control levels (**Figure 3**).

DISCUSSION

Up to date, ED induced by CN injury during RP remains a difficult-to-treat disease due to a few significant pathophysiologic conditions including cavernous apoptosis and fibrosis, although some potential therapies have been tested in animal models of post-RP ED.[5,7–10,15–18] With such background, the current study aimed to identify whether combined treatment of LIMK2i with PDE5i could further improve erectile function through both suppression of cavernous fibrosis and improvement of cavernous apoptosis in a rat model of ED induced by CN injury, compared to administration of LIMK2i alone or PDE5i alone. The present study has three main findings. First, combined administration of udenafil with LIMK2i for 2 weeks immediately after injury showed greater improvement in erectile function compared to administration of LIMK2i alone, although erectile function was not completely normalized. Meanwhile, there was no statistically significant difference in improvement of erectile function between the administration of udenafil alone and combined administration of the two drugs. Second, daily administration of udenafil alone or combined administration of udenafil with LIMK2i immediate postinjury restored cavernous apoptosis, protein expression of total eNOS, and eNOS phosphorylation to sham control levels. Third, all three treatments (LIMK2i alone, udenafil alone, and a combination of udenafil with LIMK2i) significantly alleviated cavernous fibrosis at 2 weeks after CN injury. However, the group treated with LIMK2i alone or a combination of the two drugs showed a greater degree of improvement in cavernous fibrosis by normalizing LIMK2/cofilin signaling pathway and the content of fibroblasts positive for phosphorylated LIMK2 compared to the group treated with udenafil alone.

The combined administration of udenafil with LIMK2i for 2 weeks immediate postinjury did not completely normalize erectile function. Furthermore, the combined administration of the two drugs did not show a significantly additive effect on improvement of erectile function at 2 weeks postinjury. There are some plausible explanations for this finding. First, during the acute phase (1 week to 2 weeks) after CN injury, recovery from cavernous apoptosis and endothelial dysfunction might play a more important role in the

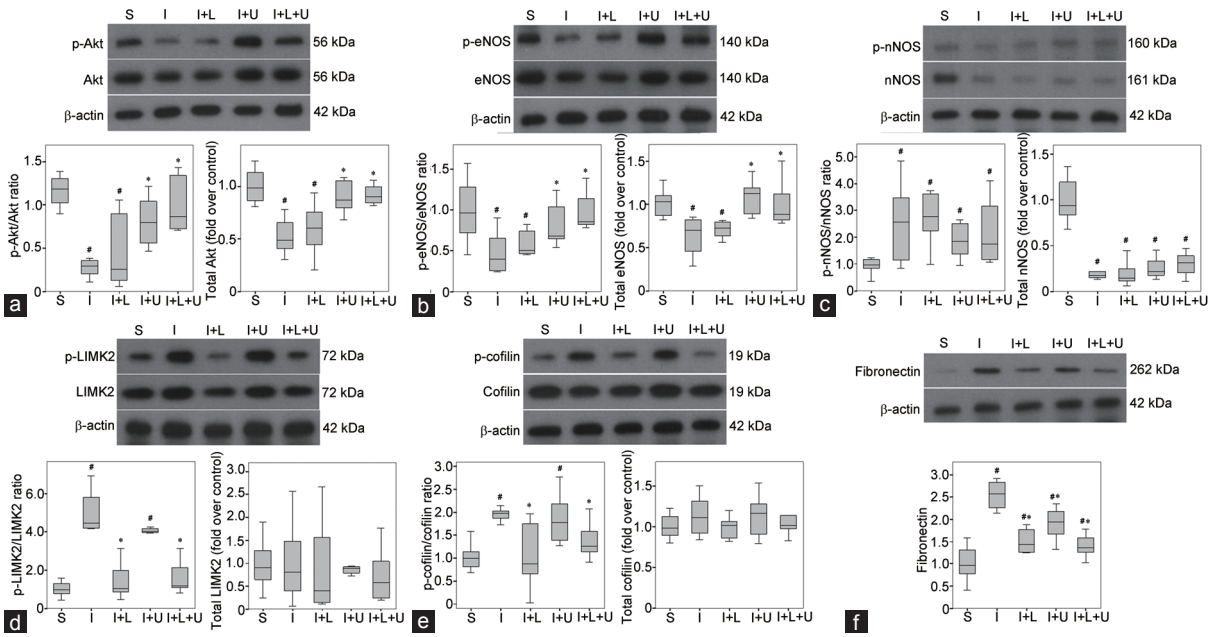

Figure 3: Representative immunoblot images and bar graphs (median and interquartile range) showing the comparison in the protein expression of (**a**) phosphorylated Akt/total Akt and total Akt, (**b**) phosphorylated eNOS/total eNOS and total eNOS, (**c**) phosphorylated nNOS/total nNOS and total nNOS, (**d**) phosphorylated LIMK2/total LIMK2 and total LIMK2, (**e**) phosphorylated cofilin/total cofilin and total cofilin, and (**f**) fibronectin from the cavernous tissues among the five groups using densitometry. The data were normalized by β-actin expression and presented as fold changes over controls. Six rats in each group were evaluated. $^{*}P < 0.05$, the indicated group compared to I group; and $^{\#}P < 0.05$, the indicated group compared to S group. CNCI: cavernous nerve crush injury; eNOS: endothelial nitric oxide synthase; nNOS: neuronal nitric oxide synthase; LIMK2i: LIM-kinase 2 inhibitor; S: sham surgery group; I: bilateral CNCI group; I + L: bilateral CNCI group treated with daily administration of 10.0 mg kg^{-1} LIMK2i; I + U: bilateral CNCI group treated with daily administration of 20.0 mg kg^{-1} udenafil; I + L + U: bilateral CNCI group treated with combined administration with 10.0 mg kg^{-1} LIMK2i and 20.0 mg kg^{-1} udenafil.

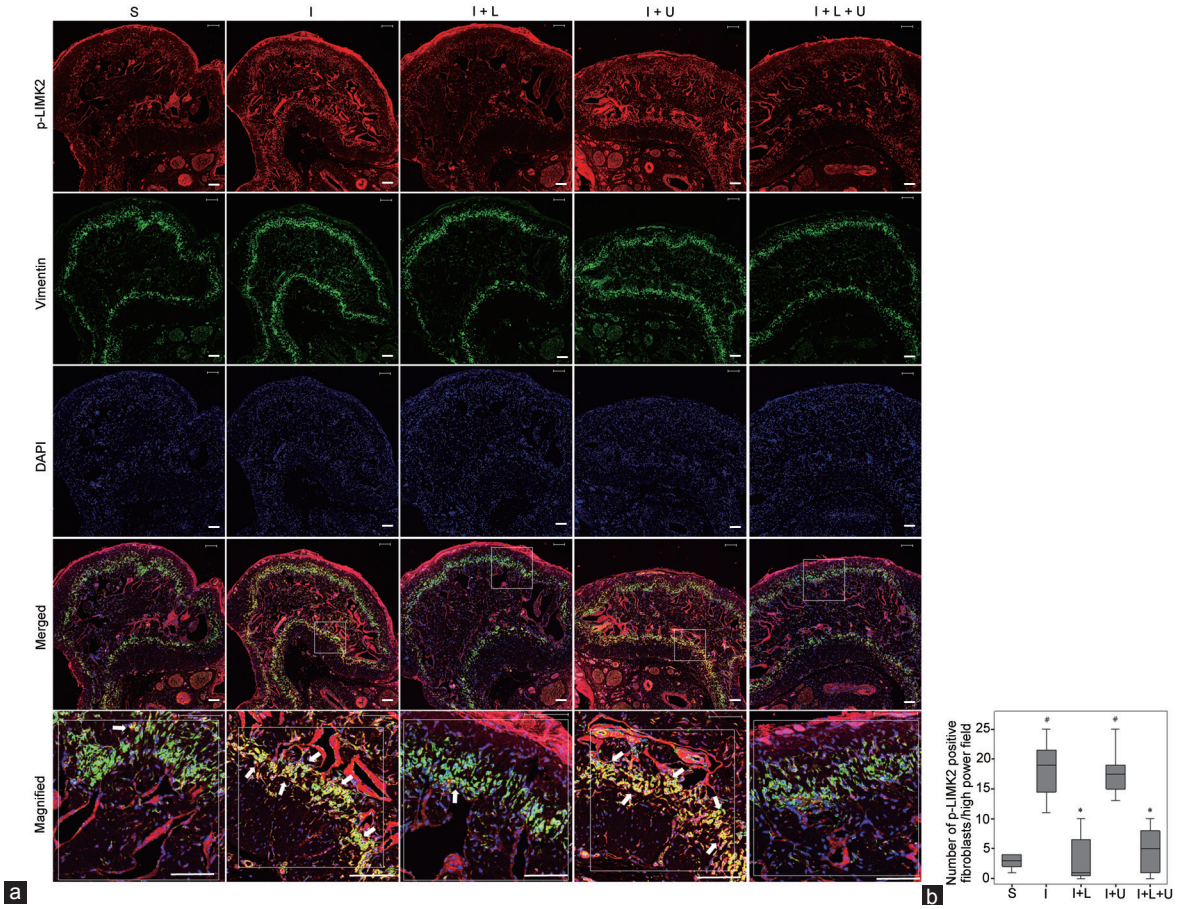

Figure 4: The comparison in the amount of fibroblasts positive for phosphorylated LIMK2 after 2 weeks of surgery among the five groups. (**a**) Representative images for double immunofluorescent staining of cavernous tissue with anti-vimentin and anti-phospho-LIMK2 using confocal microscope scanning. White arrow indicates significant expression of phosphorylated LIMK2 in cavernous fibroblasts (yellow color in merged or magnified image). Scale bar = 100 μm. (**b**) Bar graphs (median and interquartile range) showing the comparison in the number of fibroblasts positive for phosphorylated LIMK2 among the five groups. Six rats in each group were evaluated. $^{*}P < 0.05$, the indicated group compared to I group; and $^{\#}P < 0.05$, the indicated group compared to S group. CNCI: cavernous nerve crush injury; LIMK2i: LIM-kinase 2 inhibitors; S: sham surgery group; I: bilateral CNCI group; I + L: bilateral CNCI group treated with daily administration of 10.0 mg kg^{-1} LIMK2i; I + U: bilateral CNCI group treated with daily administration of 20.0 mg kg^{-1} udenafil; I + L + U: bilateral CNCI group treated with combined administration with 10.0 mg kg^{-1} LIMK2i and 20.0 mg kg^{-1} udenafil.

improvement of erectile function than recovery from cavernous fibrosis. This can be a potential explanation for our finding, given that apoptosis of cavernous SM or endothelium is well known to occur primarily during early postinjury period (1 week to 2 weeks) while cavernous fibrosis progresses over time.[2,5,19,24,25] Thus, further time-course studies with long-term follow-up are needed to elucidate relative roles of pathophysiologies (endothelial dysfunction, cavernous apoptosis, and fibrosis) in ED at chronic phase after CN injury. Second, previous studies have shown that daily administration of PDE5is from the early post-injury period can reduce SM/collagen ratio, collagen deposition, and TGF-β1 protein expression, thus improving ED.[15,23] Because PDE5is have, to some degree, anti-fibrotic effect in addition to their anti-apoptotic effect or positive effect on eNOS phosphorylation,[4,23] a significantly additive effect of combined treatment with udenafil and LIMK2i might not be observed for further improvement of erectile function during the acute phase after CN injury. Third, combined administration of LIMK2i with PDE5i did not improve protein expression of nNOS in cavernous tissue at 2 weeks after CN injury. Thus, this therapeutic strategy may not result in complete recovery of erectile function at acute phase after CN injury.

As expected, our results confirmed that daily administration of udenafil alone or combination of LIMK2i with udenafil improved ED through alleviation of cavernous apoptosis, protein expression of total Akt or eNOS and dysregulated Akt/eNOS phosphorylation in a rat model of CN injury. Our finding is consistent with results of previous studies showing that daily administration of PDE5is can mend erectile function by rectifying cavernous apoptosis and eNOS phosphorylation in rat models of CN injury.[4,15,23] Interestingly, daily administration of LIMK2i alone or combination of LIMK2i with udenafil provided greater degree of improvement in cavernous fibrosis through normalizing amounts of fibroblasts positive for phosphorylated LIMK2 and LIMK2/cofilin phosphorylation compared to daily administration of udenafil alone. Treatment with udenafil alone did not have any significant impact on amounts of fibroblasts positive for phosphorylated LIMK2 or LIMK2/cofilin phosphorylation. This suggests that rectification of dysregulated TGF-β1-driven pathways other than the LIMK2/cofilin pathway might be involved in the moderate degree of improvement in cavernous fibrosis. However, further studies are needed to elucidate the precise molecular mechanism by which PDE5i improves cavernous fibrosis in ED induced by CN injury, although previous studies have

suggested the restoration of the TGF-β1-driven pathway as a candidate mechanism.[4,23]

There are several limitations in the present study. First, the results of the present study fell short of our expectation that the combination therapy of LIMK2i with PDE5i could normalize erectile function at acute phase after CN injury in a rat. Therefore, to render our therapeutic strategy meaningful, subsequent studies are needed to determine the therapeutic effect of the combination therapy at chronic phase after CN injury. Combined therapies of LIMK2i with anti-fibrotic effect, other anti-apoptotic agents (*i.e.*, JNK inhibitor, sonic hedgehog, and dipyridamole) and agents for promoting angiogenesis/neurogenesis (*i.e.*, hepatic growth factor and basic fibroblast factor) need to be tested in the future studies. Second, the treatment with LIMK2i or PDE5i did not improve protein expression of total nNOS in cavernous tissue. Meanwhile, the phosphorylation (at Ser1417: positively regulatory site) of nNOS was increased in Group I as well as the three treatment groups after CN injury. This increase in the phosphorylation at Ser1417 of nNOS appears to function as a compensatory mechanism after CN injury. Given that a recent study showed a significant increase in phosphorylation on negatively regulatory site of nNOS after CN injury,[26] the dysregulated nNOS phosphorylation might contribute to incomplete recovery from ED induced by CN injury despite the combined treatment with LIMK2i and PDE5i. However, the status of phosphorylation on negatively regulatory site of nNOS was not evaluated in our study. Thus, in the future, the more detailed evaluation including assessment of phosphorylation status on both positively and negatively regulatory sites of nNOS in an animal model of ED induced by CN injury is needed to draw a solid conclusion regarding it. Furthermore, the effect of adding an agent for the restoration of both nNOS phosphorylation status and total nNOS protein expression to our combined regimens on the recovery of erectile function may need to be determined in animal models of ED induced by CN injury.

CONCLUSION

Our data indicate that combined treatment with LIMK2i and udenafil from the immediate postinjury period after CN injury can improve erectile function by suppressing cavernous apoptosis, improving eNOS phosphorylation and suppressing cavernous fibrosis. Rectification of Akt/eNOS and LIMK2/cofilin pathways appears to be involved in their improvement. However, such combined treatment did not completely normalize the erectile function at acute phase after CN injury, although the degree of improvement by the combined treatment was somewhat greater than that by treatment with LIMK2i alone. Furthermore, the combined treatment did not appear to provide statistically greater improvement in erectile function than treatment with udenafil during the acute phase after CN injury. Further studies are needed to determine therapeutic effects of the combined treatment on ED at a chronic phase after CN injury and determine whether other combined regimens, including other anti-apoptotic agents, agents for promoting angiogenesis/neurogenesis and an agent for the restoration of both nNOS phosphorylation status and total nNOS protein expression, could restore erectile function.

AUTHOR CONTRIBUTIONS

MCC carried out substantial contributions to conception/design, animal experiments, data acquisition, data analysis, interpretation, drafting the manuscript, and statistical analysis. JL carried out substantial contributions to conception/design and data interpretation. JP carried out substantial contributions to conception/design and data interpretation. SO carried out substantial contributions to statistical analysis and critical revision of the manuscript for scientific and factual content. JSC helped animal experiments and data acquisition. HS carried out substantial contributions to conception/design and data interpretation. JSP carried out substantial contributions to conception/design and data interpretation. SWK carried out substantial contributions to conception/design, data interpretation, and critical revision of the manuscript for scientific and factual content, and helped to draft the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

ACKNOWLEDGMENTS

This research was supported by the SNUH Research Fund (Grant No.: 04-2015-032-0) and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (Grant No.: NRF-2015R1D1A1A01060003).

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