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ORIGINAL ARTICLE

Male Sexual Dysfunction

Role of Jun amino-terminal kinase (JNK) in apoptosis of cavernosal tissue during acute phase after cavernosal nerve injury

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The present study aimed to identify which mitogen-activated protein kinase (p38 or Jun amino-terminal kinase [JNK]) was involved in cavernosal apoptosis during the acute phase after cavernosal nerve crush injury (CNCI) in rats to ameliorate apoptosis of cavernosal tissue, such as smooth muscle (SM). A total of twenty 10-week-old male Sprague-Dawley rats were divided equally into two groups: sham surgery (S) and CNCI (I). The I group approximated the clinical situation of men undergoing radical prostatectomy using two 60-second compressions of both CNs with a microsurgical vascular clamp. At 2-week postinjury, erectile response was assessed using electrostimulation. Penile tissues were harvested for immunohistochemistry analysis of alpha-SMA, western blot analysis, and double immunofluorescence analysis of alpha-SMA and phosphorylated p38 or JNK, as well as double immunofluorescent of TUNEL and phosphorylated p38 or JNK. At 2-week postinjury, the I group had a significantly lower intracavernous pressure (ICP)/mean arterial pressure (MAP) and a lower area under the curve (AUC)/MAP than the S group. The I group also exhibited decreased immunohistochemical staining of alpha-SMA, an increase in the number of SM cells positive for phosphorylated JNK, an increased number of apoptotic cells positive for phosphorylated JNK, and increased JNK phosphorylation compared with the S group. However, there was no significant difference in p38 phosphorylation expression or the number of SM cells positive for phosphorylated p38 between the two groups. In conclusion, our data suggest that JNK, not p38, is involved in cavernosal apoptosis during the acute phase after partial CN damage.

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INTRODUCTION

The main pathophysiology of erectile dysfunction (ED) after nerve-sparing radical prostatectomy (nsRP) is neuropraxia, which is induced by partial damage to cavernosal nerves (CNs) during surgery, although the etiology of post-RP ED is multifactorial.¹ During neuropraxia, which is defined as the temporary blockade of nerve transmission, structural alterations of the penis, such as cavernosal apoptosis and fibrosis, can occur after surgery.¹ Apoptosis develops in the cavernosal tissues of the penis during the acute period after CN injury.^{2,3} CN-induced apoptosis of cavernosal tissue such as smooth muscle (SM) appears to lead to cavernosal veno-occlusive dysfunction (CVOD), the main cause of post-RP ED.^{2,4} Thus, preservation of penile integrity by preventing cavernosal apoptosis is expected to play a critical role in the maintenance of erectile function after nsRP. Various strategies have been suggested for penile rehabilitation, including daily dosing of phosphodiesterase type 5 inhibitors (PDE5Is), intracavernosal injection therapy, or the use of vacuum erection devices; conclusive data, however, do not exist regarding efficacy or routine use because of insufficient efficacy, inconvenience of use, or limited high-quality studies to date.^{1,5–9} Therefore, there is a need to identify novel therapeutic targets or strategies to prevent post-RP ED. Furthermore,

although the preservation of penile integrity through early prevention of cavernosal apoptosis can have clinically significant implications for the alleviation of post-RP ED, very little is known about the molecular mechanisms related to cavernosal apoptosis during the acute phase following partial CN damage.

Mitogen-activated protein kinases (MAPKs) are involved in vital signal transduction pathways that regulate various processes, including cell death.¹⁰ Among the MAPKs, Jun amino-terminal kinase (JNK) and p38 are known to play critical roles in apoptosis.¹⁰ A previous study by Lysiak *et al.*¹¹ using a mouse model of complete CN damage (resection) reported increased cavernosal apoptosis, together with increased protein expression of phosphorylated JNK and p38. However, it remains to be determined which of the two MAPKs specifically contribute to cavernosal apoptosis during the early period following partial CN damages.

Thus, the aim of this study is to determine which MAPK (JNK or p38) is involved in early apoptosis of cavernosal tissue, such as SM, in a rat model of CN crush injury (CNCI) to provide baseline data for potential therapeutic targets for the prevention of cavernosal apoptosis.

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MATERIALS AND METHODS

Study design

All experimental procedures were approved by 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. This study followed the National Research Council guidelines for the care and use of laboratory animals. A total of twenty 11-week-old male Sprague-Dawley rats were randomized equally into two groups as sham surgery (S) and CNCI (I). For the S group, pelvic dissection was performed to identify both CNs without causing direct damage to the CNs. For the I group, which approximated the clinical situation in men undergoing nsRP, two 60-second compressions of both CNs were performed 2–3 mm distal to the major pelvic ganglion using a microsurgical vascular clamp.¹²

Measurement of erectile function

At 2-week postinjury, erectile function was assessed in anesthetized rats using erectile responses to electrical stimulation of the CNs as previously described.¹² To evaluate erectile responses to electrical stimulation of the CNs, a 24-gauge angiocatheter was introduced into the carotid artery to continuously monitor mean arterial pressure (MAP). The corpus cavernosum was cannulated with a 26-gauge needle to continuously monitor intracavernous pressure (ICP). A platinum bipolar electrode was placed around the CN, which was stimulated at a frequency of 15 Hz, width of 0.2 ms, and duration of 30 s with various voltages (1.0 V, 2.5 V, and 4.0 V). Comparisons were made for the ICP/MAP and areas under the curve (AUC) corresponding with duration of electrical stimulation. The AUC was corrected by the MAP.

After completing functional studies, the entire penis was removed from each rat. The middle section of the skin-denuded penile shaft was maintained overnight in 10% formaldehyde solution, followed by paraffin-embedding for histological studies. The remaining tissues were rapidly frozen in liquid nitrogen and stored at -80°C .

Immunohistochemical staining for α -SM actin protein expression

To assess SM content, immunohistochemical staining was performed using a primary antibody against alpha-SM actin (α -SMA, 1:100, M0851, Dako, Glostrup, Denmark) as previously described.¹² Ten rats from each group were evaluated, and two tissue sections per animal were reviewed. Quantitative image analyses were performed for each slide at $\times 40$ magnification using Image Pro Plus 4.5 software (Medica Cybernetics, Rockville, MD, USA). We analyzed $\times 40$ magnification images of the penis comprising one-half of the corpora cavernosa, and the percentage of smooth muscle fibers in a total area of one-half of the corpora cavernosa was measured. The slides were evaluated by three independent observers in a blinded fashion.

Double immunofluorescence microscopy

To assess the number of SM cells positive for phosphorylated p38 or phosphorylated JNK, paraffin-embedded sections (2.5-mm-thick) of penile tissue were incubated overnight with primary antibodies against α -SMA (a SM cell marker, 1:100, M0851, Dako, Glostrup, Denmark; red) and phosphorylated p38 (1:20, #4511, Cell-Signaling Technology, Danvers, MA, USA; green) or phosphorylated JNK (1:20, #4668S, Cell-Signaling Technology, Danvers, MA, USA; green). After washing in PBS, the sections were incubated with two secondary antibodies (goat anti-mouse IgG 488 [1:400, A-11001, Invitrogen, Camarillo, CA, USA] and goat anti-rabbit IgG 594 [1:200, ab96901, Abcam, Cambridge, CB4 0FL, UK]) in 1% bovine serum albumin at room temperature for 1 h. Digital images were acquired using a

confocal microscope (Leica TCS SP8, Leica Microsystems, Wetzlar, Germany). Ten rats from each group (two sections per animal) were analyzed. Under confocal microscopy, the number of SM cells positive for phosphorylated p38 or phosphorylated JNK (yellow) was quantified from five randomly selected high-power fields (white arrow). The slides were evaluated by three independent observers in a blinded fashion.

Detection of apoptotic cells positive for phosphorylated JNK or phosphorylated p38

To determine the number of apoptotic cells positive for phosphorylated p38 or phosphorylated JNK, terminal deoxynucleotidyl transferase-mediated 2' deoxyuridine 5'-triphosphate (dUTP) nick-end labeling (TUNEL) assay was performed using the ApopTag Red *In Situ* Apoptosis Detection Kit (S7165, Merck Millipore, Billerica, MA, USA). The sections were then incubated with antibody against phosphorylated p38 or phosphorylated JNK. Nuclear staining was performed with 4,6-diamidino-2-phenylindole (DAPI) (blue). Ten rats from each group (two sections per animal) were analyzed. Among the apoptotic cells (pink) noted in the cavernous sinusoids, the number of apoptotic cells positive for phosphorylated p38 or phosphorylated JNK (yellow) was quantified in five randomly selected high-power fields under confocal microscopy (white arrows). The slides were evaluated by three independent observers in a blinded fashion.

Western blot analysis

Western blot analyses were performed as previously described.¹² The following primary antibodies were used: anti-phospho-JNK (Thr183/Tyr185, 1:1000, #4668, Cell-Signaling Technology, Danvers, MA, USA), anti-JNK (1:1000, #9258, Cell-Signaling Technology, Danvers, MA, USA), anti-phospho-p38 (Thr180/Tyr182, 1:1000, #4511, Cell-Signaling Technology, Danvers, MA, USA), anti-p38 (1:1000, #8690, Cell-Signaling Technology, Danvers, MA, USA), anti-Bcl-2 (1:2000, #2876, Cell-Signaling Technology, Danvers, MA, USA), and anti-Bax (1:2000, #2772, Cell-Signaling Technology, Danvers, MA, USA). Results were quantified by densitometry and normalized to the β -actin expression level (internal control).

Statistical analysis

All variables are reported as mean \pm standard error of the mean (s.e.m.). Differences among groups were analyzed with the Mann-Whitney *U*-test. All reported *P* values are two-sided. $P < 0.05$ was considered statistically significant. SPSS software (version 20.0 for Windows; SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS

Effect of bilateral CN injury on erectile response and SM content

At 2-week postinjury, the I group showed a decrease in ICP/MAP and AUC/MAP under all stimulation parameters compared with the S group (1.0 V, $P = 0.016$; 2.5 V, $P = 0.021$; 4.0 V, $P = 0.028$) (Figure 1a and 1b). The differences in ICP/MAP or AUC/MAP between the two groups tended to decrease as the voltage of electrical stimulation increased from 1.0 V to 2.5 V to 4.0 V. Immunohistochemical staining for α -SMA in the cavernosum revealed that the I group had less SM content than the S group ($P < 0.001$) (Figure 1c–1e).

JNK phosphorylation, not p38 MAPK phosphorylation, is involved in cavernosal apoptosis post-CNCI

At 2-week postinjury, the I group had an increase in the number of SM cells positive for phosphorylated JNK compared with the S

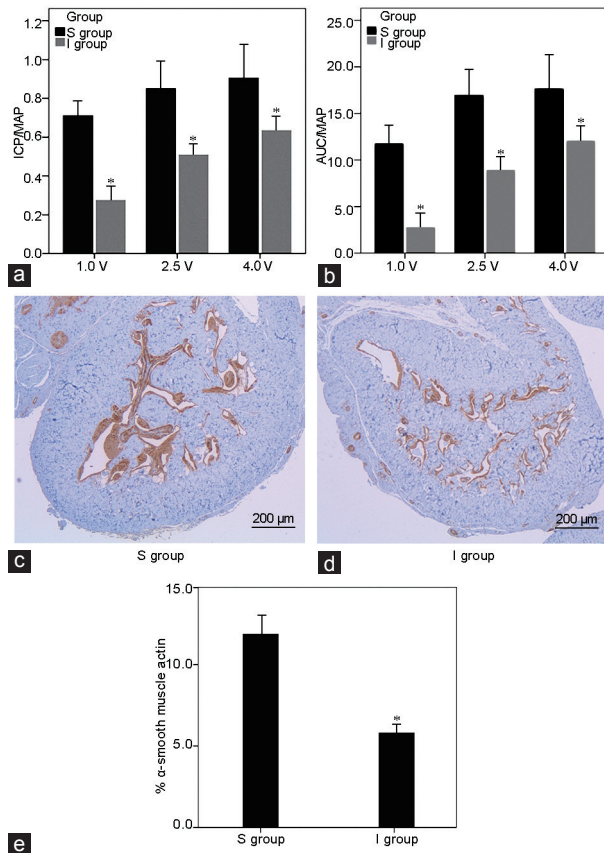


Figure 1: Effect of bilateral CN crush injury on erectile response (a: ICP/MAP, b: AUC/MAP) to electrostimulation at 2-week postinjury, and (c–e) Effect of bilateral CN crush injury on smooth muscle content at 2-week postinjury, as assessed by immunohistochemical staining of α -SMA. The smooth muscle component is shown as brown areas (magnification $\times 40$). Representative images for immunohistochemical staining of α -SMA are shown in (c) S group and (d) I group. (e) Bar graphs showing comparison of smooth muscle content (mean \pm s.e.m.) between the two groups. Data represent the percentage of smooth muscle fibers in a given area. CN: cavernous nerve; ICP/MAP: intracavernous pressure/mean arterial pressure; AUC/MAP: area under the curve corresponding to the duration of electrical stimulation/mean arterial pressure; α -SMA: α -smooth muscle actin; S: sham surgery group; I: bilateral CN crush injury group; s.e.m.: standard error of the mean. * $P < 0.05$, CNCI (I) group versus sham surgery (S) group. Scale bars = 200 μ m.

group ($P = 0.001$) (Figure 2a). However, there was no difference in the number of SM cells positive for phosphorylated p38 between the two groups ($P = 0.428$) (Figure 2b).

According to double immunofluorescent staining results of the cavernosum (TUNEL and phosphorylated JNK), the number of apoptotic cells positive for phosphorylated JNK was greater in the I group compared with the S group ($P = 0.001$) (Figure 3a). However, there was no difference in the number of apoptotic cells positive for phosphorylated p38 between the two groups ($P = 0.116$), although the number of overall apoptotic cells in the cavernosal tissue was greater in the I group compared with the S group ($P = 0.016$) (Figure 3b).

Involvement of JNK/Bcl-2/Bax pathway in cavernosal apoptosis post-CNCI

Densitometry results revealed that the I group had higher JNK phosphorylation levels and a lower Bcl-2/Bax ratio than the S group at 2-week postinjury ($P = 0.031$ and $P = 0.035$, respectively) (Figure 4). However, there was no difference in p38 phosphorylation between the

two groups, although there was a trend toward increased expression in the I group ($P = 0.085$).

DISCUSSION

Although the etiology of post-RP ED is multifactorial, neurogenic factors including CN injury appear to play a main role. Together with cavernosal fibrosis, a decrease in cavernosal tissue, such as SM, caused by apoptosis during the early postoperative period can play a critical role in the development of CVOD and post-RP ED after CN injury.^{1,4} Nevertheless, the molecular mechanisms of cavernosal apoptosis leading to ED after CN injury remain poorly understood. Thus, the present study may provide a better understanding about the mechanisms involved in apoptosis of cavernosal tissue following CN injury. There are two major findings in this study. First, JNK, not p38, appeared to be involved in apoptosis of cavernosal tissue during the early postoperative period following CNCI. Second, the increase in JNK phosphorylation caused by CNCI coincided with downregulation of Bcl2 and upregulation of Bax, suggesting that the JNK/Bcl2/Bax pathway might be involved in cavernosal apoptosis during the acute phase following CNCI.

MAPK, a stress-induced kinase, is a key component in a series of vital signal transduction pathways that regulate a variety of processes, such as cell proliferation, cell differentiation, and cell death, in eukaryotes from yeast to humans.¹⁰ In mammals, MAPK includes three main families: extracellular-signal-regulated kinases (ERK), JNK, and p38.¹⁰ Among the three MAPKs, JNK and p38 contribute to apoptosis in a variety of cells.^{10,13–15} A previous study using a rat model of CN resection showed that cavernosal apoptosis coincides with increased phosphorylated JNK and phosphorylated p38 protein expression in a multiplex screening assay at 4-week postinjury.¹¹ Densitometry analysis also revealed increased phosphorylated JNK in cavernosal tissue at 2 and 4 weeks after CN resection, together with an increase in phosphorylated JNK-positive SM cells or endothelial cells.¹¹ However, it is unknown whether JNK-positive cells are also apoptotic. Furthermore, the method of CN injury used in that study was CN resection,¹¹ not partial injury, which better approximated the clinical situation in patients undergoing nsRP. Thus, we analyzed which MAPK (JNK and p38) was involved in apoptosis of cavernosal tissue during the acute phase following CNCI. We also examined whether activated MAPK-positive SM cells were apoptotic. During the first phase of the experiment, double immunofluorescent staining of the cavernosum with antibodies specific to α -SMA and phosphorylated p38 or phosphorylated JNK revealed an increase in phosphorylated JNK, but not phosphorylated p38, in cavernosal SM at 2 weeks after CNCI. Subsequently, double immunofluorescent staining of the cavernosum with TUNEL and phosphorylated JNK-specific antibody revealed a significantly increased number of phosphorylated JNK-positive apoptotic cells in the cavernosal sinusoids during the acute phase after CNCI. Moreover, our study used a rat model of CNCI that effectively replicated nsRP, a clinically significant situation. Thus, results from the present study may provide baseline data about a candidate molecule involved in cavernosal apoptosis. Our results suggested that JNK is a potential candidate involved in apoptosis of cavernosal tissue during the acute phase after CNCI.

Results from the present study also showed that the JNK/Bcl-2/Bax pathway was involved in apoptosis of cavernosal SM during the early postoperative period after CNCI, whereas p38 MAPK was not. Previously, we showed ultrastructural alterations in cavernosal smooth muscle, such as dark shrunken cells using electron microscopy in a

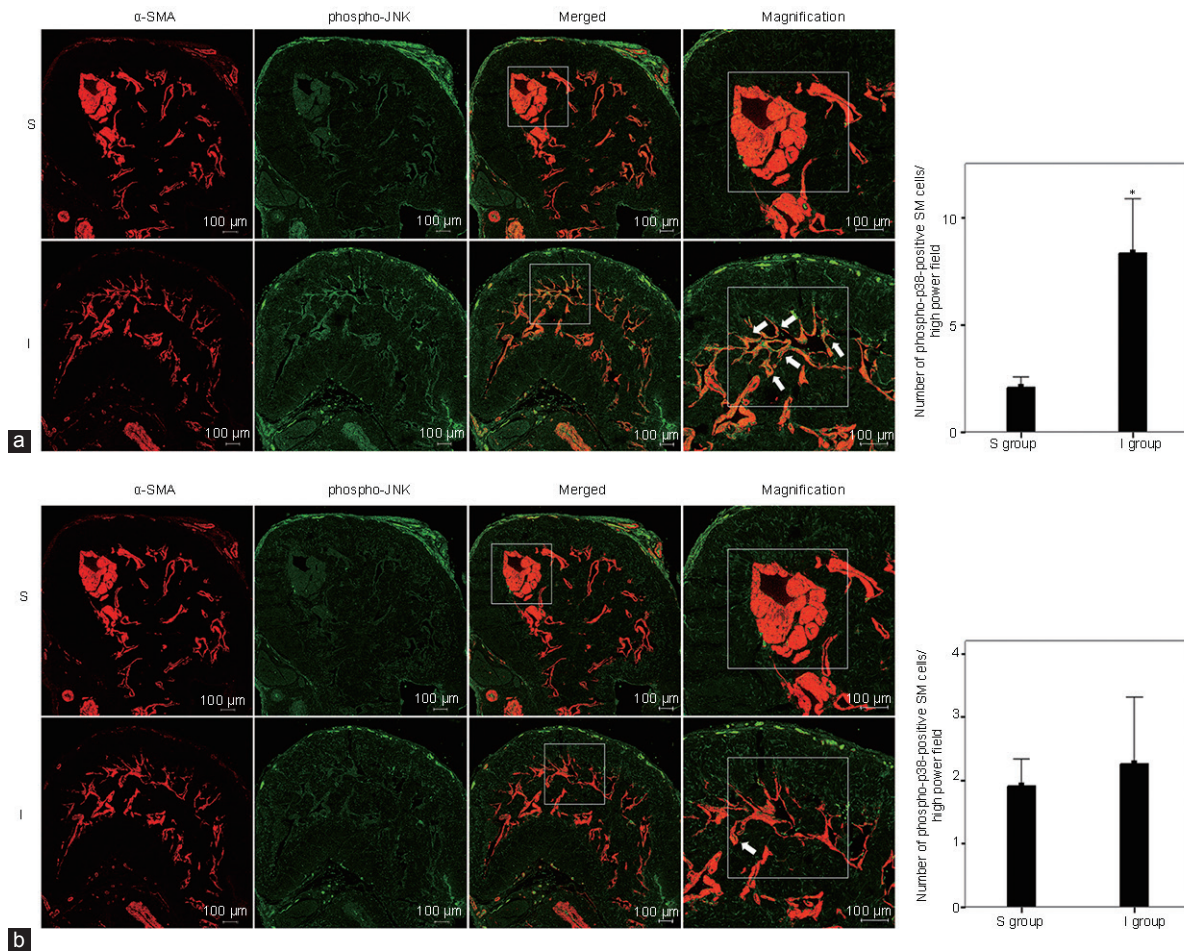


Figure 2: Effect of bilateral CN crush injury on the number of smooth muscle cells positive for phosphorylated JNK or phosphorylated p38 at 2-week postinjury. (a) Representative images and bar graphs showing comparison in the number of smooth muscle cells positive for phosphorylated JNK (mean \pm s.e.m.) between the two groups, according to double immunofluorescent staining of cavernosal tissue using anti- α -SMA and anti-phospho-JNK specific antibodies. (b) Representative images and bar graphs showing comparison in the number of smooth muscle cells positive for phosphorylated p38 (mean \pm s.e.m.) between the two groups, according to double immunofluorescent staining of cavernosal tissue with anti- α -SMA and anti-phospho-p38 specific antibodies. White arrow indicates significant expression of phospho-JNK or phospho-p38 in cavernosal smooth muscle cells (yellow color in magnified image). CN: cavernous nerve; JNK: Jun N-terminal kinase; α -SMA: α -smooth muscle actin; S: sham surgery group; I: bilateral CN crush injury group; s.e.m.: standard error of the mean. * $P < 0.05$ CNCI (I) group versus sham surgery (S) group. Scale bars = 100 μ m.

neurotomized dog model.¹⁶ We also demonstrated that cavernosal apoptosis peaked at 1 week after CNCI, gradually decreased, and then recovered to control values at 4 to 12 weeks after CNCI.¹⁷ A recent study from our group revealed that Bcl2/Bax contributes to cavernosal apoptosis as a downstream effector after CNCI.¹⁸ Furthermore, Bcl2 or Bax is a downstream molecule of JNK signaling and is related to apoptosis of various cells.^{14,18–20} JNK can phosphorylate the BH3-only family of Bcl2 proteins to antagonize anti-apoptotic activity of Bcl2 or Bcl-XL, while JNK stimulates release of cytochrome c from the mitochondrial inner membrane via a Bid-Bax-dependent mechanism, thereby promoting the formation of apoptosomes.¹⁴ Taken together, the JNK/Bcl2/Bax pathway deserves consideration as a potential target for preventing cavernosal apoptosis during the acute phase after CN injury.

p38 MAPK also plays a role in apoptosis.¹⁰ Some studies have reported that p38 activation contributed to apoptosis of cardiomyocytes and hepatocytes.^{15,21} A previous study using rats noted that the increase in phosphorylated p38 protein in a multiplex screening assay accompanied an increase in cavernosal apoptosis at 4 weeks after CN resection.¹¹ However, to date, there has been no

data from immunolocalization experiments to determine whether the numbers of phosphorylated p38-positive SM cells or phosphorylated p38 MAPK-positive apoptotic SM cells significantly increase after CNCI. In this regard, our double immunofluorescent staining showed no change in the number of phosphorylated p38-positive SM cells at 2 weeks after CNCI, suggesting that p38 activation did not contribute to apoptosis of cavernosal SM during the acute phase following partial CN damage. Rather, p38 activation might contribute to cavernosal apoptosis during the chronic phase after CN resection. This can be supported by the previous finding that protein expression of phosphorylated p38 significantly increased in conjunction with progression of cavernosal apoptosis at 4 weeks after CN injury, but not at 2 weeks.¹¹

The present study had a few limitations. First, we did not utilize a blocking method, such as using JNK inhibitors, to determine the causal relationship between a dysregulated JNK/Bcl2/Bax pathway and apoptosis of cavernosal SM after CNCI. This study was designed to identify which MAPK was involved in apoptosis of cavernosal tissue during the acute phase following partial

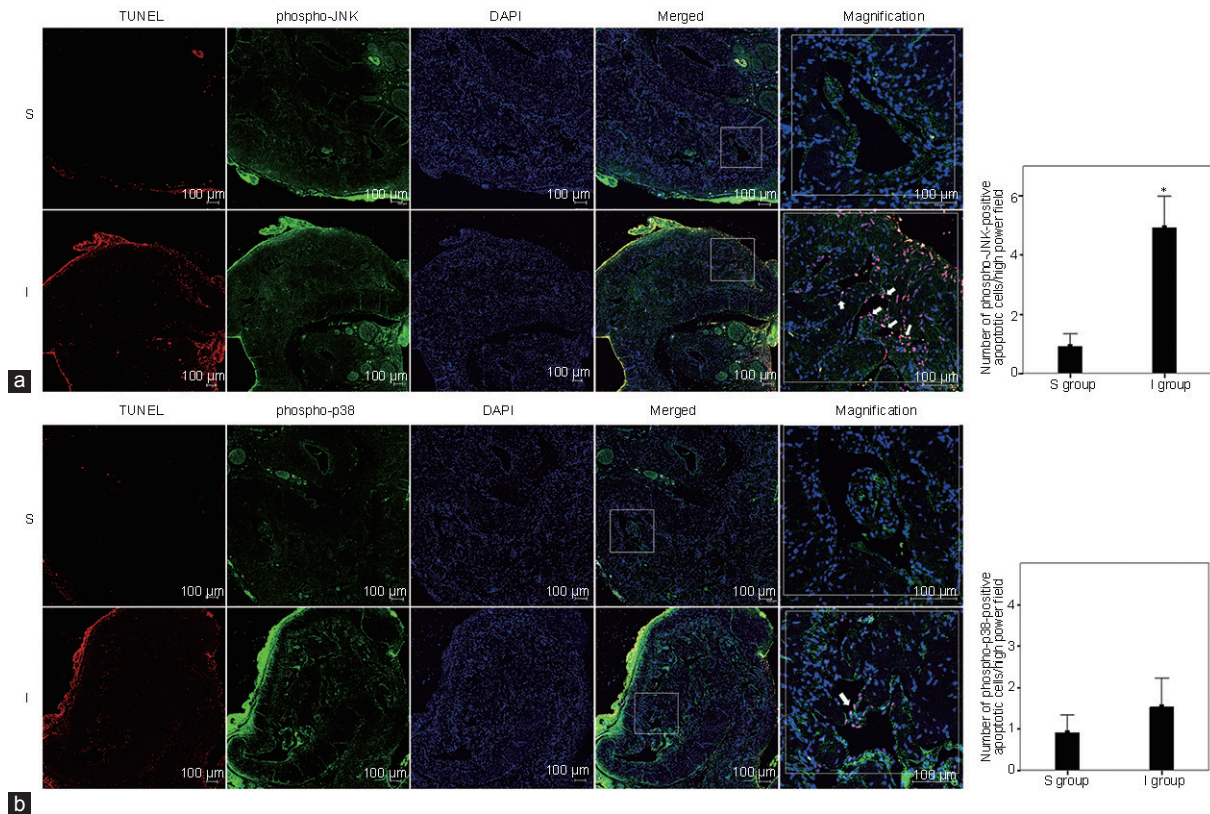


Figure 3: Effect of bilateral CN crush injury on apoptotic cells positive for phosphorylated JNK or phosphorylated p38 at 2-week post-injury. **(a)** Representative images and bar graphs showing comparison in the number of apoptotic cells positive for phosphorylated JNK (mean \pm s.e.m.) between the two groups, according to double immunofluorescent staining of cavernosal tissue with TUNEL and anti-phospho-JNK specific antibody. **(b)** Representative images and bar graphs showing comparison in the number of apoptotic cells positive for phosphorylated p38 (mean \pm s.e.m.) between the two groups, according to double immunofluorescent staining of cavernosal tissue with TUNEL and anti-phospho-p38 specific antibody. White arrows indicate apoptotic cells positive for phosphorylated JNK or phosphorylated p38 in the cavernosal sinusoids (yellow areas). CN: cavernous nerve; JNK: Jun N-terminal kinase; TUNEL: terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate (dUTP) nick end labeling; S: sham surgery group; I: bilateral CN crush injury group; s.e.m.: standard error of the mean. * $P < 0.05$, CNCI (I) group versus sham surgery (S) group. Scale bars = 100 μ m.

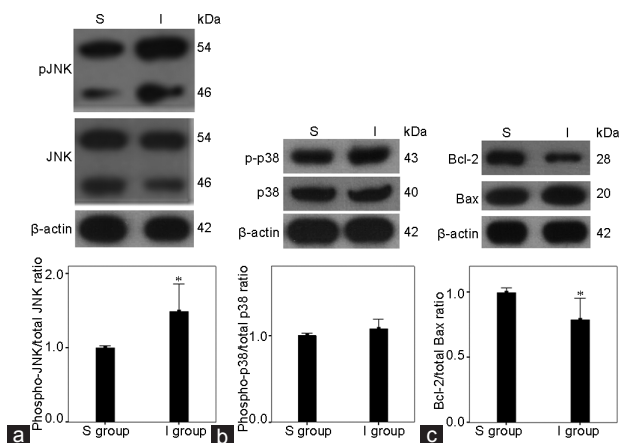


Figure 4: Representative immunoblots showing protein expression of **(a)** phospho-/total-JNK, **(b)** phospho-/total-p38, **(c)** Bcl-2, and Bax in cavernosal tissues. Bar graphs showing comparison in protein expressions (mean \pm s.e.m.) between the two groups using densitometry. The data were normalized by β -actin expression. The data were presented as fold changes over controls. JNK: Jun N-terminal kinase; S: sham surgery group; I: bilateral CN crush injury group; s.e.m.: standard error of the mean. * $P < 0.05$, CNCI (I) group versus sham surgery (S) group.

CN damages, thereby providing baseline data about potential candidates for alleviating cavernosal apoptosis. Second, our experiments were performed at only one time point (2 weeks) after CN injury. Other studies have shown progressively or persistently increased apoptosis in cavernosal tissues over time following complete CN damage (resection).^{3,11,12} However, our previous study showed that cavernosal apoptosis peaked during the acute phase after CNCI and gradually decreased to a level similar to sham controls during the chronic phase following partial CN damages.¹⁷ Because early apoptosis of cavernosal tissue after CN injury contributed to the development of CVOD, we thought that it would be clinically meaningful to determine which MAPK was responsible for cavernosal apoptosis during the acute phase following partial CN damage. Lastly, we did not completely confirm whether JNK-positive apoptotic cells were cavernosal SM cells, although double immunofluorescent staining showed an increase in phosphorylated JNK-positive SM cells and phosphorylated JNK-positive apoptotic cells in cavernosal sinusoids during the acute phase following partial CN damage.

Nevertheless, these results could have important clinical implication. From a clinical viewpoint, an early therapeutic strategy targeting the JNK-driven pathway might help alleviate cavernosal

apoptosis (e.g., immediately after nsRP). Thus, subsequent studies using JNK inhibitors are needed to confirm our findings.

CONCLUSIONS

Our data suggested that increased JNK phosphorylation caused by CNCI involves downregulation of Bcl2 and upregulation of Bax, which could play a role in apoptosis of cavernosal tissue during the acute phase following partial CN damage. To confirm these findings, time-course studies focusing on the effect of therapeutic approaches targeting JNK-driven pathways are needed in the future.

AUTHOR CONTRIBUTIONS

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

COMPETING INTERESTS

All authors declare no competing interests.

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