

# Is It Possible to Recover Erectile Function Spontaneously after Cavernous Nerve Injury? Time-Dependent Structural and Functional Changes in Corpus Cavernosum Following Cavernous Nerve Injury in Rats

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## = Abstract =

**Purpose:** There has been a scarcity of integrated, long-term (>4 week) studies on structural and functional alterations in the penis according to the period following cavernous nerve (CN) injury. The aim of this study was to investigate time-dependent structural and functional changes in the corpus cavernosum following CN injury in a rat model.

**Materials and Methods:** Ninety male Sprague-Dawley rats (10 weeks old) were divided into 4 groups: normal control (C), sham (S), bilateral CN resection (R), and bilateral CN crush injury (I) groups. At 1, 4, and 12 weeks after the procedure, erectile function was assessed by electrostimulation. The terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) assay was performed for detection of apoptosis. Masson's trichrome staining and immunohistochemistry were performed for detection of alpha smooth muscle actin ( $\alpha$ -SMA). Western blot analysis was then performed.

**Results:** The R and I groups showed persistent impairment of erectile function at all three points in time. Apoptosis peaked at 1 week after resection or crush injury and then gradually subsided. The smooth muscle cell/collagen ratio and expression of  $\alpha$ -SMA gradually decreased over time after CN resection or crush injury. Myosin phosphatase target subunit 1 phosphorylation progressively increased over time after CN resection or crush injury. On the other hand, expression of phospho-protein kinase B, phospho-endothelial nitric oxide synthase, and neuronal nitric oxide synthase transiently decreased at 1 week after resection or crush injury and then recovered to the control values.

**Conclusions:** Our results suggest that persistent up-regulation of the RhoA/Rho-kinase pathway and structural change such as decreased smooth muscle cell and increased cavernosal fibrosis might play an important role in persistent erectile dysfunction following CN injury.

**Key Words:** Nerve injury, Erectile dysfunction, Prostatectomy

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## Introduction

Erectile dysfunction (ED) continues to be a common complication after radical prostatectomy (RP) that impacts men's quality of life and self-esteem.<sup>1,2</sup> A significant percentage of men suffer from post-RP ED despite the advent of nerve sparing RP techniques. It is

generally assumed that the development of post-RP ED is due predominantly to a combination of cavernous nerve (CN) injury and damage to the erectile tissue secondary to neuropraxia, and potentially, absence of cavernosal oxygenation.<sup>3</sup> In response to CN injury, the smooth muscle and endothelium undergo structural changes such as apoptosis, loss of smooth muscle, and fibrosis, contributing to the development of venous leaking and corporal veno-occlusive dysfunction.<sup>4-7</sup>

Whereas most studies to date have focused on penile structural changes secondary to CN injury, several studies have investigated the functional consequences of CN injury. In a CN crush injury model, sildenafil treatment resulted in the preservation of erectile function through the preservation of smooth muscle contents and an increase of phosphorylation of protein kinase B (Akt) and endothelial nitric oxide synthase (eNOS), which are critical to endothelial function for penile erection.<sup>8</sup> Bilateral CN ablation was followed by transient drops in eNOS and neuronal nitric oxide synthase (nNOS), contrasting with prolonged impairment of erectile function.<sup>9</sup> Recently, it has been suggested that decreased erectile function after a CN crush injury is accompanied by the up-regulation of the RhoA/Rho-kinase (ROCK) pathway in the rat penis.<sup>10,11</sup>

However, there has been a scarcity of integrated, long-term (>4 week) studies on structural and functional alterations in the penis according to the period following CN injury. Thus, we aimed to investigate the time-dependent (1, 4, 12-week) structural and functional changes in the corpus cavernosum after CN injury (resection or crush injury) in a rat model.

## Materials and Methods

### 1. Experimental groups and treatment

Ninety 10-week-old male Sprague-Dawley rats (300 ~ 350 g) were randomly classified into four groups: a normal control group (C), sham operation group (S), bilateral CN resection group (R), and bilateral CN crush injury group (I). Within each of the four groups, three subgroups (n=5 in each group of C and S; n=10 in each group of R and I) were analyzed as a function of time (1, 4, and 12 weeks postoperatively). As de-

scribed previously,<sup>11,12</sup> for the CN resection, both CNs 5 mm distal to the major pelvic ganglion (MPG) were completely resected by removing a 2 mm segment using microsurgical scissors. For the CN crush injury, a microsurgical vascular clamp was applied with full tip closure to both CNs 5 mm distal to the MPG for 30 s, removed for 30 s, then reapplied for an additional 30 s. For the S group, both CNs were exposed, but not manipulated. All of the animals were cared for in accordance with the National Research Council publication Guide for Care and Use of Laboratory Animals. The animal experiments were approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute at Seoul National University Hospital, an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility.

### 2. In vivo assessment of erectile function and tissue procurement

Erectile function was assessed in rats at 1, 4, and 12 weeks after CN injury, as previously described.<sup>13,14</sup> After CN identification, a platinum bipolar electrode was placed around the CN, just distal to the MPG, but proximal to the location of the nerve injury. Stimulation parameters were a square wave of 0.2 ms pulse duration at 3 V and 20 Hz for 30 s. After completion of the functional studies, the entire penis was removed. The middle parts of the skin-denuded penile shaft were maintained overnight in a 10% formaldehyde solution and paraffin embedded for histological studies. The remaining penile tissues were rapidly frozen in liquid nitrogen and stored at -80°C until processing.

### 3. Detection of apoptosis

The terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) method was used as previously described for detection of apoptosis.<sup>14</sup> For each slide, five high-power (×400) fields were randomly selected and the apoptotic index was expressed as the percentage of apoptotic cells relative to the number of total cells in the given area. All histological evaluations were performed in a blinded fashion.

#### 4. Histomorphometry and Immunohistochemistry

After Masson's trichrome staining, 40×magnification images of the penis comprising one-half of the corpora cavernosa were analyzed for smooth muscle cells (SMCs, stained in red) and collagen (stained in blue) using Image Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA), and expressed as the SMC/collagen ratio. Immunohistochemical antibody staining was performed for alpha smooth muscle actin ( $\alpha$ -SMA) to estimate the SMC content, as previously described.<sup>15</sup> Two fields were randomly selected on each slide at a 100×magnification, and the percentage of smooth muscle fibers in a given area was measured.

#### 5. Western blot analysis

Western blot analysis was performed as previously described.<sup>11,14</sup> Primary antibodies included anti-phospho-myosin phosphatase target subunit 1 (MYPT1) (Thr-696, 1:1000; Millipore, Billerica, MA, USA), anti-phospho-eNOS (Ser-1177, 1:1000; Cell Signaling Technology, Beverly, MA, USA), anti-Akt (1:1000; Cell Signaling Technology), and anti-phospho-Akt (Ser-473, 1:1000; Cell Signaling Technology), or anti-nNOS (1:1000; Cell Signaling Technology). Bound antibodies were detected using peroxidase-conjugated anti-rabbit immunoglobulin G antibodies and the ECL system (Amersham Biosciences, Piscataway, NJ, USA). The results were quantified by densitometry and normalized by  $\beta$ -actin expression.

#### 6. Statistical analysis

All data are reported as the mean±standard errors of the mean. The Mann-Whitney U-test or Kruskal-Wallis test was used as indicated for analysis of differences among groups. Statistical significance was considered when  $p < 0.05$ . SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the analysis.

### Results

#### 1. Comparison of erectile function

The maximal intracavernous pressure/mean arterial pressure (ICP/MAP) ratio to the CN stimulation is displayed in Fig. 1. There was no difference in ICP/MAP

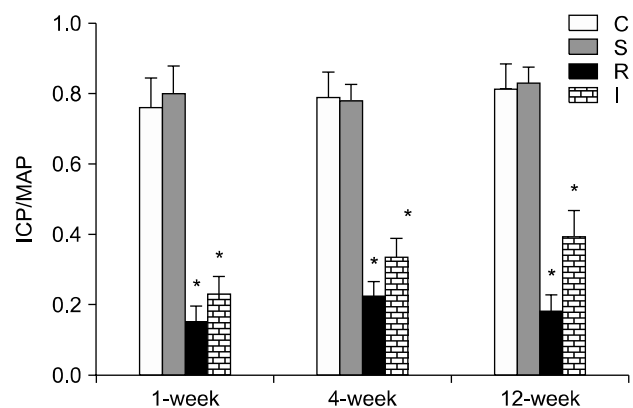
between the C and S groups in each time period (1, 4, 12 weeks) (mean max ICP/MAP: 0.78~0.85). Erectile function showed partial recovery over the course of time in the I group (1-week: 0.23, 4-week: 0.33, 12-week: 0.39); however, the R and I groups showed persistent impairment of erectile function throughout the experimental period (mean max ICP/MAP: R group=0.15~0.24, I group=0.23~0.39,  $p < 0.05$ ) (Fig. 1).

#### 2. Comparison of mean apoptosis index

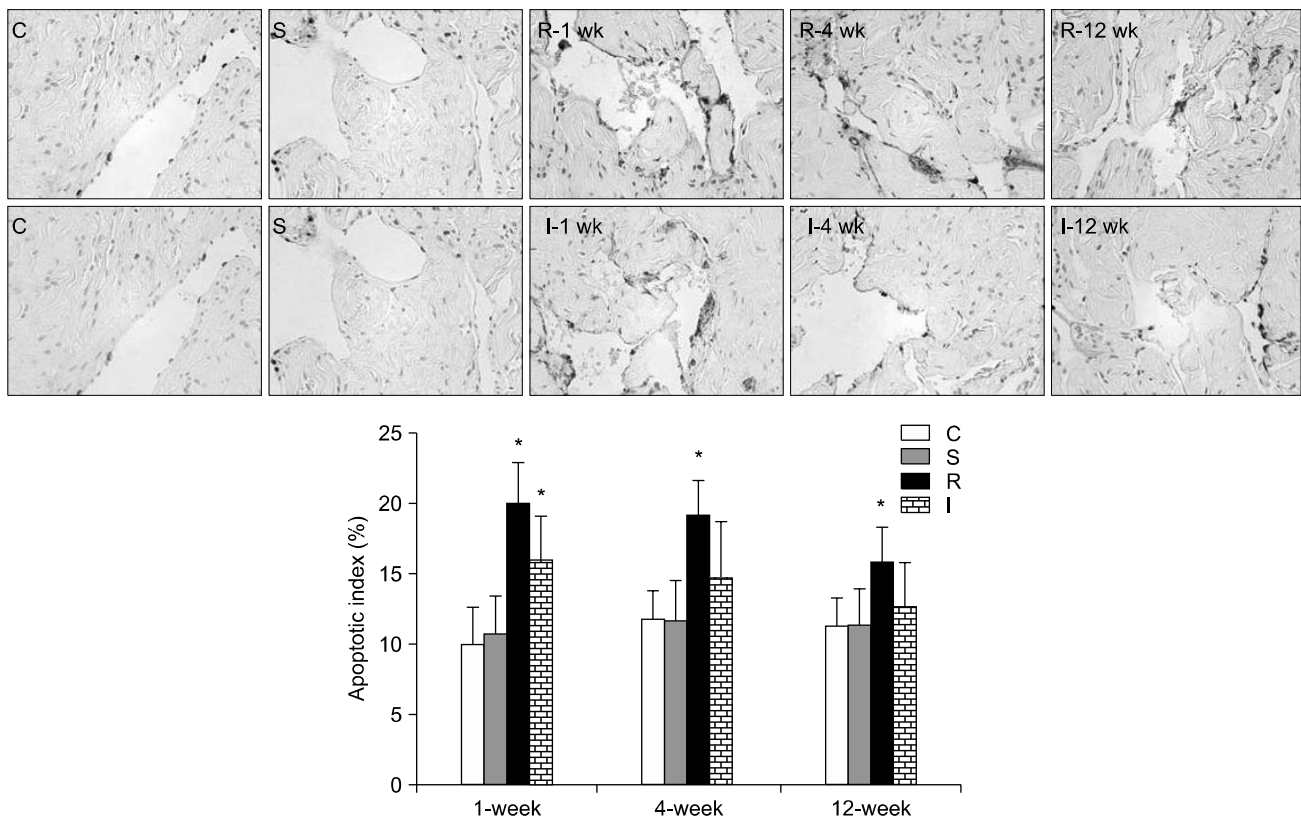
Apoptosis peaked at 1 week after resection and remained increased throughout the experimental period ( $p < 0.05$ ), despite a decreasing trend over time (1-week: 20.0%, 4-week: 19.1%, 12-week: 15.8%). Apoptosis peaked at 1 week after crush injury ( $p < 0.05$ ) and then recovered to control values (Fig. 2).

#### 3. Histomorphometry and Immunohistochemistry

At 1 week postoperatively, the R and I groups had a significantly decreased SMC/collagen ratio compared to the C or S group ( $p < 0.05$ ). The SMC/collagen ratio in the R group gradually decreased over time (1-week: 0.14, 4-week: 0.11, 12-week: 0.07) (Fig. 3). At 1 week



**Fig. 1.** Comparison of erectile function. Results of cavernous electrostimulation in the four experimental groups are expressed as percent ICP/MAP. Each bar represents the mean±standard error of the means. \* $p < 0.05$  vs. control group. ICP: intracavernous pressure, MAP: mean arterial pressure, C: control group (n=5 in each subgroup), S: sham operation group (n=5 in each subgroup), R: CN resection group (n=10 in each of the 1-, 4-, and 12-week subgroups), I: CN crush injury group (n=10 in each of the 1-, 4-, and 12-week subgroups).



**Fig. 2.** Comparison of mean apoptosis index. Representative micrographs show apoptotic cells stained black-brown by the TUNEL method in rat penile cavernosum (magnification 400 $\times$ ). Bar graphs represent quantitative image analysis. The apoptotic index was defined as the percentage of apoptotic cells within the total number of cells in a given area. \* $p < 0.05$  vs. control group. TUNEL: terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate (dUTP) nick end labeling, C: control group ( $n=5$  in each subgroup), S: sham operation group ( $n=5$  in each subgroup), R: CN resection group ( $n=10$  in each of the 1-, 4-, and 12-week subgroups), I: CN crush injury group ( $n=10$  in each of the 1-, 4-, and 12-week subgroups).

postoperatively, the R and I groups had a significant decrease in the expression of  $\alpha$ -SMA compared to the C or S group ( $p < 0.05$ ). In the R group, the decrease in the expression of  $\alpha$ -SMA was evident at 12 weeks postoperatively ( $p < 0.05$ ) (Fig. 3).

#### 4. Western blot analysis

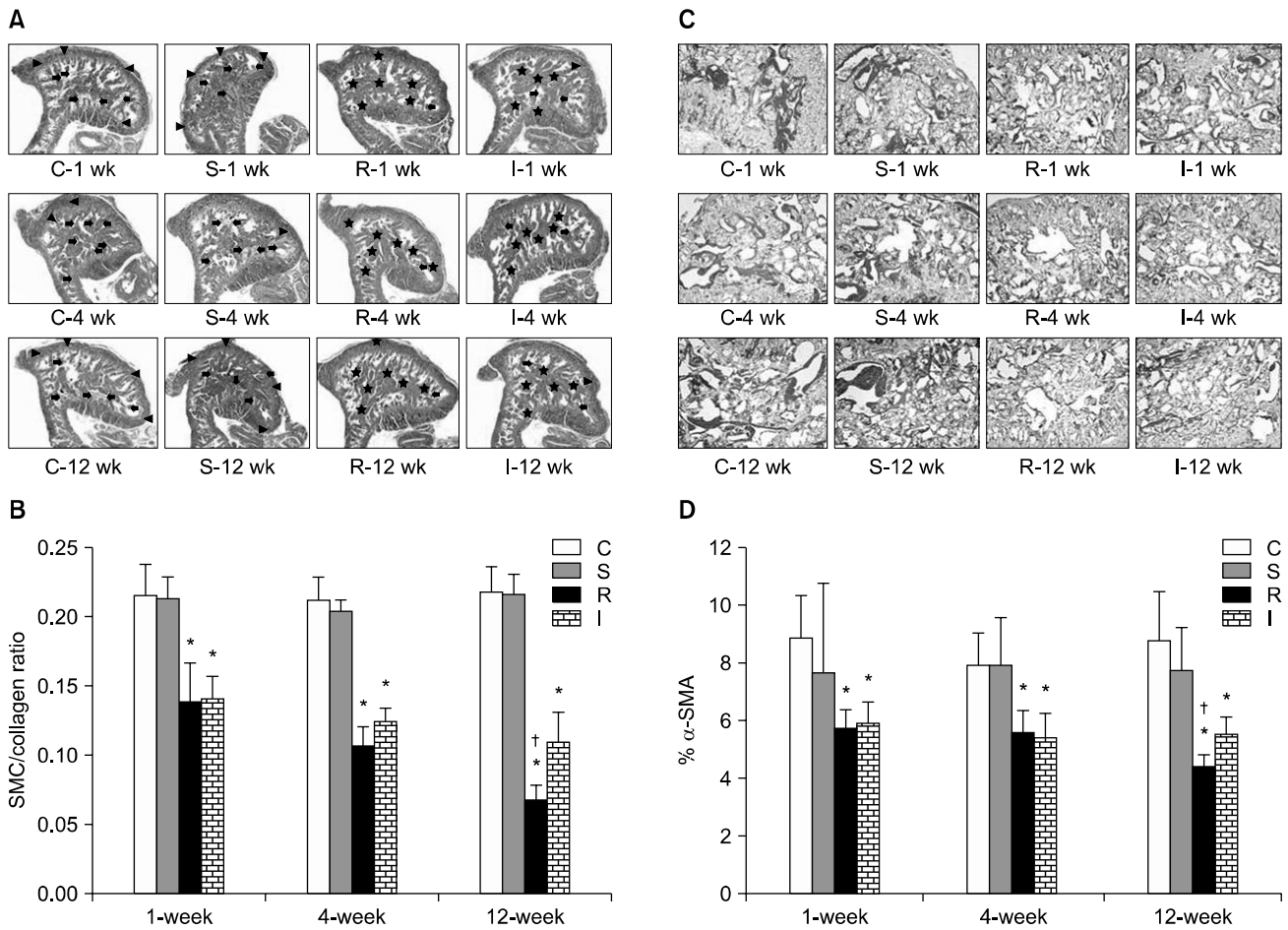
Western blot analysis is displayed in Fig. 4. MYPT1 phosphorylation at Thr-696, a marker of ROCK activity, in the R group showed a significant increase compared to that in the C or S group at 1 week after resection injury and was found to show a progressive increase throughout the study period ( $p < 0.05$ ). Expression of phospho-MYPT1 in the I group became significantly higher than that in the C or S group at 4 weeks after crush injury ( $p < 0.05$ ) (Fig. 4). On the contrary, expression of phospho-Akt, phospho-eNOS,

and nNOS transiently decreased at 1 week after resection or crush injury ( $p < 0.05$ ) and then recovered to control values, contrasting with prolonged impairment of erectile function (Fig. 4).

#### Discussion

The present study showed the structural and functional changes integrated with time (acute, sub-acute, and chronic phases) in corpus cavernosum after CN injury. In the present study, we chose 1-, 4-, and 12-week intervals to analyze. Based on the assumption that 3 weeks in rats approximates 2 chronologic years in humans, the 1-, and 4-, and 12-week intervals represent the acute, sub-acute, and chronic phases following CN injury, respectively.<sup>16</sup>

Many studies have shown partial recovery over time

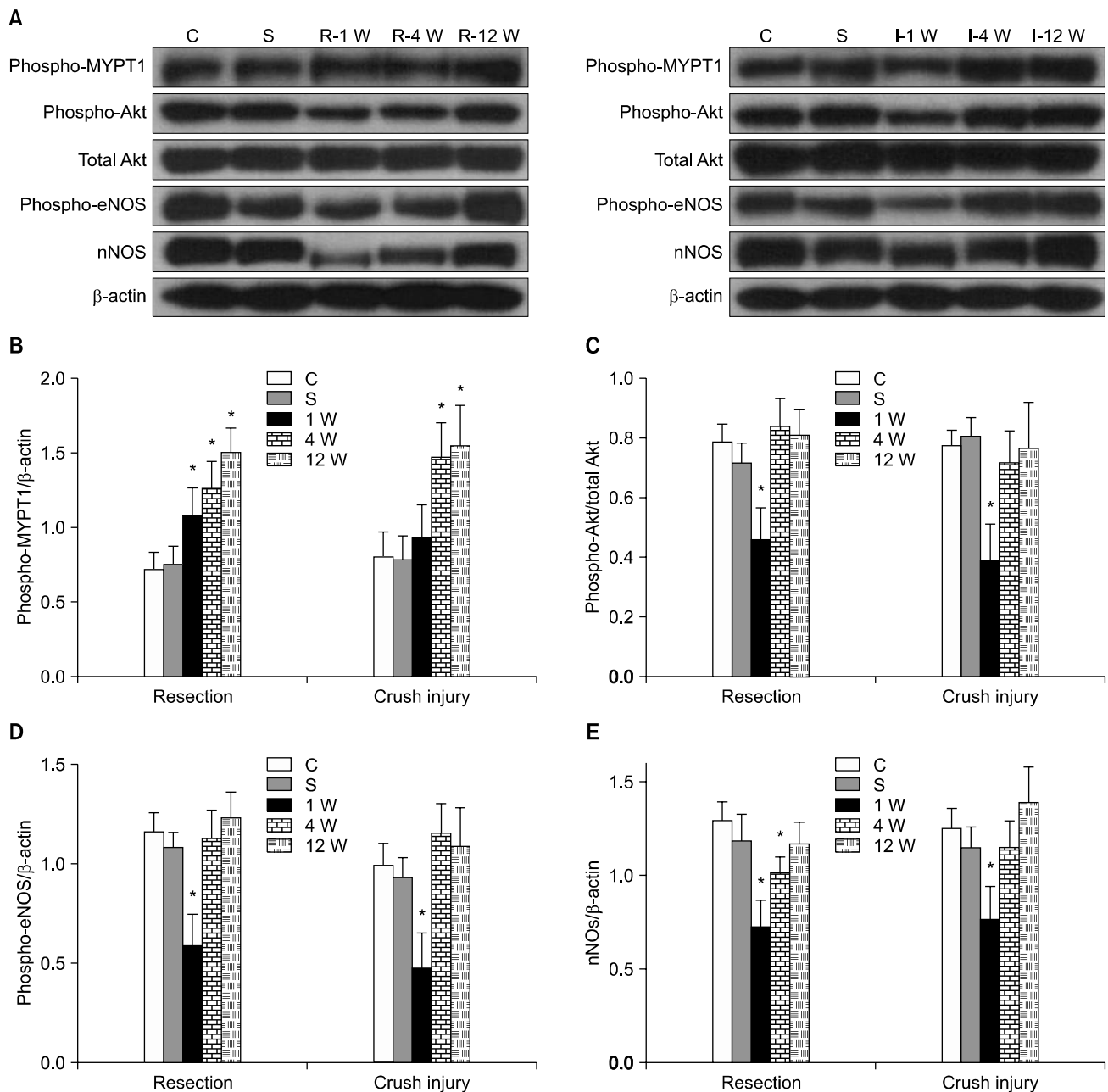


**Fig. 3.** Masson's trichrome staining and immunohistochemical analysis of smooth muscle content in rat penile sections. Smooth muscle and collagen fibers were stained in red and blue, respectively (magnification 40 $\times$ ). (A) Representative images for Masson's trichrome staining. (B) Comparison of Masson's trichrome staining results among four experimental groups. The results are presented as the smooth muscle cell (SMC)/collagen ratio (mean $\pm$ standard error of the means). Smooth muscle cells in the endothelial lining of sinusoids (black arrows), SMCs in the cavernous tissue beneath the tunica albuginea (black-head arrows), collagen fibers in the peri-sinusoidal area or the cavernous tissue beneath the tunica albuginea (asterisks). The smooth muscle component is shown as brown areas (magnification 100 $\times$ ). (C) Representative images for immune-stained alpha smooth muscle actin ( $\alpha$ -SMA). (D) Comparison of the expression of  $\alpha$ -SMA among four experimental groups. The results are presented as the percentage of smooth muscle fibers in a given area. \* $p < 0.05$  vs. control group,  $^{\dagger}p < 0.05$  vs. crush injury group. C: control group (n=5 in each subgroup), S: sham operation group (n=5 in each subgroup), R: cavernous nerve (CN) resection group (n=10 in each of the 1-, 4-, and 12-week subgroups), I: CN crush injury group (n=10 in each of the 1-, 4-, and 12-week subgroups).

after CN crush injury. However, most of the studies did not actually demonstrate the complete recovery of erectile function. For example, Mullerad et al<sup>17</sup> reported that the ICP/MAP ratios for 3, 10, and 28 days after bilateral hemostat CN crush were 18%, 31%, and 32%, respectively. These values were statistically significantly lower compared with that of a control group (70%,  $p < 0.0001$ ). Jin et al<sup>12</sup> showed that there was no difference in the ICP/MAP between the C and S

group at each point in time, which is similar to the results of the present study. They also reported that the ICP/MAP of the crush injury group gradually increased after 4 weeks and was significantly higher than that of the neurectomy group after 12 weeks ( $p < 0.05$ ). However, it was still lower than that of the C or S group ( $p < 0.05$ ).

Similar to the results from previous studies,<sup>12,17</sup> the erectile response in the present study showed partial



**Fig. 4.** Western blot analysis demonstrating corporal expression of phospho-myosin phosphatase target subunit 1 (MYPT1), phospho-protein kinase B (Akt), phospho-endothelial nitric oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS). (A) Representative immunoblots show expression of phospho-MYPT1, phospho-Akt, total Akt, phospho-eNOS, nNOS, and  $\beta$ -actin from the corporal tissues of the four groups (according to period). (B) Bar graphs demonstrating the comparison of phospho-MYPT1 protein expression among the four experimental groups using densitometry. Results were normalized by  $\beta$ -actin expression. (C) Bar graphs demonstrating the comparison of phospho-Akt protein expression among the four experimental groups using densitometry. Results were normalized by total Akt expression. (D) Bar graphs demonstrating the comparison of phospho-eNOS protein expression among the four experimental groups using densitometry. Results were normalized by  $\beta$ -actin expression. (E) Bar graphs demonstrating the comparison of nNOS protein expression among the four experimental groups using densitometry. Results were normalized by  $\beta$ -actin expression. Results were presented as fold changes over controls. \* $p < 0.05$  vs control group. C: control group ( $n=5$  in each subgroup), S: sham operation group ( $n=5$  in each subgroup), R: cavernous nerve (CN) resection group ( $n=10$  in each of the 1-, 4-, and 12-week subgroups), I: CN crush injury group ( $n=10$  in each of the 1-, 4-, and 12-week subgroups, respectively).

recovery over time after CN crush injury (mean max ICP/MAP: 1-week=0.23, 4-week=0.33, 12-week=0.39). However, these values were still lower than those of the C or S group (mean max ICP/MAP: 0.78~0.85,  $p<0.05$ ). Therefore, one of the important findings in this study was that the erectile impairment persisted throughout the experimental period after both CN resection and crush injury. This means that it is impossible to recover the erectile response spontaneously. However, recently, Kim et al<sup>18</sup> reported the spontaneous recovery from CN crush injury. They showed that the ICP/MAP of a crush injury group was significantly lower than that of a sham group after 12 weeks (51.7% vs. 71.9%;  $p<0.05$ ). However, there was no difference in the ICP/MAP between the crush injury and sham groups after 24 weeks (63.3% vs. 70.5%;  $p>0.05$ ). In that study, they performed electrical stimulation for 60 s with a bipolar electrode at 1.5 mA, 20 Hz, and 7.5 V with a square-wave pulse duration of 5 ms. We believe that this is excessive compared with the stimulation parameters of the present study (3 V at 20 Hz with a square wave pulse duration of 0.2 ms for 30 s). Though the ideal conditions of electrical stimulation have not been determined definitively, it is assumed that each of the stimulation parameters (electric current, voltage, frequency, and pulse duration) should increase the ICP in a dose-dependent manner. Therefore, we believe that the minimal electrical stimulation that can induce erection is ideal for an animal study measuring ICP by CN stimulation.

In the present study, apoptosis peaked at 1 week after CN resection. The apoptotic index then showed a gradual decline, but was still significantly higher than that in the C or S group. This supports the study by Ferrini et al<sup>6</sup> Persistent cavernosal apoptosis after CN resection might explain the absence of functional recovery in the R group. A similar trend was observed after CN crush injury; however, the level of apoptosis was only significantly higher at 1 week after injury. Furthermore, we also evaluated a detailed structural alteration after CN injury, such as a loss of SMC or an increase in collagen content. In the present study, the SMC/collagen ratio and the expression of  $\alpha$ -SMA de-

creased in a sustained fashion after CN injury. These results mean that it is impossible to restore SMC spontaneously after CN injury and that a normal apoptotic index does not result in the normal contents of SMC.

In this study, similar to the results from previous studies,<sup>8,9,19</sup> western blot analysis showed a transient decrease in expression of penile phospho-Akt and phospho-eNOS at 1 week after CN resection or crush injury. On the other hand, our results showed that MYPT1 phosphorylation, a surrogate for ROCK activity, progressively increased over the course of time after bilateral CN resection or crush injury, possibly contributing to persistent impairment of erectile function throughout the experimental period. Furthermore, many studies have shown that the RhoA/Rho-associated kinase pathway plays an important role in tissue fibrosis and that Rho-kinase inhibition prevents and ameliorates tissue fibrosis.<sup>11,20-25</sup> Therefore, our results may raise the possibility of targeting the RhoA/ROCK pathway to prevent corporal fibrosis and preserve the corpus cavernosum in the early period after partial or incomplete nerve injury.

One of the novel findings of this study was that the erectile response had not spontaneously normalized at 4 or 12 weeks after CN injury, despite recovery of Akt and eNOS phosphorylation. Although the erectile response showed partial recovery over time after CN crush injury, the erectile impairment persisted throughout the experimental period after both CN resection and CN crush injury. There are several plausible explanations for these findings. Elevated levels of eNOS protein in the penis may be adaptive and serve as a compensatory response to decreased endothelial NO production in an effort to maintain a certain level of erectile function, although the active form of eNOS is reduced.<sup>26</sup> Consistent elevation of ROCK activity following CN injury might be another explanation.

Previous rat studies found no evidence of nerve regeneration up to 6 months after CN injury.<sup>27,28</sup> However, the present study showed that the expression of nNOS protein transiently decreased at 1 week after CN resection or crush injury and then recovered to control values, which supports the study by Fall et al<sup>9</sup> suggesting spontaneous nerve regeneration after bilateral CN

ablation.

Absence of functional recovery despite spontaneous nerve regeneration strongly suggests that other pathophysiologic mechanisms, such as increased corporal apoptosis or ROCK activity or structural alteration such as a loss of SMC or an increase in collagen content, leading to corporal fibrosis, may be key factors for post-RP ED.

One of the limitations of this study is that we did not investigate sequential functional or structural alterations that would occur with the use of therapeutic approaches, such as Rho-kinase inhibitor, at various points in time after CN injury. However, we think that the present study extends the current state of knowledge in the study of ED after CN injury by further exploring the time-dependent derangements in penile hemodynamics and subsequent structural and functional changes in the corpus cavernosum after CN injury in a rat model.

### Conclusions

Our data reconfirmed that it is impossible to recover the erectile response spontaneously after either CN resection or crush injury. Our results also provide evidence that the RhoA/ROCK pathway and structural changes such as a loss of SMC or an increase in collagen content might play an important role in the persistent ED following CN injury.

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