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## ORIGINAL ARTICLE

Erectile Function

# Losartan improves erectile function through suppression of corporal apoptosis and oxidative stress in rats with cavernous nerve injury

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This study aimed to investigate the functional and morphological changes in the corpus cavernosum after cavernous nerve (CN) injury or neurectomy and then reveal whether treatment with the angiotensin II Type 1 receptor antagonist losartan would improve erectile function as well as its potential mechanisms. A total of 48 10-week-old Sprague–Dawley male rats, weighing 300–350 g, were randomly divided into the following four groups ( $n = 12$  per group): sham operation (Sham) group, bilateral cavernous nerve injury (BCNI) group, losartan-treated BCNI (BCNI + Losartan) group, and bilateral cavernous neurectomy (Neurectomy) group. Losartan was administered once daily by oral gavage at a dose of  $30 \text{ mg kg}^{-1} \text{ day}^{-1}$  for 4 weeks starting on the day of surgery. The BCNI and the Neurectomy groups exhibited decreases in erectile response and increases in apoptosis and oxidative stress, compared with the Sham group. Treatment with losartan could have a modest effect on erectile function and significantly prevent corporal apoptosis and oxidative stress. The phospho-B-cell lymphoma 2 (Bcl-2)-associated death promoter (p-Bad)/Bad and phospho-the protein kinase B (p-AKT)/AKT ratios were substantially lower, while the Bcl-2-associated X protein (Bax)/Bcl-2 ratio, nuclear factor erythroid 2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap-1), transforming growth factor- $\beta$  1 (TGF- $\beta$ 1) and heme oxygenase-1 (HO-1) levels, and caspase-3 activity were higher in the BCNI and Neurectomy groups than in the Sham group. After 4 weeks of daily administration with losartan, these expression levels were remarkably attenuated compared with the BCNI group. Taken together, our results suggested that early administration of losartan after CN injury could slightly improve erectile function and significantly reduce corporal apoptosis and oxidative stress by inhibiting the Akt/Bad/Bax/caspase-3 and Nrf2/Keap-1 pathways. *Asian Journal of Andrology* (2019) 21, 452–459; doi: 10.4103/aja.aja\_8\_19; published online: 15 March 2019

**Keywords:** angiotensin II; apoptosis; erectile dysfunction; fibrosis; losartan; oxidative stress

## INTRODUCTION

Erectile dysfunction (ED), defined as the inability to attain or sustain erection satisfactory for sexual intercourse, has been predicted to affect 322 million individuals by the year 2025.<sup>1,2</sup> Over 30 million men are diagnosed with ED every year, seriously diminishing their quality of life.<sup>3</sup> Despite the application of robotic surgery and advances in nerve-sparing techniques, irreversible ED resulting from cavernous nerve (CN) injury remains an important quality of life issue after radical prostatectomy (RP).<sup>4</sup> It has been reported that the rates of complete ED and partial erectile function after RP for clinically localized prostate cancer are 26%–100% and 16%–48%, respectively.<sup>5</sup> Moreover, these patients are less responsive to phosphodiesterase Type 5 (PDE5) inhibitors, the first-line treatment for ED.<sup>6</sup>

The pathophysiology of CN injury-induced ED remains poorly understood. Following CN injury caused by traction, compression, or use of electrocautery during RP, the damaged nerve will undergo Wallerian degeneration, leading to a temporary block of neurotransmission, which can result in morphological changes in the corpus cavernosum tissue, including cavernous fibrosis from increased

collagen synthesis, a reduction in nitric oxide synthase-positive nerve density, and a decrease in smooth muscle and endothelial cells from apoptosis.<sup>7–10</sup> Cavernous hypoxia, increased reactive oxygen species, and the upregulation of profibrotic factors such as transforming growth factor- $\beta$  (TGF- $\beta$ ) have also been implicated in the pathophysiology of ED.<sup>11–13</sup>

Angiotensin II (Ang II), as the main active metabolite of the renin–angiotensin system, has been reported to cause endothelial dysfunction by increasing levels of reactive oxygen species and vasoconstrictive eicosanoids, which can counteract the effects of nitric oxide.<sup>14,15</sup> Moreover, physiological amounts of Ang II and its receptors could be produced and identified in the corpus cavernosum. As demonstrated by Kifor *et al.*,<sup>16</sup> intracavernosal injection of Ang II could terminate erection in anesthetized dogs. In addition, Ang II activation might also be involved in apoptosis and fibrosis of the corpus cavernosum through Smad and non-Smad pathways, resulting in corporal veno-occlusive dysfunction (CVOD) and ED in diabetic rats.<sup>17</sup> On the other hand, silencing of Ang II could alleviate ED via downregulating the Ras homolog

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Received: 25 July 2018; Accepted: 18 December 2018

family member A (RhoA)/Rho associated kinase (ROCK) signaling pathway in diabetic rats.<sup>18</sup>

Based on the functions of Ang II and its potential involvement in ED, we hypothesized that Ang II might also take part in apoptosis, oxidative stress, and fibrosis of the corpus cavernosum in rats with CN injury. Hence, the subsequent histologic changes in the corpus cavernosum tissue after CN injury were studied, and meanwhile, losartan, the Ang II Type 1 receptor antagonist, was utilized to examine whether daily administration of it would improve erectile function. The outcomes of these were anticipated to provide some references for future clinical management.

## MATERIALS AND METHODS

### *Animals and experimental design*

This study was conducted in strict accordance with the approved animal protocols and guidelines established by the Animal Care and Use Committee of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). All surgeries were performed under anesthesia, and all efforts were made to minimize the number and sufferings of experimental animals. A total of 48 10-week-old Sprague–Dawley male rats weighing 300–350 g were randomly divided into four groups ( $n = 12$  per group): sham operation (Sham) group, bilateral cavernous nerve injury (BCNI) group, losartan-treated BCNI (BCNI+Losartan) group, and bilateral cavernous neurectomy (Neurectomy) group.

All rats were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg kg<sup>-1</sup>, P3761, Sigma-Aldrich, Copenhagen, Denmark) before surgery. The major pelvic ganglion (MPG) and the CNs were identified dorsolateral to the prostate by blunt dissection with the aid of a dissecting microscope (Zeiss, Göttingen, Germany). In the Sham group, rats underwent only MPG and CN exposure without further manipulation. In the BCNI group, the bilateral CNs were crushed using a nonserrated hemostat (Karl Storz Co., Tuttlingen, Germany) about 2 mm distal to the ganglion for 2 min with full tip closure. In the neurectomy group, all rats underwent excision of a 2-mm section of the CN. In the BCNI + Losartan group, losartan was prepared by dissolution in distilled water and administered once daily by oral gavage at a dose of 30 mg kg<sup>-1</sup> day<sup>-1</sup> (MSD, Hangzhou, China) for 4 weeks starting from the day of surgery. The rats in the Sham, BCNI, and Neurectomy groups were given treatment with vehicle only (distilled water). Moreover, the individual performing the crush injury or neurectomy was blinded to treatment.

### *Erectile function evaluation*

Four weeks postoperatively, erectile function was evaluated in anesthetized rats, and the bilateral MPGs and CNs were exposed with the abovementioned method. Intracavernosal pressure (ICP) and mean arterial pressure (MAP) were measured as described previously.<sup>19,20</sup> For electrical stimulation of the CN, a bipolar hook electrode attached to a SDZ-II stimulator (Hwato, Suzhou, China) was placed around the injured or sham-treated CN proximal to the injury site. The stimulation parameters were 10 V at a frequency of 15 Hz with a square wave duration of 1.2 ms for 1 min. PowerLab/4SP (AD Instruments, Bella Vista, Australia) was used to acquire data during the experiment. The ratio of the maximal ICP to the corresponding MAP (ICP/MAP) was calculated and recorded.

At the end of the experiment, the corpus cavernosum, MPGs, and CNs were harvested. The midshaft of the penis was immediately fixed in 4% paraformaldehyde (Servicebio, Wuhan, China) overnight and then embedded in paraffin for histologic analysis. The MPGs and CNs were prefixed in 0.01 mol l<sup>-1</sup> PBS (pH 7.3) containing 2.5% glutaraldehyde

(Servicebio) for 2–4 h and then embedded in EMBED 812 (Servicebio) for transmission electron microscopy. The left cavernous tissue was rapidly frozen in liquid nitrogen and stored at –80°C until protein extraction.

### *Transmission electron microscopy (TEM)*

For TEM analysis, fresh CNs were carefully isolated to avoid physical damage, with the size of the tissue sample no more than 1 mm<sup>3</sup>. The tissues were fixed in 0.01 mol l<sup>-1</sup> PBS (pH 7.3) containing 2.5% glutaraldehyde for 2–4 h, washed in 0.1 mol l<sup>-1</sup> PBS, postfixed with 1% osmium tetroxide (OsO<sub>4</sub>, Servicebio) in 0.1 mol l<sup>-1</sup> PBS for 2 h at room temperature, and washed in 0.1 mol l<sup>-1</sup> PBS. The tissue was then dehydrated in a graded ethanol series, treated with propylene oxide (Servicebio), embedded in EMBED 812, and cut into ultrathin sections (approximately 60–80 nm). The sections were subsequently stained with uranyl acetate (Servicebio) and lead citrate (Servicebio) and photographed on a Hitachi HT7700 transmission electron microscope (Hitachi, Tokyo, Japan).

### *Apoptotic cell detection*

The TdT-mediated dUTP Nick-End Labeling (TUNEL) method was used to detect apoptosis with the ApopTag<sup>®</sup> Red *In Situ* Apoptosis Detection Kit (Merck Millipore, Billerica, MA, USA), as previously described.<sup>21,22</sup> Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Under confocal microscopy (Nikon Corporation, Tokyo, Japan), five high-power (×400) fields were randomly selected, and the apoptotic index was calculated as the percentage of apoptotic cells among the total number of cells in the given area. Analysis was performed in a blinded fashion with the same standards in all groups. By covering the label on each slide, microscopic viewing, reading, and counting were performed blindly, and only thereafter, the label of each slide was uncovered.

### *Histological and immunohistochemical analyses*

Masson's trichrome staining and immunohistochemistry were performed as previously described.<sup>23</sup> The midshaft of the penis was immediately fixed in 4% paraformaldehyde overnight and then embedded in paraffin for further histologic studies. To assess fibrosis, Masson's trichrome staining was used to evaluate the smooth muscle-to-collagen ratio using images at ×200 magnification. Alpha smooth muscle actin (α-SMA; 1:50; Cell Signaling Technology, Danvers, MA, USA) was used to assess smooth muscle content at ×200 magnifications. For apoptosis, the tissue sections were incubated with antibody to caspase-3 (1:200; Cell Signaling Technology) at ×200 magnification. To assess oxidative stress, the tissue sections were incubated with antibodies to nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) (both 1:100; Cell Signaling Technology) at ×200 magnification. Ten sections of the corpus cavernosum were evaluated for each group, and six fields were counted for each section at ×200 magnification on a fluorescence microscope (Olympus, Tokyo, Japan) by an observer blinded to group allocation. Images were analyzed using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA). The expression levels were standardized as a ratio to control.

### *Western blot and caspase-3 activity assay*

The corpus cavernosum was homogenized in ice-cold radioimmune precipitation assay (RIPA) Lysis and Extraction Buffer (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Western blot analysis was performed as previously described.<sup>24</sup> After blocking in 5% nonfat milk in Tris-buffered saline Tween (TBST, 20 mmol l<sup>-1</sup> Tris-HCl, pH 7.5;

150 mmol l<sup>-1</sup> NaCl, 0.1% Tween 20) for 2 h at room temperature, the membranes were incubated overnight at 4°C with antibodies against Nrf2 (1:1000, Cell Signaling Technology), Kelch-like ECH associated protein 1 (Keap-1, 1:1000, Cell Signaling Technology), HO-1 (1:1000, Servicebio), TGF-β1 (1:500, Abcam, Cambridge, UK), B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax, 1:1000, Servicebio), Bcl-2 (1:1000, Servicebio), β-actin (1:5000, Cell Signaling Technology), Bcl-2-associated death promoter (Bad, 1:1000, Servicebio), phosphor-Bad (1:1000, Servicebio), the protein kinase B (AKT, 1:1000, Cell Signaling Technology), and phosphor-AKT (1:1000, Cell Signaling Technology). Then, the membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:4000, Cell Signaling Technology) at room temperature for 90 min. After washing with TBST, immunoreactive bands were visualized with the GeneGnome gel imaging system (Syngene, Cambridge, UK). Densitometric analysis of the protein bands was performed with Image-Pro Plus software.

Caspase-3 activity in the penile tissue was quantified by a colorimetric enzyme-linked immunosorbent assay using a caspase-3 Activity Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. The relevant absorbance was measured at 405 nm with a microplate reader (Thermo Scientific, Waltham, MA, USA). The corresponding activity was calculated according to the standard curve and normalized by the protein concentration.

### Statistical analyses

All data were shown as the mean ± standard deviation (s.d.), and differences among groups were analyzed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Given the skewed distribution of data and the modest sample size, the nonparametric Kruskal–Wallis test and Mann–Whitney U test were used to assess statistical significance.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Anatomy of CNs and ultrastructural changes

The MPGs and CNs were identified dorsolateral to the prostate by blunt dissection and then stimulated with a bipolar hook electrode with parameters of 10 V at a frequency of 15 Hz with a square wave duration of 1.2 ms for 1 min (Figure 1). To validate the model of CN injury, TEM was used to identify the ultrastructural changes of CNs. Four weeks after nerve lesioning, disordered ultrastructure and damaged myelin sheath could be found in the BCNI group, compared with the Sham group (Figure 2a and 2b). In the group given bilateral cavernous neurectomy, both intact and damaged myelin sheaths were observed (Figure 2c). In other words, our model might be effective.

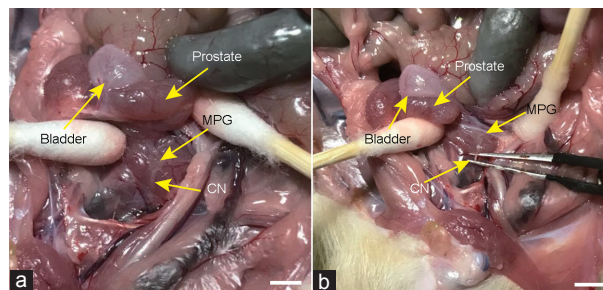
### Effect of daily losartan on erectile response

ICP/MAP was widely used to evaluate erectile function. No significant differences ( $P = 0.7224$ ) were found in baseline MAP among the four groups, and electrical stimulation of the CN induced a frequency-dependent increase in ICP in the other three experimental groups, except for the Neurectomy group. Four weeks postoperatively, the BCNI group had a significantly lower ICP/MAP ratio than the Sham group ( $P < 0.01$ ). After treatment with losartan, the ICP/MAP was slightly restored compared with no treatment ( $P = 0.0211$ ). However, the value was still lower than that in the Sham group ( $P < 0.01$ ) (Figure 3). On the other hand, these results validated our model again.

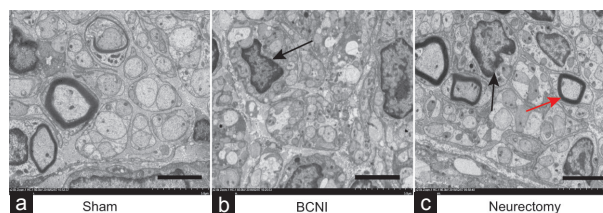
### Effect of daily losartan on fibrosis in the corpus cavernosum

Four weeks postoperatively, the ratio of smooth muscle to collagen detected by Masson's trichrome staining was lower in the BCNI

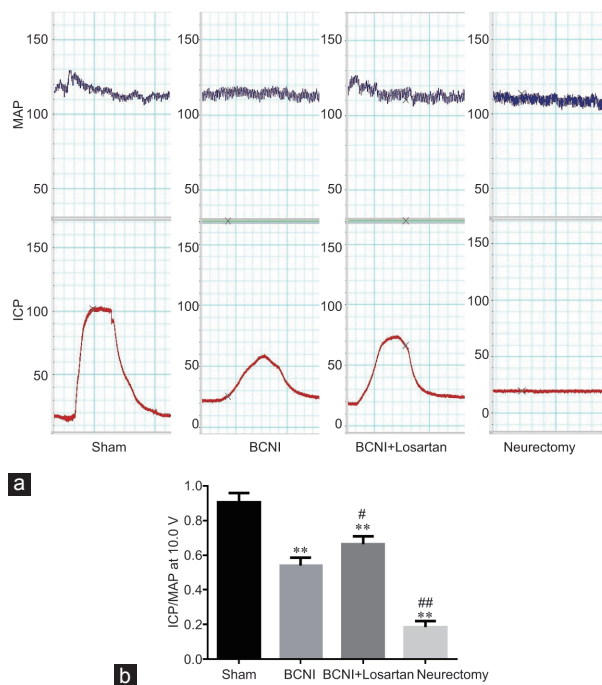
group and the Neurectomy group compared with the Sham group (both  $P < 0.01$ ). After daily treatment with losartan, the ratio



**Figure 1:** Anatomy of the MPG and CN. (a) MPG and CNs were identified dorsolateral to the prostate by blunt dissection. (b) Dissociated CNs were shown by blunt dissection. Scale bars = 5 mm. MPG: major pelvic ganglion; CN: cavernous nerve.



**Figure 2:** Ultrastructural changes of CN by TEM. (a) The Sham group; (b) the BCNI group; (c) the Neurectomy group. Black arrows represent damaged myelin sheath and red arrow represents intact myelin sheath. Scale bars=5 μm. BCNI: bilateral cavernous nerve injury; CN: cavernous nerve; TEM: transmission electron microscopy.



**Figure 3:** Evaluation of erectile response at 10.0 V. (a) The Sham group, the BCNI group, the BCNI + Losartan group, and the neurectomy group;  $n = 12$  in each group. (b) Results of cavernous electrostimulation in the four experimental groups are expressed as ICP/MAP. Bar graphs represent mean ± standard deviation. \*\* $P < 0.01$ , the indicated group compared with the Sham group; # $P < 0.05$  and ## $P < 0.01$ , the indicated group compared with the BCNI group. BCNI: bilateral cavernous nerve injury; ICP: intracavernosal pressure; MAP: mean arterial pressure.

significantly recovered (increased) compared with the BCNI group ( $P = 0.0353$ ). However, it was still markedly lower than that in the Sham group ( $P = 0.0247$ ) (Figure 4a and 4b).  $\alpha$ -SMA immunohistochemical staining revealed that the content of smooth muscle in the corpus cavernosum was substantially lower in the BCNI and Neurectomy groups than in the Sham group (both  $P < 0.01$ ). After treatment with losartan for 4 weeks, smooth muscle content was significantly restored compared with the BCNI group ( $P < 0.01$ ), but still lower than in the Sham group ( $P < 0.01$ ) (Figure 4c and 4d).

By western blot analysis, compared with the Sham group, the expression levels of TGF- $\beta$ 1 were significantly increased in the BCNI and Neurectomy groups. These increases were dramatically attenuated by daily treatment with losartan for 4 weeks. However, expression levels were still significantly higher than that in the Sham group (both  $P < 0.01$ ) (Figure 4e and 4f). Meanwhile, our model was also reconfirmed to be effective.

#### Effect of daily losartan on apoptosis in the corpus cavernosum

Four weeks postoperatively, the BCNI and Neurectomy groups had a significantly higher apoptotic index than the Sham group (both  $P < 0.01$ ). After treatment with losartan, the apoptotic index dramatically decreased compared with no treatment ( $P = 0.0124$ ) and was not statistically different from that in the Sham group (Figure 5a and 5b). Cavernous caspase-3 immunohistochemical staining revealed a higher expression level in the BCNI and Neurectomy groups than that in the Sham group (both  $P < 0.01$ ). After daily administration of losartan, caspase-3 expression significantly decreased, compared with the BCNI group ( $P = 0.0179$ ), although it was still higher than that in the Sham group ( $P = 0.0147$ ) (Figure 5c and 5d).

As for western blot analysis and caspase-3 activity assay, the p-Bad/Bad and p-AKT/AKT ratios were substantially lower (both  $P < 0.01$ ), while the Bax/Bcl-2 ratio and caspase-3 activity were higher (both  $P < 0.01$ ), in the BCNI and Neurectomy groups compared with the Sham group. After daily administration of losartan for 4 weeks, their values significantly recovered compared with the BCNI group ( $P < 0.01$ ) but were still not comparable to those in the Sham group, except for caspase-3 activity ( $P = 0.2116$ ) (Figure 5e–5i).

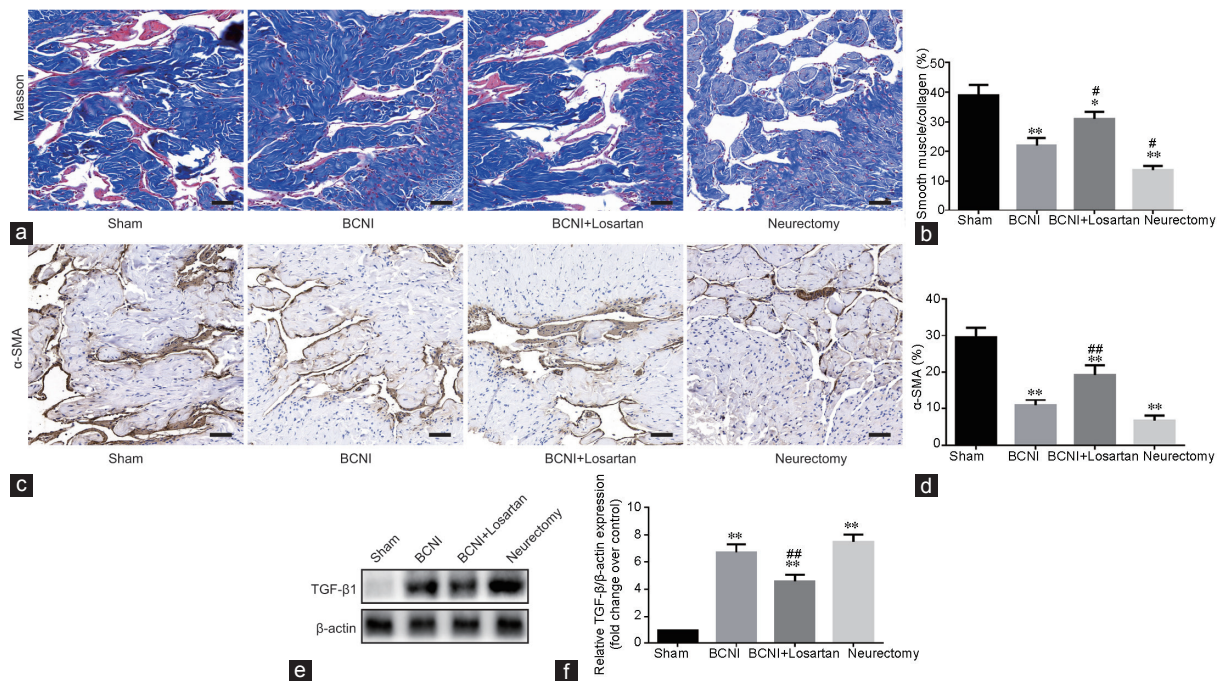
#### Effect of daily losartan on oxidative stress in the corpus cavernosum

Relevant Nrf2 and HO-1 immunohistochemical staining was used to assess the levels of oxidative stress in the corpus cavernosum. Nrf2 and HO-1 expression levels were significantly higher in the BCNI and Neurectomy groups than that in the Sham group (all  $P < 0.01$ ). After daily administration of losartan for 4 weeks, their expression levels dramatically decreased compared with the BCNI group ( $P = 0.0278$  and  $P = 0.0312$ , respectively). However, they were still higher than that in the Sham group ( $P = 0.0349$  and  $P = 0.0291$ , respectively) (Figure 6a–6d).

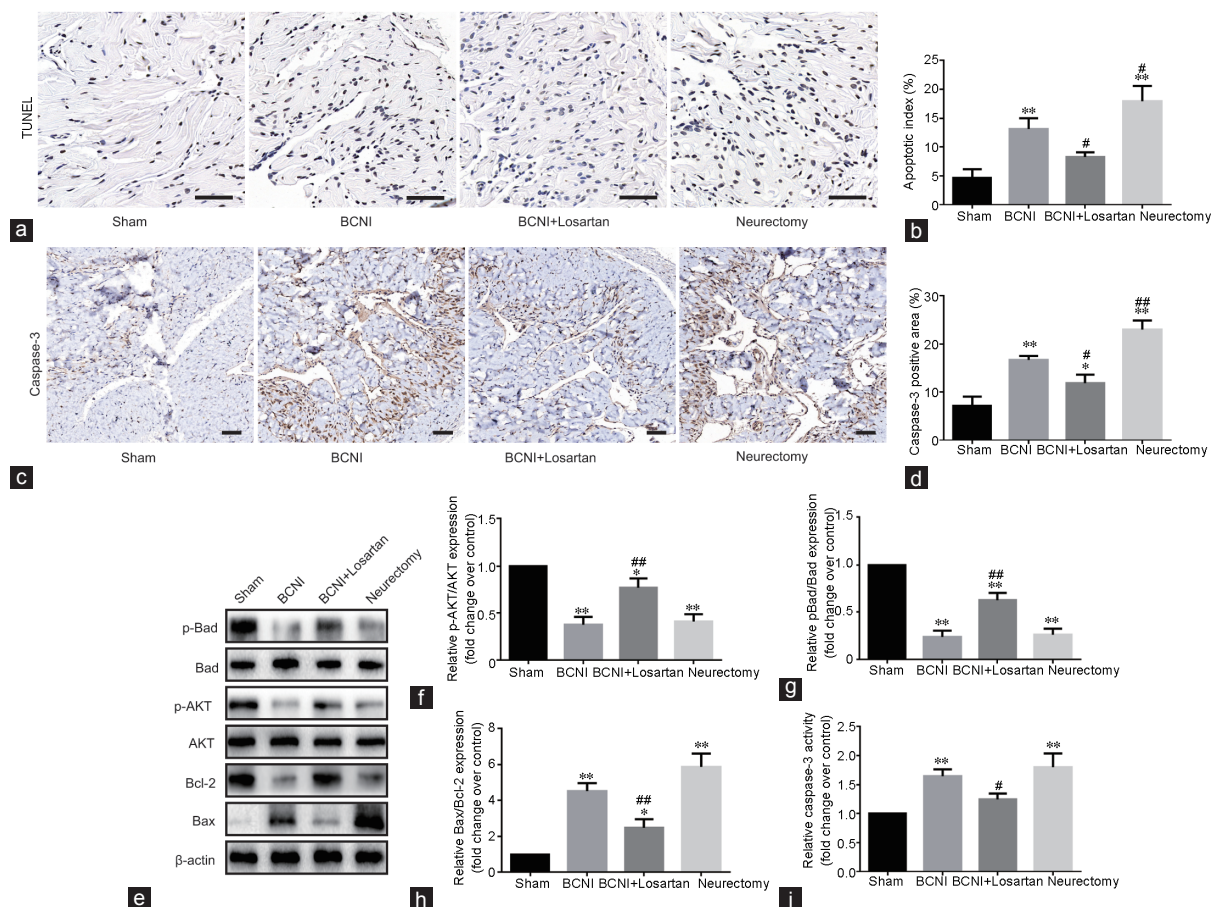
In the case of western blot analysis, the expression levels of Nrf2/Keap-1 and HO-1 in the BCNI and Neurectomy groups were higher than that in the Sham group. After losartan treatment for 4 weeks, their expression levels were significantly attenuated compared with the BCNI group, but still not comparable to those in the Sham group (Figure 6e–6g).

## DISCUSSION

As reflected in clinical findings, the response to PDE5 inhibitors is poor in male patients with post-RP ED resulting from CN injury.<sup>25</sup> Although attempts have been made to regenerate injured nerves by neurotrophic factors<sup>26,27</sup> or immunophilin ligands,<sup>28,29</sup> their responsiveness of



**Figure 4:** Detection of fibrosis by Masson's trichrome staining, cavernous  $\alpha$ -SMA immunohistochemical staining and western blot. (a) Masson's trichrome staining; (b) bar graph of smooth muscle/collagen; (c) cavernous  $\alpha$ -SMA immunohistochemical staining; (d) bar graph of cavernous  $\alpha$ -SMA immunohistochemical staining; (e) the expression of TGF- $\beta$ 1 in corpus cavernosum of Western blot; (f) bar graph of the expression of TGF- $\beta$ 1 in corpus cavernosum. Scale bars = 50  $\mu$ m;  $n = 6$  in each group. Representative micrographs of Masson's trichrome staining show smooth muscle component as red areas, collagen component as blue areas ( $\times 200$ ). Representative micrographs of cavernous  $\alpha$ -SMA immunohistochemical staining show penile smooth muscle alpha-actin as brown areas ( $\times 200$ ). Data of western blot analysis are shown as the fold changes over the control group. Bar graphs represent mean  $\pm$  standard deviation. \* $P < 0.05$  and \*\* $P < 0.01$ , the indicated group compared with the Sham group; # $P < 0.05$  and ## $P < 0.01$ , the indicated group compared with the BCNI group.  $\alpha$ -SMA: alpha smooth muscle actin; BCNI: bilateral cavernous nerve injury; TGF- $\beta$ 1: transforming growth factor beta 1.



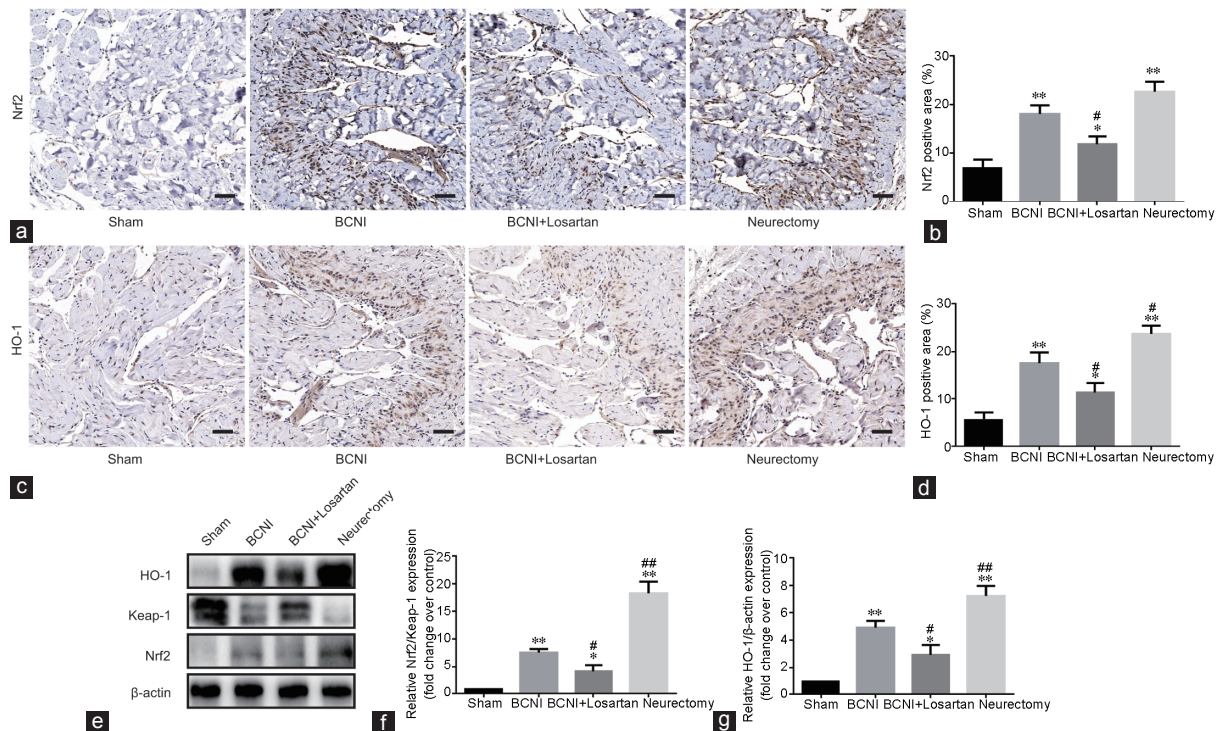
**Figure 5:** Detection of apoptosis by TUNEL, cavernous caspase-3 immunohistochemical staining and western blot. (a) The TUNEL method; (b) bar graph of apoptotic index; (c) cavernous caspase-3 immunohistochemical staining; (d) bar graph of caspase-3 positive area; (e) western blot of the Akt/Bad/Bax/caspase-3 pathways; (f) bar graph of relative p-AKT/AKT expression; (g) bar graph of relative p-Bad/Bad expression; (h) bar graph of relative Bax/Bcl-2 expression; (i) bar graph of relative caspase-3 activity; scale bars = 50  $\mu$ m;  $n = 6$  in each group. Representative micrographs of TUNEL show apoptotic cells as black-brown dots ( $\times 400$ ). Representative micrographs of cavernous caspase-3 immunohistochemical staining show apoptotic cells as brown areas ( $\times 200$ ). Bar graph depicts caspase-3 activity in penile tissues measured by a Caspase-3 Activity Assay Kit, which was calculated according to a standard curve and normalized by the protein concentration. Data of western blot analysis and caspase-3 activity assay are shown as the fold changes over the control group. Bar graphs represent mean  $\pm$  standard deviation. \* $P < 0.05$  and \*\* $P < 0.01$ , the indicated group compared with the Sham group; # $P < 0.05$  and ## $P < 0.01$ , the indicated group compared with the BCNI group. BCNI: bilateral cavernous nerve injury; TUNEL: terminal dextrynucleotidyl transferase(TdT)-mediated dUTP nick end labeling; Bcl-2: B-cell lymphoma 2; Bax: Bcl-2-associated X protein; Bad: Bcl-2-associated death promoter; p-Bad: phosphor-Bad; AKT: the protein kinase B; p-AKT: phosphor-AKT.

cavernous tissue would be compromised even if successful neural regeneration is achieved, due to the histologic changes in the target organ. Moreover, based on our experience, erectile function is unlikely to be restored after CVOD because of apoptosis and fibrosis. Hence, an early intervention to preserve the integrity of corpus cavernosum is likely critical for erectile functional recovery.<sup>30</sup> Hence, this study was aimed to identify the functional and morphological characterization of the corpus cavernosum after CN injury or neurectomy and then reveal whether early treatment with losartan would improve erectile function as well as its potential pathophysiologic mechanisms.

As for fibrosis, a previous study by Canguven *et al.*<sup>31</sup> had fully demonstrated the antifibrotic mechanisms after bilateral CN injury and our outcomes merely reconfirmed its role. On the other hand, our model was also reconfirmed to be effective. Hence, this article mainly focused on the aspects of apoptosis and oxidative stress. A major finding of the current study was that Ang II increased apoptosis and oxidative stress in the corpus cavernosum, resulting in an impaired erectile response. Despite a modest effect on erectile function, the Ang

II Type 1 receptor antagonist losartan could significantly reduce corporal apoptosis and oxidative stress via inhibiting the Akt/Bad/Bax/caspase-3 and Nrf2/Keap-1 signaling pathways in the rat model of CN injury.

Ang II, produced and secreted by the human corpus cavernosum, was present in this tissue at a concentration about 200-fold higher than in plasma and played a key role in the pathogenesis of ED.<sup>32,33</sup> A study by Kifor *et al.*<sup>16</sup> showed that intracavernous injection of Ang II could terminate spontaneous erection, whereas treatment with losartan resulted in smooth muscle relaxation and erection in anesthetized dogs. In normotensive aged rats, losartan restored age-related ED attributable to increased oxidative stress and diminished endothelial nitric oxide synthase (eNOS) expression.<sup>34</sup> In spontaneously hypertensive rats, long-term combined therapy with losartan and sildenafil was revealed as a useful tool for functional and structural modification in cavernous tissue.<sup>35</sup> However, the definite role of Ang II in the case of CN injury was seldom involved. Hence, the Ang II Type 1 receptor antagonist losartan was chosen to discover its potential mechanisms in a rat model of CN injury.



**Figure 6:** Detection of oxidative stress by cavernous Nrf2, HO-1 immunohistochemical staining and western blot. (a) Cavernous Nrf2 immunohistochemical staining; (b) bar graph of Nrf2 positive area; (c) cavernous HO-1 immunohistochemical staining; (d) bar graph of HO-1 positive area; (e) western blot of the Nrf2/Keap-1 pathways; (f) bar graph of relative Nrf2/Keap-1 expression; (g) bar graph of relative HO-1 expression; scale bars = 50  $\mu$ m;  $n = 6$  in each group. Representative micrographs of cavernous Nrf2 immunohistochemical staining show areas of oxidative stress as brown ( $\times 200$ ). Representative micrographs of cavernous HO-1 immunohistochemical staining show areas of oxidative stress as brown ( $\times 200$ ). Data of western blot analysis are shown as the fold changes over the control group. Bar graphs represent mean  $\pm$  standard deviation. \* $P < 0.05$  and \*\* $P < 0.01$ , the indicated group compared with the Sham group; # $P < 0.05$  and ## $P < 0.01$ , the indicated group compared with the BCNI group. BCNI: bilateral cavernous nerve injury; HO-1: heme oxygenase-1; Keap-1: Kelch-like ECH associated protein 1; Nrf2: nuclear factor erythroid 2-related factor 2.

The underlying pathogenic mechanisms in terms of CN injury-induced ED had not been fully understood. Canguven *et al.*<sup>31</sup> revealed that Ang II induced activation of SMAD signaling by Ang II Type 1 receptors contributing to penile fibrosis after CN injury, whereas losartan treatment effectively restores erectile function through antifibrotic mechanisms. Cho *et al.*<sup>25</sup> suggested that early inhibition of Rho-kinase after CN injury might restore erectile function and prevent corporal apoptosis, fibrosis, and CVOD by suppressing the Akt/Bad/Bax/caspase-3 and LIMK2/cofilin pathways. Through normalizing the LIMK2/Cofilin pathway, Park *et al.*<sup>36</sup> showed that corporal fibrosis and erectile function could be improved by inhibition of LIMK2. Oxidative stress had also been implicated in the pathogenesis of ED,<sup>37,38</sup> and Ang II was also reported to cause endothelial dysfunction by increasing reactive oxygen species levels.<sup>14,34</sup> Further studies are required to identify key genes and signaling pathways. Advances in high-throughput sequencing methods and bioinformatics analysis should help in the identification of changes in gene expression in the rat model of CN injury.<sup>39</sup>

Here, we found that daily treatment with losartan after CN injury only had a slight effect on erectile function, but it did significantly reduce corporal apoptosis and oxidative stress. Hence, an early administration of losartan might inhibit histopathologic changes in the corpus cavernosum. As presented in previous studies, the low efficacy of PDE5 inhibitors on the erectile response was strongly associated with its relatively small anti-apoptotic and anti-fibrotic effects, although it might improve erectile function via activation of the NO/cGMP pathway.<sup>17,23</sup> Therefore, to enhance therapeutic effectiveness, combined

therapy with losartan and PDE5 inhibitors had been recommended, and indeed, better performance had been shown in other models of ED.<sup>35</sup> Along with the advances in technology and new therapeutic methods, numerous studies had focused on regenerating of the injured CN. However, because of the histologic changes, the compromised cavernous tissue limited the effectiveness of regenerative strategies. Based on our results, we hypothesized that combined therapy with losartan and these new therapeutic methods might produce better outcomes. Notably, losartan treatment should be started as soon as possible after injury.

In the case of diabetic rats with ED, primary study by Chen *et al.*<sup>33</sup> found that angiotensin Type 1 receptor blocker could improve their erectile function. Therefore, they explored losartan in clinical treatment for diabetic patients suffering from ED. They found that losartan seemed to be effective and well-tolerated in diabetic ED patients, especially in mild-to-moderate cases, and meanwhile, combined losartan and tadalafil therapy appeared to be more effective than monotherapy.<sup>33</sup> In this study, our results had indicated that an early administration of losartan could slightly restore erectile function and dramatically prevent corporal apoptosis and oxidative stress in the rat model of CN injury. In future studies, we would investigate the clinical effectiveness of losartan for postprostatectomy ED.

To the best of our knowledge, the present study comprehensively shed light on losartan reduced corporal apoptosis and oxidative stress following CN injury by suppressing the Akt/Bad/Bax/caspase-3 and Nrf2/Keap-1 pathways. Although our results are of clinical importance,

several limitations should be noted. For example, combined therapy with losartan and PDE5 inhibitors was not used, and side effects were not examined. Our subsequent studies will focus on these issues. In addition, although we have revealed the role of apoptosis and oxidative stress in CN injury, there will be existence of other mechanisms remaining to be discovered. By means of high-throughput sequencing methods and bioinformatics analysis, we shall investigate more fully the genes and signaling pathways involved in CN injury-induced ED in future studies.

## CONCLUSIONS

Taken together, our results shed light on that Ang II contributed to apoptosis and oxidative stress in the corpus cavernosum after CN injury, resulting in an impaired erectile response. Despite a modest effect on erectile function, the Ang II Type 1 receptor antagonist losartan significantly prevented corporal apoptosis and oxidative stress by inhibiting the Akt/Bad/Bax/caspase-3 and Nrf2/Keap-1 signaling pathways in the rat model of CN injury. Therefore, early administration of losartan might lessen histopathologic changes in the corpus cavernosum. Our novel findings helped advance our understanding of the pathophysiology and molecular mechanisms of CN injury-induced ED and also provided new insights for developing improved therapeutic strategies.

## AUTHOR CONTRIBUTIONS

YW, XHM, and NHS designed the study, analyzed and interpreted the experimental data, and drafted and revised the manuscript. YW, XHM, QJZ, and YMW carried out the studies and collected the experimental data. CC, YCW, XZ, and CJJ performed the statistical analysis. NHS conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

## COMPETING INTERESTS

All authors declared no competing interests.

## ACKNOWLEDGMENTS

This article was funded by the Medical key talent of Jiangsu Province (No. ZDRCA2016009), which had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in this study. We would like to thank the researchers and study participants for their contributions.

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