N-acetylcysteine maintains penile length and erectile function in bilateral cavernous nerve crush rat model by reducing penile fibrosis

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Penile length shortening and erectile dysfunction are common complications after radical prostatectomy. Various methods have been used to maintain erectile function, but less attention has been paid to preserving penis length. N-acetylcysteine (NAC) has the effect of antioxidation and antifibrotic, which may be beneficial to improve those postoperative complications. This study investigated the effect of NAC on maintaining the penile length and the erectile function after bilateral cavernous nerve crush (BCNC) and its underlying mechanism. Twenty-four male rats were randomly divided into three groups: control group, BCNC group, and BCNC + NAC group. NAC or equal volume of saline was daily administrated by intragastric gavage for 4 weeks. The initial and end penile lengths were measured. Intracavernosal pressure/mean arterial pressure (ICP/MAP) ratio was calculated to assess erectile function. Hematoxylin–eosin staining, Masson’s trichrome staining, immunohistochemistry, and Western blot were performed to explore cellular and molecular changes of the penis. Compared to the BCNC group, the penile length, ICP/MAP ratio and smooth muscle/collagen ratios in the BCNC + NAC group were improved significantly (all P < 0.05), along with the decreased expressions of hypoxia-inducible factor-1α, transforming growth factor-β1, collagen I, collagen III, collagen IV, malonaldehyde, and lysine oxidase (all P < 0.05). This study demonstrated that NAC could maintain penile length and partly improve erectile function. Possible mechanism is directly and/or indirectly related to antihypoxic and antifibrosis. Asian Journal of Andrology (2021) 23, 215–221; doi: 10.4103/aja.aja_17_20; published online: 12 May 2020

Keywords: erectile dysfunction; N-acetylcysteine; penile fibrosis; penile length
To improve the erectile function by inhibiting fibrosis in bilateral cavernous nerve injury rat model. Also, as the most effective fibrogenic factor, transforming growth factor beta 1 (TGF-β1) has been widely considered to play an important role in penile fibrosis. N-acetylcysteine (NAC), as an acetylated derivative of natural amino acid L-cysteine, is a precursor of glutathione (GSH), which plays an essential role in regulating the redox environment of cells. However, the impact of NAC on penile length and erectile function after radical prostatectomy has not been studied. Based on the effect of NAC and the pathological mechanism of penile length shortening and ED after radical prostatectomy, we assumed that NAC might be a potential drug for the maintenance of erectile function and penile length. In this study, we would like to verify our hypothesis in bilateral cavernous nerve crush (BCNC) rat model and investigate the underlying mechanism.

**MATERIALS AND METHODS**

**Animals**

Twenty-four adult male Sprague–Dawley rats (8 weeks old, 250.00–265.00 g, Chengdu Dossy Experimental Animals, Co., Ltd., Chengdu, China) were housed in cages. All these animals were raised under strict guidelines. Then those 24 rats were randomly divided into three groups after adaptive feeding for 3 days: (1) control group (sham surgery, saline); (2) BCNC group (BCNC surgery, saline); and (3) BCNC + NAC group (BCNC surgery, NAC). The BCNC rat model was based on previous studies. NAC (Hainan Zambon Pharmaceutical Co., Ltd., Haikou, China) was dissolved with normal saline, and the intragastric dose was 500.00 mg kg⁻¹, and 100.00 mg ml⁻¹. NAC or equal volume of saline, which was started on the day after 3 days of BCNC surgery, was daily administered by intragastric gavage for 4 weeks. The dosage had been fine-tuned based on previous studies. The initial (0 day) and end (28 days) body weight and penis length of rats were measured. The new method for measuring the length of rat penis is based on the method to measure the penis length of newborns and infants. Briefly, a modified 2.5 ml disposable syringe (inner diameter: 9.0 mm) connected to a negative pressure device (Chengdu Xin Wei Cheng Technology Co., Ltd., Chengdu, China), and the flanged end was placed over the penis and firmly pressed onto the pubis. After a unified vacuum (~200 mmHg, 2 min), penile length was recorded, with head of the ruler close to syringe flanged end. All experimental procedures were followed by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of West China Hospital, Sichuan University, Chengdu, China (No. 2016057A).

**Erectile function evaluation**

Erectile function was evaluated in all rats before euthanized. Intracavernosal pressure (ICP) under electrical stimulation of the cavernous nerve and mean arterial blood pressure (MAP) were measured by the BL-420F functional biological experiment system (Chengdu TME Technology Co., Ltd., Chengdu, China). Briefly, after being anesthetized with isoflurane inhalation, the cavernous nerve was exposed on the left side of prostate. The left carotid artery was exposed and cannulated with a PE-50 tube and filled with 250.00U ml⁻¹ heparin sodium solution (Changzhou Qianhong Bio-pharma Co., Ltd., Changzhou, China) was inserted into the left penile crus for measurement of ICP. Stimulation was performed at parameters of 20.0 Hz, 5.0 V, and pulse width of 5.0 ms for 50.0 s. The maximal increase of ICP during electrical nerve stimulation was selected for statistical analysis. The ICP/MAP ratios were analyzed to evaluate the erectile function. After the measurement of erectile function, all penises were harvested from the symphysis pubis for histopathology and molecular assessments.

**Histopathology**

The Pathology Platform of West China Hospital assisted us in the processing of pathological sections. Briefly, the middle shaft of the penile tissues was rinsed with phosphate buffer solution and fixed in 4.0% paraformaldehyde overnight. After that, these tissues were routinely processed and paraffin embedded. Tissue samples were sliced into 5.0 μm sections. Then, the tissue slides were prepared for hematoxylin-eosin (HE) staining, Masson’s trichrome (MT) staining, and immunohistochemistry (IHC). Smooth muscle/collagen ratios were quantitatively analyzed by MT staining (Baso Diagnostics Inc., Zhuhai, China) and those data were collected by Image-Pro Plus 6.0 software (National Institutes of Health, Bethesda, MD, USA). Three sections of each rat were taken for statistical analysis at ×200 magnification, and sections from eight rats per group were analyzed.

IHC was performed for analysis of endothelial nitric oxide synthesis (eNOS), hypoxia-inducible factor-1alpha (HIF-1α), alpha-smooth muscle actin (α-SMA), TGF-β1, LOX, collagen I, collagen III, and collagen IV. The streptavidin-peroxidase method was performed according to the manufacturer’s instructions (SP-9000, Beijing ZSbio, Beijing, China). Briefly, samples were treated with 3.0% hydrogen peroxide and blocked by superblock (either 10.0% goat serum or horse serum, depending on the primary antibody) for 30 min at 37°C respectively. Then the sections were incubated with primary antibodies at 4°C overnight: anti-eNOS (ab76198, 1:100, Abcam, Cambridge, MA, USA), anti-HIF-1α (ab2185, 1:100, Abcam), anti-α-SMA (ab32575,1:200,Abcam),anti-TGF-β1 (ab92486,1:100,Abcam),anti-LOX (ab174316,1:100, Abcam), anticollagen I (ab34710, 1:100, Abcam), anticollagen III (ab7778, 1:100, Abcam), and anticollagen IV (ab6586, 1:100,Abcam). After washing with phosphate buffer solution three times, the sections were incubated with anti-rabbit or antimouse IgG-conjugated biotin, followed by streptavidin-conjugated horseradish peroxidase. Finally, 3,3′-diaminobenzidine (ZLI-9018, Beijing ZSbio) was used as a chromogen to visualize the positive brown signal. Then the stained sections were counterstained with hematoxylin, dehydrated, and mounted with clear mounting medium. Three sections per rat were quantified at ×200 magnification by Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). Eight rats per group were quantified.

**Western blot**

The nerve bundle and urethra were stripped from the corpora before homogenization. Radio immunoprecipitation assay (RIPA) lysis buffer (MA0151, Meilunbio, Dalian, China) containing protease inhibitor cocktail set I (Merck Millipore, Billerica, MA, USA) was added to the corpora cavernosum. The samples were cut and homogenized, and then centrifuged (Sorvall Legend Micro 17R Centrifuge, Thermo Electron LED GmbH, Osterode, Germany) at 12 000g for 20 min at 4°C. Protein concentration was determined by Coomassie brilliant blue G-250 (Amresco Inc., Solon, OH, USA) working buffer. Equal amounts of protein were loaded on 10.0% Tris-glycine gels for electrophoresis. Proteins were wet-transferred to polyvinylidene fluoride membranes (Merck Millipore) and then primary antibodies were blocked with 5% non-fat dry milk in Tris buffered saline-Tween (TBST) for 30 min at room temperature. The membranes were washed three times with TBST for 20 min at room temperature. The membrane was incubated at 4°C overnight. After washing with TBST three times, the membranes were washed with 3.0% hydrogen peroxide, and then blocked by 5% non-fat dry milk in TBST. Membranes were incubated at 4°C overnight with the following primary antibodies: anti-eNOS (ab76198, 1:100, Abcam), anti-α-SMA (ab32575,1:200,Abcam),anti-TGF-β1 (ab92486,1:100,Abcam),anti-LOX (ab174316,1:100, Abcam), anticollagen I (ab34710, 1:100, Abcam), anticollagen III (ab7778, 1:100, Abcam), and anticollagen IV (ab6586, 1:100,Abcam). After washing with phosphate buffer solution three times, the membranes were incubated with anti-rabbit or antimouse IgG-conjugated biotin, followed by streptavidin-conjugated horseradish peroxidase. Finally, 3,3′-diaminobenzidine (ZLI-9018, Beijing ZSbio) was used as a chromogen to visualize the positive brown signal. Then the stained sections were counterstained with hematoxylin, dehydrated, and mounted with clear mounting medium. Three sections per rat were quantified at ×200 magnification by Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). Eight rats per group were quantified.
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**GSH, malonaldehyde (MDA) and LOX activity**

GSH and MDA expressions were assessed in rat corpus cavernosum. The expressions were measured with a GSH and MAD assay kit (Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer’s instruction. LOX activity was determined using an Amplite TM Fluorimetric Lysyl Oxidase Assay kit (AAT Bioquest Inc., Sunnyvale, CA, USA). Tissues were homogenized in 1.2 mol l⁻¹ urea and 50.0 mmol l⁻¹ sodium borate buffer (pH 8.0). The supernatants were collected after centrifugation (15 000 g, 30 min; Sorvall Legend Micro 17R Centrifuge, Thermo Electron LED GmbH). Then, fluorescence was measured using BioTek Synergy Mx (BioTek Instruments Co., Ltd., Winooski, VT, USA) with excitation and emission wavelengths at 560.00 nm and 590.00 nm, respectively. LOX activity was normalized to total protein and expressed as relative fluorescent units per µg protein (RFUs µg⁻¹ protein).

**Statistical analyses**

Data were analyzed by GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA) and presented as mean ± standard deviation (s.d.). Statistical differences among multiple groups were compared using the one-way ANOVA analysis, followed with a Tukey test to compare all pairs of columns. For some groups, we performed the Student’s t-test to obtain P values. A value of P < 0.05 was considered significant.

**RESULTS**

**Body weight and penile length**

As shown in **Table 1**, there was no significant difference in initial body weight (P = 0.1307) or penile length (P = 0.9214) among different groups. Twenty-eight days later, BCNC surgery significantly reduced penile length by 7.28%, but NAC therapy suppressed this reduction (control vs BCNC and BCNC + NAC: 33.25 ± 0.82 mm vs 30.83 ± 1.17 mm and 34.33 ± 1.72 mm, respectively, P < 0.05).

**Erectile function**

**Figure 1** shows the results of erectile function. Compared with the BCNC group (0.37 ± 0.03), the ICP/MAP ratio in the NAC treated group (0.49 ± 0.07, P < 0.05) was significantly improved, but it was only partially recovered when compared with the control group (0.66 ± 0.10, P < 0.05).

**Table 1**: Effect of N-acetylcysteine treatment on body weight and penile length

|        | Body weights | Penile lengths |
|--------|--------------|---------------|
|        | Initial (g)  | End (g)       | Initial (mm) | End (mm) |
| Control| 255.48±5.05  | 380.83±9.75   | 29.25±0.69   | 33.25±0.82|
| BCNC   | 259.22±2.23  | 385.00±21.63  | 29.00±1.18   | 30.83±1.77 |
| BCNC + NAC | 260.00±3.79 | 391.33±28.39  | 29.17±1.29   | 34.33±1.72 |

All data were expressed as mean±s.d.; *P*<0.05, significant difference; BCNC: bilateral cavernous nerve crush; NAC: N-acetylcysteine; s.d.: standard deviation

**Smooth muscle/collagen ratio and collagen content**

**Figure 2a** shows representative pictures of HE and MT staining in each group. The penile smooth muscle/collagen structure was disordered in the BCNC group, and a large amount of extracellular matrix was deposited. NAC treatment alleviated the above lesions. In **Figure 2b**, the smooth muscle/collagen ratio of the control group was 0.37 ± 0.13, which was significantly higher compared with the BCNC group (0.24 ± 0.07, P < 0.05). NAC had improved the rate considerably to 0.35 ± 0.13, which was 48.80% higher than that in the BCNC group (P < 0.05).

**Expression of eNOS, α-SMA, TGF-β1, and HIF-1α**

IHC was performed to assess the expression of collagen content, including collagen I, collagen III, and collagen IV. According to **Figure 2c** and 2d, when compared with the control group, the BCNC group had significant increases of collagen I (29.92%, P < 0.05), collagen III (38.85%, P < 0.05) and collagen IV (98.50%, P < 0.05) in corpus cavernosum, while NAC administration could significantly decrease the expression of collagen I (17.61%, P < 0.05), collagen III (24.81%, P < 0.05), and collagen IV (36.22%, P < 0.05).

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**DISCUSSION**

As an animal model simulating the injury of corpus cavernosum nerve caused by radical prostatectomy, BCNC rat model has gradually become a classical model for the study of penile rehabilitation. In this study, penile length and erectile function decreased in the BCNC group, which was consistent with our previous studies. Daily administration of NAC maintained penile length after BCNC, and the increased ICP/MAP ratio represented the improvement of erectile function. The expression of eNOS, a key enzyme in nitric oxide synthesis, playing a vital role in penile erectile, was significantly higher in NAC treated group than that in the BCNC group. HE staining showed that the structure of penile cavernosum was disordered with a large amount of extracellular matrix deposition, and the MT staining showed that the smooth muscle/collagen ratio was decreased after BCNC. Moreover, the pathological changes of penile cavernosum fibrosis were correlated with the down-expression of α-SMA and...
over-expression of hypoxia-related factors, such as TGF-β1, collagens and LOX. Furthermore, the up-regulation of HIF-1α and MDA expression, and the down-regulation of GPX1 and GSH expression suggested that oxidative stress would also be involved in penis shortening and ED after BCNC.

In this study, NAC, a commonly used clinical drug, was proved to be effective in the maintenance of penile length and erectile function after BCNC for the first time. The mechanism may be directly and/or indirectly related to antihypoxic and antifibrosis (Figure 5). Oxidative stress caused by cavernous nerve injury and hypoxia is closely related...
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Figure 3: Western blot analysis. (a) Typical bands of Western blot; bands from up to down represent eNOS, α-SMA, TGF-β1, HIF-1α, and GAPDH. (b) Column figures of eNOS, α-SMA, TGF-β1, and HIF-1α, respectively. Each figure denotes mean ± standard deviation of each protein expression determined by densitometry relative to GAPDH. Compared with the BCNC group, the NAC therapy group significantly increased the expression of eNOS and α-SMA (P < 0.05), and decreased the expression of HIF-1α and TGF-β1 (P < 0.05). *P < 0.05. α-SMA: alpha smooth muscle action; BCNC: bilateral cavernous nerve crush; eNOS: endothelial nitric oxide synthase; GAPDH: glyceraldehyde phosphate dehydrogenase; HIF-1α: hypoxia-inducible factor 1α; NAC: N-acetylcysteine; TGF-β1: transforming growth factor beta 1.

Penile fibrosis is a significant contributor to penile fibrosis. In this study, cavernous nerve crush broke the balance between oxidation and antioxidation. MDA, a lipid oxidation production, was increased in BCNC rats. While the main antioxidants, GSH and its catalysis enzyme GPX1, were decreased. GSH is a primary substrate for enzymatic antioxidant functions and is capable of nonenzymatic radical scavenging. The reduced GSH and GPX1 can promote the production of reactive oxygen species (ROS), and the over-expression of ROS can promote the stability and expression of HIF-1α. As a highly conserved transcription factor, HIF-1α can promote the over-expression of other hypoxia-related substrates during hypoxia, such as TGF-β1, LOX and collagen, eventually aggravated penile fibrosis. Besides, HIF-1α can activate target genes and initiate apoptosis, cell death and autophagy. In this study, HIF-1α increased significantly in the BCNC group, while NAC down-regulated the over-expression. Many studies have confirmed that administration of NAC could suppress the ROS-dependent expression of HIF-1α. Thus, we speculate that the down-regulation may be attributed to the direct clearance of ROS by GSH, because NAC is a precursor in the formation of the antioxidant GSH. Whether this signaling pathway is involved or not needs to be explored in the follow-up study.

Penile fibrosis was thought to play an essential role in the pathological mechanisms of penile length shortening and ED after BCNC. Previous studies have proved that penile vein occlusion resulted from fibrosis leads to the occurrence of ED, and the decreased compliance of cavernous tissue finally leads to the shortening of penile length. Studies have demonstrated that adjuvant administration of NAC is more effective than standard therapy in patients with idiopathic pulmonary fibrosis with retention of vital capacity and single-breath carbon monoxide diffusing capacity. The underlying mechanism might be that NAC significantly inhibited pulmonary fibrosis and improved pulmonary function. In this study, the results of HE, MT and IHC staining also suggested that NAC may ameliorate the structural disorder of corpus cavernosum in BCNC rats by increasing smooth muscle and decreasing collagen components. Therefore, we hypothesized that NAC might retain the erectile function and penis length through a common antifibrotic mechanism.
In recent years, the role of LOX in penile fibrosis has attracted more attention. LOX is an extracellular and copper-dependent monoamine oxidase, which works through catalyzing the crosslinking of lysine residues in collagen and elastin, promoting deposition of collagen and elastin fibers, finally resulting in fibrosis. Studies have demonstrated that LOX plays essential roles in tissue fibrosis, such as myocardium, lung, and liver. Our previous study has found that LOX also plays an important role in penile fibrosis in priapism rat model, and aminopropionitrile can improve penile structure and erectile function through anti-LOX and antifibrotic mechanisms, which is consistent with that of Wan et al. Therefore, anti-LOX therapy shows a good prospect in antifibrotic. Unfortunately, aminopropionitrile is a chemical, not being directly applied in clinic. In this study, the penile fibrosis is closely related to the up-regulation of LOX expression and activity, and NAC could down-regulate the expression and activity of LOX to inhibit fibrosis. As discussed above, the down-regulation of HIF-1α can reduce the expression of LOX. Besides, TGF-β1, as an essential activating factor of LOX, whose reduction can lead to the inhibition of LOX expression, was also suppressed by NAC in this study. The increased expression of GSH by NAC may be related to the destruction of the disulphide bond of TGF-β1. Furthermore, as a co-enzyme of LOX, Cu²⁺ plays a crucial role in the activity of LOX, while GSH can form a complex with the intracellular Cu²⁺ and transferred to metallothionein. The reduced concentration of Cu²⁺ and the reduced expression of LOX may attribute to the declined LOX activity.

There are some limitations of this study. NAC can be used in the treatment of a variety of diseases because of its various pharmacological effects. In this study, NAC was administrated by intragastric gavage for 4 weeks. NAC improved the structure of the corpus cavernosum of the penis through antifibrosis and antioxidation, and retained the length and erectile function of the penis. However, it is not clear which is the central role of the above antifibrotic effect. The erectile function was partly maintained, suggesting that NAC may not be used alone, but may become an important auxiliary drug for other penile rehabilitation therapy. Moreover, it is regretful that we only focused on the effects of NAC on penile cavernosum, but did not detect the effect of NAC on the repair or regeneration of the injured cavernous nerve. In the follow-up experiments, further studies are needed to explore the molecular mechanism in more detail, and explain whether NAC has an impact on the structure and function of the nerve, and explore its mechanism.

AUTHOR CONTRIBUTIONS
MM, CJW, and PZ carried out the experiments. MM and CJW contributed to the statistical analyses and the manuscript preparation. BTY, SZW, and FQ participated in the article screening, and critical revision of the manuscript. MM, CJW, PZ and TL participated in the experiments design. JHY conceived of this study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declare no competing interests.

ACKNOWLEDGMENTS
This work was supported by the Natural Science Foundation of China (No. 81671453 and No. 81871147), the Sichuan Science and Technology Program (No. 2018SZ0019 and No. 2018TJPT0018).

Supplementary Information is linked to the online version of the paper on the Asian Journal of Andrology website.
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Supplementary Figure 1: Representative pictures of immunohistochemistry. From up to down represent eNOS, α-SMA, TGF-β1, and HIF-1α. The result is consistent with that of Western blot. NAC therapy group significantly increased the expression of eNOS, α-SMA ($P < 0.05$), and decreased the expression of HIF-1α, TGF-β1 ($P < 0.05$) in penis after BCNC surgery. *Means significant difference ($P < 0.05$).

eNOS: endothelial nitric oxide synthase; GAPDH: glyceraldehyde phosphate dehydrogenase; HIF-1α: hypoxia-inducible factor 1α; NAC: N-Acetylcysteine; TGF-β1: transforming growth factor beta 1.