Original Article

Botulinum toxin A improves neurogenic bladder fibrosis by suppressing transforming growth factor β1 expression in rats

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Background: Intradetrusor botulinum toxin A injection is recommended for the treatment of refractory detrusor overactivity (DO) in patients with neurogenic bladder, however, whether it could inhibit neurogenic bladder fibrosis is uncertain. This study aimed to investigate the effect of botulinum toxin A on neurogenic bladder fibrosis and the underlying mechanism.

Methods: Forty eight Female Wistar rats were evenly randomized into 4 groups: Sham, T10 transection, Early and Late groups. The last three groups were subjected to T10 spinal cord transection, while the Sham group was treated with sham surgery. 0.9% saline was injected into the detrusor in the Sham and T10 transection groups simultaneously with the surgery, while 2 U/rat botulinum toxin A was injected into the detrusor simultaneously with the surgery in the Early group and 4 weeks following the surgery in the Late group. Body/bladder weight, cystometric parameters, bladder Hematoxylin-eosin staining were used to evaluate the bladder fibrosis. Western blot and quantitative Real-time PCR were used to evaluate the expression of bladder transforming growth factor β1.

Results: Compared with the T10 transection group, the bladder/body weight was decreased significantly in the Early and Late groups ($P<0.05$), along with the significant inhibition of non-voiding contraction (NVC) frequency and amplitude ($P<0.05$), and the significant increase of bladder volume ($P<0.05$). The detrusor connective tissue percentage ($P<0.05$) and the expression of transforming growth factor β1 ($P<0.05$) also decreased significantly in the Early and Late groups. Those changes were more obviously in the Early group than in the Late group.

Conclusions: Intradetrusor botulinum toxin A injection reduced bladder fibrosis in rats with spinal cord injury (SCI), which was more obviously in the Early group than in the Late group. The mechanisms might be mediated by suppression of transforming growth factor β1 (TGF-β1) expression.

Keywords: Spinal cord injury (SCI); neurogenic bladder; bladder fibrosis; botulinum toxin A; transforming growth factor β1 (TGF-β1)

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Introduction

Supraspinal cord injury usually induces detrusor overactivity (DO), detrusor-sphincter dyssynergia and bladder fibrosis, resulting urinary dysfunction, such as urgency, frequency, incontinence, retention, and renal impairment in some cases (1). Intradetrusor botulinum toxin A (BoNTA) injection is a safe, cost-effective and long-term therapy for patients with neurogenic DO (1,2). However, few studies have explored the effect of intradetrusor BoNTA injection on neurogenic bladder fibrosis and the answer remains unclear. In patients

with neurogenic DO, BoNTA injection did not increase extracellular matrix or collagen deposition (3-5), but repeated injections reduced bladder fibrosis (6). In rats with spinal cord injury (SCI), BoNTA injection decreased bladder fibrosis with short-term and small-scale observation (7). Transforming growth factor β1 (TGF-β1) is a key cytokine in promoting fibrosis (8), but few studies explored the relationship between BoNTA injection and TGF-β1 expression in the treatment of neurogenic bladder. The study aimed to explore the effect of intradetrusor BoNTA injection on bladder fibrosis and TGF-β1 expression in rats with T10 spinal cord transection. As fibrosis is usually irreversible, we divided the rats received BoNTA injection into the Early group and the Late group to further explore the benefit of early application of BoNTA on bladder fibrosis in this study.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/tau-21-62).

Methods

Rat models

This study was performed in compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Xuanwu Hospital Capital Medical University (No. 20190128). Forty eight female Wistar rats (200±10 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and randomly divided into four groups (n=12, respectively): Sham, T10 transection, Early and Late. The Sham group underwent T10 sham operation, which opened the spinal canal and the dura mater, but did not damage the spinal cord and then closed the wound. The other three groups underwent T10 spinal cord transection. For intradetrusor injection, agents (totally 80 μL) were injected into four sites around the bladder base and four sites around the middle of the bladder between the serosal layer and the muscle layer via a midline abdominal incision using a syringe needle (30G). A total of 0.9% saline was injected into the detrusor in the Sham and T10 transection groups simultaneously with the surgery, while 2 U/rat BoNTA (Hengli®, Lanzhou Biological Products, China) was injected into the detrusor simultaneously with the surgery in the Early group and 4 weeks following the surgery in the Late group. T10 spinal cord transection and intradetrusor injection were made under 2% isoflurane anesthesia. After operation, rats were kept alone and provided free access to food and water. The bladders were urinated manually 3 times a day for 14 days and then once a day.

Cystometry in conscious rats

Conscious cystometry was recorded following the measurement of body weight in all rats by 8 weeks post spinalization. First, the bladder dome was exposed via a midline abdominal incision and the ureters were ligated at the level of the aortic bifurcation under 2% isoflurane anesthesia, then, cystometric parameters was recorded according to previous report after two stable micturition cycles at an infusing speed of 0.08 mL/min with 0.9% saline after the rats recovered from anesthesia for 1 h. Cystometric parameters included bladder volume, non-voiding contraction (NVC) frequency and NVC amplitude (9). Bladder volume was defined as the infusion volume when the fluid releases from urethra, and NVCs were defined as rhythmic intravesical pressure increases over 7 cmH_2O from baseline pressure without release of fluid from the urethra (9). After cystometry, the rats were sacrificed and the bladders were weighed after removal and divided into 4 pieces via the sagittal and coronal planes. Three pieces of the detrusor were separated and used for Western blot and quantitative real-time polymerase chain reaction (qRT-PCR) analysis, while one piece was used for paraffin embedding.

Evaluation of detrusor fibrosis with hematoxylin-eosin (HE) staining

HE staining and Masson staining were performed with 4 μm sections of bladder strips after dewaxing and rehydration. The sections were scanned with APERIO AT TURBO slide scanning system (Aperio Technologies, USA) at 20× magnification and saved as digital images. Three areas of muscle layer were randomly selected for evaluation at 10× magnification and saved as JPG files. The abundance of connective tissue in the HE staining were evaluated with Fiji-win64: connective tissue percentage (%) = connective tissue area/detrusor area ×100. The experiments were done using randomized blind selection.

Evaluation of TGF-β1 protein expression with Western blot

The supernatant of homogenized detrusor was separated with 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membrane.

Table 1 Changes of bladder weight and bladder function (n=12 each group)

Variables	Sham	T10 transection	Early	Late
Body weight (g)	272±12	264±13	267±8	258±15*
Bladder weight (g)	0.2±0.1	0.6±0.1*	0.4±0.1*&	0.5±0.1*&
Bladder/body weight (×10^4)	9.1±2.3	24.0±5.2*	14.3±4.1*&	18.5±2.9*&#

*, P<0.01 compared with Sham; &, P<0.05 compared with T10 transection; #, P<0.05 compared with Early.

The membrane was incubated with rabbit anti-TGF-β1 monoclonal antibody (1:5,000; Abcam, Shanghai, China) and mouse anti-GAPDH monoclonal antibody (1:1,000; ZSGB Biotechnology, Beijing, China) at 4 ℃ overnight, and then with HRP-conjugated goat anti-rabbit IgG (1:500; ZSGB Biotechnology, Beijing, China) and HRP-conjugated goat anti-mouse IgG (1:4,000; ZSGB Biotechnology, Beijing, China) at room temperature for 1 h. Immunoreactive bands were visualized using an enhanced chemiluminescence system. The relative TGF-β1 expression was normalized to GAPDH. Each experiment was performed in triplicate.

Evaluation of TGF-β1 mRNA expression with quantitative real-time PCR

Total RNA was extracted from homogenized detrusor with the TRIzol reagent (Invitrogen, Grand Island, NY, USA) and synthesized to cDNA with the Prime-Script RT reagent kit (TaKaRa, Dalian, China). Roche LightCycler® 480 instrument II (Roche Diagnostics Ltd., Shanghai, China) was used for qRT-PCR. The primers were as following: GCTGAAGCCGTTCATTTAGC (forward) and GAGGAGGCCAAATTCAACAA (reverse) for TGF-β1; ACAAGATGGTGAAGGTCGGTG (forward) and AGAAGGCAGCCCTGGTAACC (reverse) for GAPDH. The reaction system was as following: 1 μL cDNA (10 ng), 2 μL 10× Ex Taq Buffer (TaKaRa, Dalian, China), 1 μL dNTP Mixture (TaKaRa, Dalian, China), 0.2 μL Ex Taq® Hot Start Version (TaKaRa, Dalian, China), 2 μL SYBR Green I (BioTek, Beijing, China), 1 μL (10 μM) forward primer, 1 μL (10 μM) reverse primer and 11.8 μL DEPC-treated water. The temperature program encompassed 95 ℃ for 2 min, 95 ℃ for 10 s, and 40 cycles of 60 ℃ for 40 s, and 72 ℃ for 30 s. TGF-β1 mRNA expression was normalized to GAPDH. Each experiment was performed in triplicate.

Statistical analysis

Data are expressed as means ± SD. One-way ANOVA with LSD or Dunnett's test was used for comparisons between groups. SPSS 22.0 software (IBM, Armonk, NY, USA) was used for data analysis and differences with P<0.05 were considered statistically significant.

Results

Changes of bladder/body weight

There was no significant difference about body weight between the four groups, except for the Sham group and the Late group (P<0.01) (*Table 1*). The bladder weight in the SCI rats was increased significantly compared with the Sham group (P<0.01), meanwhile, it was intermediate between the Sham group and the T10 transection group in the two BoNTA-treated groups (*Table 1*). The bladder/body weight ratios were in line with the changes of bladder weight among the groups. Notably, it decreased significantly in the Early group compared with the Late group (P<0.05; *Table 1*).

Changes of cystometric parameters

The NVC frequency increased significantly in the SCI rats compared with the Sham group (P<0.05), meanwhile, it was intermediate between the Sham group and the T10 transection group in the two BoNTA-treated groups (*Table 2*, *Figure 1*). Notably, the NVC frequency decreased significantly in the Early group compared with the Late group (P<0.05) (*Table 2*, *Figure 1*). The NVC amplitude had the similar tendency to the changes of NVC frequency, however, there was no significant difference between the Early and Late groups (P=0.053; *Table 2*, *Figure 1*). The bladder volume had the opposite tendency to the changes of NVC frequency among the groups (*Table 2*, *Figure 1*).

Changes of bladder fibrosis

The detrusor connective tissue percentage increased

Table 2 Changes of bladder function (n=12 each group)

Variables	Sham	T10 transection	Early	Late
DO frequency (n)	5.1±1.7	17.1±2.6*	9.6±1.8*&	13.4±2.9*&#
DO amplitude (cmH_2O)	8.1±0.8	16.9±3.0*	10.0±1.7*&	12.8±2.8*&
Bladder volume (mL)	2.0±0.2	1.0±0.3*	1.6±0.4*&	1.3±0.3*&#

*, P<0.05 compared with Sham; &, P<0.05 compared with T10 transection; #, P<0.05 compared with Early. DO, detrusor overactivity.

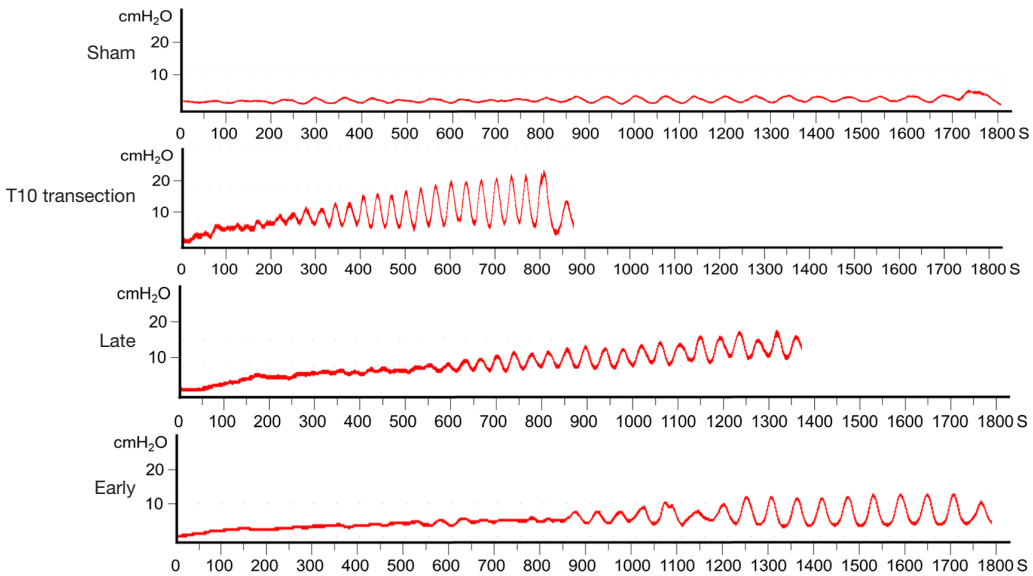

Figure 1 Representative traces of bladder cystometry.

significantly in the SCI rats compared with the Sham group (P<0.05), meanwhile, it was intermediate between the Sham group and the T10 transection group in the two BoNTA-treated groups (*Figure 2*). Notably, the detrusor connective tissue percentage was tended to decrease in the Early group compared with the Late group (P=0.133; *Figure 2*).

Changes of TGF-β1 expression

TGF-β1 protein expression increased significantly in the SCI rats compared with the Sham group (P<0.05), meanwhile, it was intermediate between the Sham group and the T10 transection group in the two BoNTA-treated groups (*Figure 3*). Notably, TGF-β1 protein decreased significantly in the Early group compared with the Late group (P<0.05) (*Figure 3*). TGF-β1 mRNA was in line with the changes of TGF-β1 protein expression among the groups (*Figure 3*).

Discussion

The prevalence of SCI is as high as 236–1,298/1 million in the world (10), and as many as 95% patients suffer from neurogenic bladder, but less than 1% patients' bladder function could fully recover when discharged from hospital (11). The improvement of DO and bladder fibrosis could reduce detrusor pressure and increase bladder volume, thus improving urinary dysfunction, reducing the risk of hydronephrosis and protecting renal function in SCI patients (12).

In patients with neurogenic DO refractory to anticholinergics/antimuscarinics, BoNTA injection should be considered as the next step to restore a low pressure, high-capacity reservoir (12). A dose of 300 U Hengli® BoNTA is usually used for the treatment of DO in patients with supraspinal cord transection, which is approximately 5 U/kg. This dose was considered to correspond to 1 U/rat in this study. The fibrosis-inhibiting effect was evaluated by some arbitrarily comparing with 2 U/rat of BoNTA. Previous

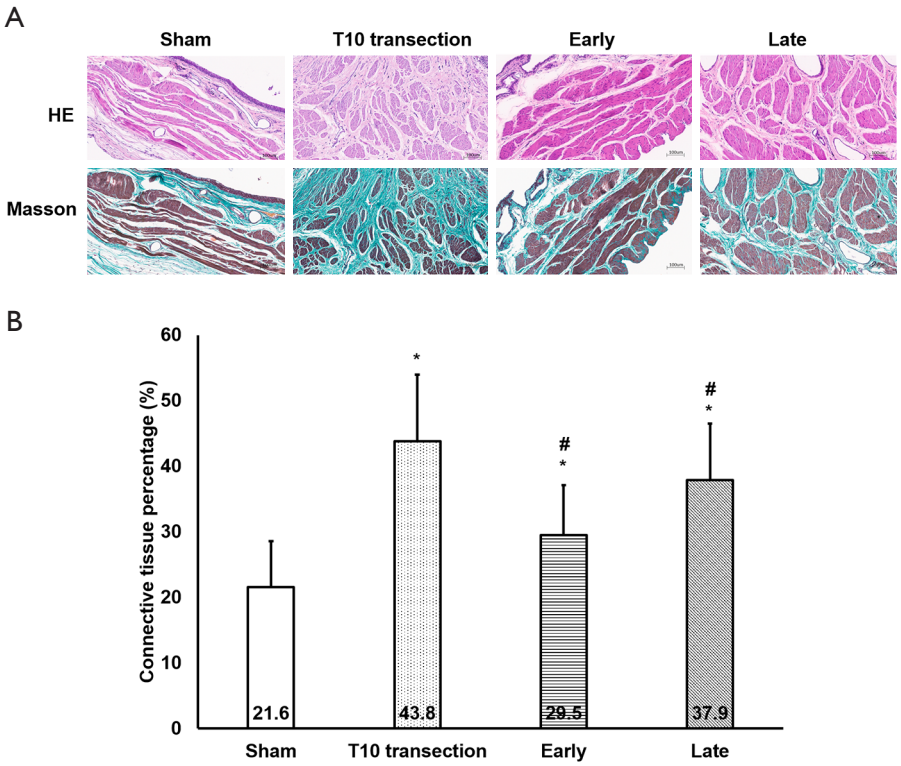

Figure 2 Changes of detrusor fibrosis (n=12, respectively). (A) Bladder HE and Masson staining; (B) changes of detrusor connective tissue percentage. *, P<0.05 compared with Sham; #, P<0.01 compared with T10 transection. Scale bar =100 μm.

studies on bladder injection used BoNTA doses ranging from 1–5 U/rat (7,13,14). The BoNTA used in our research is the same serotype A of botulinum toxin as those used in previous studies (7,13,14), but the manufacturers are different.

To clarify whether intradetrusor BoNTA injection could prevent bladder fibrosis makes sense to the usage of BoNTA in patients with supraspinal cord injury. Histologically, bladder fibrosis is characterized by excessive deposition of connective tissue between and within the detrusor muscle bundles (15-17), usually accompanied by hypertrophy/hyperplasia of detrusor muscle cells, increased bladder weight and disordered bladder function (18). In this study, we found the inhibited NVCs, increased bladder volume, decreased detrusor connective tissue deposition and bladder weight following BoNTA injection, which implies BoNTA injection is effective to inhibit bladder fibrosis caused by supraspinal cord injury. Neurogenic bladder fibrosis in those patients is usually irreversible, and early BoNTA injection might prevent the fibrosis. Previous study showed BoNTA injection decreased bladder fibrosis in SCI rats independent of the time interval between SCI and BoNTA treatment (7). However, we found that the bladder/body weight, DO frequency and bladder volume improved significantly in the Early group than in the Late group, while there was no significant difference between them in the bladder weight, DO amplitude and connective tissue percentage. Thus, this study showed early BoNTA injection had a tendency to prevent bladder fibrosis, but further research is needed to provide a definite answer.

It was proposed that the action of BoNTA on neurogenic bladder fibrosis is through reducing detrusor contractility by inhibiting the release of acetylcholine from presynaptic nerve terminals (7). However, TGF-β1 might play an important role in this process. TGF-β1 is a major driver of human fibrotic pathologies, including hepatic, renal, cardiac and pulmonary fibrosis (19), which induces collagen production and fibrocyte-myofibroblast differentiation through binding to serine/threonine kinase receptors on the cell surface that phosphorylates intracellular Smad2/3 transcription factors (20). Reports showed that BoNTA

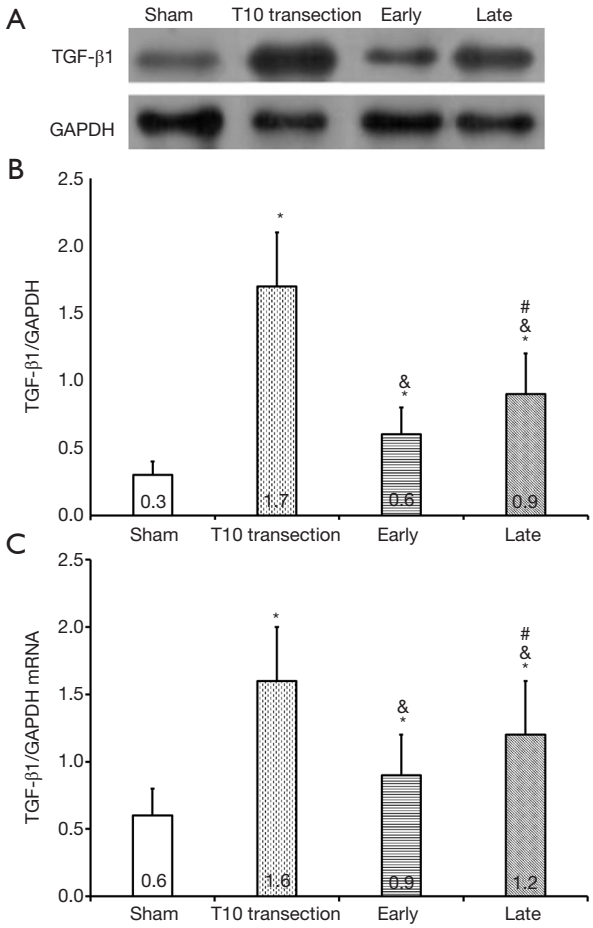

Figure 3 Changes of detrusor TGF-β1 expression in rats with BoNTA treatment (n=12, respectively). TGF-β1 expression was examined with Western blot (A and B) and qRT-PCR (C). *, P<0.05 compared with Sham; &, P<0.05 compared with T10 transection; #, P<0.05 compared with Early. TGF-β1, transforming growth factor β1.

could reduce the expression of TGF-β1 in fibroblasts derived from hypertrophic scars *in vitro* (21-23). Our study showed that TGF-β1 expression was inhibited in SCI rats following BoNTA injection, which demonstrates a mechanism of BoNTA action by downregulating TGF-β1 expression.

The main limitation of this study was the short-term observation. The bladder fibrosis progresses gradually, and DO improvement with BoNTA injection begins within 1 to 2 weeks of injection and lasts for 9 to 10 months in patients with neurogenic bladder (24), thus, 8 weeks might be too short to evaluate the agent's effect.

Conclusions

Bladder fibrosis aggravates urinary dysfunction and endangers kidney function in patients with suprasacral SCI. However, there is currently no effective treatment. Our research showed that the bladder fibrosis and TGF-β1 expression could be suppressed by intradetrusor BoNTA injection in SCI rats. The results suggest the usage of BoNTA to prevent bladder fibrosis in SCI patients with DO and demonstrate a mechanism of BoNTA action by downregulating TGF-β1 expression.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experimental procedures were implemented in compliance with the National Institute of Health Guidelines for the Care

and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Xuanwu Hospital Capital Medical University (No. 20190128).

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