Manual acupuncture alleviates bladder dysfunction by up-regulating expression of NGF and its receptors in bladder tissue of diabetic neurogenic bladder rats

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Research Article

Keywords: Manual acupuncture; diabetic neurogenic bladder (DNB); nerve growth factor (NGF); tropomyosin receptor kinase A (TrkA), p75neurotrophinreceptor (p75NTR); substance P (SP); calcitonin gene related peptides (CGRP)

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Abstract

Objective

To investigate the effect of manual acupuncture on diabetic neurogenic bladder (DNB) rats through the protein and mRNA expression of nerve growth factor (NGF), tropomyosin receptor kinase A (TrkA), p75neurotrophinreceptor (p75NTR) in bladder tissue, as well as the levels of substance P (SP) and calcitonin gene-related peptides (CGRP).

Methods

A DNB rat model was induced using intraperitoneal injection of streptozotocin (STZ). The rats were randomly divided into a blank control group, a model group, and a manual acupuncture group (n = 10). For the manual acupuncture group, the manual acupuncture was applied after modeling. These groups were compared regarding body weight, fasting blood-glucose (FBG), and bladder wet weight. The histomorphology of muscle fibers were observed after hematoxylin and eosin (H&E) staining of bladder tissue sections. Protein and mRNA expression of NGF, TrkA and p75NTR in the bladder tissue were determined by Western blot and real-time PCR analysis, respectively. Levels of SP and CGRP in the bladder tissue were determined by ELISA.

Results

Compared to the blank control group, the model group and manual acupuncture group both showed higher FBG and lower body weight after STZ injection (P< 0.05). Compared to the blank control group, the model group and manual acupuncture group both showed higher bladder wet weight (P< 0.05). Histopathological evaluation indicated that manual acupuncture improved muscle fiber alignment disorders and detrusor cells compensatory hypertrophy in bladder tissue. The protein and mRNA expression of NGF, TrkA, p75NTR and the levels of SP and CGRP in bladder tissue of the manual acupuncture group were significantly higher than those of the model group (P< 0.01).

Conclusions

The therapeutic effect of manual acupuncture on bladder dysfunction in DNB may be mediated by up-regulating of the protein and mRNA expression of NGF, TrkA, p75NTR and the levels of SP and CGRP in bladder tissue.

1. Introduction

Diabetes mellitus is a common chronic metabolic disease, which is expected to affect 578 million people worldwide by 2030 (1). Diabetic neurogenic bladder (DNB), also called diabetic bladder dysfunction
(DBD), diabetic voiding dysfunction (DVD), or diabetic cystopathy (DCP), is a common chronic complication of diabetes. According to a survey, about 40%-80% of diabetic patients will suffer from DNB (2). It is caused by the long-term metabolic disorder of diabetes, which harms peripheral nerve including sympathetic and parasympathetic nerves. The symptoms are reporting one or more of: urinary frequency, urgency, or hesitancy of urinary voiding, difficulty in initiating voiding, decreased sensation of bladder fullness and urinary incontinence (3–5). Numerous physiological insults, such as neuropathy, vasculopathy and detrusor atrophy, may occur in DNB, which can damage the function of kidney in severe cases (6). Investigations of affected patients can show increased bladder capacity, impaired detrusor contractility and incomplete voiding (7). A urodynamic study of individuals with diabetes mellitus and persistent dysfunctional voiding reported 55% with detrusor smooth muscle hypercontractility, 23% with reduced contractility, and 10% with areflexia (8). DNB not only has severe physical and mental health hazards, but also imposes a heavy medical burden on the patients.

Nerve growth factor (NGF) serve as a potential therapeutic option to increase neural repair and recovery as they promote neuroprotection and regeneration (9). There are two receptors for NGF, tropomyosin receptor kinase A (TrkA) and p75neurotrophin receptor (p75NTR). When NGF is combined with TrkA or p75NTR, it can promote the growth of axons and maintain the growth and survival of neuronal cells (10, 11). According to the latest studies, the levels of NGF and TrkA, p75NTR decreased in the bladder tissue of DNB rats (12–14). NGF regulates the levels of neuropeptides such as substance P (SP) and calcitonin gene-related peptides (CGRP) to some extent, which are associated with the micturition reflex. The levels of these neuropeptide were also decreased in DNB bladder tissue (15–18). Therefore, increasing the expression of NGF, TrkA, p75NTR, SP, CGRP may be considered as therapeutic targets.

As a traditional Chinese medicine therapy, acupuncture is widely used for its high curative effect, low side effect and price, and only requires simple operations. Some research suggested that acupuncture can improve the clinical symptoms of DNB patients (19). However, to our knowledge, there has been no thorough investigation of the mechanism of how acupuncture can improve their clinical symptoms. This study is the first to investigate the mechanism of acupuncture in DNB.

Using a streptozotocin (STZ)-induced rat model of DNB, the aim of the present study was to investigate the effect of acupuncture on DNB. We also aimed to investigate the role of the NGF and NGF-receptors, neuropeptides (including CGRP and SP) in the mechanism of underlying acupuncture treatment.

2. Methods

Experimental animals

Thirty SPF male Sprague–Dawley (SD) rats aged 2 months old and weighing (250 ± 20) g were bought from HUNAN SJA LABORATORY ANIMAL CO., LTD. [China; license No: SCXK (XIAN) 2019-0004]. Experiments were performed under a project license granted by Guangxi University of Chinese Medicine Institution Animal Ethical and Welfare Committee [ethical clearance No: DW20220310-031], in compliance with institutional guidelines for the care and use of animals.
Drugs and reagents

The main drugs and reagents included the following: STZ (Sigma, USA). 0.1 mol/L sodium citrate buffer, PH4.5 (Beijing Soledad Bao Technology Co.). Rabbit anti-NGF monoclonal antibody (ab52918) and rabbit anti-TrkA monoclonal antibody (ab76291) (Abcam). Rabbit anti-P75NTR monoclonal antibody (55014-1-AP) (Proteintech). SP and CGRP ELISA kits (Lunchangshuo, Biotech Co. Ltd, China). Isoflurane (R510-22, Shenzhen Ruiwode Life Technology Co., Ltd.). Glucometer and blood glucose test strips (Sannuo, Guangxi, China). Acupuncture needles (model: 0.25 mm × 25 mm, Wuijiang Yunlong Medical Instrument Co., Ltd). Refrigerated centrifuge 5418R (Eppendorf, Hamburg, Germany). Microplate reader (MD, USA). Tanon-5200 automatic gel imaging system (Shanghai Tianneng Corporation, China). AriaMx Real-Time PCR System (Agilent Technologies, UK). ultra-micronucleic acid detector (SuiZheng, FC-1100). SYBR Green qPCR Mix (Biosharp Co., Ltd, China). Electronic Balance and electronic analytical balance (Sedolis Scientific Instruments Co., Ltd.). Olympus BX53 microscope (Olympus Optical Co., Tokyo, Japan).

Establishment of the DNB model

After 1 week of adaptive feeding, the rats were weighed and the models were made by intraperitoneal injection of STZ (20). Briefly, type I diabetes was induced in rats by a single intraperitoneal injection of 60 mg/kg STZ diluted in sodium citrate buffer (0.1 mol/L, pH4.5). Three days later, the tail vein blood glucose levels were measured, and fasting blood glucose (FBG) ≥ 16.7 mmol/L indicates successful diabetes modeling. After 8 weeks of normal feeding, the DNB was successfully molded (since the evaluation of the DNB model requires the execution of rats, we evaluated the model by comparing the model group with the blank control group after the completion of treatment at the 13th week). The success criteria of the DNB model contain the following components: fasting blood glucose ≥ 16.7 mmol/L; increased bladder wet weight; pathological section of the bladder shows disorganized arrangement of the muscle fibers of the detrusor muscle and hypertrophy of the detrusor cells.

Experimental grouping

The rats were randomly divided into a blank control group, a model group, and a manual acupuncture group (n = 10). At the 2nd week, the model group and manual acupuncture group were given intraperitoneal injection of STZ for modeling, while the blank control group was injected intraperitoneally with the same dose of sodium citrate buffer. These groups were then fed normally for eight weeks until the completion of the modeling at the 9th week, after which the intervention was administered to each group for 4 weeks as follows.

Manual acupuncture group: manual acupuncture was applied at the Zhongji (CV3), Sanyinjiao (SP6, bilateral), Lieque (LU7, bilateral), Taichong (LR3, bilateral). These acupoints were positioned with reference to *Experimental Acupuncture and Moxibustion* (21), and combined with anatomical knowledge and anthropoid comparison method. After positioning and disinfection of the acupuncture points, manual acupuncture was performed as follows: we inserted the needle into the acupoint quickly, gently twisted the needle clockwise into the superficial tissue, made a small downward thrusting maneuver in...
place (frequency of downward thrusting: 3–5 times/s), pulled out the needle after 1-2s of rapid shaking, and finally pressed the needle hole for 1-2s. These rats were administered acupuncture once a day for 4 weeks. The rats will be restrained in the supine position during the needling procedure to ensure that acupuncture is performed smoothly.

Blank control group and model group: the rats were given the same restraint as the manual acupuncture group daily and all were fed normally.

**Measurement of body weight and FBG**

Body weight of rats was measured every 4 weeks using a weighing balance. FBG was measured with a blood sample from the tail vein every 2 weeks using a glucometer.

**Measurement of bladder wet weight**

After the treatment, the rats were killed by bloodletting of the femoral artery under isofluorane anesthesia, then we incised in the middle of the lower abdomen. Finally, the complete bladder was excised and weighed using an electronic analytical balance.

**Hematoxylin and eosin (H&E) staining**

Bladder tissue was fixed with paraformaldehyde (4%) for 24–48 hours at room temperature, then embedded in paraffin, sliced, stained with H&E and observed under a light microscope. Bladder tissue morphology and computer images were acquired using a microscopic imaging system.

**Measurement of NGF, TrkA and P75NTR protein expression**

After the bladder wet weight measurement, the bladder is quickly frozen in liquid nitrogen and then stored at -80°C. Western Blot (WB) determined the protein expression of NGF, TrkA and P75NTR in rat bladder tissue. Bladder tissue 20 mg was lysed by RIPA-PMSF and homogenized at 4 °C. After centrifugation, the supernatant was taken for protein content determination by BCA method, and 5 x protein loading buffer was added for denaturation at 100 °C for 10 min. After loading, electrophoresis was performed at 120 V and 70 min – 100 min. The index of 10% separation glue was 320mA constant current membrane for 70 min, and the index of 12% separation glue was 300mA constant current membrane for 35 min. At the end of membrane transformation, 5% skim milk powder was prepared with TBST as blocking solution, and the membrane was blocked in the blocking solution for 1.5 h. Then they were incubated with NGF, TrkA and p75NTR for 12 h at 4°C. After primary antibody incubation, the membranes were washed with TBST for 3 times, 15 min each time. Secondary antibodies of corresponding species were prepared with 5% blocking solution and incubated at 37 °C for 90min. The membranes were washed with TBST for 3 times, 15 min each time. ECL was used to color the film, and exposure imaging was performed in a gel imager. The gray values of the protein bands were analyzed by Image J software and recorded. The gray value ratio of target band and reference band was used as the relative protein expression level.
Measurement of NGF, TrkA and P75NTR mRNA expression

Real-time PCR (RT-PCR) determined the levels of NGF, TrkA and P75NTR mRNA in rat bladder tissue. 20 mg bladder tissue was taken, 1 mL TriQuick Reagent was added, 0.2 mL chloroform was added after homogenization, centrifuged at 4 °C, 12 000 g×15 min, and 0.5 mL supernatant was transferred to another clean 1.5 mL centrifuge tube. Then 0.5 mL isopropanol was added, centrifuged at 12 000 g×10 min at 4 °C, and the supernatant was discarded. The precipitate was gently washed by adding 1 mL 75% ethanol, centrifuged at 12 000 g×5 min at 4 °C, and the supernatant was discarded. Then 1 mL absolute ethanol was added, centrifuged at 12 000 g×5 min at 4 °C, and the supernatant was discarded. The precipitate was dried on a metal bath at 65 °C and dissolved in appropriate amount of nuclease-free water. RNA concentration and purity were detected with an ultra-micro nucleic acid detector. Reverse transcription was performed according to the reverse recording kit instruction, and PCR amplification was performed with the reverse transcribed cDNA as template. Reaction procedure: predenaturation 95 °C for 30 s; PCR amplification (40 cycles) was performed at 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 30 s. Melting curve: Standard melting curve procedure. The relative mRNA expressions of NGF, TrkA and p75NTR were calculated by 2- △△ct method with β-actin as the internal reference. The primer sequences of the target genes are shown in Table 1.

Table 1
Primers used for real-time PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence</th>
<th>Product length (base pairs)</th>
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<tbody>
<tr>
<td>NGF</td>
<td>F: AGAGAGCGCCTGGAGCC &lt;br&gt; R: TAGAAAGCTGCGTCCTTGGC</td>
<td>154</td>
</tr>
<tr>
<td>TrkA</td>
<td>F: GACCCCATCCCTGTCTCCTT &lt;br&gt; R: CCACAGAGACCCCCAAAGGT</td>
<td>97</td>
</tr>
<tr>
<td>p75NTR</td>
<td>F: AGAGAAACTGCACAGCGACA &lt;br&gt; R: CCAGATGTCGCCAGGTATCC</td>
<td>185</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: CGTAAAGACCTCTATGCCAACA &lt;br&gt; R: TAGGAGCCAGGGCAGTATC</td>
<td>120</td>
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Measurement of SP and CGRP protein expression

ELISA determined the levels of SP and CGRP in rat bladder tissue. The operation steps were strictly carried out according to the instructions of the kit.

Statistical Methods
GraphPad Prism 8.0 program software and SPSS 26.0 Statistical Software were performed using for statistical analysis. Measurement data were expressed as mean ± standard deviation. All data were tested for normality and homogeneity of variance. For the data with normality, one-way analysis of variance was used for multiple groups of measurement data, the least significant difference (LSD) method was selected for homogeneity of variance, and the Games-Howell method was selected for heterogeneity of variance. $P < 0.05$ indicated statistical significance.

3. Results

Three rats died in the model group, one rat died in the manual acupuncture group, and no death in the blank control group. The cause of death in rats is thought to be hyperglycemia and toxicity of streptozotocin.

**Generation of a DNB model via large-dose STZ injection**

We created a DNB model using a single intraperitoneal STZ injection (60 mg/kg body weight) to trigger type 1 insulin deficiency in rats (Fig. 1a). Compared to the blank control group, the model group and manual acupuncture group both showed higher FBG and lower body weight after STZ injection ($P < 0.05$), and there was no statistically significant difference between the model group and manual acupuncture group ($P > 0.05$) (Fig. 1b, c). This phenomenon persisted during the experiment, suggesting that the development of diabetes. After measuring the bladder wet weight at the 13th week, we found that the bladder wet weight was significantly higher in the other 2 groups compared to the blank control group ($P < 0.05$); compared with the model group, the bladder wet weight of the manual acupuncture group was significantly lower ($P > 0.05$) (Fig. 1d). After HE staining of bladder tissues, we found that no pathological changes were observed in the blank control group, and minor histopathological changes such as disorganized alignment of some muscle bers and detrusor cells compensatory hypertrophy were observed in the manual acupuncture groups, however, the disorganized alignment of muscle bers and detrusor cells compensatory hypertrophy were most serious in the model group (Fig. 1e). Together, these observations showed successful establishment of the DNB model.

**Changes in protein expression levels of NGF, TrkA, and p75NTR in rat bladder tissue measured by Western blotting**

After treatment, the protein expression levels of NGF, TrkA, and p75NTR in bladder tissue of the model group were significantly lower than those of the blank control group ($P < 0.01$). The expression levels of these proteins in the manual acupuncture group were significantly higher than those of the model group ($P < 0.01$) (Fig. 2).

**Changes in mRNA expression levels of NGF, TrkA, and p75NTR mRNA in bladder tissue by RT-PCR**

After treatment, the mRNA expression levels of NGF, TrkA, and p75NTR in bladder tissue of the model group were significantly lower than those of the blank control group ($P < 0.001$). The expression levels of
these mRNAs in the manual acupuncture group were significantly higher than those of the model group ($P < 0.001$) (Fig. 3).

**Changes in the levels of SP and CGRP in bladder tissue by ELISA**

After treatment, the levels of SP and CGRP in bladder tissue of the model group were significantly lower than those of the blank control group ($P < 0.001$). These levels in the manual acupuncture group were significantly higher than those of the model group ($P < 0.001$) (Fig. 4a, b).

**Discussion**

Diabetes mellitus (DM) is one of the major causes of chronic urologic disease and there are more studies on diabetic nephropathy (22). In contrast, bladder disease associated with DM has not been well studied. In fact, DNB may be underestimated because its symptoms usually appear gradually and can be attributed to other causes (e.g. prostate disease) (23). DNB mainly manifests as abnormalities in urinary function, which is an unhealthy state of the human body. With the rapid development of society, the incidence and prevalence rates of DNB have shown a significant upward trend. Currently, the treatment of DNB is mainly based on the control of blood glucose with nutritional nerve therapy, but the efficacy is not satisfactory. Therefore, finding an effective therapy has attracted widespread attention.

Acupuncture is widely used in the treatment of DNB. Recent studies showed that acupuncture can significantly improve the urodynamic symptoms of DNB patients, including maximal detrusor pressure, bladder compliance, maximal bladder capacity, bladder volume at desire to void, rate for the urgency of urination, frequency of micturition and so on (19). A meta-analysis (24) published in China showed that acupuncture had a superior therapeutic effect than drug alone in the treatment of DNB. Although the effectiveness of acupuncture for DNB treatment has been demonstrated by many clinical studies, the mechanisms of acupuncture remain unclear. To our knowledge, this is the first study to investigate the possible mechanism of acupuncture in treating DNB.

In this study, we used Zhu Lian's "excited" type acupuncture method. This method can excite the nervous system very well (25). In view of the characteristics of DNB, we chose this acupuncture method to stimulate the nervous system. This method requires the needle to be inserted quickly and gently twisted the needle clockwise into the superficial tissue, made a small downward thrusting maneuver in place (frequency of downward thrusting: 3–5 times/s), then pulled out the needle after 1-2s of rapid shaking, and finally pressed the needle hole for 1-2s. According to traditional Chinese medicine theory, the causes of DNB are related to the lung, spleen, kidney and bladder (26, 27). Combined with the acupoint selection requirements of Zhu Lian's "excited" type acupuncture method, we selected Zhongji, Sanyinjiao, Lieque, and Taichong for acupuncture.

This study was designed to investigate the effects of acupuncture treatment in DNB using STZ-induced DNB rat models (Fig. 5). We found that manual acupuncture can effectively increase the expression of
the protein and gene of NGF and its receptors (TrkA and P75NTR), and the levels of substance P (SP), calcitonin gene-related peptides (CGRP) in bladder tissue.

NGF belongs to the family of neurotrophic factors, which is composed of α, β, and δ subunits and is distributed in the brain, ganglion, intestine, and other organs (28). NGF is produced by urothelium and smooth muscle in the bladder and is considered to be an important mediator of various urothelial responses in diabetic bladder lesions (15, 29). The level of NGF decreased in the bladder and L6 - S1 dorsal root ganglion after STZ injection in a time dependent fashion, and the dysfunctional bladder could be reversed by increasing the level of NGF (30). There are two NGF receptors: the higher-affinity TrkA receptor and the lower-affinity receptor p75NTR. NGF binding to the receptor TrkA and p75NTR will produce biological functions (28, 31). Some studies found that the protein and mRNA expressions of TrkA and p75NTR were significantly decreased in the bladder of diabetic rats, which are potential targets in the treatment of DNB (13, 32).

NGF can up-regulate the levels of SP and CGRP because it is directly related to their synthesis and release (33, 34). SP reportedly plays an important role in the micturition reflex as well as in nociceptive responses (35, 36). The decrease in SP clearly pointed to reduced synthesis in the bladder of diabetic rats, and the expression of NGF gene mediates the synthesis of SP (15, 16). CGRP initiates a biological response by binding to a specific CGRP receptor located on cell surfaces and serves a key role in a variety of physiological and pathological processes, including cell proliferation, differentiation, apoptosis, inflammatory and immune responses (37), which is the main transmitters affiliated with bladder sensory nerves (38); Up-regulation the levels of SP and CGRP in bladder tissue can significantly improve the urine contractility and ameliorate the feeling of bladder fullness in DNB (17, 18). Decreased SP and CGRP may lead to the loss of neurotrophic support and detrusor contraction dysfunction. In this study, we found that manual acupuncture significantly increased SP and CGRP levels in the bladder tissue of DNB model rats, which may be the mechanism of acupuncture in treating DNB.

As the third leading cause of mortality, diabetes seriously threatens human health worldwide. In recent decades, the prevalence of DM has increased rapidly in almost all countries, especially in low-income, middle-income countries (39). DNB, as one of the common complications of diabetes, imposes a heavy burden on patients' families and society. As one of the traditional Chinese medicine treatments, manual acupuncture is easy to perform with minimal risk and has proven to be effective in the treatment of DNB.

**Conclusion**

In summary, this study found that manual acupuncture could up-regulate the expression of the protein and gene of NGF, TrkA, p75NTR in bladder tissue of DNB rats, as well as the levels of SP and CGRP.

**Declarations**
Authors’ Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yujun He, Furui Miao and Ningjing Qin. Acupuncture treatment and data processing were performed by Yujun He, Rui Lin, Jingwen Huang and Hui Zhang. The first draft of the manuscript was written by Yujun He and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article.

Acknowledgments

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Patient consent for publication: Not applicable.

Ethics Approval All procedures performed in studies were in accordance with the ethical standards of the guide for the care and use of laboratory animals. The study was approved by the Guangxi University of Chinese Medicine Institution Animal Ethical and Welfare Committee [ethical clearance No: DW20220310-031].

Competing Interests The authors have no conflicts of interest to declare that are relevant to the content of this article.

References


Tables

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Figure 1

Generation of the DNB rat model. a Schematic of the DNB rat model creation process; b time course effect of STZ injection on FBG; c time course effect of STZ injection on body weight; d time course effect of STZ injection on bladder wet weight; e HE staining of bladder tissues; *P<0.05, **P<0.01, ***P<0.001 vs. blank control group; #P<0.05, ##P<0.01, ###P<0.01 vs. model group
protein expression levels of NGF, TrkA, and p75NTR in rat bladder tissue *Compared with the blank control group, *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. blank control group; # $P<0.05$, ## $P<0.01$, ### $P<0.01$ vs. model group
Figure 3

mRNA expression levels of NGF, TrkA, and p75NTR in rat bladder tissue *Compared with the blank control group, \( *P < 0.05, **P < 0.01, ***P < 0.001 \) vs. blank control group; \( #P < 0.05, ##P < 0.01, ###P < 0.01 \) vs. model group
Figure 4

a expression levels of SP in rat bladder tissue; b expression levels of CGRP in rat bladder tissue;
*Compared with the blank control group, *P<0.05, **P<0.01, ***P<0.001 vs. blank control group; #P<0.05, ##P<0.01, ###P<0.01 vs. model group
Figure 5

The research route of this study