Long-term anodal block stimulation at sacral anterior roots promoted recovery of neurogenic bladder function in a rabbit model of complete spinal cord injury

Xiaoran Wang¹, Qi Gao², Xiaoyu Yang³, Weihua Wang⁴, Xinquan Gu⁵, Guifeng Liu⁶, Peng Yan⁷, Ge Gao⁸, Xin Yu⁹, Yongjie Wang⁵, Jihu Lian⁵, Chaoling Shi⁷, Yao Wang⁴, Li Fan¹

¹Department of Urology, China-Japan Union Hospital of Jilin University, Changchun 130033, Jilin Province, China
²Department of Orthopedics, China-Japan Union Hospital of Jilin University, Changchun 130033, Jilin Province, China
³Department of Radiology, China-Japan Union Hospital of Jilin University, Changchun 130033, Jilin Province, China
⁴Department of Pathology, China-Japan Union Hospital of Jilin University, Changchun 130033, Jilin Province, China
⁵Department of Critical Care Medicine, People’s Hospital of Jilin Province, Changchun 130021, Jilin Province, China
⁶Department of Urology, Jilin Provincial Hospital, Changchun 130021, Jilin Province, China
⁷Department of Urology, the Fourth Hospital of Jilin University, Changchun 130011, Jilin Province, China

Abstract
A complete spinal cord injury model was established in experimental rabbits using the spinal cord clip compression method. Urodynamic examination was performed 2 weeks later to determine neurogenic bladder status. The rabbits were treated with anodal block stimulation at sacral anterior roots for 4 weeks. Electrical stimulation of sacral anterior roots improved urodynamic parameters of neurogenic bladder in rabbit models of complete spinal cord injury, effectively promoted urinary function, and relieved urinary retention. Immunohistochemistry results showed that a balance was achieved among expression of muscarinic receptor subunits M2, M3, ATP-gated ion channel P2X3 receptors, and β2-adrenergic receptor, and nerve growth factor expression decreased. These results suggested that long-term sacral anterior root stimulation of anodal block could be used to treat neurogenic bladder in a rabbit model of complete spinal cord injury.

Key Words: anode block; electrical stimulation; immunohistochemistry; neurogenic bladder; urodynamics; spinal cord injury

INTRODUCTION
The level of spinal cord injury (SCI) is not always in accordance with damage to the bladder and upper urinary tract. Therefore, it is necessary to perform urodynamic examinations for the evaluation of bladder functions⁴-⁸. SCI above the micturition center level of the sacral nerve results in detrusor hyperreflexia and detrusor-sphincter-dyssynergia (DSD)⁹. In addition, increased intravesical pressure and decreased bladder function accordingly damage storage function, resulting in urinary incontinence and chronic renal failure⁴. Neurogenic bladder treatment via a Finetech-Brindley bladder controller has been shown to be effective for short⁴ and long-term⁴ treatment. Electrical stimulation of the sacral roots has been used in the clinic for years and has been shown to be efficient⁴; it improves voiding and storage functions, decreases infection and incontinence⁴, and is especially effective in detrusor hyperreflexia and urgency syndrome with related upper urinary dilatation⁸, ¹⁰-¹¹. These voiding methods involve post-stimulation voiding, and many studies have made efforts to identify a method that mimics the human physical voiding process. The anodal block utilizes suitable pulse width and electrical current to stimulate sacral nerve roots, which then selectively activate the detrusor¹². Stimulation prevents simultaneous contraction of the sphincter and detrusor, which subsequently protects the upper urinary tract. To the best of our knowledge, few studies have reported on long-term anodal block stimulation at sacral anterior roots, and the present study hypothesized that stimulation would restore bladder function via regular contraction-relaxation of the detrusor. The present study analyzed the long-term effects of anodal block stimulation at sacral anterior roots on bladder function. In...
addition to urodynamic examinations, immunohistochemical analysis was performed to analyze expression of neurotransmitter receptors and nerve growth factor (NGF) in bladder tissue.

**RESULTS**

**Quantitative analysis of experimental animals**

Fifty rabbits were initially included in the study and were randomly assigned to sham-surgery (n = 10) and SCI (n = 40) groups. To establish complete SCI rabbit models, the spinal cord at T10 was completely resected in the SCI group. Successful SCI rabbit models were randomly assigned to neurogenic bladder (n = 16) and anodal block (neurogenic bladder + anodal block stimulation; n = 15) groups. Following 4-week stimulation, 24 rabbits were enrolled, with 8 rabbits in sham-surgery, neurogenic bladder, and anodal block groups, respectively. The remaining 26 rabbits were excluded due to various reasons, such as death, nerve root damage, or anorexia.

**Urodynamic index changes**

Following 4-week accumulated stimulation, intravesical pressure returned to normal levels in SCI rabbits (Figure 1). Following long-term electrical stimulation, maximum bladder pressure, maximum detrusor pressure, bladder leak-point pressure, and resting pressure decreased in SCI rabbits (P < 0.05; Figure 2). There was no statistical difference in voiding efficiency between prior to SCI and post-anodal block stimulation (P > 0.05; Figure 3).

Following long-term electrical stimulation, bladder capacity and voiding volume significantly improved in SCI rabbits, although values remained less than the sham-surgery group (P < 0.05). Residual urine volume in the anodal block group was less than after SCI, but remained greater than the sham-surgery group (P < 0.05; Figure 4).

(Figures 5A–C). Two weeks after SCI, the main features of urodynamics were as follows: urinary bladder resting pressure increased, bladder leak-point pressure increased and the detrusor was hyperactive during bladder filling (most consisted of invalid contraction), DSD occurred during voiding, urine output decreased, residual urine volume increased, bladder volume decreased, and voiding efficiency and bladder compliance decreased (Figures 5D, F). Urodynamic features in SCI rabbits after long-term electrical stimulation were as follows: increased resting bladder pressure and decreased detrusor leak-point pressure (Figures 5G–H). In addition, invalid detrusor contraction decreased during bladder filling, sphincter electromyography was stable during urination, urine output increased, residual urine decreased, bladder volume increased, urination efficiency increased, and bladder compliance increased.

![Maximum intravesical pressure (Max Pves), maximum detrusor pressure (Max Pdet), detrusor leak-point pressure (DLPP), and resting pressure after long-term electrical stimulation (ES) in neurogenic bladder dysfunction rabbits significantly decrease (\(P < 0.05\), vs. after ES). No statistical difference exists between before injury and after ES (\(P > 0.05\)). 1 cm H2O = 0.098 kPa. Data were expressed as mean ± SD and were analyzed by paired-samples t-test.](image1)

In the sham-surgery group, pressure curves during the bladder-filling phase were smooth before and after SCI, voiding reflex was normal, and there was no detrusor hyperreflexia or differences in urodynamic parameters (Figures 5A–C). Two weeks after SCI, the main features of urodynamics were as follows: urinary bladder resting pressure increased, bladder leak-point pressure increased and the detrusor was hyperactive during bladder filling (most consisted of invalid contraction), DSD occurred during voiding, urine output decreased, residual urine volume increased, bladder volume decreased, and voiding efficiency and bladder compliance decreased (Figures 5D, F). Urodynamic features in SCI rabbits after long-term electrical stimulation were as follows: increased resting bladder pressure and decreased detrusor leak-point pressure (Figures 5G–H). In addition, invalid detrusor contraction decreased during bladder filling, sphincter electromyography was stable during urination, urine output increased, residual urine decreased, bladder volume increased, urination efficiency increased, and bladder compliance increased.

![Voiding efficiency of spinal cord injury (SCI) rats treated by electrical stimulation (ES). After 4-week anodal block ES, there is no statistical difference in voiding efficiency before SCI and after accumulated ES (\(P > 0.05\)). Voiding efficiency = voided volume/(voided volume + post-void residual volume) × 100%. Data were expressed as mean ± SD and were analyzed by paired-samples t-test.](image2)
Effect of electrical stimulation on neurotransmitter receptors and NGF expression

As shown in Table 1, expression of the M2 receptor, P2X3 receptor, and NGF significantly increased in bladder tissue following neurogenic bladder dysfunction (P < 0.05), but significantly decreased after 4-week electrical stimulation (P < 0.05). M3 receptor and β2-adrenergic receptor expression significantly decreased in bladder tissue following neurogenic bladder dysfunction (P < 0.05), but significantly increased after 4-week electrical stimulation (P < 0.05). The corresponding immunohistochemical results are shown in Figure 6.

DISCUSSION

DSD results in increased intravesical pressure and vesicoureteral reflux, which is associated with kidney damage [13] and eventually leads to renal dysfunction and renal failure [14]. Myoelectrogram results of the external sphincter was not weakened during the voiding phase and demonstrated irregular polyphasic waves (direction changed five times in one electric activity) during resting, which indicated that the sphincter was in a pathological state. Detrusor leak-point pressure or intravesical pressure > 4 kPa has been deemed a risk factor for upper urinary tract injury [15], and damage is dependent on lasting time of high pressure [16]. The mean value of detrusor leak-point pressure decreased following long-term electricity stimulation.

Table 1 Expression of neurotransmitter receptors and nerve growth factor (NGF) (integrated absorbance) in bladder tissue

| Group          | M3 receptor | M2 receptor | P2X3 receptor |
|----------------|-------------|-------------|---------------|
| Sham-surgery   | 0.170±0.057 | 0.109±0.079 | 0.089±0.035   |
| Neurogenic bladder | 0.065±0.019a | 0.321±0.068a | 0.228±0.050a |
| Anodal block   | 0.211±0.041 | 0.114±0.029 | 0.103±0.029   |

| Group          | β2-adrenergic receptor | NGF           |
|----------------|------------------------|---------------|
| Sham-surgery   | 0.228±0.045            | 0.105±0.019   |
| Neurogenic bladder | 0.075±0.021a       | 0.316±0.096a  |
| Anodal block   | 0.236±0.066            | 0.110±0.025   |

*P < 0.05, vs. sham-surgery and anodal block groups. Data were expressed as mean ± SD and were analyzed by paired-samples t-test. M3 and M2 receptors: Muscarinic receptor subunits M2 and M3; P2X3 receptor: ATP-gated ion channel P2X3 receptor.
Figure 6  Expression of neurotransmitter receptors and nerve growth factor (NGF) in bladder tissue, as detected by immunohistochemistry (optical microscopy).

Following neurogenic bladder, NGF expression increases in the detrusor, intercellular M2 receptor expression increases in smooth muscle stroma, and P2X3 receptor increases in bladder subepithelium; expression of these molecules decrease following anodal block electrical stimulation.

Following neurogenic bladder, M3 receptor decreases in smooth muscle stroma and β2-adrenergic receptor decreases in bladder epithelium, but expression of both receptors increases post-anodal block electrical stimulation.
Although decreased intravesical pressure and detrusor leak-point pressure effectively prevented vesicoureteral reflux and protected renal function, DSD was not reversed. The sphincter electromyogram revealed a double-phase stable curve, and amplitude significantly decreased following 4-week electrical stimulation, suggesting that the sphincter was under repair. In addition, urethra intrinsic pressure was likely reduced.

Improved urodynamic parameters following electrical stimulation suggested repair of voiding function in SCI rabbits. Decreased residual urine volume reduced resting intravesical pressure, improved voiding efficiency, reduced invalid contraction, and prevented vesicoureteral reflux. A previous study\(^1\) demonstrated that early sacral nerve modulators prevent urinary incontinence following SCI, reduce urinary tract infection, and prevent detrus or hyperactivity, thereby maintaining normal bladder capacity. These findings suggest action of neurotransmitters between sympathetic trunk ganglia and the brain following complete SCI. However, the exact mechanisms by which anodal block improved bladder function remain unclear.

The M2 receptor indirectly mediates smooth muscle contraction by inhibiting cyclic adenosine monophosphate; it reduces β-adrenergic receptor-induced relaxation and inhibits soluble guanylyl cyclase by Gβγ-dependent activation of c-Src kinase\(^1^9\). The inhibitory effect of the adrenergic receptor on detrusor relaxation is suppressed following long-term stimulation, which aids recovery of bladder relaxation and restoration of detrusor hyperactivity. The M3 receptor is activated by a series of proteases, and activation leads to calcium channel opening and enhanced signal transcription of second messenger molecules, such as inositol 1, 4, 5-trisphosphate and diacylglycerol, thereby inducing detrusor contraction\(^1^9\). M3 receptor expression decreases following SCI, but increases following long-term electrical stimulation in the detrusor; results have confirmed that activation of muscarinic receptors by the cholinergic agonist carbachol, or by endogenous acetylcholine, induces a cascade of events that suppress purinergic responses and consequently inhibit the bladder non-adrenergic non-cholinergic response\(^2^0\).

Therefore, increased M3 receptor expression suppressed the purinergic response of bladder detrusor, although further studies are needed to determine whether M3 receptor expression suppresses purinergic receptors.

ATP activates P2X3 receptors in bladder afferent nerves, which helps to maintain normal bladder function in patients with detrusor overactivity. In P2X3 receptor knock-out mice, bladder activity and voiding efficiency is decreased\(^2^1\). The decrease in P2X3 receptor expression corresponds with improved frequent micturition, and the absence of P2X3 receptor subtypes is associated with weakened detrusor contractility and urgent incontinence. Increased expression of purinergic receptors in the bladder lead to bladder hypersensitivity and lower micturition threshold, which subsequently results in increased bladder pressure and damage to the upper urinary tract\(^2^1\). Electrical stimulation reduces P2X3 expression in bladder regions, which in turn alleviates bladder high sensitivity and reduces invalid contraction\(^2^1\). Detrusor relaxation is achieved via the β2 subtype, and relaxation of the detrusor muscle is regulated by β1 and β2 subtypes\(^2^0\). Studies have shown that β-adrenergic receptor agonist induce relaxation and can antagonize a wide range of contraction stimuli. β-adrenergic receptor agonists also inhibit overactivity of the detrusor without affecting physiological micturition; the antagonizing effect on non-cholinergic stimulation is greater than with muscarinic agonists, which suggests a potential method for treating overactive bladder\(^2^3\).

β2 adrenergic receptor agonist antagonizes the detrusor contractile response to electrical stimulation and ATP by regulating β2-adrenergic receptor and ATP released from the purinergic nerve\(^2^4\). In the present study, β2-adrenergic receptor expression decreased after 4-week electrical stimulation, which further decreased inhibition of bladder hyperactivity. However, electrical stimulation increased expression, which suggested that electrical stimulation decreased detrusor hyperreflexia via the adrenergic pathway.

NGF injection into the bladder has been shown to induce bladder overactivity and increase frequency of action potential in bladder afferent neurons in vitro\(^2^5\). NGF expression increases in the lumbosacral spinal cord, dorsal root ganglion, and bladder following SCI\(^2^6\). Due to DSD and weakened voiding efficiency, NGF could also lead to excessive bladder expansion. Results have suggested that NGF is involved in afferent C-fiber excitability and pathological changes in bladder reflex activity following spinal cord injury\(^2^7\), which leads to detrusor hyperreflexia and DSD. In addition, endogenous NGF leads to decreased urinary tract dysfunction following SCI. Recent studies have shown that bladder overactivity is inhibited by NGF antibodies, and intrathecal injection of NGF antibodies inhibits DSD and detrusor hyperreflexia after SCI\(^2^8\), as well as blocks abnormal bladder reflex\(^2^9\). Therefore, NGF and its receptors in the bladder or spinal cord could serve as targets for detrusor hyperreflexia and DSD treatments. In the present study, electrical stimulation reduced NGF expression, suppressed formation of the C-fiber afferent pathway, inhibited DSD and bladder overactivity, improved bladder compliance, and regulated redistribution of neurochemical transmitters within the bladder. These findings suggested that long-term sacral anterior root stimulation of anodal block in rabbits following complete SCI could repair urinary function, as well as restore urine storage function. Most urodynamic parameters of bladder detrusor were improved, and the recovery neurotransmitter receptor expression and decreased NGF expression could be one of the
mechanisms of action.

MATERIALS AND METHODS

Design
A randomized, controlled, animal study.

Time and setting
The present study was performed at the Department of Center Laboratory and Department of Pathology, China-Japan Union Hospital, Jilin University, China from January 2010 to March 2011.

Materials
A total of 50 female, adult, New Zealand rabbits, weighing 2.2–2.4 kg, were fed standard rabbit food and water for 2 weeks prior to experimentation. All animals were provided by the Animal Experiment Campus of Jilin University School of Medicine, China (license No. SCXK (Ji) 2008-0005). Experimental treatment of animals was performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China.

Methods
Urodynamic analysis and animal model establishment
Prior to surgery, all rabbits underwent urodynamic examination after hypnosis without anesthesia. Briefly, a 6F double-lumen catheter was placed into the bladder via the urethra, and a 6F double-lumen catheter was placed into the rectum. Three 28G needle electrodes were gently inserted into the external sphincter through the perineum and gastrocnemius of both lower extremities, respectively. Intravesical pressure, rectal pressure, and external sphincter electromyogram were simultaneously recorded before and after spinal cord transaction, as well as after long-term stimulation. Multichannel electrophysiology instruments (BL420E Biologic Function Experiment System; Chengdu TME Technology, Chengdu City, Sichuan Province, China) were connected to a computer. The pressure-transducers were calibrated to atmospheric pressure, and 10 mL saline/minute was injected into the bladder via a bladder-inserted 4Fr urethral catheter with a syringe pump to elicit bladder contraction. Infusion was terminated when micturition reflex appeared, and detrusor leak point pressure was recorded when saline voided from the urethral meatus. During the rise-up phase of intravesical pressure, maximal detrusor pressure was recorded, and saline was collected and measured to determine voided volume (VV). Post-void residual volume (RV) was determined by means of gravity, urethral catheter, or abdominal pressure. Bladder capacity (BC) was calculated as BC = RV + VV, and voiding efficiency (VE) was calculated as VE = VV/BC × 100%.

Rabbits were anesthetized with Sumianxin II (Veterinary Institute of Military Need University, Changchun City, China; 0.10 mL/kg) prior to surgery. In 50 animals, laminctomy was performed at the T₁₀ segment, and in the SCI group, the spinal cord was completely clamped with a vascular clamp for 1 minute, until the lower limbs stopped trembling. After the incisions were cleaned and sutured, 100 000 units penicillin/kg body weight was intramuscularly injected daily for 3 days. Bladder emptying was performed by manually pressing on the abdomen until automatic micturition recovery. Two weeks after complete SCI, urodynamic studies were performed, and neurogenic bladder criteria were as follows: urodynamics with unstable detrusor pressure during bladder infusion, frequent invalid contraction, detrusor hyperreflexia, and DSD.

Implantation of stimulating electrodes
In the anodal block group, the bilateral S₄ sacral nerve dorsal roots were resected to abolish detrusor reflection, and electrodes were used to stimulate sacral nerve anterior roots. The main nerve roots dominating bladder detrusor were identified according to increased bladder pressure. Two electrodes were implanted and fixed in both S₂ sacral anterior nerve roots in each rabbit. The following stimulation parameters were used to induce selective bladder contraction: 1.05 mA electrical current; 20 Hz stimulation frequency; 300 μs stimulation pulse width; 5-second stimulus time; and 10-second off-time. Long-term electrical stimulation was performed, with a stimulus time of 30 minutes/administration for six administrations/day for 4 weeks. After 4 weeks, urodynamic testing was performed again to evaluate bladder function.

Immunohistochemistry of neurotransmitter receptors and NGF expression
Bladder tissues were fixed in neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Samples were observed under a light microscope (Olympus, Tokyo, Japan). The remaining paraffin blocks were de-waxed, sectioned, incubated in diluted antibody (1: 50; anti-mAChR M2 receptor, anti-β2-adrenergic receptor, anti-mAChR M3 receptor, anti-P2X3 receptor, anti-NGF, respectively) at 4°C for 24 hours, rinsed with phosphate-buffered saline three times for 10 minutes each, followed by horseradish peroxidase-conjugated secondary antibody (1: 100) and DAB coloration. Immunohistochemistry results were observed under a light microscope (Olympus, Tokyo, Japan) with a 20-fold objective, using five random fields of view. An image-pro plus analysis system (Quantimet Q570; Leica, Bensheim, Germany) was used to perform semi-quantitative image analysis. The ratio of staining integrated absorbance/intercepted area in each field of view was calculated, and the ratios served as statistical numerical data.

Statistical analysis
Data were expressed as mean ± SD and were analyzed by one-way analysis of variance, followed by least significant difference-t test, using SPSS 13.0 software (SPSS, Chicago, IL, USA). P < 0.05 was considered statistically significant.
Author contributions: Xiaoran Wang and Xinquan Gu designed the study. Weihua Wang and Xiaoyu Yang served as the research directors. Xiaoran Wang, Qi Gao, Guifeng Liu, Peng Yan, Xin Yu, Yongjie Wang, Jihu Lian and Chaoling Shi performed the study. Li Fan, Yao Wang, and Ge Gao evaluated results in a blinded fashion.

Conflicts of interest: None declared.

Ethical approval: The study was approved by the Experimental Animal Ethics Committee of Jilin University in China.

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