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Prolonged electrical stimulation causes no damage to sacral nerve roots in rabbits

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Abstract

Previous studies have shown that, anode block electrical stimulation of the sacral nerve root can produce physiological urination and reconstruct urinary bladder function in rabbits. However, whether long-term anode block electrical stimulation causes damage to the sacral nerve root remains unclear, and needs further investigation. In this study, a complete spinal cord injury model was established in New Zealand white rabbits through T_{9-10} segment transection. Rabbits were given continuous electrical stimulation for a short period and then chronic stimulation for a longer period. Results showed that compared with normal rabbits, the structure of nerve cells in the anterior sacral nerve roots was unchanged in spinal cord injury rabbits after electrical stimulation. There was no significant difference in the expression of apoptosis-related proteins such as Bax, Caspase-3, and Bcl-2. Experimental findings indicate that neurons in the rabbit sacral nerve roots tolerate electrical stimulation, even after long-term anode block electrical stimulation.

Key Words: *nerve regeneration; spinal cord injury; sacral nerve root; electrical stimulation; anode block; spinal cord reconstruction; bladder function; nerve prosthesis; neural regeneration*

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Introduction

Neurogenic bladder refers to bladder dysfunction caused by the central nervous system that dominates urination or occurs following spinal cord injury (Lee et al., 2007). Spinal cord injury is one of the leading causes of neurogenic bladder (Nosseir et al., 2007; Schalow, 2010). Following neurogenic bladder, the resultant urinary tract infection, uremia, renal failure, and other serious complications are potentially lethal in patients with spinal cord injury. Thus, bladder reconstruction can improve quality of life and reduce complications after spinal cord injury. Existing treatment for neurogenic bladder includes drugs, intermittent catheterization, bladder fistula, and other auxiliary surgical treatments. However, these therapies have poor efficacy, a long treatment period, a high cost, many complications, and result in a high recurrence rate (Yan et al., 2010a). Electrical stimulation of the sacral nerve root has been shown to restore urination and storage functions (Hagerty et al., 2007; Huber et al., 2007; Lewis and Cheng, 2007). Presently, neural prosthesis grafting is set to become the main method for reconstructing bladder decompensation after spinal cord injury. Currently, the only neural prosthesis used in the treatment of neurogenic bladder after paraplegia is the Finetech-Brindley bladder system (Finetech Medical, Welwyn Garden, UK). It stimulates urination according to different biological characteristics of the bladder detrusor and urethral sphincter, through intermittent pulses of electrical stimulation (Brindley, 1974). However, the voiding pattern of the neural prosthesis is artificial, and only achieved with stimulation intermittently. In addition, intravesical pressure rises after voiding, leading to upper urinary tract damage. It is estimated that intravesical pressure of some patients with the Finetech-Brindley system was up to 80–90 cmH_2O (7.84–8.82 kPa), whereas normal pressure is only 35.0–43.7 cmH_2O (3.43–4.28 kPa) (Egon et al., 1998). Anode technology can effectively prevent the above issues. Specifically, when electrical stimulation is applied, the current at the cathode excites all nerve fibers, and the remote anode leads to hyperpolarization. Thus, action potentials cannot cross this area and conduction is blocked; even a large diameter nerve can be blocked by a small current. Consequently, action potentials are only allowed to cross small diameter nerves, causing contraction of the detrusor alone, and resulting in physiological urination (Jezernik et al., 2002).

Preliminary studies of our research group have successfully developed a new anode block stimulator and stimulating electrodes for the sacral nerve root. Moreover, we have shown that the sacral nerve root stimulator mediated physiological urination (Yan et al., 2010b, 2013; Wang et al., 2012), reduced residual urine volume after spinal cord injury, increased urination volume and bladder volume, improved voiding efficiency, reduced detrusor leak point pressure and

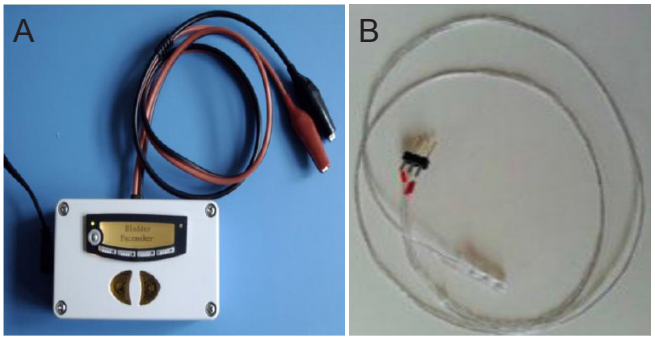

Figure 1 Sacral nerve root stimulator (A) and stimulating electrode (B).

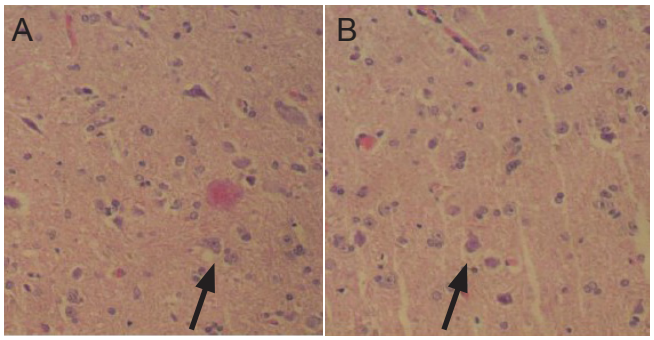

Figure 2 Effect of continuous anode block electrical stimulation on the morphology of the anterior sacral nerve root in rabbits with complete spinal cord injury (hematoxylin-eosin staining, × 40).
The morphology of nerve cells (arrows) was similar between the control group (A) and electrical stimulation group (B). There was no bleeding, inflammatory infiltration, or glial scar formation.

resting bladder pressure, improved bladder compliance, ameliorated urinary incontinence and urinary reflux symptoms caused by detrusor spasm, and significantly prolonged survival time after spinal cord injury in rabbits. Further animal experiments are needed to verify the safety of this stimulation technique and its parameters. Rabbits cannot survive for a long period following paraplegia, and it is thus difficult to detect the effect of long-term electrical stimulation on sacral nerve roots. In the current experiment, we further explored the morphology of the sacral nerve root and the expression of apoptosis related proteins after electrical stimulation, in an attempt to explore the safety of electrical stimulation parameters and provide evidence for the clinical application of the sacral nerve root stimulation electrode system.

Materials and Methods

Experimental animals

Thirty adult New Zealand white rabbits, weighing 2.2–2.4 kg, were provided by the Experimental Animal Center of Jilin University, China (license No. SCXK (Ji) 2008-0005). The rabbits were housed at 20°C and 65% humidity, and then randomly divided into a control group and a stimulation group. All rabbits were fed for 2 weeks prior to experiments. This study conformed to *the Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996), and the protocol was approved by the Institutional Animal Care Committee of Jilin University in China.

In the electrical stimulation group, the rabbit model was established according to the previous methods (Fehlings and Tator, 1995; Wang et al., 2012). Briefly, rabbits were fasted and deprived of food for 6 hours before surgery, and anesthetized using 0.15 mL/kg Sumianxin II (Military Veterinary Institute, Quartermaster University, Changchun, Jilin Province, China). Next, rabbits were fixed in a supine position, and infused with saline at 100 mL/hour. The skin was shaved and disinfected. A 1.5 cm long vertical incision was made at $T_{9–10}$ and then the skin, subcutaneous tissue, and deep fascia were cut open, exposing the spinous process and lamina. The spinal cord was completely occluded with forceps for 1 minute, until the lower limbs of rabbits' stopped trembling, which indicated the success of establishing the complete spinal cord injury model. After surgery, rabbits were placed in a separate cage, and forced to urinate and stool by 4–6 pressings every day. Intramuscular injection of 1×10^6 U/kg penicillin was given for 5 days after surgery. The incision dressing was changed every day. After surgery, the tissue below the complete spinal cord injury level showed no response to pain stimuli, the rabbits had poor appetite, and both hind limbs and the tail were paralyzed.

Electrical stimulation of the sacral nerve root

The sacral nerve root stimulator is composed of two synchronous power sources, used to control the current at 0–5 mA. The two power sources share a cathode, and are equipped with symmetrical electrodes. The anode current ratio was set at 1:1; stimulation frequency was 3–35 Hz, and the stimulation pulse was 50–700 μs. Using bidirectional rectangular pulses, the stimulation duration was set at 0–10 seconds, with a resting interval of 0–20 seconds (**Figure 1A**).

Stimulating electrodes contained one cathode and two anodes, equipped with three titanium ring contact points. The contact points were 0.01 cm thick, 0.1 cm wide, and were spaced at an interval of 0.2 cm. The titanium ring was embedded into the cylindrical insulating tube, which is made of medical silicone, at a diameter of 0.05 cm, and the total length of the electrode was 0.7 cm. Electrode material was provided by Professor Nico from the Department of Biological Engineering, Aalborg University, Denmark (**Figure 1B**).

At 10 days after electrical stimulation, rabbits underwent urodynamic examination, to exclude model failure (such as non-spastic bladder). The successful models were anesthetized, and an incision was made along the $S_{1–4}$ spinous process, and the skin, subcutaneous tissue, and vertebral plate were removed, exposing the anterior and posterior sacral nerve roots. Two stimulation electrodes were inserted into the bilateral sacral nerve roots, which govern the detrusor muscle, then fixed and sutured after gently pressing the metal ring. The wires were fixed in subcutaneous tissue. The position of stimulation electrodes was determined using lumbar X-ray examination 2 days postoperatively. The anterior

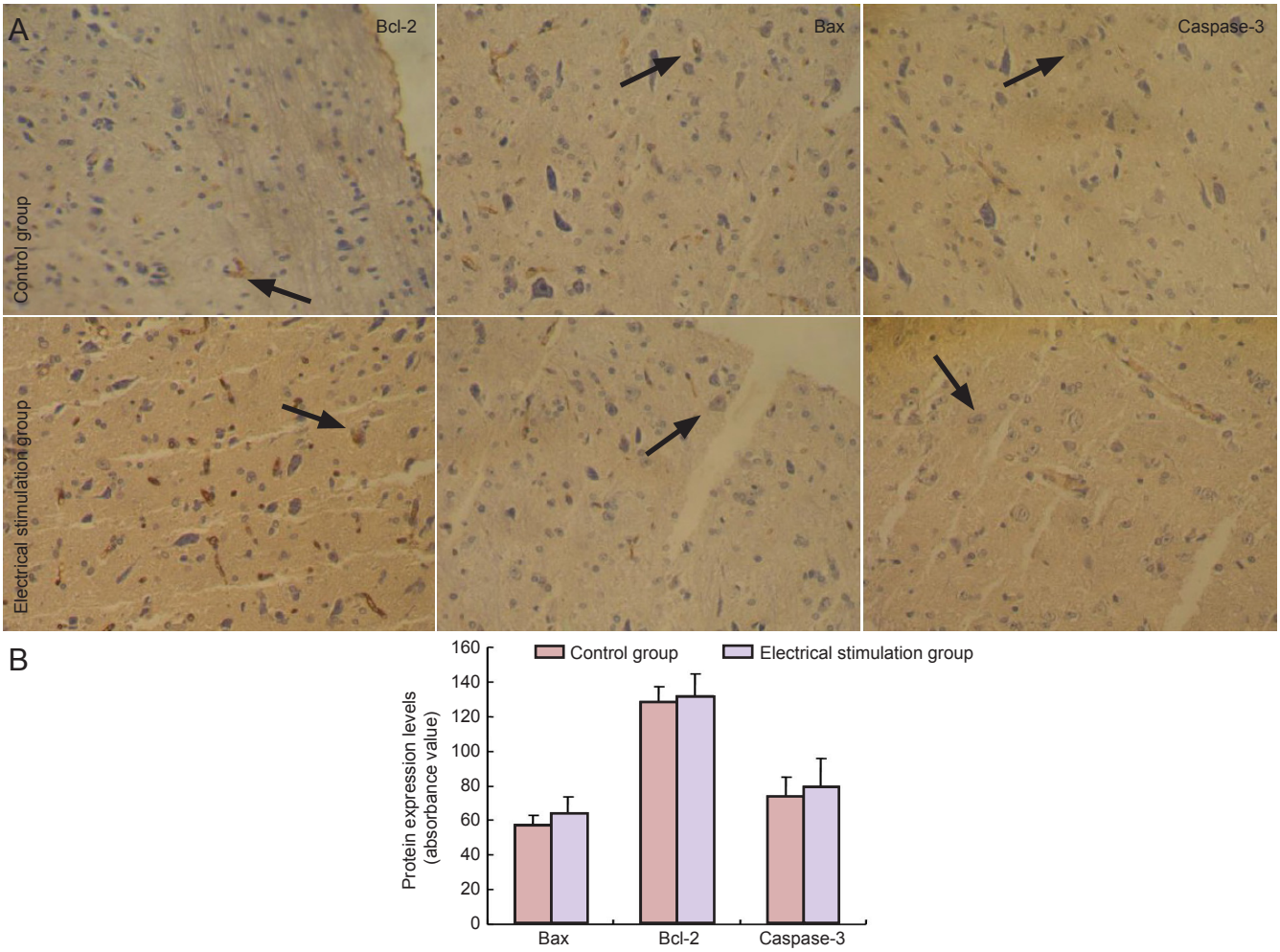

Figure 3 Effect of continuous anode block electrical stimulation on Bcl-2, Bax, and Caspase-3 expression in the anterior sacral nerve root in rabbits with complete spinal cord injury.
(A) Bcl-2, Bax, and Caspase-3 expression (× 400). Arrows indicate positive expression. (B) Quantitative analysis of Bcl-2, Bax, and Caspase-3 expression. Data are expressed as mean ± SD. There were 15 rabbits in the control group and 8 rabbits in the electrical stimulation group. Differences between groups were compared using one-way analysis of variance, and further comparison was performed using the paired *t*-test.

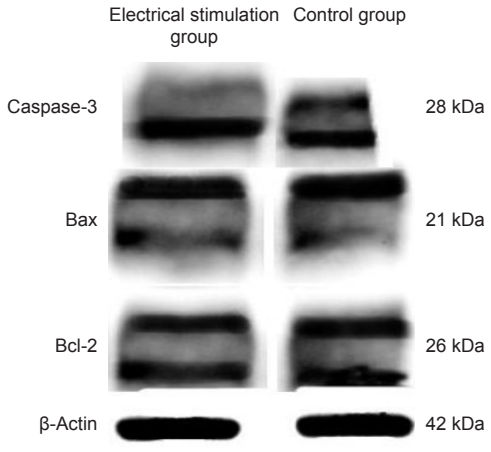

Figure 4 Western blot analysis of the effect of continuous anode block electrical stimulation on Bcl-2, Bax, and Caspase-3 expression in the anterior sacral nerve root in rabbits with complete spinal cord injury.
Bcl-2, Bax, and Caspase-3 expression were similar between the control group and electrical stimulation group.

sacral nerve root was given continuous anode block electrical stimulation, at a pulse width of 300 μs, current intensity of 1.05 mA, frequency of 20 Hz, duration of 5 seconds, and at intervals of 10 seconds. Stimulation was performed 4 hours per day, for 60 days. There were 15 rabbits in the electrical stimulation group, 6 of them died during electrical stimulation and one was excluded after urodynamic examination. Thus, 8 rabbits were available for final analysis.

Specimen harvesting

After 60 days of continuous electrical stimulation, the incision in the rabbits was cut open after electrical stimulation, exposing the vertebral canal. The S_2 sacral nerve roots were carefully dissected under microscope, taking the electrode as the center. The harvested nerve was fixed with glutaric dialdehyde and neutral formalin, and then stored in liquid nitrogen. The rabbits in the control group were anesthetized with Sumianxin and bilateral S_2 and S_3 anterior roots were directly harvested for observation.

Pathological observation

Using hematoxylin-eosin staining, paraffin sections of the anterior sacral root tissue were cut into continuous slices at 4 μm thickness, followed by dewaxing, immersion, staining, dehydration, transparent treatment, and mounting. The slices were observed under a light microscope (Olympus, Tokyo, Japan) to detect the presence of neural structural damage, bleeding and inflammatory cell infiltration, nerve cell swelling, cyst formation, and glial scar formation.

Immunohistochemistry of Bcl-2, Bax, and Caspase-3 expression

Using SP staining, specimens were blocked with bovine serum albumin (ABCAM, Cambridge, Massachusetts, USA), and incubated with mouse anti-rabbit Bcl-2, Bax, and Caspase-3 monoclonal antibodies (1:500, ABCAM) at 4°C overnight. Subsequently, specimens were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,000, ABCAM) at 37°C for 1 hour, and visualized using DAB (ABGENT, San Diego, CA, USA). Images were semi-quantitatively analyzed using the IDA2000 image analysis system (Beijing Kong Hai Science and Technology Development Co., Ltd., Chinese Academy of Sciences, Beijing, China). The integrated absorbance value of positive expression at each field of view was calculated among five fields of view of each slice at 40 × magnification.

Western blot analysis of Bcl-2, Bax, and Caspase-3 expression

Tissue stored in liquid nitrogen was homogenized in PIPA buffer (centrifugation at 15,000 r/min for 1 minute). Cells were collected and protein lysate was added, and the mixture stored at –20°C. Specimens were then subjected to electrophoresis for 90 minutes at 300 mA using 12% separating gel and 5% stacking gel, blocked with skimmed milk powder, and incubated with mouse anti-rabbit Bcl-2, Bax, and Caspase-3 monoclonal antibodies (1:500) at 4°C overnight. After rinsing 3 × 10 minutes, the specimens were incubated with goat anti-mouse IgG (1:1,000) at 37°C for 1 hour, rinsed with PBS, and visualized using electro-chemiluminescence. The specimens were photographed and observed using an image processing system (Canon, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Measurement data are expressed as mean ± SD. Differences between groups were compared using one-way analysis of variance, and further comparisons were performed using the paired *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of electrical stimulation on the morphology of the anterior sacral nerve root

Hematoxylin-eosin staining results revealed that, in the control and electrical stimulation groups, the nerve root cross-section was well preserved. Additionally, the nucleolus and cytoplasm Nissl bodies were clearly visible, the structure of nerve cells was intact, no axonal degeneration occurred, and no bleeding, inflammatory infiltration, and glial scar formation was observed (**Figure 2**).

Effect of electrical stimulation on Bcl-2, Bax, and Caspase-3 expression in the anterior sacral nerve root

Immunohistochemistry revealed that in the control group, Bax and Caspase-3 were minimally expressed, while Bcl-2 was highly expressed. The positive particles were brownish-yellow stained and mainly located in the cytoplasm. In the electrical stimulation group, Bax, Caspase-3, and Bcl-2 expression showed no significant differences in the number of particles compared with the control group ($P < 0.05$; **Figure 3**).

Western blot analysis showed that, Bax, Caspase-3, and Bcl-2 expression was not significantly different between the electrical stimulation and control groups (**Figure 4**).

Discussion

Accumulating evidence shows that electrical stimulation contributes to restoration of motor function in tissues and organs. However, the effect of stimulation intensity, frequency, and pulse width on nerve tissue remains unclear. Generally, greater intensity, higher frequency, and longer pulse width stimulation lead to more severe damage in nerve cells (McCreery et al., 2004). In addition, although short-term electrical stimulation is not damaging to nervous tissue, chronic electrical stimulation can damage nerve structure. After the ultrastructure of neurons is altered, neuronal function may be disturbed. Neuronal injury is mainly mediated by cytotoxicity caused by excessive electrical activity and change to the local microenvironment. Lee et al. (1997) reported 1 case of unexplained nerve root degeneration at 6 months after 11 patients were treated with sacral nerve root stimulation. Wang et al. (2004) found myelin sheath loosening, and rough endoplasmic reticulum and collagen secretion after prolonged electrical stimulation of the sacral nerve in dogs, suggesting fibroblast proliferation. Thus, for any type of electrical stimulator and stimulation parameters for use in humans, a safety assessment of prolonged stimulation is critical.

It is widely recognized that functional electrical stimulation (lower than nociceptive stimuli) does not cause damage in all animals. Bamford et al. (2010) demonstrated that in the restoration of lower limb function in paraplegic rats, spinal cord tissue tolerated 120 hours of intraspinal micro-stimulation. Creasey et al. (1993) reported a group of cases carrying a bladder controller for 10–15 years. The patients showed no neurological degeneration, and the stimulated nerve root showed normal structure upon histological examination. There were only two deaths at 3 and 5 years owing to reasons not associated with the bladder controller. In the above studies, there were no specified criteria for distinguishing functional electrical stimulation from noxious electrical stimulation, thus making it difficult to select stimulation parameters. Therefore, defining appropriate stimulation parameters is urgently required.

Electrical stimulation to the nerve root may trigger a series of pathophysiological processes that lead to secondary injury. Apoptosis is an important mechanism of cell death following spinal cord injury and a very complex pathophysiological process involving Bcl-2, Bax, P53, C-myc, and Caspase-3 proteins. Among them, Caspase-3 is considered a major inducer of apoptosis (Ahn et al., 2006), and it plays a crucial role in the execution phase of cell death. Bax and Bcl-2 are important members of the Bcl-2 protein family; Bax is a pro-apoptotic gene, whereas Bcl-2 is an anti-apoptotic gene. The Bax/Bcl-2 ratio determines neuronal apoptosis and this family of proteins also plays an important role in apoptosis (Cook et al., 1999). Growing evidence shows that neuronal apoptosis is an inevitable phenomenon following spinal cord injury (Cook et al., 1999). In the present study, we determined the expression of Bcl-2, Bax, and Caspase-3 by immunohistochemistry after prolonged electrical stimulation and compared expression results with a control group, to verify whether such stimulation could damage nerve tissue. In summary, we found no significant difference in Bcl-2, Bax, and Caspase-3 expression levels between the electrical stimulation group and control group, suggesting that rabbit sacral nerve root neurons tolerate stimulation with the parameters used in the current study.

Author contributions: *All authors were responsible for designing the experiment, implementing the experiments and evaluating the study. Yan P wrote the manuscript. All authors approved the final version of the manuscript.*
Conflicts of interest: *None declared.*

References

Ahn YH, Lee G, Kang SK (2006) Molecular insights of the injured lesions of rat spinal cords: Inflammation, apoptosis, and cell survival. Biochem Biophys Res Commun 348:560-570.

Bamford JA, Todd KG, Mushahwar VK (2010) The effects of intraspinal microstimulation on spinal cord tissue in the rat. Biomaterials 31: 5552-5563.

Brindley GS (1974) Emptying the bladder by stimulating sacral ventral roots. J Physiol 237:15P-16P.

Cook SA, Sugden PH, Clerk A (1999) Regulation of bcl-2 family proteins during development and in response to oxidative stress in cardiac myocytes association with changes in mitochondrial membrane potential. Circ Res 85:940-949.

Creasey GH (1993) Electrical stimulation of sacral roots for micturition after spinal cord injury. Urol Clin North Am 20:505-515.

Egon G, Barat M, Colombel P, Visentin C, Isambert JL, Guerin J (1998) Implantation of anterior sacral root stimulators combined with posterior sacral rhizotomy in spinal injury patients. World J Urol 16: 342-349.

Fehlings MG, Tator CH (1995) The relationships among the severity of spinal cord injury, residual neurological function, axon counts, and counts of retrogradely labeled neurons after experimental spinal cord injury. Exp Neurol 132:220-228.

Hagerty JA, Richards I, Kaplan WE (2007) Intravesical electrotherapy for neurogenic bladder dysfunction: a 22-year experience. J Urol 178: 1680-1683.

Huber ER, Kiss G, Berger T, Rehder P, Madersbacher H (2007) The value of intravesical electrostimulation in the treatment of acute prolonged bladder overdistension. Urologe A 46:662-666.

Jezernik S, Craggs M, Grill WM, Creasey G, Rijkhoff NJ (2002) Electrical stimulation for the treatment of bladder dysfunction: current status and future possibilities. Neurol Res 24:413-430.

Lee BB, Haran MJ, Hunt LM, Simpson JM, Marial O, Rutkowski SB, Middleton JW, Kotsiou G, Tudehope M, Cameron ID (2007) Spinal-injured neuropathic bladder antisepsis (SINBA) trial. Spinal Cord 45:542-550.

Lee T (1997) Electrical stimulation techniques to restore bladder function. Neurosurg Q 7:264-272.

Lewis JM, Cheng EY (2007) Non-traditional management of the neurogenic bladder: tissue engineering and neuromodulation. Scientific World Journal 7:1230-1241.

McCreery D, Pikov V, Lossinsky A, Bullara L, Agnew W (2004) Arrays for chronic functional microstimulation of the lumbosacral spinal cord. IEEE Trans Neural Syst Rehabil Eng 12:195-207.

Nosseir M, Hinkel A, Pannek J (2007) Clinical usefulness of urodynamic assessment for maintenance of bladder function in patients with spinal cord injury. Neurourol Urodynam 26:228-233.

Schalow G (2010) Cure of urinary bladder functions in severe (95%) motoric complete cervical spinal cord injury in human. Electromyogr Clin Neurophysiol 50:155-179.

Wang SB, Hou CL, Lei B, Zhang SM, Diao YM, Zhang W, Chen AM, Yin CH (2004) Structure observation and clinical significance of sacral roots after long term electric stimulation in dogs. Zhonghua Xianwei Waike Zazhi 27:268-270.

Wang XR, Gao Q, Yang XY, Wang WH, Gu XQ, Liu GF, Yan P, Gao G, Yu X, Wang YJ (2012) Long-term anodal block stimulation at sacral anterior roots promoted recovery of neurogenic bladder function in a rabbit model of complete spinal cord injury. Neural Regen Res 7: 352-358.

Yan P, Yang XY, Gao Q, Liu J, Yang MG (2010a) Neuroprostheses and neurogenic bladder. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu 14:10026-10030.

Yan P, Yang XY, Gao Q, Wang XR, Liu J, Yang MG (2010b) Electrical stimulation of the sacral nerve anterior root following induced bladder detrusor contraction. Neural Regen Res 5:1900-1904.

Yan P, Zheng WD, Zhang JK, Tan YB, Li GF, Li GC, Song C, Yang XY (2013) Histological changes and safety of long-term acral nerve root stimulation electrode placement in rabbits. Zhongguo Zuzhi Gongcheng Yanjiu 17:6587-6593.

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