Research Article

Novel method for restoration of anorectal function following spinal cord injury via nerve transfer in rats

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Objectives: Nerve transfer has been developed to restore partial function after serious nerve injuries, for example, restoring bladder control after spinal cord injury (SCI). Our aim here was to establish a preclinical proof-of-concept model using nerve transfer for restoring anorectal function after SCI.

Setting: We used laminectomy to model SCI, and bilateral spinal ventral and dorsal nerve root anastomosis to re-establish connectivity to the anorectal musculature.

Methods: Multidisciplinary methods were used to assess the anatomical and functional integrity of the alternative spinal-to-anorectal nerve circuit. Adult rats were used to establish the model. Bilateral anterior and posterior L5 nerve roots were surgically matched with anterior and posterior of S1 nerve roots by microscopic anastomosis to establish an artificial rectal reflex arc with complete sensory and motor pathways. Twelve weeks later, we used retrograde nerve tracing and neurohistomorphological analysis to assess anatomical integrity of the new artificial rectal reflex arc. Anorectal manometry was used to assess the function of the new nerve circuit.

Results: Retrograde tracing with recombinant attenuated pseudo rabies virus indicated that the new neural pathway was successfully established to the anorectal musculature after experimental SCI. Toluidine blue-stained sections of the anastomosis site revealed normal-appearing nerve fiber morphology and regeneration, and transmission electron microscopy revealed myelinated axons. Anorectal manometry revealed significant anorectal functional recovery.

Conclusion: These results suggest that our model is a feasible first step in developing an alternative reflex pathway after laminectomy at L4 to S2 and shows promise for effective restoration of anorectal function.

Keywords: Spinal cord injury, Animal model, Cauda equina, Regeneration, Bowel incontinence

Introduction

Spinal cord injuries (SCIs) affect more than 6 million people worldwide, with an estimated global prevalence of 23 cases per one million people, based on regional data from the World Health Organization.^{1,2} Although mortality is relatively low, SCI is associated with high morbidity, high complication rates, and high cost of treatment and rehabilitation.³ It is well known SCI can lead to both sensory and motor dysfunction below the

plane of injury, and to sexual dysfunction and incontinence.^{1,4} Neurogenic bowel dysfunction (NBD) following SCI is associated with difficulty in voiding, abdominal distension, fecal impaction, and fecal incontinence.⁵ These conditions severely affect patient quality of life and are a primary healthcare challenge for SCI patients. Treating defecation dysfunction caused by trauma-related SCI or disease remains a clinical challenge.^{6,7}

Over the past two decades, nerve transfers have been performed successfully and modified for different scenarios.⁸ With advancements in surgical design and management, nerve transfers are producing better functional outcomes in patients. In attempts to restore bladder function after SCI, prior studies focused on using the

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normal somatic reflex above the injury level to accomplish reinnervation. For example, Xiao *et al.*^{9,10} performed intradural anastomosis of the central end of the L4 spinal nerve anterior motor root with the peripheral end of the bladder-governing L6 spinal nerve anterior motor root in a rat model of L5 paraplegia. Using this method, they successfully established an artificial skin → CNS → bladder reflex pathway and confirmed its integrity using electrophysiological and neuroanatomical tracing techniques. Similarly, Hou *et al.*^{11,12} established an artificial bladder reflex arc via nerve root transfer involving abdominal reflex pathways above the paraplegic plane. Subsequent studies have used this nerve transfer approach to successfully restore control of bladder function in both SCI model rats and patients with SCI.¹³

Although much progress has been made in treating disorders of micturition via nerve transfer, functional restoration of bowel control following SCI is a greater challenge. The aim of the present study was to develop a new nerve-transfer method to more robustly restore anorectal function after SCI.

Methods

Experimental animals

All procedures were approved by the Institutional Animal Care and Use Committee of the Medical

College of Shanghai Jiao Tong University, in compliance with the Guide for the Care and Use of Laboratory Animals.¹⁴ Sixty 8-week-old adult Sprague-Dawley female rats, weighing about 220 g (range, 200–250 g), were provided by the Animal Center of the Medical College of Shanghai Jiao Tong University, and were housed at 20–25°C and 50 ± 5% humidity with ad libitum access to food and water and 12:12 hour light/dark cycle. Animal housing and husbandry followed standard international procedures for small rodents.

Experimental nerve transfer model and laminectomy surgical procedure

Rats were anesthetized with sodium pentobarbital (40 mg/kg; i.p). After exposing the spinal nerves via a curved dorsal incision under aseptic conditions, we performed laminectomy of the L4 to S2 vertebrae. Briefly, the cauda equina was opened to expose the L5 and S1 nerve roots bilaterally. The nerves were carefully separated end-to-end prior to making an anastomosis via atraumatic suture (size: 12/0) at the level of the L5 nerve root. Finally, the cauda equina was cut between L5 and L6 (except S1) (Fig. 1). This entire procedure was performed under an operating microscope (Shanghai Eder Medical Technology Inc., Shanghai, China) using 10× magnification. Following wound

Figure 1 Animal model. (A) Bilateral L5 and S1 ventral and dorsal nerve root anastomosis. (B) An 8-cm curved incision was made to expose the muscles of the back and spinous process. (C) An electric drill (Seayang Company, Korea) was used to grind back the spinous process, articular process, and a portion of the lamina. (D) The lamina was opened to expose the cauda equina, following which the bilateral S1 nerve roots were separated. (E) End-to-end anastomosis of the bilateral L5 and S1 nerve roots was performed using a 12/0 atraumatic suture kit.

closure, 0.2 million U/d penicillin was administered intramuscularly to the rats for three consecutive days. We refer to the rats with the laminectomy and nerve transfer as the “model rats.”

For analysis of the nerve anastomosis, we subjected model and control rats to different kinds of analyses. Twelve weeks after the experimental reinnervation procedure, rats were allocated to the following groups, named for the kind of analysis: retrograde axonal tracing of nerve connections to anorectal musculature ($n = 5$), light histological and morphometric analyses of anastomosis nerves ($n = 5$), ultrastructural morphometric analyses of anastomosis nerves ($n = 5$), electrophysiological nerve root measurements and anorectal manometry ($n = 15$). Similarly, 30 healthy naïve rats were used as controls and were allocated similarly to the four groups (Table 1).

Retrograde axonal tracing and analysis

Twelve weeks following the initial nerve transfer procedure, we used retrograde labeling of axons to assess for reinnervation of the anorectal musculature. Recombinant attenuated pseudo rabies virus (PRV)-Bartha expressing enhanced green fluorescent protein (EGFP) (Shanghai Veterinary Research Institute, Shanghai, China) was used as a retrograde tracer.

Briefly, under aseptic conditions, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). A 2-3 cm midline incision was made on the lower abdomen to expose the anal canal and rectum. PRV suspension (10^8 pfu/ml, 1.0 μ l/site) was injected at four sites throughout the surface of the anal canal and rectal mucosa using a glass micropipette connected to a 1- μ l Hamilton microsyringe (Microinjector, Shanghai, China). An operating microscope was used (10X magnification) to guide injections and to avoid puncturing the wall of the rectum and anal canal. The micropipette tip was maintained in place at

the injection site for 2 minutes after each injection to prevent PRV leakage from the pipette tip. After the micropipette was withdrawn, the injection site was cleaned with saline-soaked swabs, the abdominal wall incision was sutured, and topical antibiotic was applied. Healthy control rats ($n = 5$) received the same injections and procedures.

Four days after the PRV injections, the model rats and control rats were deeply anesthetized with sodium pentobarbital and perfused to prepare the tissue for histological analyses. Briefly, the thoracic cavity and heart were exposed, a perfusion cannula was inserted into the left ventricle and ascending aorta, and 100 mL of chilled 0.9% saline (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was immediately perfused into the left ventricle at a pressure of 130 cmH₂O. This was followed by 500 mL of 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.4, 4°C). Following perfusion, the anastomotic nerve endings (2 cm) were harvested and postfixed overnight in 4% paraformaldehyde at 4°C. Samples of the nerve endings were then cryoprotected in 30% sucrose for 24 hours at 4°C, embedded in Tissue-Tek O.C.T. compound (SAKURA 4583, USA), and frozen at -20°C. Serial transverse sections (20-mm thickness) and vertical sections (20-mm thickness) of the L5, S1 root anastomosis tissue blocks were cut on a cryostat (Leica, CM 1900, Germany).

Sections were stained briefly with DAPI to label cell nuclei, then were rinsed, and mounted on gel-coated slides, which were then dried for 30 minutes in the dark. They were coverslipped and then examined under an epifluorescent microscope (Olympus IX71, Tokyo, Japan) equipped with a digital camera (DP-70, Tokyo, Japan). Fluorescent labeling was surveyed at 10X magnification and images documented at higher magnifications. Tissue from healthy control rats was processed similarly and examined and documented similarly.

Electron microscopy and morphometric analysis

Twelve weeks the initial nerve transfer procedure, 10 model rats and 10 control rats were euthanized with an overdose of sodium pentobarbital. A 10-mm-long nerve segment of the anastomotic site of the laminectomized rats was resected under an operating microscope. Similar nerve segments from control rats were also harvested. Specimens were postfixed in cacodylate-buffered 4% glutaraldehyde for 24 hours at 4°C, and then in 1% osmium tetroxide for 24 hours at room temperature. The tissue was then dehydrated through ascending concentrations of ethanol (Sinopharm Chemical Reagent, Shanghai, China), ending with propylene oxide (SPI Supplies/Structure Probe, Inc., West Chester, PA,

Table 1 Overview of the experimental study groups and analyses.

Group	No. of animals	Postoperative procedure
Model	5	Retrograde nerve tracing
	5	Histo-morphological assessment
	5	Electron microscopy of nerve anastomosis
Control	15	Anorectal manometry
	5	Retrograde nerve tracing
	5	Histo-morphological assessment
	5	Electron microscopy of nerve roots
	15	Anorectal manometry

USA). The tissue was then embedded in Epon 812 (SPI Supplies/Structure Probe, Inc.), and cured for 24 hours at 60°C.

Semi-thin sections (1 µm thick) were cut from the tissue blocks using an ultramicrotome (PowerTome-XL, RMC, USA), collected, mounted onto glass slides, and stained with Toluidine Blue (Sigma Aldrich, Munich, Germany). Toluidine Blue is commonly used to stain semi-thin sections that will ultimately be re-sectioned and analyzed for electron microscopy. It is generally accepted that the quality of Toluidine Blue staining of plastic-embedded tissue is not as good as Toluidine Blue staining of frozen sections.¹⁵ However, for our purposes, the staining quality suffices for our analysis of nerve tissue. The Toluidine Blue sections were used for gross examination of nerves at the light level, and areas of interest were cut out and re-embedded in Epon 812 for TEM examination.

Re-embedded tissues block embedded in Epon according to standard procedures were prepared for ultrastructural analysis by TEM. Ultrathin sections (50 nm thick) were collected onto 200 mesh grids and stained with uranyl acetate followed by lead acetate. The ultrathin sections were examined on a TEM (Tecnai G2, The Netherlands).

Fluorescent and electron digital micrograph image data were adjusted only for global brightness across the entire image.

Anorectal distension studies

The efficacy of the anastomosed nerve to respond to induced rectum distension was assessed by means of an intrarectal balloon, stimulation of the anastomosed nerve, and a pressure transducer to measure pressure imposed on the balloon by the intrarectal muscles following electrical stimulation of the nerve transfer.

Twelve weeks following the experimental nerve transfer procedure, 15 model rats were anesthetized with sodium pentobarbital (40 mg/kg) for anorectal function testing. Using sterile techniques, we reopened the original surgical site for the nerve transfer to examine the anastomosis and to electrically stimulate the anastomosed portion of the nerve. To measure the pressure exerted by rectal muscles stimulated through electrical stimulation of the anastomosed nerve, a warm water-filled balloon (ChengDu TME Technology Co., Ltd., Chengdu, China) was inserted into the rat's anal canal. One end of a flexible polyethylene catheter was connected to the balloon, and the other end was attached to a pressure transducer (PowerLab, AD Instruments, New South Wales, Australia). The balloon was left in place in the

rectum for 50 seconds to ensure muscular and rectal accommodation. To stimulate the anastomosed nerve, a bipolar electrode was placed on the proximal nerve root anastomosis in model rats. Electric stimulation (Power Lab System Labchart 5.0) was applied as square-wave pulses (1.5 mA, 20 Hz; pulse width, 0.2 ms; duration, 10 seconds). Pressure was recorded continuously as the stimulation pulse trains were repeated five times. The mean of the recorded pressures was calculated. This procedure was also performed in 15 healthy control rats prior to electrophysiological measurements.

Data analysis

Statistical analysis was performed using SPSS (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS, Inc.). One-way analysis of variance (ANOVA) and the Tukey HSD tests for *post hoc* assessments were used. When appropriate, paired sample t-tests were used to evaluate differences between groups. Statistical significance was set at $P < 0.05$.

Results

Retrograde labeling of anastomosed nerves

Following injection of PRV into the wall of the anal canal and rectum in healthy control rats, we observed discrete EGFP-labeled neurons in S1 nerve roots. In the model rats, we observed discrete EGFP-labeled neurons throughout the longitudinal extent of the proximal region of the anastomosed nerve (Fig. 2).

Morphometric analysis and EM

Toluidine Blue staining revealed appropriate organization of nerve root fibers in normal control rats. In model rats, we observed nascent nerve fibers at the site of the anastomosis (Fig. 3). Electron microscopic examination of the ultrastructure of the anastomosed nerves revealed that the nerve fibers appear normal in the model rats (Fig. 4).

Anorectal manometry assessment

Electrophysiological stimulation of the L5, L6, and S1 nerve roots in control rats revealed obvious induced pressures in the rectum and anal canal, as assessed by anorectal manometry (28.660 ± 2.157 cmH₂O, 37.047 ± 3.909 cmH₂O, 31.520 ± 3.005 cmH₂O, respectively). We also observed significant electrically induced pressures in the rectum and anal canal in model rats 12 weeks post-nerve transfer. Pressures reached as high as 30.447 ± 2.359 cmH₂O (Fig. 5). Pressures were significantly greater after stimulation of the L6 nerve root than after stimulation of the L5

Figure 2 Retrograde-labeled neurons under ultraviolet fluorescence microscopy. Green fluorescence was observed in a longitudinal section of L5 roots in healthy rats. (A, B, C; 200×). Green fluorescence was observed in the longitudinal section of the anastomosed (white arrow) roots in model rats (D, E, F; 100×). Green fluorescence was observed in the proximal of part of the anastomosis in model rats (G, H, I; 400×).

or S1 roots ($P < 0.001$, $n = 15$). Although the pressures were remarkable in model rats, this change was not as obvious at the L6 nerve root ($P < 0.001$, $n = 15$). However, pressure change was restored to the L5 and S1 nerve roots, there are no statistically significant difference in pressure change was observed between L5/S1 and the proximal anastomosis ($P = 0.608$, $P = 1.000$, $n = 15$) (Fig. 6).

Discussion

SCI may lead to NBD, which is mainly manifested as constipation, fecal incontinence, or both may appear alternately.¹⁶ There is no definitive treatment for this problem. Motivated by Zheng *et al.*'s¹¹ success in using

a “somatic nerve → autonomic nerve → bladder” strategy to reconstruct an artificial bladder reflex arc to restore urinary function in dogs with SCI, we attempted a similar strategy to restore bowel dysfunction in rats with experimentally induced SCI. After experimental laminectomy at the level of L4 to S2 vertebrae, we used an existing healthy somatic reflex to construct a defecation reflex arc via nerve anastomosis. Bilateral L5 anterior motor and posterior spinal sensory roots were anastomosed with bilateral S1 anterior motor and posterior sensory roots. This produced the defecation reflex, partially restoring functional anorectal movement and sensory function. With this new pathway, defecation function improved.

Figure 3 Representative Toluidine Blue staining of nerve roots in semi-thin sections. (A) L5 nerve root from a control rat. (B) S1 nerve root from a control rat. (C) Site of anastomosis in model rats (Magnification, 400×).

Figure 4 Ultrastructure of nerve fibers. Representative electron micrographs from an L5 nerve root from a control rat have a normal-appearing nerve fiber morphology (A, B, C). The ultrastructure of the anastomosis of model rats also has normal-appearing nerve fibers (D, E, F) (Magnification, 5000 $\times$).

The artificial defecation reflex arc, however, was not robust during the early postoperative period. Damage to the dominant nerve root that governs anorectal function might have contributed to this lack of robustness, and allowing the anastomosis to heal and become reinnervated might lead to better stool storage. Indeed, anorectal function testing conducted 12 weeks after surgery showed that nerve roots regenerated and connections within the artificial reflex arc were established to the extent where the artificial defecation reflex arc could serve as a trigger for inducing anorectal defecation.

In the present study, we aimed to determine whether the anatomical integrity of this newly constructed defecation reflex arc in laminectomized rats would be demonstrable through functional and anatomical experiments. To do this, we used PRV retrograde tracing, Toluidine Blue staining, electron microscopy, and anorectal pressure measurements. Electrical stimulation of the proximal anastomosis produced anorectal systolic and diastolic activity comparable to stimulation of sites located between the anastomosis and L5/S1 nerve roots, indicating that some axons passed through the anastomosis and made contact with the anorectal nerve. This led to partial recovery of anorectal function. Injection of the retrograde tracer PRV into anorectal muscles labeled neurons in the proximal anastomosis, indicating that the somatic nerve fibers of L5 roots grew into the myelin sheath of the parasympathetic nerve fibers of S1 roots through the anastomosis and

established a new, alternative nerve connection with its target organ.

Our neurotracing and histology experiments showed that numerous nerve fibers had grown through the anastomosis and that S1 autonomic nerve fibers can be re-innervated by L5 somatic nerve fibers. This demonstrates that our animal model is anatomically and functionally effective. This promising new approach has theoretical implications and potential clinical applications. It could represent the first step in mitigating the high morbidity, high complication rates, and high treatment and rehabilitation costs associated with SCI.³

The novel artificial defecation reflex arc we employed in our animal model has several advantages. First, unlike the approaches of Xiao and Godec,⁹ Chuang *et al.*,¹⁷ and Zheng *et al.*,¹¹ who used a single motor nerve root or unilateral nerve root to repair bladder function after SCI, in the present study, we anastomosed bilateral L5 anterior motor and posterior sensory roots with bilateral S1 anterior motor and posterior sensory roots to repair anorectal movement and sensory function. Our alternative approach provided a sensory trigger point and way for more axons to cross the anastomosis, leading to more robust nerve impulses.

Second, we chose an anastomosis site that would maximize re-innervation and minimize collateral damage to lower extremities. The anastomosis site we employed differs markedly from that of Sun *et al.*,¹⁸

Figure 5 Changes in rectal and anal canal pressure. (A) Changes in pressure following stimulation of the L5 nerve root in healthy rat. (B) Changes in pressure following stimulation of the L6 nerve root in healthy rat. (C) Changes in pressure following stimulation of the S1 nerve root in healthy rat. (D) Changes in pressure following stimulation of the proximal anastomosis in a model rat.

who anastomosed the anterior L4 and L6 nerve roots to restore anorectal function after SCI in rats. Our earlier studies showed that the S1 nerve root provides the greatest contribution to anorectal control during defecation in rats,¹⁹ while the L2-L4 nerve roots are associated with movement of the quadriceps femoris and the L5-L6 nerve roots are associated with the sciatic nerve and calf muscles.²⁰ This prompted us to select the S1 nerve root as the receptor nerve to maximize restoration of anorectal function, and to select the L5 nerve root as the donor nerve to minimize postoperative impact on lower extremity function. Thus, our animal model produces less damage in efforts to restore more functionality. Importantly, our model-innervation approach establishes a connection with the brain, because the cauda equina was cut between L5 and L6 (except for S1) after anastomosis of the bilateral L5 and S1 nerve roots. This is significant because the spinal cord and brain control physical defecation. Previous studies

rarely mention the brain's attempts to take control of a new bladder or defecation reflex arc after SCI. Further research is needed to better understand interactions between the defecation reflex arc and the brain.

The present study had the following limitation. The complexity of physical defecation remains unclear at present, and our study did not address how the brain and spinal cord interacts with the artificial defecation reflex arc. Indeed, experimental evidence on restoration of anorectal function and CNS control of bowel function is limited. Future studies are required to address these issues and identify the mechanisms underlying CNS control of bowel function.

Conclusions

The present results suggest that our model is feasible and effective for restoring anorectal function following SCI. This method could serve as a new clinical approach for restoring anorectal function in patients with SCI.

Figure 6 Pressure changes. (A) Pressure measured in the anal and rectal canal following electrical stimulation of the L5, L6, or S1 nerve roots or the proximal anastomosis in model rats. Pressures were 28.660 ± 2.157 cmH₂O, 37.047 ± 3.909 cmH₂O, 31.520 ± 3.005 cmH₂O, and 30.447 ± 2.359 cmH₂O, respectively. (B) Significant differences were observed in pressures resulting from the stimulation of the L6 and L5/S1 roots, and proximal anastomosis ($P < 0.001$, $n = 15$). However, pressures resulting from stimulation of the proximal anastomosis and that of L5/S1 did not differ significantly ($P = 0.608$, $P = 1.000$, $n = 15$).

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Disclaimer statements

Conflicts of Interest: There are no conflict of interests relating to the specific study.

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