Reconstruction of atonic bladder innervation after spinal cord injury: A bladder reflex arc with afferent and efferent pathways

Jun He¹, Guitao Li², Dixin Luo², Hongtao Sun², Yong Qi², Yiyi Li², Xunjie Jin²

¹Department of Orthopaedics, the Affiliated Nanhua Hospital of University of South China, Hengyang, China,

²Department of Orthopaedics, Guangdong No. 2 Provincial People's Hospital, No. 1 Shiliugang Road, Guangzhou, China

Background: Establishing bladder reflex arcs only with the efferent pathway to induce micturition after spinal cord injury (SCI) has been successful. However, the absence of sensory function and micturition desires can lead to serious complications.

Objectives: To reconstruct a bladder reflex arc with both afferent and efferent pathways to achieve atonic bladder innervation after SCI.

Methods: A reflex arc was established by microanastomosis of the S2 dorsal root to the peripheral process of the L5 dorsal ganglion and the L5 ventral root to the S2 ventral root. The functions of the reflex arc were evaluated using electrophysiology, wheat germ agglutinin–horseradish peroxidase (WGA–HRP) tracing, and calcitonin gene-related peptide (CGRP) immunocytochemistry analysis. Hind-paw motion was evaluated by CatWalk gait.

Results: Compound action potentials and compound muscle action potentials were recorded at the right L5 dorsal root following electrical stimulation of right S2 dorsal root. Similar to the control side, these were not significantly different before or after the spinal cord destruction between L6 and S4. WGA–HRP tracing and CGRP immunocytochemistry showed that construction of the afferent and efferent pathways of the bladder reflex arc encouraged axonal regeneration of motor and sensory nerves, which then made contact with the anterior and posterior horns of the spinal cord, ultimately reestablishing axoplasmic transportation. Gait analysis showed that at 3 months following the operation, only the regularity index was significantly different as compared with 1 day before the operation, other parameters showing no difference.

Conclusion: Bladder reflex arc with the afferent and efferent pathways reconstructs the micturition function without great influence on the motion of leg.

Keywords: Spinal cord injury, Bladder, Afferent pathways, Efferent pathways, Reflex arc

Introduction

Spinal cord injury (SCI) followed by thoracolumbar fracture is a common clinical condition that can cause the bladder to become atonic due to injury to the sacral bladder micturition center.¹ An atonic bladder is not only inconvenient to a patient, but also causes severe urinary retention, urinary tract infection, and even death due to renal failure.² Regenerative therapy for spinal cord injuries is not yet possible, while potential treatments such as olfactory ensheathing, cell transplantation, and stem cell transplantation are not yet

available in humans due to ethical issues. Therefore, attempts to reduce mortality after SCI focus on neuro-pathic bladder dysfunction treatment.

Current bladder dysfunction treatment methods include medication, nerve blockade, electrical stimulation therapy, intermittent catheterization, and urinary diversion surgery.^{3–4} Brindley⁵ performed sacral anterior root electrostimulation to recover controllable urination in SCI patients, and this method achieved clinical success in the United States and Europe. However, it is difficult to carry out due to its expense and extensive secondary injury occurrences.⁶

In recent years, Wang *et al.*^{3,4,6–12} established the abdominal reflex–spinal cord–central bladder pathway

Correspondence to: Xunjie Jin, Department of Orthopaedics, Guangdong No. 2 Provincial People's Hospital, No. 1 Shiliugang Road, Guangzhou 510317, China. Email: jxj_2012@163.com

to induce regular micturition via the healthy ventral root above the level of injury. This method has been successful in animal experiments and has been useful in clinical applications.^{13,14} However, this kind of the bladder reflex pathway only reconstructs the efferent pathway of bladder micturition, which is a trigger for urination but does not establish the afferent pathway. Thus, a continuous stimulatory trigger is needed for urination.¹² Owing to the lack of any sensory function in the bladder, the desire for micturition cannot be detected, which can lead to serious complications.¹⁵

Owing to the aforementioned problems, repairing the sensory afferent pathway of the bladder reflex arc is essential. We can therefore think about reconstructing the afferent pathway with a functional dorsal root. However, dorsal root damage may seriously inhibit dorsal root axon regeneration in the dorsal root enter zone (DREZ).^{16–20} To alleviate dorsal root axon regeneration difficulties, our team previously investigated using peripheral processes that originated from the spinal ganglion instead of the dorsal root, thus avoiding any inhibition from the DREZ. When the proximal end of a peripheral process from the L4 spinal ganglion was connected to the distal end of the L6 dorsal root, the L6 dorsal root axon regenerated, the fiber contacted with the L4 spinal cord, and it recovered the ability to undergo axoplasmic transport.²¹ Based on these results, we were able to construct a bladder reflex arc with sensory pathways of the peripheral process from the L5 spinal ganglion.

Furthermore, establishing a bladder reflex arc requires the use of healthy spinal nerves, such as the L5 spinal nerve, which is an important sciatic nerve component. Whether motor function in the experimental side hind paw is affected after L5 nerve use has not been studied. In this study, we attempted to demonstrate that autonomic micturition restoration in the atonic bladder is achievable by re-establishing a bladder reflex arc consisting of afferent and efferent pathways. We also evaluated the effects on hind-paw motor function after establishing the reflex arc.

Materials and methods

Animals

Twenty 3-month-old Sprague-Dawley male rats, weighing 220–250 g, were provided by Guangdong Medical Lab Animal Center, Guangzhou, China. Throughout the study, these animals were individually housed in a temperature (20–24 °C) and humidity (50–60%) controlled room, with a normal 12:12 hour light/dark cycle in barrier facilities at the animal center of the Institute of Respiratory Disease of Guangzhou, China.

For each rat, the right side was the experimental side and the left side was the control side.

Wheat germ agglutinin-horseradish peroxidase (WGA–HRP) and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St Louis, MO, USA), polyclonal rabbit calcitonin gene-related peptide (CGRP) was obtained from Enzo Life Sciences (Lausen, Switzerland), and a streptavidin-peroxidase test kit and a 3,3'-diaminobenzidine chromogenic kit were obtained from Beijing Golden Biotechnology Company Ltd (Beijing, China).

The instruments used included a surgical microscope from the Olympus Corporation (Tokyo, Japan), a 32-channel Cadwell Cascade intra-operative nerve electrophysiological monitor from the Cadwell Company (Holliston, TX, USA), and a CatWalk gait analysis system from the Noldus Information Technology Company (Wageningen, Netherlands).

Surgical procedure for bladder reflex arc reconstruction

The animals were anesthetized before surgery by intraperitoneal injection of 1% sodium pentobarbital solution (40 mg/kg). Under sterile conditions, a lateral hemilaminectomy was performed on the right side to expose the cauda equina right side and the dorsal root ganglia from L4 to S3. The dorsal and the ventral roots of S2 were transected at the L5 level intradurally, and the peripheral processes and ventral root of L5 were transected in the immediate post-ganglionic site (Fig. 1A). The proximal end of the right L5 peripheral processes was anastomosed to the distal end of the right S2 dorsal root. Subsequently, the proximal end of the right L5 ventral root was intradurally anastomosed to the distal end of the S2 ventral root. The stumps and nerve graft were anastomosed end to end with four stitches using 11–0 monofilament nylon under a 16-power microscope (Fig. 1B). To protect the spinal cord neurons from damage, 1% local closed lidocaine treatment was performed before the nerves were transected. The left side of the spinal nerves was kept intact and served as an internal control. We observed rat survival, eating, and urination following the operation. After a 3-month period, the surviving rats were evaluated before and after destruction of the L6–S4 spinal cord segments.

CatWalk gait analysis

All live rats were prepared for CatWalk gait analysis. During the 2 weeks preceding surgery, and for 12 weeks after the operation, animals were trained daily to make consecutive uninterrupted runs on the

Figure 1 A schematic diagram (dorsal view) of the bladder reflex arc with afferent and efferent pathways. (A) Illustration of the normal spinal cord and nerve root. The short straight line shows the cut position of the L5 and S2 dorsal root, ventral root, and the L5 peripheral process of the dorsal ganglion. (B) Illustration of a reconstructed bladder reflex arc with afferent and efferent pathways. 1, L5 spinal cord; 2, S2 spinal cord; 3, S2 dorsal root; 4, S2 ventral root; 5, L5 ventral root; 6, L5 peripheral process; 7, bladder; 8, site of cutoff; 9, site of anastomosis.

CatWalk runway. The methodology for CatWalk training is described elsewhere.^{22,23} During the training period, a 12–14 g/day food deprivation protocol was used to motivate the animals. CatWalk gait analysis was performed 1 day before surgery and 3 months after the operation, with the values recorded on the day before the operation used as baseline. Data were collected and analyzed with CatWalk software version 9.1. To avoid human error, the independent researcher

performing the CatWalk gait analysis was blind to the rats' condition. We selected the experimental parameters according to the published literature,^{23,24} and the definitions of these parameters are included in Table 1.

Electrophysiological studies

Three months after the operation, following CatWalk gait analysis completion, the skin was incised along the original incision after abdominal anesthesia and

Table 1 Definition of parameters

CatWalk parameter	Definition
Base of support	Average width of the hind paws, as measured perpendicular to the waking direction
Stride length	Distance between the placement of a hind paw and the subsequent placement of the same paw
Print length	Length (horizontal direction) of the complete print
Print width	Width (vertical direction) of the complete paw print
Print area	Surface area of the complete print
Maximum area	Total surface area of the glass floor contacted by the hind paw at the moment of maximum paw-floor contact during the stance phase of the hind paw
Regularity index	RI = (NSSP × 4)/number of paw placements × 100%. It expresses the number of normal step sequence patterns relative to the total number of paw placements
Coupling diagonal LF → RH	The coupling between the left fore paw (anchor) and right hind paw (target)
Coupling diagonal RH → LF	The coupling between the right hind paw (anchor) and left fore paw (target)
Coupling girdle LH → RH	The coupling between the left hind paw (anchor) and right hind paw (target)
Coupling girdle RH → LH	The coupling between the right hind paw (anchor) and left hind paw (target)
Coupling ipsilateral RF → RH	The coupling between the right fore paw (anchor) and right hind paw (target)
Coupling ipsilateral RH → RF	The coupling between the right hind paw (anchor) and right fore paw (target)
Mean intensity	The mean intensity of the complete paw
Stance duration	The time of contact of the hind paw with the glass floor
Swing duration	The time that the hindpaw is not in contact with the glass floor
Duty cycle	Expressed stand (stance duration) as a percentage of step cycle

the surgical scars used to locate the anastomosis. The following steps were then performed: (1) the stimulation electrode was placed in the right S2 dorsal root 5 mm distal to the anastomosis, while the recording electrode was placed in the right S2 ventral root 5 mm distal to the anastomosis. The stimulation and recording electrodes were then connected to the Cadwell Cascade and stimulated at 3 mA for 0.3 ms. Compound action potentials (CAPs) were then recorded. (2) The stimulation electrode was placed at the left S2 dorsal root, and the recording electrode was placed at the left S2 ventral root, which was stimulated using the same parameters as on the right side. CAPs were recorded. (3) Finally, the spinal cord was damaged bilaterally and absolutely between L6 and S4, leaving the spinal nerves intact, and the CAPs were recorded again on both the right and left sides as described above. Subsequent to recording the bladder detrusor muscle compound muscle action potentials (CMAPs), the following steps were taken: (1) the stimulation electrode was placed at the right S2 dorsal root 5 mm distal to the anastomosis and was connected to the Cadwell Cascade. (2) Then, the rat was placed in a supine position and the bladder exposed by a small vertical incision on the lower abdominal wall. A muscular electrophysiology-recording electrode was placed in the anterior wall of the bladder detrusor muscle close to the top of the bladder and was sutured to the bladder wall with a 4-0 nylon suture. (3) The bladder detrusor muscle CMAPs were recorded before and after the L6-S4 spinal cord segment transection using a 3-mA stimulating intensity, a 0.3 ms pulse width, a 20-Hz frequency, and a 5-second duration. (4) As a control, an electrode was placed in the left S2 dorsal root, stimulated similarly to the right side, and CMAPs were recorded.

WGA-HRP tracing

After measuring the electrophysiological studies, half of the rats were randomly selected and given abdominal anesthesia for WGA-HRP tracing. Both sides of the dorsal and ventral roots of L4, L6, S1, and S3 were cut, and the residual liquid in the bladder was pumped out. Using a microinjector, 4% WGA-HRP was injected to label all parts (dome, body, base, and neck) of the urinary bladder wall's right side. The total injection volume was 10 μ l. Immediately after the injections, the bladder surface was thoroughly cleaned with saline to remove any excess tracer. Thereafter, the incision on the lower abdominal wall was closed. Finally, 3 days post-operation, the animals were anesthetized and perfused through the ascending aorta with 250–300 ml warm (37°C) 0.9% saline. This was followed by 500 ml

cold (4°C) fixative consisting of 1% glutaraldehyde and 1.25% formaldehyde in 0.1 mol/l phosphate-buffered saline (PBS; pH = 7.4). The L5 spinal cord segments were dissected out, post-fixed in the same fixatives for 2 hours at 4°C, and stored overnight in 20% sucrose in PBS at 4°C. Then, 30- μ m thick serial transverse sections were cut from the spinal cord segments on a freezing microtome. Finally, the sections were processed histochemically using TMB as the chromogen, examined, and photographed on a Leica microscope.²⁵

Immunohistochemistry

After completing electrophysiological measurements, the other half of the rats to be used for CGRP immunocytochemical analysis were anesthetized, and the bilateral dorsal and ventral roots of L4, L6, S1, and S3 were cut off. Two weeks after this root cutting operation, the rats were deeply anesthetized and perfused through the ascending aorta with 250–300 ml warm (37°C) 0.9% saline. This was followed by 300 ml fixative consisting of 4% formaldehyde in 0.1 mol/l PBS (4°C, pH = 7.4). The fixed L5 spinal cord segments were removed and post-fixed in the same fixative for 3 hours at 4°C. All tissues were then transferred to 30% sucrose in 0.1 mol/l PBS at 4°C for cryoprotection. Tissues were embedded in optimal cutting temperature solution compound. The spinal cords were sliced coronally at a 20- μ m thickness on a freezing microtome, and every third section was collected for immunocytochemical processing.²⁶ Five sections were randomly selected from each specimen. The area covering the immunopositive region (IR) of the spinal cord's posterior–anterior horn was photographed using a Leica microscope (magnification 10 $\times$ 20), and the staining was measured with Image-ProPlus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Data on print length, print width, maximum area, print area, mean intensity, stance duration, swing duration, swing speed, duty cycle, and couplings were collected during the CatWalk gait analysis. They are shown as the ratio between the values for the right hind paw and the left hind paw. The other data are presented as mean $\pm$ standard deviation (SD). Statistical differences were analyzed using Student's paired *t*-test in SPSS 16.0 (SPSS Inc., Chicago, IL, USA). A P-value less than 0.05 was regarded as statistically significant.

Results

Seventeen out of 20 rats survived for 3 months after surgery. They ate, drank, and urinated normally

during this period. We used these rats ($n = 17$) for CatWalk gait analysis. The nerves were successfully separated in eight rats. The nerve separations failed in the remaining rats due to nerve adhesion presence. Therefore, we used these eight rats for electrophysiological analysis, WGA–HRP tracing, and immunocytochemistry analyses.

CatWalk gait analysis

Seventeen rats successfully performed the CatWalk gait analysis. With respect to reliable gait parameter comparison, body weight and speed of gait are the two very important aspects. Body weight may seriously confound CatWalk parameter interpretation, such as the mean intensity parameter of gait. The speed of gait may have a strong influence on many gait parameters, including print area, stance, and swing duration.²³ So, the animals were continuously trained and their food restricted throughout the entire experimental period to ensure consistent weight gain and performance between animals. A comparison of the body weight and speed of gait between rats who were 1-day pre-operation and 3-month post-operation showed no statistical difference between the two groups (Fig. 2A and B), indicating that the influence on weight and speed of gait was negligible.

General parameters, individual paw parameters, and pain-related gait parameters

In this study, the general, individual paw, and pain-related gait parameters were not significantly different

before and after L5 nerve root injury (Fig. 2C–L). Base of support was 4.01 ± 0.24 cm before and 4.01 ± 0.28 cm after surgery ($n = 17$, $P = 0.59$); hind-paw stride length was also not significantly different ($n = 17$, $P = 0.59$), with a mean value of 105.5 ± 2.81 and $102.7 \pm 4.51\%$ before and after surgery, respectively.

Coordination-related parameters

The regularity index (RI) parameter is often used to evaluate coordination. As seen in Fig. 3, there were obvious differences in the RI between 1-day pre-operation and 3-month post-operation. The RI value at 3-month post-operation was 86.9% of the RI before surgery, at $83.6 \pm 4.4\%$ compared to $96.2 \pm 1.6\%$ ($n = 17$, $P < 0.05$). Coupling is another parameter used to assess coordination, but there were no obvious changes in coupling (Fig. 4).

Electrophysiological studies

Using electrical stimulus to excite the right S2 dorsal root, CAPs were recorded at the right S2 ventral root both before and after SCI between L6 and S4. The evoked potentials' mean amplitude was 0.120 ± 0.005 mV before the transection and 0.110 ± 0.005 mV after the transection, which was not significantly different ($n = 8$, $P = 0.309$). The curve shapes were similar to those recorded on the control side before the transection (Fig. 5). The evoked potentials' mean amplitude on the right side went up to 71.9%, but was lower than that recorded on the control side, which was $0.160 \pm$

Figure 2 Statistical analysis of weight, crossing time, general parameters, individual paw parameter, and pain-related gait parameters. Comparisons of the (A) body weight and (B) crossing time between 1-day pre-operation and 3-month post-operation showed no statistical differences. General gait parameters included the (C) base of support (BOS) and (D) stride length of the hind paw. Individual paw parameters include (E) print length, (F) print width, (G) maximum area, and (H) print area. The pain-related gait parameters included (I) mean intensity, (J) stance duration, (K) swing duration, and (L) duty cycle, which have been linked to mechanical hyperalgesia. In our study, there was no significant difference in all these parameters (C–L) at 3-month post-operation compared with 1-day pre-operation.

Figure 3 Statistical analysis of the regularity index (RI). The parameter of RI is often used to evaluate coordination. In this figure, there were obvious differences in RI between 1-day pre-operation and 3-month post-operation. The value of RI at 3-month post-operation was 86.9% of the RI before surgery, at $83.6 \pm 4.4\%$ compared with $96.2 \pm 1.6\%$ ($n = 17$, $P = 0.0107$).

0.009 mV ($P < 0.01$). CAPs were not detected on the control side after spinal cord transection.

When the right S2 dorsal root was stimulated, the differences in the bladder detrusor muscle CMAPs both before and after spinal cord transection were not significant ($n = 8$, $P = 0.078$). The curve shape was similar to the control sides before transection, and the mean amplitude reached 82.4% of that recorded when the same stimulus was applied to the left S2 dorsal root before transection. However, the CMAPs failed to record when the stimulus was applied to the left S2 dorsal root after paraplegia (Fig. 6).

WGA-HRP tracing

Four rats underwent WGA-HRP tracing. Following WGA-HRP injection into the experimental (right) urinary bladder wall, cyan positive labeling of WGA-HRP-labeled fibers and motoneurons was found in laminae I–VI, IX, and X on the right side of the spinal gray matter (Fig. 7).

Immunocytochemistry

Immunocytochemistry was performed on four rats. CGRP-immunopositive nerve fibers were present in lamina I, II, and III of the L5 spinal cord on both the control and experimental side (Fig. 8A). The dense CGRP-immunopositive nerve fibers displayed no obvious differences between either side ($n = 4$, $P = 0.30$). The immunopositive sensory nerve fibers extended through the dorsal root entry zone (DREZ)

and into the bilateral dorsal horn of the spinal cord (Fig. 8B).

SCI is one of the most common conditions observed in the clinic. Survey data from Hackler show that the 25-year mortality in patients with SCI is 49%, of which 43% of deaths are due to renal failure caused by neuropathic bladder dysfunction.²⁷

The “abdominal reflex–spinal cord–central bladder” reflex arc model was recently constructed and shows promising results at the experimental stage and during clinical research.^{5,6,13} Studies proved that, after SCI, the normal body reflex innervation above the injury level can be used to reconstruct the atonic bladder’s micturition function. However, due to the lack of any sensory function in the bladder, the desire for micturition cannot be detected, which can lead to serious complications. Therefore, there is a need to reconstruct a reflex arc with the afferent pathway, using the peripheral process to prevent inhibition in DREZ.

Pascual *et al.*²⁵ injected WGA-HRP into the urinary bladder wall dome, body, neck, and base on one side of the rats and found positive neurons in the L6–S4 segment dorsal horn. S1 and S2 segment staining was the most intense. Similarly, Wang *et al.*⁴ performed electrostimulation on SD rat nerve roots and found that the largest CMAPs could be recorded from S2. Therefore, in this experiment, we chose to connect the S2 ventral and dorsal roots with another ventral root and peripheral processes of the functional spinal cord segment. Because the L5 ventral root and peripheral processes are of similar thickness and property as the S2 ventral and dorsal roots, we anastomosed them to reduce the axon mismatch rate during the nerve regeneration process and to help regrow the axon. Furthermore, the L6–S4 spinal cords are the primary micturition center,²⁵ so we damaged L6–S4 to construct an atonic bladder animal model, which is used in similar previous research.^{3,4}

According to Ramer,²⁰ the regeneration rate of spinal ventral root is 1–2 mm/day, and that of peripheral processes is 4–7 mm/day. Because the distance from bladder to anastomoses is always shorter than 80 mm, 3 months is enough for the nerve regeneration. So, 3 months after surgery, we used WGA-HRP for anterograde and retrograde tracing to demonstrate neural pathway integrity. To avoid false-positive results due to intradural conjoined nerve roots and collateral sprouting,²⁸ we cut off the ventral and dorsal roots of the L4, L6, S1, and S3 spinal nerves before the WGA-HRP experiment. The results showed cyan-positive WGA-HRP particles present in the right side of the ventral horn and dorsal horn of the spinal

Figure 4 Statistical analysis of coupling. Coupling was a parameter used to assess coordination. Six related parameters (A–F) should be detected when assessing the affected right hind paw's coordination. In our study, these parameters were not significantly different before and after the operation.

cord's L5 segment. This meant that following axonal regeneration, the afferent and efferent pathways were reconstructed, axoplasmic transport was recovered, and the sensory and motor nerve pathways were successfully established.

CGRP is a 37-amino acid peptide that is a member of the calcitonin family and is widely expressed in the central and peripheral nervous systems. It is used to study the distribution and functional status of the dorsal root axons in the dorsal horn.^{29–31} Three

Figure 5 The compound action potentials (CAPs) of the bladder. Three-month post-operation, the same electrical stimulus was used to excite the distal end of the right L5–S2 dorsal root anastomosis in four rats. CAPs were recorded both before (B) and after (C) spinal cord injury between L6 and S4. The mean amplitude of the evoked potentials was 0.120 ± 0.005 mV before the transection and 0.110 ± 0.005 mV after the transection, which was not significantly different ($n = 8$, $P = 0.309$). The curve's shape and amplitude were similar to those recorded on the control side when the left S2 dorsal root was excited (A), but CAPs were not detected when the same stimulus was used on the control side after the transection.

months after surgery, we observed dense CGRP-immunopositive nerve fibers distributed in layers I, II, and III of the L5 posterior horn of the spinal cord and the DREZ of the adjacent L5 posterior horn of the spinal cord. There was no difference in the CGRP staining compared to the control side, indicating that the S2 dorsal root had regenerated, its axon made contact with the L5 posterior horn, and axoplasmic transport had recovered.

In addition to WGA–HRP and histological studies, CAPs and CMAPs were used to further confirm that a bladder reflex arc had been successfully constructed. Based on these experiments, we can conclude that somatic nerves' motor and sensory nerve fibers can regenerate into the parasympathetic nerve fiber, recover axoplasmic transport, and transmit afferent and efferent nerve impulses.

Although we confirmed the above model's feasibility, we also needed to assess the effect on hind-paw function, as the nerve used for the reconstruction included the L5 spinal nerve, a part of the sciatic nerve. Unfortunately, few previous studies exist on the subject. In recent years, CatWalk gait analysis has been applied to assess hind-limb function before and after successful sciatic

nerve injury repair.^{23,24} Thus, we used this method to identify the effects of cutting the L5 spinal nerve on hind-limb function.

Body weight may seriously confound interpretation of CatWalk parameters, such as the mean intensity parameter of gait. Therefore, the rats were continuously trained, and their food restricted, throughout the entire experimental period to ensure consistent weight gain and performance among the animals. We observed, while performing gait analysis, an abnormal gait appeared occasionally when the different gaits alternated at 3-month post-operation. This gait led to a reduction in RI to 86.9% of the value at 1 day before surgery. We think the reason for this excess gait is right paw hyperalgesia.

The difference between pain-related parameters before and after injury was not statistically significant. However, it is possible that pain was present, and thus, when the right hind-paw contacted the glass, a brief lift to escape the pain occurred.

Although the RI of coordination-related parameters showed a small decline compared to the pre-operative state, walking function did not significantly change. The general, individual, coupling, and pain-related

Figure 6 The compound muscle action potentials (CMAPs) of the bladder detrusor muscle. Three-month post-operation, the bladder detrusor muscle CMAPs were recorded in response to distal end stimulation of the S2 dorsal root anastomosis on the right side both before (B) and after (C) paraplegia. This was caused by transecting the L6–S4 spinal cord segment. The differences between the conditions were not significant ($n = 8$, $P = 0.078$). The curve shapes were similar to those of the control side before transection (A). The mean amplitude reached 82.4% of that recorded when the same stimulus was applied to the left S2 dorsal root before transection. However, the CMAPs failed to record when the stimulus was applied to the left S2 dorsal root after paraplegia.

gait parameters were not significantly different before and after the L5 spinal nerve dorsal and ventral root injury. These results showed that using the L5 spinal nerve for neural reconstruction would not cause enormous influence on normal walking function in rats.

Although we have proved the possibility and feasibility of the bladder reflex arc, there are some issues worth noting. First, the experimental results confirmed that the afferent and efferent pathways were reconstructed, but there was no way to quantitatively evaluate sensory function recovery. Clinically, the patient's subjective sensory function can be tested, but this is not possible in animal models. For this reason, common examination methods, such as the

urodynamics test and the sympathetic skin response, cannot be used in rats.^{32,33} In recent years, Blok *et al.*³⁴ determined bladder function by using positron emission tomography to detect the changes in neuronal excitability of the frontal lobe and cingulate gyrus during bladder filling. It remains to be determined whether this technique can be used in the experiment type described here.

Secondly, because it is difficult to keep a paraplegic animal alive for 3 months,²² we used a rat model based on a previous report^{3–6} with a paradigm that allowed us to evaluate reconstructed bladder reflex arc functionality. However, this kind of model is not completely in accordance with clinical practice, which may be one

Figure 7 Injected WGA–HRP into the experimental (right) urinary bladder wall 3-month post-operation. (A) The cyan-positive labeling of WGA–HRP-labeled fibers and motoneurons (arrows) was found in laminae I–VI, IX, and X on the right side of the spinal gray matter (magnification 5×10). (B) The positive neurons (arrows) were identified in the anterior horn of the L5 spinal cord on the experimental side (magnification 10×20). (C) The positive fibers (arrows) were identified in the posterior horn of the L5 spinal cord on the experimental side (magnification 10×20).

reason for the differences between the experimental results and clinical outcomes.³⁵ Thus, further research is required to successfully establish a realistic model.

Thirdly, detrusor sphincter dyssynergia is the key problem after SCI. We proved that bladder detrusor function was restored by this bladder reflex arc with electrophysiological methods. Whether the sphincter function recovered, and whether the disorder between two muscles improved, could not be proved here due to limited conditions. In subsequent experiments, we will try our best to perfect it.

In conclusion, a bladder reflex arc with afferent and efferent pathways in an SCI rat model was successfully reconstructed. We used microanastomosis of the L5 ventral root and the L5 peripheral process of the dorsal ganglion to the S2 ventral and S2 dorsal roots. This bladder reflex arc reconstructs the atonic bladder's micturition function, avoids inhibiting dorsal root regeneration by the DREZ, and has no enormous influence on leg motion in rats. This new approach may show considerable positive clinical prospects for SCI patients with atonic bladder.

Figure 8 Expression of calcitonin gene-related peptide (CGRP) in the L5 spinal cord 3-month post-operation. (A) The dense CGRP-immunopositive nerve fibers were distributed in layers I-III of the bilateral posterior horn of the spinal cord. The left side is the control group and the right side is the experimental side (magnification 10×20). (B) As the red circle shows, the immunopositive sensory nerve fibers extended through the dorsal root entry zone and into the bilateral dorsal horn of the spinal cord (magnification 10×40). (C) The area of the CGRP-immunopositive nerve fibers on the left side was 842.20 ± 17.60 and on the right side was 827.40 ± 15.24 . There was no obvious difference between either side ($n = 20$, $P = 0.30$).

Disclaimer statements

Contributors Conceived and designed the experiments: XJ, GL and JH. Performed the experiments: JH, DL, YL and YQ. Analyzed the data: XJ, JH, GL and HS. Wrote the manuscript: JH and XJ.

Conflicts of interest None.

Ethics approval This study was conducted based on the recommendations in the *Guide for the Care and Use of Laboratory Animals of the National Institutes of Health*. Animal studies were approved by the Institutional Animal Care and Use Committee, Guangzhou Medical College (protocol number: 2011-0603). Sodium pentobarbital anesthesia methods and other efforts were taken to minimize animal suffering.

Funding None.

References

- Chen SL, Huang YH, Wei TY, Huang KM, Ho SH, Bih LI. Motor and bladder dysfunctions in patients with vertebral fractures at the thoracolumbar junction. *Eur Spine J* 2012;21(5):844-9.
- Sievert KD, Amend B, Gakis G, Toomey P, Badke A, Kaps HP, et al. Early sacral neuromodulation prevents urinary incontinence after complete spinal cord injury. *Ann Neurol* 2010;67(1):74-84.
- Wang JW, Zhao YW, Hou CL, Ni WF, Rui BY, Guo SC, et al. An experimental study of artificial murine bladder reflex arc established by abdominal reflex. *Chin Med J (Engl)* 2011;124(3):413-8.
- Wang J, Hou C, Jiang J, Li Q, Zhang F. Selection of the sacral nerve posterior roots to establish skin-CNS-bladder reflex pathway: an experimental study in rats. *Microsurgery* 2007;27(2):118-24.
- Brindley GS. The first 500 patients with sacral anterior root stimulator implants: general description. *Paraplegia* 1994;32(12):795-805.
- Xiao CG, Godec CJ. A possible new reflex pathway for micturition after spinal cord injury. *Paraplegia* 1994;32(5):300-7.
- Zheng XY, Hou CL, Zhong HB, Xu RS, Chen AM, Xu Z, et al. Reconstructed bladder innervation below the level of spinal cord injury: the knee-tendon to bladder artificial reflex arc. *J Spinal Cord Med* 2009;32(1):79-85.
- Lin H, Hou C, Zhen X, Xu Z. Clinical study of reconstructed bladder innervation below the level of spinal cord injury to produce urination by Achilles tendon-to-bladder reflex contractions. *J Neurosurg Spine* 2009;10(5):452-7.
- Wang J, Hou C, Zheng X, Xu Z, Wang W, Lin H. Major influential factors of the micturition alert device dedicated to neurogenic bladders. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2008;22(8):964-8.
- Wang J, Hou C, Zhang W, Zheng X, Xu Z, Wang W, et al. Micturition alert device dedicated to neurogenic bladders. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2008;22(5):597-601.
- Wang J, Hou C, Wang W, Zheng X, Xu Z. Biocompatibility of micturition alert device dedicated to neurogenic bladder. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2008;22(9):1108-12.
- Xiao CG, de Groat WC, Godec CJ, Dai C, Xiao Q. "Skin-CNS-bladder" reflex pathway for micturition after spinal cord injury and its underlying mechanisms. *J Urol* 1999;162(3 Pt 1):936-42.
- Xiao CG. Reinnervation for neurogenic bladder: historic review and introduction of a somatic-autonomic reflex pathway procedure for patients with spinal cord injury or spina bifida. *Eur Urol* 2006;49(1):22-8; discussion 28-9.
- Lin H, Hou CL, Zhong G, Xie Q, Wang S. Reconstruction of reflex pathways to the atonic bladder after conus medullaris injury: preliminary clinical results. *Microsurgery* 2008;28(6):429-35.
- Frankel HL, Coll JR, Charlifue SW, Whiteneck GG, Gardner BP, Jamous MA, et al. Long-term survival in spinal cord injury: a fifty year investigation. *Spinal Cord* 1998;36(4):266-74.
- Carlstedt T. Nerve fibre regeneration across the peripheral-central transitional zone. *J Anat* 1997;190 (Pt 1):51-6.
- Murray M, Wang SD, Goldberger ME, Levitt P. Modification of astrocytes in the spinal cord following dorsal root or peripheral nerve lesions. *Exp Neurol* 1990;110(3):248-57.
- Fawcett JW, Geller HM. Regeneration in the CNS: optimism mounts. *Trends Neurosci* 1998;21(5):179-80.
- Onose G, Angheliescu A, Muresanu DF, Padure L, Haras MA, Chendreau CO, et al. A review of published reports on neuroprotection in spinal cord injury. *Spinal Cord* 2009;47(10):716-26.

- 20 Ramer MS, McMahon SB, Priestley JV. Axon regeneration across the dorsal root entry zone. *Prog Brain Res* 2001;132:621–39.
- 21 Tang XJ, Li GT, Xu WY, Qi Y, Luo DX, Jin XJ. Study on reconstruction of afferent pathway after repairing transected dorsal root. *Chin J Orthop* 2011; 31:175–83.
- 22 Hamers FP, Lankhorst AJ, van Laar TJ, Veldhuis WB, Gispens WH. Automated quantitative gait analysis during overground locomotion in the rat: its application to spinal cord contusion and transection injuries. *J Neurotrauma* 2001;18(2):187–201.
- 23 Deumens R, Jaken RJ, Marcus MA, Joosten EA. The CatWalk gait analysis in assessment of both dynamic and static gait changes after adult rat sciatic nerve resection. *J Neurosci Methods* 2007;164(1):120–30.
- 24 Bozkurt A, Deumens R, Scheffell J, O'Dey DM, Weis J, Joosten EA, *et al.* CatWalk gait analysis in assessment of functional recovery after sciatic nerve injury. *J Neurosci Methods* 2008;173(1): 91–8.
- 25 Pascual JJ, Insausti R, Gonzalo LM. Urinary bladder innervation in male rat: termination of primary afferents in the spinal cord as determined by transganglionic transport of WGA–HRP. *J Urol* 1993;150(2 Pt 1):500–4.
- 26 Chen LJ, Zhang FG, Li J, Song HX, Zhou LB, Yao BC, *et al.* Expression of calcitonin gene-related peptide in anterior and posterior horns of the spinal cord after brachial plexus injury. *J Clin Neurosci* 2010;17(1):87–91.
- 27 Tanagho EA, Schmidt RA. Electrical stimulation in the clinical management of the neurogenic bladder. *J Urol* 1988;140(6): 1331–39.
- 28 Phillips LH, 2nd, Park TS. The frequency of intradural conjoined lumbosacral dorsal nerve roots found during selective dorsal rhizotomy. *Neurosurgery* 1993;33(1):88–90; discussion 91.
- 29 Zheng LF, Wang R, Xu YZ, Yi XN, Zhang JW, Zeng ZC. Calcitonin gene-related peptide dynamics in rat dorsal root ganglia and spinal cord following different sciatic nerve injuries. *Brain Res* 2008;1187:20–32.
- 30 Li XQ, Verge VM, Johnston JM, Zochodne DW. CGRP peptide and regenerating sensory axons. *J Neuropathol Exp Neurol* 2004; 63(10):1092–103.
- 31 Zinck ND, Rafuse VF, Downie JW. Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury. *Exp Neurol* 2007;204(2): 777–90.
- 32 Ockrim J, Laniado ME, Khoubehi B, Renzetti R, Finazzi Agro E, Carter SS, *et al.* Variability of detrusor overactivity on repeated filling cystometry in men with urge symptoms: comparison with spinal cord injury patients. *BJU Int* 2005;95(4):587–90.
- 33 Elie B, Guiheneuc P. Sympathetic skin response: normal results in different experimental conditions. *Electroencephalogr Clin Neurophysiol* 1990;76(3):258–67.
- 34 Blok BF, Sturms LM, Holstege G. A PET study on cortical and subcortical control of pelvic floor musculature in women. *J Comp Neurol* 1997 22;389(3):535–44.
- 35 Nouhaud FX, Caremel R, Leroi AM, Grise P. Bladder reinnervation with creation of a 'somato-autonomic' reflex pathway in spinal cord injured or spina bifida, a new way for treatment? *Prog Urol* 2011;21(8):501–7.