

## ORIGINAL ARTICLE

# Optimal timing of operation for repairing atonic bladder after medullary cone injury: an experimental study in rats

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**Objective:** To determine optimal timing of operation for repairing atonic bladder after medullary cone injury in rats.

**Materials and methods:** In all, 56 adult female Sprague–Dawley rats were equally randomized into seven groups: normal control group, and 4w, 6w, 8w, 10w, 12w and 16w groups after medullary cone injury, assigned as groups A–G. The model was established by sharp transaction of spinal cord at the level of L<sub>4/5</sub> vertebral body. Bladder weight, cross-sectional area and ultrastructure of the detrusor muscle and its neuromuscular junction (NMJ), fibrotic change, and  $\alpha$ -smooth muscle antibody ( $\alpha$ -SMA) expression in the detrusor muscle were examined individually.

**Results:** Bladder weight in groups E–G was significantly increased than that in group A ( $P < 0.05$ ). And cross-sectional area of detrusor muscle fiber in groups E–G was significantly smaller than that in group A ( $P < 0.05$ ). Transmission electronic microscopy showed that the number of synaptic vesicles, mitochondria and other organelles in NMJ decreased markedly in group E. In groups F and G, NMJ further degenerated with synaptic vesicles and organelles decreased or even disappeared. Masson's stain showed that the proportion of connective tissue in the detrusor muscle of groups E–G was significantly different from that of group A ( $P < 0.05$ ).  $\alpha$ -SMA expression in the detrusor muscle decreased with the lapse of time.

**Conclusions:** The 10th week after rat medullary cone injury can be regarded as the time node when the detrusor muscle and NMJ undergo changes, and therefore surgical nerve repair should be performed before this.

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**Keywords:** medullary cone injury; atonic bladder; detrusor muscle; neuromuscular junction; treatment; rat

## Introduction

Thoracolumbar fracture is one of the most common clinical SCI (spinal cord injury), often causing medullary cone injury and resultant functional impairment to the sensory and motor nerves, or associated atonic bladder.<sup>1–3</sup> Severe urinary retention, refractory urinary infection or chronic renal failure, thus arising, is the main cause of death in SCI patients.<sup>4,5</sup> Functional reconstruction of controllable urination is, therefore, of vital significance with respect to decreasing mortality and improve the quality of life in these patients.

The basic principle of reconstructing the function of atonic bladder is intradural autogeneic neural anastomosis

with the normal somato-reflex above the paraplegic level.<sup>6–8</sup> Xiao *et al.*<sup>8</sup> successfully established the skin–spinal cord–bladder reflex path in rats by anastomosing the central end of the ventral root of L<sub>4</sub> and the peripheral end of the ventral root of L<sub>6</sub>, with the dorsal root of L<sub>4</sub> kept intact. This new concept of establishing an artificial bladder reflex with somatic nerve-to-autonomic nerve anastomosis has far-reaching influence on the reconstruction of atonic bladder. Our earlier studies had successfully established an abdominal reflex–spinal cord–bladder reflex path to restore controllable urination either in rats or in Beagle dogs,<sup>9</sup> based on which we anastomosed the ventral root of T<sub>11</sub> and the ventral root of S<sub>2</sub> with sural nerve transplantation and restored self-controlled urination successfully in a woman patient with medullary cone injury induced atonic bladder 4 month after the injury.<sup>10</sup> A 55-month follow-up showed that the patient had controllable urination trigger by constriction of the detrusor muscle as was confirmed by urodynamic testing.

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Above all, both experiments and clinical trials have demonstrated the feasibility of using abdominal reflex–spinal cord–bladder reflex path to reconstruct controllable urination in medullary cone injury induced atonic bladder.

However, previous studies mostly focused on the nerve reflex pathway of atonic bladder, and there are few studies about related morphological and functional changes of the target organ, that is, the detrusor muscle. For example, what changes may occur in the detrusor muscle and its neuromuscular junction (NMJ) after medullary cone injury? Do the unaffected lowest level intact neurons in or near the bladder wall have any nourishing function on the detrusor muscle? If so, how long can this function last? The present study was designed to observe morphological changes of the detrusor muscle and NMJ by light or electron microscopy and Masson's stain in a rat atonic bladder models in an attempt to find out the laws of degeneration in the detrusor muscle and NMJ and thus provide experimental data for selecting an optimal timing for reconstruction of atonic bladder in SCI patients.

## Materials and methods

### *Animals and treatment*

A total of 56 adult female 7-week-old Sprague–Dawley rats were equally randomized into seven groups: normal control group, and 4w, 6w, 8w, 10w, 12w and 16w groups after medullary cone injury, assigned as groups A–G. All rats were housed in cages individually.

The rat medullary cone injury model of groups B–G was prepared as follows: the rat was weighed and anesthetized with intraperitoneal injection of 1% sodium pentobarbital ( $40 \text{ mg kg}^{-1}$ ). The animal was placed in a prone position with the four extremities immobilized abductly and sterilized routinely. Laminotomy was performed at L4–5 vertebral plain under an operation microscope and the spinal cord was epidurally transected sharply with microsurgical scissors. The wound was sterilized postoperative before healing. The paraplegic animals were assisted with urination by pressing the abdomen 2–3 times daily.

The animals in all groups survived, without occurrence of wound infection or self-eating of the limbs. Digital ulceration was observed in three rats in groups E–G, which improve after disinfection and dressing. The animals in each group were killed at the designated time points to procure the detrusor muscle for observation.

### *Macroscopic observation of bladder*

After laparotomy, the macroscopic appearance of bladder was observed. Then, after removing the fatty tissues around the bladder and absorbing the water completely, the bladder was weighted individually by electronic balance.

### *Histological observation of the detrusor muscle*

*Calculation of the cross-sectional area of the muscular fibers.* The detrusor muscle was fixed in 10% formalin for 40 h, dehydrated, paraffin embedded, sliced along the middle of the smooth muscle into  $5 \mu\text{m}$  sections,

hematoxylin and eosin stained, and analyzed by the FW4000 digital imaging workstation (Leica Inc., Solms, Germany) to calculate the mean value of the cross-sectional areas of the muscle fibers.

*Electron microscopic observation of the ultrastructure of the detrusor muscle and NMJ.* The detrusor muscle was double fixed in 4% taraldehyde and osmic acid, dehydrated with pyroracemic acid, embedded with EPON812, sliced by LKBNOVA ultramicrotome (LKB Inc., Bromma, Sweden), double dyed with lead and uranium and observed by the Philips CM120 transmission electron microscope (Philips Inc., Amsterdam, The Netherlands) for the ultrastructure of the detrusor muscle and NMJ, mainly including changes of the synaptic vesicles and organelles.

### *Analysis of fibrotic components in the detrusor muscle*

The detrusor muscle was dyed with Masson's trichrome as follows: (1) the muscle was fixed with alcohol–glacial acetic acid–formaldehyde solution, routinely dehydrated, paraffin embedded, sliced into  $2 \mu\text{m}$  sections and deparaffinized; (2) the sections were fixed with Bouin's solution for 30 min and rinsed with lotic water; (3) the nucleus was dyed with hematoxylin for 10 min and rinsed with lotic water; (4) the sections were dyed with ponceau 2R, fuchsin acid and biebrich scarlet for 10–20 min and rinsed with distilled water; (5) the sections were dyed with 1% phosphomolybdic acid for 3–5 min, and without wash, dyed with 2% aniline blue for 5–10 min; (6) the sections were differentiated with 0.5% acetic acid solution, dehydrated with 95–100% absolute alcohol, hyalinized with xylene and mounted with neutral gum.

*Conclusion on the results.* Collagen fibers were blue; muscular fibers and endochylema were red; and the nuclei and red blood cells were purple. The percentage of the smooth muscle and the connective tissue against the total area of the specimen was calculated by Leica FW4000 digital imaging workstation for analysis of fibrosis of the atonic bladder.

### *Immunohistochemical staining of $\alpha$ -SMA in the detrusor muscle*

$\alpha$ -Smooth muscle antibody ( $\alpha$ -SMA) of the detrusor muscle, was stained by the automatic immunohistochemical staining system (Ventana Medical Systems Inc., Tucson, AZ, USA), using anti- $\alpha$ -SMA (Santa Cruz Inc., Santa Cruz, CA, USA) antibody as the primary antibody, and mouse anti-rabbit antibody as the secondary antibody (Ventana Medical Systems Inc.). The integral optical density (IOD) of the  $\alpha$ -SMA was analyzed by FW4000 digital imaging workstation. Ten fields ( $10 \times 10$ ) were chosen in each section to measure the mean IOD of the  $\alpha$ -SMA.

### *Statistics*

Means and standard deviations were calculated from individual values using standard procedures. One-way analysis of variance was used to determine significant differences between the groups, and with Student–Newman–Keuls test

for multiple comparisons. Differences were considered significant when  $P < 0.05$ .

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

## Results

### Macroscopic observation of bladder

The macroscopic observation showed bladder volume increased, bladder wall thickened and congestion of bladder aggravated with the lapse of time. Also, there was bladder stone formation in three cases of group G due to urinary tract infection.

The bladder weight in groups B–F was gradually increased with the lapse of time. The bladder weight in groups A–G was  $0.138 \pm 0.019$ ,  $0.286 \pm 0.025$ ,  $0.362 \pm 0.018$ ,  $0.412 \pm 0.082$ ,  $0.496 \pm 0.022$ ,  $0.589 \pm 0.023$  and  $0.721 \pm 0.020$  g, respectively. Bladder weight in groups E–G significantly increased than that in group A ( $P < 0.05$ ). Furthermore, multiple comparisons revealed that there was significant difference of bladder weight between groups E and G ( $P < 0.05$ ) (Table 1).

### Histological observation of the detrusor muscle

In group A, normal detrusor fibers were fusiform in shape and funicular in arrangement; the muscular cells were well arranged and parallel to cell bundles. No infiltration of the connective tissue was seen in muscle bundles. In groups B–G, the muscular cells were crescent or irregular and deranged. Infiltration of the connective tissue was seen in muscle bundles and became worse with the lapse of time (Figure 1).

The detrusor muscle in groups B–F were gradually atrophic with the lapse of time. The cross-sectional area of muscle fibers in groups A–G was  $7265.86 \pm 114.36$ ,  $5156.31 \pm 310.42$ ,  $5398.47 \pm 453.08$ ,  $4146.49 \pm 284.06$ ,  $3067.40 \pm 130.91$ ,  $2123.56 \pm 235.66$  and  $1675.98 \pm 322.09 \mu\text{m}^2$ , respectively. The cross-sectional area of muscle fibers in groups E–G was significantly smaller than that of group A ( $P < 0.05$ ), although it was not significantly different between group A and groups B, C and D. Furthermore, multiple comparisons indicated that there was significant difference of cross-sectional area of muscle fibers between groups E and G ( $P < 0.05$ ) (Table 1).

### Ultrastructure of the detrusor muscle

In group A, normal detrusor cells were well arranged and well distributed with consistent contours and size; the distance between muscular cells was much smaller than the abnormal specimens with proper intermediate junction. Few collagen fibers and elastic fibers were seen in the matrix. Organelles such as mitochondria and endocyttoplasmic reticulum (ER), myofilament and dense body were well organized in smooth muscle cells. In groups B–G, degeneration of the ultrastructure of detrusor cells deteriorated with the lapse of time, presenting as inconsistent contours, malalignment and derangement of cells with wider space between them.

Abundant irregular collagen fibers and few elastic fibers were seen in the interstitial. The rough endoplasmic reticulum was dilated markedly, and the mitochondria were edematous, or even underwent vacuolar or globular changes. The course of myofilaments was malaligned. The nuclei were umbilicate, with few nucleoles seen in them.

### Ultrastructures of NMJ

In group A, the normal structure of NMJ was seen between cells of the detrusor muscle. Lots of organelles including mitochondria and ER and synaptic vesicles were seen in the sympathetic nerve endings. The ultrastructure of NMJ in groups B–D was similar to that in group A, except that the number of mitochondria and synaptic vesicles was a bit smaller. In group E, the structure of NMJ was apparently degenerated, where the reducti were deranged or disappeared, and the number of synaptic vesicles and organelles including mitochondria and ER in sympathetic nerve endings reduced greatly. In group F, the structure of NMJ further deteriorated, where synaptic vesicles in sympathetic nerve endings, mitochondria and ER further decreased in number or even disappeared, and degenerated corpuscles were seen. NMJ degeneration in group G was even more severe, with deficiency of synaptic vesicles, and large numbers of degenerated corpuscles were seen (Figure 2).

### Analysis of fiber components

In group A, normal detrusor cells were funicular and well arranged; the arrangement of nuclei was parallel to muscular bundles; small amounts of collagen was seen between the muscle bundles without evidence of infiltration and well distributed with consistent contours and size. In groups B–G,

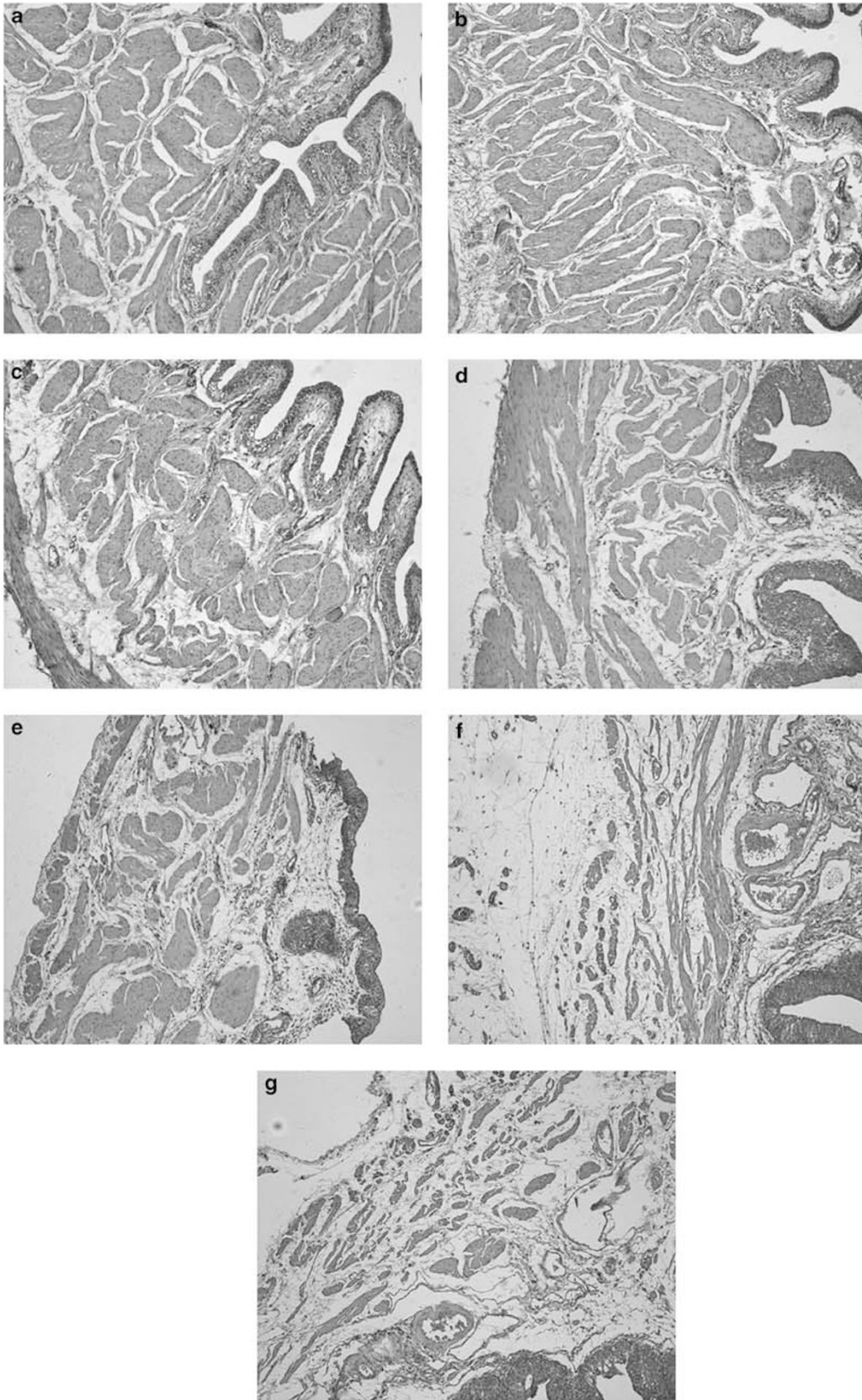
**Table 1** Detrusor muscle changes in rats after medullary cone injury ( $n = 8$ ,  $\bar{x} \pm s$ )

Group	Bladder weight (g)	Cross-sectional area of muscle fibers ( $\mu\text{m}^2$ )	Percentage of connective tissue (%)	$\alpha$ -SMA activity (IOD)
A	$0.138 \pm 0.019$	$7265.86 \pm 114.36$	$12.08 \pm 1.45$	$0.96 \pm 0.03$
B	$0.286 \pm 0.025$	$5156.31 \pm 310.42$	$18.47 \pm 1.32$	$0.85 \pm 0.10$
C	$0.362 \pm 0.018$	$5398.47 \pm 453.08$	$23.24 \pm 2.76$	$0.74 \pm 0.06$
D	$0.412 \pm 0.082$	$4146.49 \pm 284.06$	$26.29 \pm 1.88$	$0.69 \pm 0.09$
E	$0.496 \pm 0.022^{\text{a,b}}$	$3067.40 \pm 130.91^{\text{a,b}}$	$35.67 \pm 1.12^{\text{a,b}}$	$0.44 \pm 0.08^{\text{a}}$
F	$0.589 \pm 0.023^{\text{a}}$	$2123.56 \pm 235.66^{\text{a}}$	$47.22 \pm 2.38^{\text{a,b}}$	$0.39 \pm 0.10^{\text{a}}$
G	$0.721 \pm 0.020^{\text{a}}$	$1675.98 \pm 322.09^{\text{a}}$	$55.56 \pm 3.12^{\text{a}}$	$0.36 \pm 0.07^{\text{a}}$

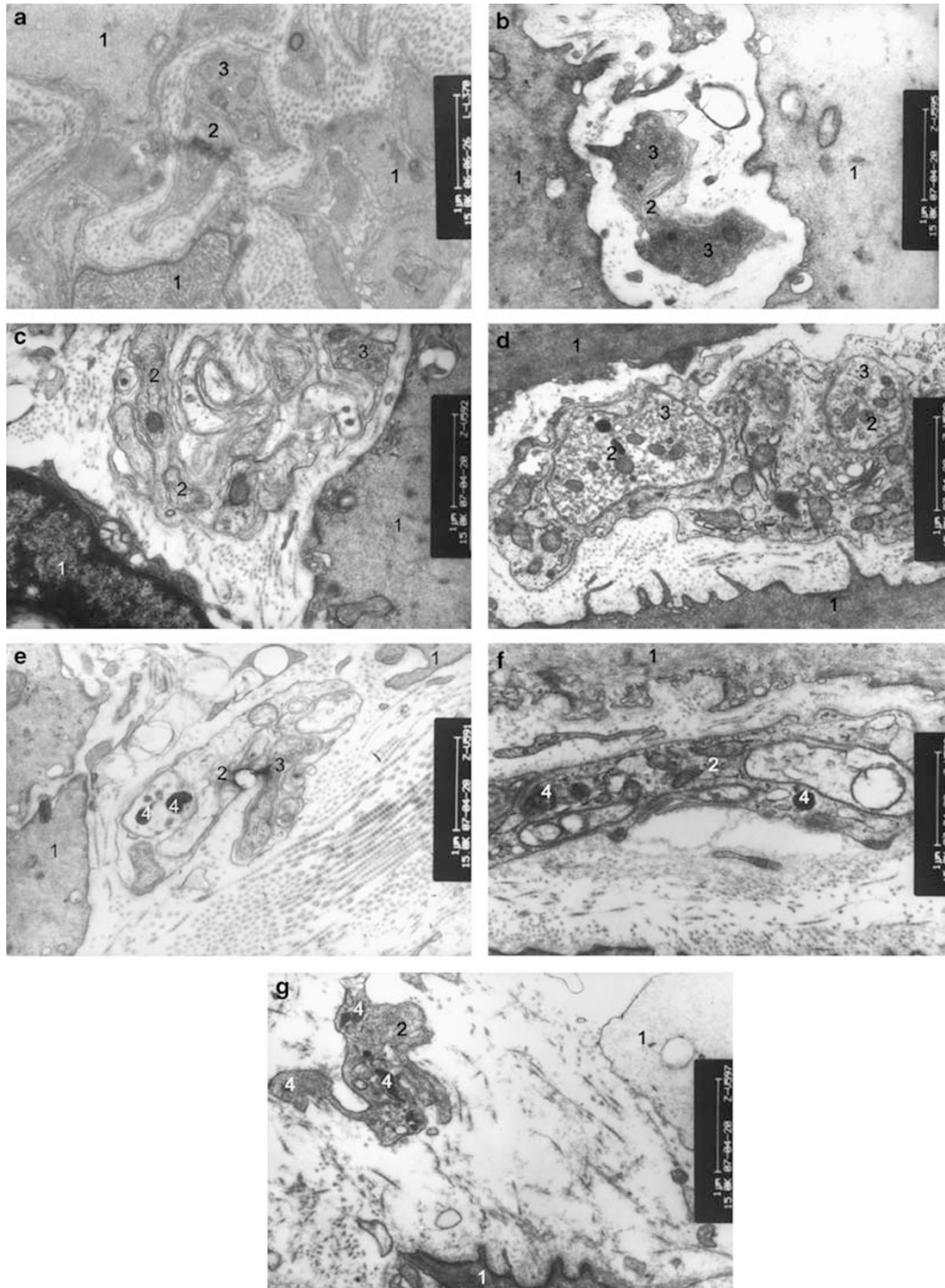
Abbreviations: IOD, integral optical density;  $\alpha$ -SMA,  $\alpha$ -smooth muscle antibody.

<sup>a</sup>Compared with group A,  $P < 0.05$ .

<sup>b</sup>Compared with group G,  $P < 0.05$ .



**Figure 1** Transverse sections of the detrusor muscle in Sprague-Dawley rats of normal control (a); 4w (b), 6w (c), 8w (d), 10w (e), 12w (f) and 16w (g) after the medullary cone injury stained by hematoxylin and eosin for analysis of the cross-sectional areas of muscle fibers ( $\times 100$ ).

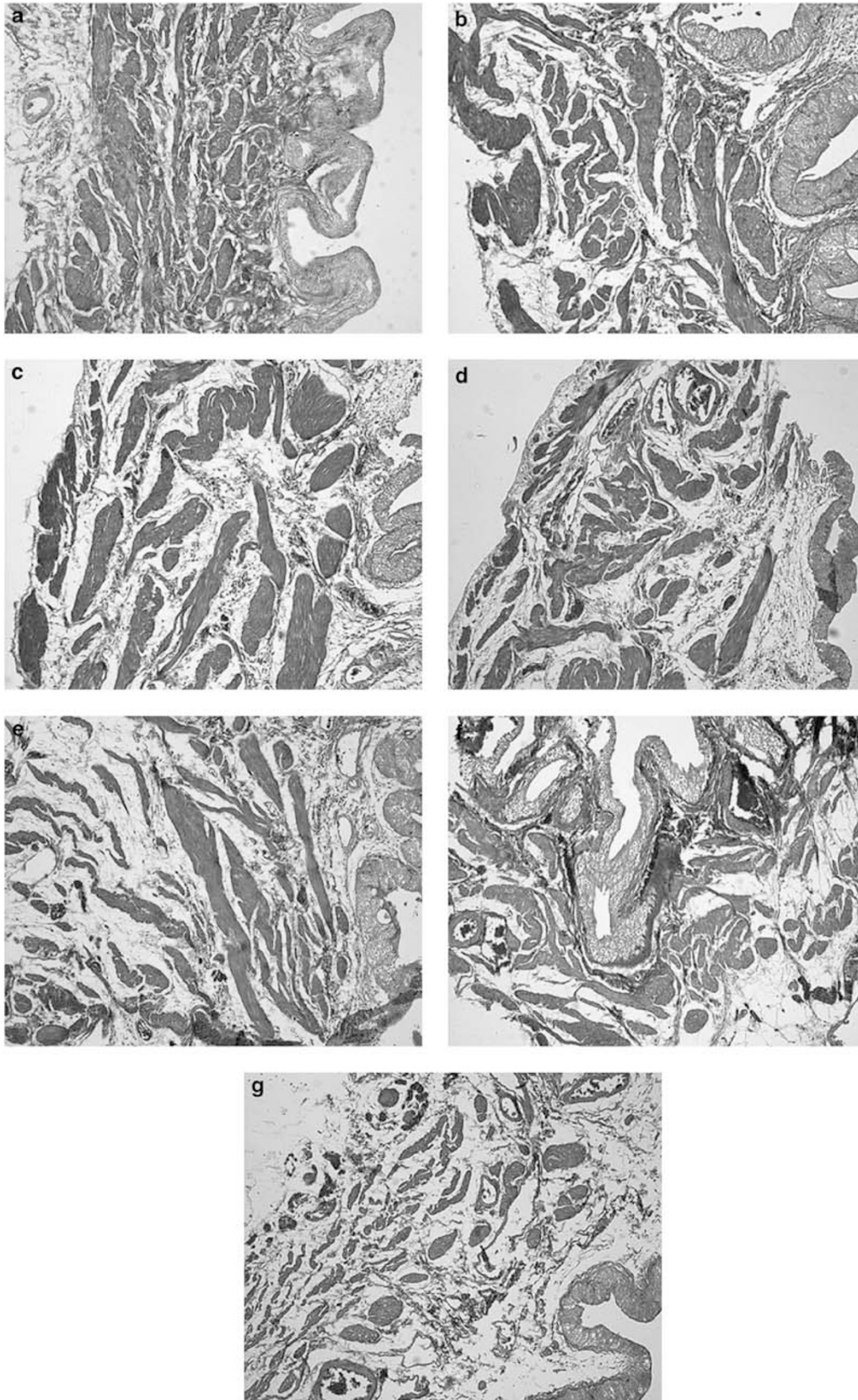


**Figure 2** Ultrastructures of the detrusor muscle and its neuromuscular junction observed by the Philips CM120 transmission electron microscope in Sprague-Dawley rats of normal control (a); 4w (b), 6w (c), 8w (d), 10w (e), 12w (f) and 16w (g) after the medullary cone injury. (1) Detrusor muscle; (2) neuromuscular junction; (3) synaptic vesicles; (4) degenerative corpuscle. ( $\times 15\,000$ ).

fibrosis of the detrusor muscle became worse and the bundles became smaller with the lapse of time. The smooth muscle cells were deranged, and large numbers of collagen fibers were seen between the muscle bundles, with collagen infiltration (Figure 3).

Fibrosis of the detrusor muscle deteriorated in varying degree with the lapse of time in all postoperative groups. The

percentage of connective tissue in the detrusor muscle in groups A-G was  $12.08 \pm 1.45$ ,  $18.47 \pm 1.32$ ,  $23.24 \pm 2.76$ ,  $26.29 \pm 1.88$ ,  $35.67 \pm 1.12$ ,  $47.22 \pm 2.38$ ,  $55.56 \pm 3.12\%$ , respectively. There difference between group A and groups E, F and G was significant ( $P < 0.05$ ), and insignificant between group A and groups B, C and D ( $P > 0.05$ ). Furthermore, multiple comparisons revealed that there were significant



**Figure 3** Transverse sections of the detrusor muscle in Sprague-Dawley rats of normal control (a); 4w (b), 6w (c), 8w (d), 10w (e), 12w (f) and 16w (g) after the medullary cone injury stained by Masson's trichrome for analysis of the fibrosis in detrusor muscle ( $\times 100$ ).



differences of the percentage of connective tissue between group G and groups E and F individually ( $P < 0.05$ ) (Table 1).

#### Expression of $\alpha$ -SMA

Immunohistochemical staining demonstrated that brown-stained  $\alpha$ -SMA distributed mainly in the detrusor muscle. After transection of the spinal cord,  $\alpha$ -SMA of the detrusor muscle turned light brown.

The expression of  $\alpha$ -SMA of the detrusor muscle gradually decreased with the lapse of time. The mean IOD of  $\alpha$ -SMA in groups A–G was  $0.96 \pm 0.03$ ,  $0.85 \pm 0.10$ ,  $0.74 \pm 0.06$ ,  $0.69 \pm 0.09$ ,  $0.44 \pm 0.08$ ,  $0.39 \pm 0.10$  and  $0.36 \pm 0.07$ , respectively. The mean IOD of  $\alpha$ -SMA in groups E–G was significantly lower ( $P < 0.05$ ) than that of group A, but there was no significant difference between group A and groups B, C and D. However, multiple comparisons revealed that there was no significant difference of IOD of  $\alpha$ -SMA in groups E–G ( $P > 0.05$ ) (Table 1).

## Discussion

Medullary cone injury often leads to occurrence of atonic bladder.<sup>11,12</sup> At present, clean intermittent catheterization is a commonly recommended procedure for people with this condition, whereas the urination is still uncontrollable.<sup>13</sup> Fortunately, the feasible technique to restore controllable urination is autogeneic neural anastomosis intradurally with the normal somato-reflex above the paraplegic level. Previous studies had revealed the efficiency of the artificial reflex path both in experiments and in clinical trials.<sup>6–8</sup> The concomitant problem is the optimal timing of operation for these patients. Atonic bladder is one of the manifestations of the lower motoneurons paralysis, with which the target organ can be degenerated because of denervation and loss of nourishment by the elementary central nerves. Therefore, nerve anastomotic procedures should be performed before irreversible changes of the detrusor muscle and NMJ occur.

A review of the literature shows that most studies focus on the motor end plate and the NMJ of skeletal muscle, and studies about the NMJ of smooth muscle mainly involve gastrointestinal motility disorders and gastrointestinal tract tumors.<sup>14–16</sup> There have been few studies reporting the NMJ of detrusor muscle. On the one hand, previous studies suggested that the NMJ of smooth muscle was different from that of skeletal muscle. Also, the NMJ of smooth muscle without independent ending structures was distributed surrounding the target muscle in the form of thin neuroplexus. Furthermore, the neurotransmitter releasing from the nerve terminal might stimulate more adjacent muscular fibers in a diffusive way.<sup>17–20</sup> These findings were consistent with those in our present study. On the other hand, the detrusor muscle is innervated by the parasympathetic nerve through a model of twice neuron exchanging. So in patients with medullary cone injury, the lowest level neurons localized in or near the bladder wall, which might offer neurotrophs to some extent, were often intact. However, our present study revealed that this neurotrophs of the lowest level neurons is limited.

Consistent with our hypothesis that the detrusor muscle and its NMJ would sooner or later irreversibly degenerate after medullary cone injury along with the prolonging of denervation, we observed that obvious degeneration occurred in the 10th week postoperatively. In this study, we did not find obvious degeneration of the NMJ but a slight reduction in the number of mitochondria and synaptic vesicles within 8 weeks after medullary cone injury in the rats, and from the 10th week on conspicuous degeneration of the NMJ appeared, presenting as derangement or disappearance of the reductus, and a marked reduction in the number of synaptic vesicles, organelles including mitochondria and ER in the sympathetic nerve endings. In the 12th or 16th week after injury, the number of synaptic vesicles and mitochondria reduced markedly or even disappeared, and degenerated corpuscles were seen. So, significant degeneration of the NMJ occurred in the 10th week postoperatively. In addition, the results indicated that the cross-sectional area of muscular fibers and the percentage of connective tissue in the detrusor muscle were significantly different from the control group from the 10th postoperative week. These findings suggest that gradual muscular atrophy and fibrosis of the detrusor muscle were consistent with the degeneration of NMJ. Finally,  $\alpha$ -SMA was a microfilamin with contractibility. Immunohistochemical staining demonstrated that brown  $\alpha$ -SMA were distributed mainly in the detrusor muscle. After transection of the spinal cord,  $\alpha$ -SMA of the detrusor muscle decreased and turned light brown. In our study, the expression of  $\alpha$ -SMA in the detrusor muscle decreased along with the prolonging of denervation. There was significant difference between the control group and group E from the 10th postoperative week. Decreased expression of  $\alpha$ -SMA may lead to declined contractibility of the detrusor muscle and occurrence of a dynamic and big bladder, which might play a significant role in the development of atonic bladder.

In conclusion, conspicuous changes of the detrusor muscle and its NMJ occurred from the 10th week after medullary cone injury in rats, and, therefore, the nerve repairing procedure should be performed before this time point. As there may be species differences between human beings and rats, more studies are needed to find out optimal operation timing for restoring autogeneic urination in patients with atonic bladder.

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