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ORIGINAL ARTICLE

Expression of autophagy in different stages of neurogenic bladder after spinal cord injury in rats

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Study design: Experimental study.
Objectives: To investigate the expression of autophagy in different stages of the neurogenic bladder after spinal cord injury (SCI) in rats.
Setting: Second Hospital of Shandong University, Jinan, China.
Methods: A total of 36 Wistar rats were divided into the SCI and control groups. In total, six animals were killed and sampled from each group at 1, 4 and 14 days after surgery of T10–T11 level. BBB scale, residual urine volume and urinary bladder function score were estimated at each time point. The expression of microtubule-associated protein 1 light chain 3 (LC3) and P62 was detected using western blot analysis, immunofluorescence staining or real-time PCR (RT-PCR).
Results: The locomotor functions of the hindlimbs and the bladder function of the SCI group rats were lost after surgery, but gradually recovered from 1 day. Western blot showed that the LC3-II/actin was higher in the SCI than in the control group. Immunofluorescence staining revealed that LC3 and P62 were expressed in bladder smooth muscle cell. RT-PCR showed a remarkably increased LC3 mRNA expression at 1, 4 and 14 days in the SCI than in the control group. The P62 mRNA level of the SCI bladder tissues did not differ from that of the control group at 1 day but decreased at 4 and 14 days after surgery.
Conclusions: Autophagy is activated during the recovery of the bladder after SCI and sustained. Autophagy may play an important role in bladder neurogenesis and may represent one of the mechanisms of bladder self-repair.
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INTRODUCTION

Autophagy is a normal physiological process that maintains homeostasis and normal functioning via a pathway by which cytoplasmic constituents are sequestered in a double-membrane-bound structure, the autophagosome, and delivered to the lysosome for degradation. Autophagy is a highly conserved protein degradation pathway.[1] It includes alterations of the autophagy-related proteins and pathways that are involved in pathophysiological processes of various diseases of organs and tissues, such as inflammation, aging, metabolism and infection. During these diseases, the protective function of autophagy is blocked and reduced, indicating that the autophagy disorder and the occurrence of these diseases are highly correlated.[2,3] Autophagy is involved not only in the maintenance of the balance between protein synthesis and degradation but also in the execution of cell death, known as autophagic cell death.[4] Autophagy has been demonstrated to be increased in some experimental models of traumatic brain injury, excitotoxicity and in patients with Alzheimer's disease or critical illness.[5] A number of proteins that regulate autophagy have been reported, including Beclin-1, microtubule-associated protein 1 light chain 3 and Cathepsin D.[6,7]

Autophagy can also occur in the smooth muscle, such as blood vessels, respiratory tract and corpus cavernosum, suggesting that it plays a vital role in tissue protection.[8–10] Autophagy exists at low level in the bladder smooth muscle, thereby involved in the maintenance of homeostasis.[11]

Spinal cord injury (SCI) is a central nervous system injury, which always leads to serious neurological complications in the limbs and body below the damaged spinal cord segments.[12] Neurogenic bladder is one of the sensory and autonomic dysfunctions, which is a serious problem for those with SCI.

Neurogenic bladder after SCI often manifest as bladder detrusor hyperactivity and poor bladder compliance clinically, which leads to bladder deformity, bladder capacity decrease, hydronephrosis and vesicoureteral reflux, and renal failure, thereby affecting the quality of life and even leading to death.[13,14] Although the molecular mechanisms involved in the impairment of the affected spinal cord or neural tissue have been well-studied, little has been reported about the expression and changes in mechanism underlying the sensory and autonomic dysfunctions induced by SCI, such as neurogenic bladder.[15,16]

However, little is known about the role and function of autophagy in detrusor disorder of neurogenic bladder after SCI. In the present study, we established a neurogenic bladder model after SCI and observed the role of autophagy in the process of neurogenesis of the bladder in different stages.

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MATERIALS AND METHODS

Experimental animals

The adult female Wistar rats (8–9 weeks, 270–290 g, $N=36$) were provided by The Experimental Animal Center of Shandong University. The animals were housed four or five per cage at 24 °C with free access to water and food before and after surgery. All the experimental procedures were in compliance with the guidelines for animal scientific procedures approved by Shandong University Ethics Committee.

SCI model and grouping

The rats were randomly divided into the SCI group ($N=18$) and the control group ($N=18$). Both groups were subjected to kinematic scoring at 1, 4 and 14 days after surgery. Six experimental animals were killed and sampled from each group at every time point.

The control group underwent only the laminectomy, which was performed at the T10–T11 level, but not SCI. The experimental rats in the SCI group underwent laminectomy, following which the SCI model was established with a modified Allen's method.

The rats were anesthetized with 10% chloral hydrate (3 ml kg^{-1} body weight) administered by intraperitoneal injection. After successful anesthetization, the rats were shaved on the back and placed in a prone position on the operating table. The T10–11 spinous processes at the back of the rats were located, and the T10–11 spinal cord area was exposed. The SCI was induced using a modified Allen's method. A 5 g rod was dropped from a vertical distance of 20 cm onto the T10–11 level of the exposed spinal cord. In this process, the hindlimbs and tails of the rats showed spastic convulsions, which last for a few seconds. After the surgery, the muscle, subcutaneous tissue and skin were sutured in layers. During the surgery, the temperature was monitored and maintained at 37.0 ± 0.5 °C by a heating pad.

To prevent the infection, all rats received an intraperitoneal injection of normal saline (1 ml) immediately after surgery at once and antibiotics for 3 days.

Evaluation of the hindlimb locomotor function

To evaluate the bilateral hindlimb locomotor function, we used the Basso, Beattie and Bresnahan (BBB) scale in an open field.[17] The BBB score is 0 for no hindlimb movement and 21 for normal movement. The BBB scores of the rats were determined at 1, 3, 5, 7 and 14 days on a smooth and open floor. All the locomotor tests were recorded for a minimum of 4 min with a digital camcorder and were interpreted by two observers cognizant of the score but did not participate in the study.

Evaluation of the bladder function

The urodynamic study was used to evaluate the bladder function in several assays; however, this method was invasive and injurious to the rat bladder. The damage could cause unnecessary interference in the experimental study. Thus, we selected two noninvasive methods to evaluate the bladder function.

(1) Residual urine volume. Bladder expression was performed twice per day. The bladder emptying was carried out by applying pressure to the lowermost central part of the belly using the index and the middle finger. The expelled urine is collected in a tube, and the volume is recorded.[18]
(2) Urinary bladder function score (UBFS). We used the four-point UBFS,[19] which was evaluated daily. The standard for evaluation was as follows: 0 = complete loss of function (manual expression of bladder 3× a day, the urine contains blood), 1 = partial recovery of UBF (manual expression 2× a day, urine may contain blood), 2 = partial recovery of UBF (partial/complete release of sphincter spasm, manual expression 1× a day, blood persistent in urine), 3 = advanced recovery (manual expression 1× a day, no blood in urine), 4 = physiological UBF.

Western blot analysis

To detect the expression of the autophagy-related proteins, we applied western blot analysis. The rats were killed at 1, 4 or 14 days after operation. The bladders were homogenized and collected in radio-immunoprecipitation assay lysis buffer with supplementary 1 mM phenylmethanesulfonyl fluoride (Beyotime Biotechnology, Shanghai, China), sonicated and centrifuged to collect the supernatant. The protein concentration was estimated using a bicinchoninic acid (BCA) assay. An equivalent of 40 μg total protein was resolved on a 5–12% SDS-PAGE and transferred to the polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). Then, the membrane was blocked with 5% non-fat milk in TBS with 0.1% Tween 20 for 2 h, followed by incubation overnight at 4 °C with primary antibodies according to the manufacturer's recommendations. Subsequently, the membranes were washed thrice with TBST for 5 min each, incubated with horseradish peroxidase-conjugated secondary antibody in 5% non-fat milk-TBST for 1 h and washed with TBST four times for 7 min each. The intensity of the immunoreactive bands of LC3 were quantified by Image J (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence staining

To confirm the locations of the autophagy-related proteins, we used immunofluorescence staining. The rats in the SCI and control groups were killed at each time point and their bladders fixed with 4% paraformaldehyde for >24 h. The bladder tissues were paraffin-embedded, and then sliced transversely into 5 μm. The sections were deparaffinized, rehydrated and incubated in 3% hydrogen peroxide for 10 min, followed by antigen retrieval for 13 min. Then, the sections were blocked with 5% albumin from goat serum in a 37 °C oven for 1 h, followed by incubation with primary antibodies overnight at 4 °C. The incubation with the fluorescent Alexa488 goat anti-rabbit secondary antibody was 1 h at room temperature, followed by DAPI for 10 min. The sections were finally washed in PBS and sealed with a coverslip. The images were captured on a Nikon ECLIPSE Ti microscope (Nikon, Tokyo, Japan) and analyzed by Image Pro Plus (Media Cybernetics, Inc, Rockville, MD, USA).

Quantitative RT-PCR

To assess the expression of the autophagy-related genes, we applied quantitative RT-PCR. Tissues were homogenized in ice-cold TRIzol reagent (Takara, Shiga, Japan). RNA was isolated by chloroform phase separation and alcohol precipitation and estimated using the Biowave DNA UV–Vis spectrophotometer (Biochrom, Cambridge, UK). cDNA was synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (Takara). The amplification was carried out using SYBR Premix Ex Taq (Takara). The PCR primers were synthesized (Takara) as follows: GAPDH forward: 5′-GGCACAGTCA AGGCTGAGAATG-3′; reverse: 5′-ATGGTGGTGAAGACGCCAGTA-3′. LC3 forward: 5′-CGAGAGCGAGAGAGATGAAGACGG-3′; reverse: 5′-GGTAAC GTCCCTTTTTGCCTTGGTA-3′. P62 forward: 5′-AGAATGTGGGGGAGAG CGTGGC-3′; reverse: 5′-GGGTGTCAGGCGGCTTCTCTT-3′.

Statistical analysis

All data are presented as the mean ± s.e.m. from three independent experiments. Statistical significance was examined using Student's *t*-test in the case of two experimental groups. $P<0.05$ was considered statistically significant. Statistics were calculated using SPSS version 17 (IBM, Chicago, IL, USA).

Statement of Ethics

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

RESULTS

Locomotor function assessment

The locomotor functions of both hindlimbs of the rats were assessed by the BBB rating scale.

The BBB scores for the experimental groups are summarized in Figure 1. The hindlimbs of the rats of the SCI group lost locomotor functions after surgery and were dragged behind. Starting at 1 day, the locomotor functions of the rats in the SCI group were gradually recovered. The BBB rating increased over time. However, the BBB

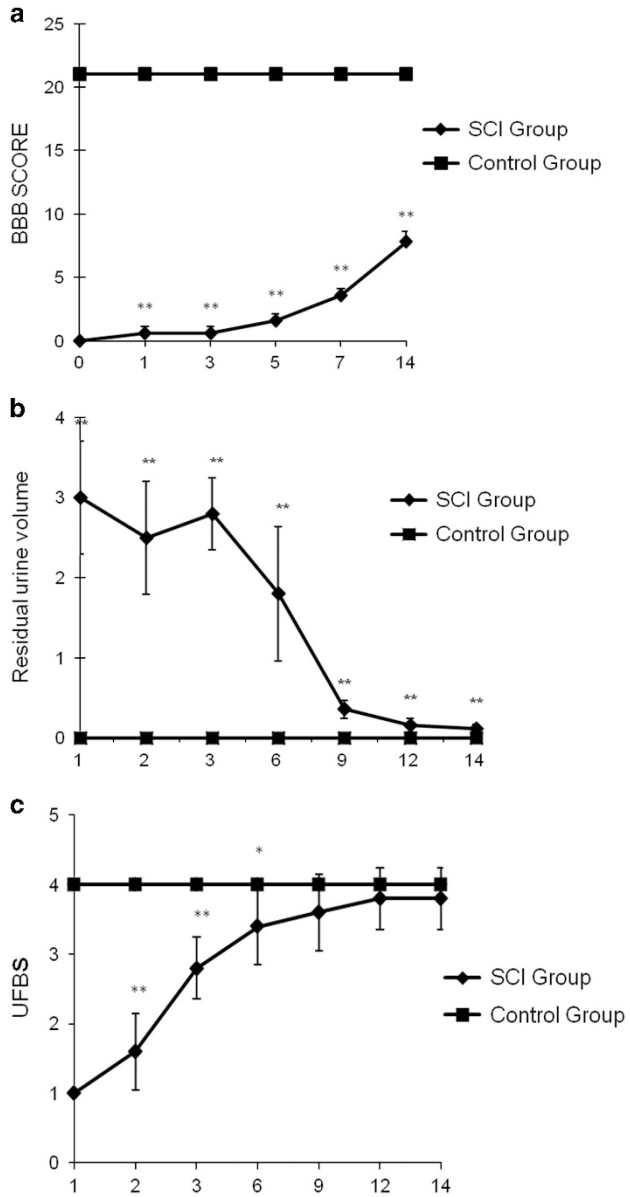

Figure 1 Assessment of motor functions of hindlimbs of the rats by the BBB rating scale, the bladder function of the rats by the residual urine volume and urinary bladder function scores (UBFSs) after experimental spinal cord injury. (**a**) The BBB scores of the rats were determined at 1, 3, 5, 7 and 14 days on a smooth and open floor. (**b**) The residual urine volume at 1, 2, 3, 6, 9, 12 and 14 days. (**c**) The UBFSs. Data presented as mean ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$.

scores were lower in the SCI groups as compared with those in the Control group ($P<0.05$).

Bladder function assessment

The bladder function of the rats was assessed by the residual urine volume and UBFS.

The residual urine volume for all rats is analyzed in Figure 1. The residual urine volumes were significantly increased in the SCI groups as compared with those in the sham group ($P<0.05$). However, the residual urine volume reduced gradually from 1 to 14 days after surgery, especially among 3 to 9 days.

The UBFSs for all the rats are summarized in Figure 1. The urinary bladder's detrusor activity was damaged after surgery but recovered gradually. The UBFSs were lower in the SCI group as compared with those in the control group at 1, 2, 3 ($P<0.01$) and 6 days ($P<0.05$). Moreover, the UBFSs showed no significant difference in the SCI group as compared with that in the control group at 9, 12 and 14 days.

Western blot analysis

The altered expression of the autophagy-related proteins such as LC3, in the bladder tissues of the rats at 1, 4 and 14 days after SCI, was analyzed by western blot.

Protein electrophoresis and optical density scanning and analysis revealed almost no expression in the control group. The LC3-II/actin expression was higher in the SCI group compared with that in the control group ($P<0.01$). The expression of the LC3-II was at maximum at 4 days in the SCI group (Figure 2).

Immunofluorescence staining

To observe the activation of autophagy in bladder after SCI, immunofluorescence staining of LC3 and P62 was performed. The LC3-expressing cells showed that punctate LC3 dots were located in the cytoplasm, and the P62-expressing cells showed that punctate P62 dots were also in the cytoplasm (Figure 3).

RT-PCR analysis

To further evaluate the expression of autophagy-related proteins, RT-PCR was employed to detect the mRNA level of LC3 and P62 (Figure 2). The RT-PCR analysis results showed that LC3 mRNA was significantly increased in the rats of the SCI group as compared with the rats of the control group at 1, 4 ($P<0.01$) and 14 days ($P<0.05$) after surgery. The expression level of LC3 mRNA was higher at 4 days as compared with that at 1 day and was lower at 14 days than at 4 days.

The RT-PCR analysis of P62 mRNA demonstrated no significant difference in the bladder tissues of the SCI group as compared to the bladder of the control group at 1 day after surgery. However, it was found that the P62 mRNA level was significantly decreased at 4 days in the SCI group compared with the control group, and was at a minimum at 14 days ($P<0.05$). Nevertheless, the expression level of P62 mRNA was higher at 14 days than at 4 days.

DISCUSSION

The SCI of the segment above the thoracic region affects the lower urinary tract function, as the normal descending control system was damaged.[20] Because of the continued excitement of the low-level micturition in the spinal cord, the detrusor convulsion was caused by the continuous contraction of the bladder, and the compliance of the bladder was decreased.[21] In addition, the neurogenic bladder dysfunction could be induced including bladder areflexia, hyperreflexia and detrusor-sphincter dyssynergia.[16] The bladder pathologies after SCI comprised the changes in the mechanical function and the tissue morphology such as hypertrophy and fibrosis.[22] Some studies found that proteins that are continuously expressed with different patterns in the bladder after SCI, such as transgelin and S100-A11, potentially play a role in the process of neurogenesis of the bladder.[16] However, the mechanisms underlying the observed changes in the bladder following SCI have not yet been elucidated.

Autophagy was detected in the vascular system, respiratory system and the corpus cavernosum,[8–10] suggesting its critical role in tissue protection. During autophagy, the cellular components that are required to be degraded are wrapped by the monolayer or bilayer

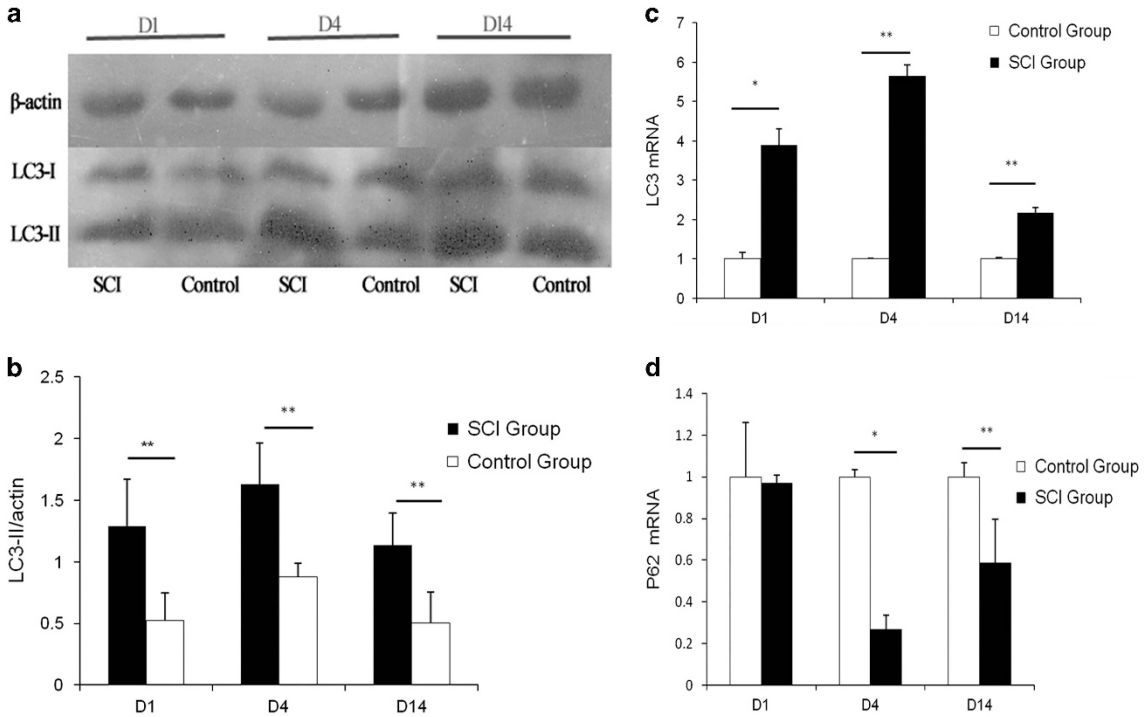

Figure 2 The altered expression of the markers of the autophagy in the bladder tissues of the rats at 1, 4 and 14 days after spinal cord injury. (**a**) Representative image of a western blot. (**b**) Quantifications of proteins LC3-II. (**c**) Quantifications of LC3 mRNA. (**d**) Quantifications of P62 mRNA. Data presented as mean ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$.

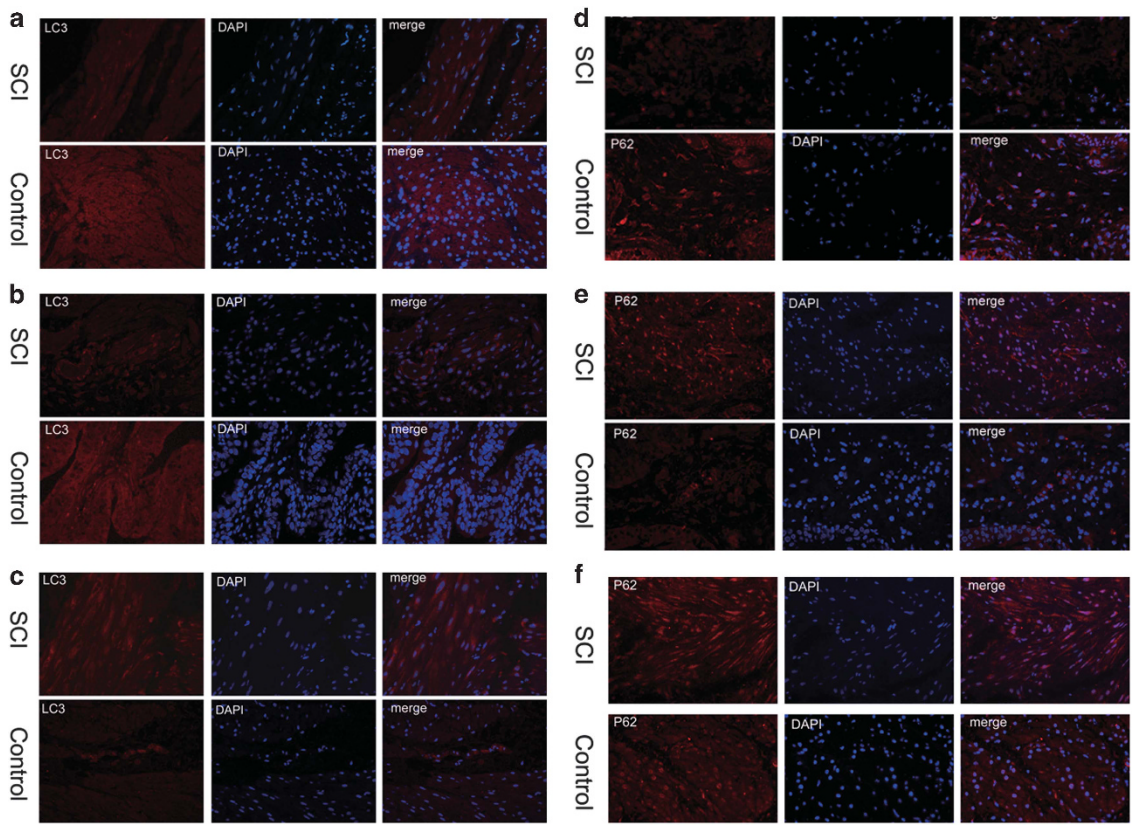

Figure 3 Representative photomicrographs of staining for the markers of the autophagy in the bladder detrusor muscle of rats at 1, 4 and 14 days after spinal cord injury (scale bar = 5 μm). Punctate LC3 dots were located in the cytoplasm (**a**–**c**), and punctate P62 dots were also in the cytoplasm (**d**–**f**).

membranes, forming autophagosomes, which are then transported to the lysosomes to generate autophagic lysosomes.[23] In the autophagic lysosomes, the digestion and degradation are conducted by multiple enzymes, thereby fulfilling the metabolic requirements of the cells, thereby renewing certain organelles.[24,25] Therefore, autophagy plays a pivotal role in preventing diseases, such as myopathy, cancer, microorganism infection, inflammation and neurogenic disease. Through autophagy, certain toxins and pathogens are wrapped, degraded and then eliminated. Thus, the activation of autophagy prevents the progression of the diseases.

Microtubule-associated protein 1 light chain 3 is a mammalian homolog of the yeast autophagy-related protein 8 (ATG8) gene. In the absence of autophagy, the intracellularly synthesized LC3 is processed and converted to LC3 type I, which is soluble in the cytoplasm and regularly expressed. During autophagy, LC3 type I undergoes ubiquitin-like modification and binds to phosphatidylethanolamine on the surface of the autophagic vacuole membranes, thereby forming LC3-II.[26] LC3-II is recruited from the cytosol and associated with the phagophore early in autophagy. This localization serves as a marker for autophagic membranes in order to monitor the development of the process.[27]

P62 is a receptor for cargo destined to be degraded by autophagy, including ubiquitinated protein aggregates designated for clearance.[28] The P62 protein can interact with ubiquitin and also LC3, thus targeting the autophagosome and facilitating clearance of the ubiquitinated proteins.[29] P62 is a multifunctional protein that interacts with LC3 and transports the altered proteins to degradation by autophagy.[30]

The presence of autophagy in the normal rat bladder smooth muscle cells is at a low level[5] and may play a vital role in the maintenance of homeostasis in bladder smooth muscle cells. However, it was unclear whether autophagy was involved in the process of bladder neurogenesis. In order to illustrate the role of detrusor myocytes autophagy, samples from a trauma-induced SCI neurogenic bladder model were used. In the present study, the neurogenic bladder was successfully induced by the T10–11 trauma of rats, as evidenced by the results of the BBB score, the residual urine volume and the UBFS.

The result of the BBB score, which was used to evaluate the locomotor function, showed that the function was damaged first, then recovered gradually after SCI shock stage. To evaluate the bladder function, the residual urine volume was estimated, which was increased after SCI and reduced gradually, and the UBFSs were decreased after SCI and increased gradually, especially in the period of 3–6 days after SCI. However, though there was recovery, both locomotor and bladder functions were abnormal in the 14 days, which was a long period after SCI to rats. These results indicated that the bladder function, as well as the motor function, were damaged at 1 day after SCI, and recovered spontaneously with prolonged time, especially in 3–6 days.

Western blot analysis showed that the bladder of the rats started to express LC3-II at 1 day after SCI and reached a peak at 4 days. In addition, the RT-PCR results showed that the LC3 mRNA level increased as compared with that in the control group, and reached a peak at 4 days after SCI, which was consistent with the western blot analysis. These results indicate that the autophagy level of detrusor myocytes was enhanced after SCI, and was consistent with the recovery.

The LC3-II expression in the SCI-affected urinary bladder showed different profiles according to the recovery phase after injury. At 1 day after injury, the autophagy was initiated in the early stage. At 4 days, the level of autophagy reached a peak, which was in agreement with the motor and bladder recovery. Thus, we can conclude that autophagy played a major role in the recovery of the bladder after SCI. Therefore, autophagy may serve as a mechanism of cell self-repair, renewal and protection in the neurogenic bladder after SCI. In the present study, western blot analysis and RT-PCR assays showed that the expression level of the autophagy-related proteins LC3-II was significantly elevated in the SCI group as compared with that in the control group. In addition, the RT-PCR results showed that the expression level of P62 mRNA was decreased at 4 days after SCI compared with that in the control group, which was another evidence to prove the role of the autophagy. The immunofluorescence staining showed that the autophagy occurred in the cytoplasm. Thus, we can conclude that the autophagic lysosomes were generated more in the bladder after SCI. Currently, the studies that focus on the role of autophagy in the SCI-induced neurogenic bladder are rare. Therefore, the mechanism underlying autophagy in the process of neurogenesis of the bladder necessitates further investigation.

CONCLUSION

In this study, we established a neurogenic bladder model after SCI, and detected the expression of autophagy-related proteins in the different stages of neurogenic bladder. We found that autophagy is activated in the recovery of the bladder after SCI and sustained over a period of time. Autophagy may play a critical role in the process of neurogenic bladder and may represent one of the mechanisms of bladder self-repair.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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