EFFECT OF 3-MA ON THE NEUROGENIC URINARY BLADDER AFTER SPINAL CORD INJURY IN RATS BY INHIBITING THE AUTOPHAGY REACTION OF URINARY BLADDER DETRUSOR MUSCLE CELLS

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ABSTRACT

Objective: *To analyze the effects of 3-methyladenine (3-MA) on the neurogenic bladder after spinal cord injury (SCI) in rats via the inhabitation of the autophagic response of bladder detrusor muscle cells.*

Methods: *Fifty, 8-week-old male Sprague-Dawley (SD) rats were selected and prepared by spinal cord transection to generate a neurogenic bladder model after SCI. The hindlimb motor function of the rats was evaluated 3, 9, and 18 d after modeling using the BB behavior score. The levels of autophagy-associated protein microtubule-associated protein 1 light chain 3 type II (LC3-II) and basic myelin sheath protein (MBP) were detected. The effects of 3-MA on the neurogenic bladder after SCI, via the inhibition of the autophagic response of bladder detrusor muscle cells, were analyzed.*

Results: *The BBB scores of both the model group and the 3-MA group were lower than those of the sham operation group, at each time point, and the BBB scores for the 3-MA group were higher than those for the model group, the difference is statistically significant ($P<0.05$). At each time point, the LC3-II level of rats in the model group was significantly higher than that in the sham operation group, and the LC3-II level of the 3-MA group at each time point was significantly lower than that of the model group ($P<0.05$). In the model group, 3 and 18 d after the operation, the MBP levels in the model group were significantly lower than those in the sham operation group. The MBP levels in the 3-MA group, 3 and 18d after the operation, were significantly higher than those in the model group ($P<0.05$).*

Conclusion: *An obvious autophagy reaction occurs in the detrusor myocytes in rats with neurogenic bladder after SCI, and the autophagy inhibitor 3-MA can reduce the MBP loss and inhibit bladder detrusor in rats by inhibiting the autophagy reaction of the bladder detrusor muscle cells.*

Keywords: *3-MA, inhibition, bladder detrusor muscle cells, autophagy response, rats, spinal cord injury, neurogenic bladder.*

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Introduction

Spinal cord injury (SCI) often leads to the severe dysfunction of the limbs below the injured segment, and can also cause severe urination disorders and upper urinary tract damage, resulting in renal failure[(1)]. In patients with neurogenic bladder after SCI, the detrusor muscles showed different degrees of apoptosis due to the lack of nerve stimulation. Increased collagen fibers in the detrusor muscles reduce bladder compliance, leading to bladder fibrosis, urine storage, and urinary dysfunction[(2)]. SCI not only causes physical and psychological harm to the patients but also imposes a huge economic burden on society[(3)].

A recent study reported the obvious activation of autophagy in oligodendrocytes in the spinal cord after SCI, which may play a role in the pathogenesis of neurogenic bladder after SCI[(4)]. 3-Methyladenine (3-MA), an inhibitor of phosphatidylinositol-3

kinase (PI3K), can specifically block the formation of autophagic vesicles and has been used as an autophagy inhibitor in various experiments[5]. The present study aimed to analyze the effects of 3-MA on neurogenic bladder after SCI by inhibiting the autophagic response in bladder detrusor muscle cells.

Materials and methods

General information

Seventy 8-week-old, specific-pathogen-free grade, Sprague-Dawley (SD) rats with body weights of 220–260 g were provided by Shanghai SLAC [Certificate No.: SCXK (Shanghai): 2012-002] The rats were maintained in a temperature-controlled environment, at 23.5±1.5°C, with natural light, humidity maintained at 55.00%±5.00%, and food and water ad libitum. This experiment strictly followed the relevant provisions of the "Regulations for the Management of Laboratory Animals".

Methods

Preparation of animal models

A total of 50 rats were prepared as models of the neurogenic bladder after SCI using the spinal cord transection method. The rats were intraperitoneally anesthetized with 10% chloral hydrate after fasting for 12 hours. Rats underwent a longitudinal incision in the back, followed by the performance of a total transection at the T9 segment of the spinal cord. The 4 mm of spinal cord tissue above the transection was excised to remove any residual nerve tissue in the injured cavity, and implant-bone wax was used as a filler in the defect cavity. The incision was sutured layer by layer.

The criteria for successful modeling were as follows:

- The tail spastically wobbled when the spinal cord was removed, and both hind limbs crawled;
- The rats were in the spinal shock stage immediately after SCI, and an enlarged bladder at the upper margin of the pubic symphysis could be detected by touch;
- Abdominal compression was initially used to assist urination;
- Rats that became infected or died were excluded during the modeling process.

A total of 46 rats were successfully modeled and were randomly divided into a model group and a 3-MA intervention group, with each group containing 23 rats. An additional 20 rats were anesthetized by the same method to comprise a sham-operated group. A sham operation was performed, in which only the lamina was removed without damaging the spinal cord.

Method of administration

The rats in each group were fixed on the operating table, with the back arched, and the anterior iliac spine was marked to determine the space between the spinal cord.

A micro-syringe was inserted into the spinal sheath, vertically, at the positioning point, and the drug was slowly pushed. After 30 s the needle was slowly removed.

The 3-MA group was injected with 1 μL 3-MA, at a concentration of 200 nmol/L into the spinal sheath. The model group and the sham operation group were injected with 1 μL saline.

Observation indicators

The hindlimb motor function of rats was evaluated using the BB behavior score 1, 9, and 18 after modeling. Each rat was placed on an enclosed, circular plate, with a diameter of 90 cm.

The rat's behavior and activities were continuously observed for 5 minutes. The behavior was captured on video, and behavior scores were recorded, which was 21 points for normal.

The rats in each group were sacrificed to perform hematoxylin and eosin (HE) staining, and a long spinal cord segment, approximately 15 mm long, was removed under aseptic operation using the original surgical approach.

Spinal cord segments were fixed in 4% paraformaldehyde, dehydrated with an ethanol gradient, treated with xylene, and embedded in paraffin. Sections were prepared at a slice thickness was 6 μm, and HE staining was performed, followed by neutral resin mounting.

The morphological changes of the spinal cord were observed under 40 x magnification using a light microscope.

Van Gieson's (VG) collagen and amaranth red staining

Rats were sacrificed by decapitation, and their bladder tissues were sliced to a thickness of 4 μm. Paraffin-embedded sections were dewaxed with xylene for 15 min, gradient alcohol rehydrated, and stained with Van Gieson's (VG) collagen stain and 0.1% saturated picric acid-Sirius red solution.

Western blot detection

Five rats in each group were sacrificed by decapitation, and a long spinal cord segment, approximately 15 mm in length, was removed using sterile operation procedures through the original surgical approach and quickly placed into liquid nitrogen for preservation. The stored tissue was removed and ground quickly, and a tissue lysis buffer containing protease inhibitors was added. The lysate was incubated on ice, centrifuged at 4°C, and the supernatant was carefully separated.

The protein was quantified by the bicinchoninic acid (BCA) method, and equal amounts of lysed spinal cord tissue lysates were subjected to polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was rinsed with 25 mL of Tris-buffered saline (TBS) at room temperature for approximately 5 minutes. The membrane was blocked with a blocking solution at room temperature for approximately 2 hours.

Combining with a primary antibody and secondary antibody. A chemiluminescent substrate was spread uniformly on the PVDF film, fixed with plastic wrap, and quickly developed in the darkroom. After the bands appeared, it was immediately placed in the fixing solution to treat PVDF film, it was washed with water and dried. The levels of autophagy associated protein microtubule-associated protein 1 light chain type 3-II (LC3-II) and basic myelin protein (MBP) were detected.

Statistical methods

All experimental data were analyzed using SPSS, version 20.0, software.

All measurement data were represented as the mean ± standard deviation ($\bar{x}\pm s$), and a t-test was used for comparison between groups. Enumeration data are presented as percentages, and the χ^2 test was used to compare these data. The results were considered significant at $P<0.05$.

Results

Comparison of hindlimb motor function among the rats in each group

The postoperative BBB scores of the rats in each group showed an increasing trend over time. The BBB scores for the model group and the 3-MA group, at each time point, were lower than those for the sham operation group, and the BBB scores for the 3-MA group, at all time points, were significantly higher than those for the model group (<0.05, Table 1).

Group	BBB scores		
	1 d	9 d	18 d
The sham operation group	19.56±2.06	19.85±2.16	21.09±2.03
The model group	5.12±0.25[a]	6.79±0.35[a]	8.25±0.35[a]
3-MA group	5.39±0.28[ab]	8.59±0.85[ab]	12.46±1.85[ab]

Table 1: Comparison of hindlimb motor function among the rats in each group ($\bar{x}\pm s$).
Note: a is compared with the sham operation group, [a]P<0.05; b is compared with the sham operation group, [b]P<0.05.

Comparison of rat spinal cords and bladder muscles

HE-stained spinal cord tissues showed that the gray and white matter structures of the spinal cord tissues in the sham-operated group remained intact, and the nerve cells were evenly distributed in the gray matter. In the model group, the space around the nerve cells in the spinal cord tissue was enlarged, the nucleolus was blurred, the cells were swollen, and vacuoles were visible. In the 3-MA group, the number of necrotic nerve cells and vacuoles in the spinal cord tissue was reduced compared with the model group, and the degree of inflammatory infiltration was reduced.

The VG collagen staining of rat bladder detrusor muscles revealed that collagen fibers and muscle fibers existed between the bladder smooth muscle cells in the sham-operated group. The collagen fibers between the smooth muscle cells of the model group increased significantly compared with the sham-operated group, whereas the muscle fibers were significantly reduced. The collagen fibers between the smooth muscle cells of the 3-MA group were significantly reduced compared with those of the model group, and the cell arrangement appeared relatively regular.

The urinary bladder detrusor muscle, stained with Sirius erythrosin red, showed that the type I collagen was arranged in parallel between the bladder smooth muscle cells of the sham-operated group, and the density was uniform. Type III collagen was distributed around type I collagen. In the model group, the type I collagen appeared disordered, and the density distribution was uneven among the bladder smooth muscle cells of the model group.

The proportion of type III collagen increased significantly, and it was scattered around the type I collagen in a reticular shape. The ratio of type I/III

collagen between bladder smooth muscle cells in the 3-MA group was significantly lower than that in the model group, and the cells in the 3-MA group were arranged regularly (Figures 1-3).

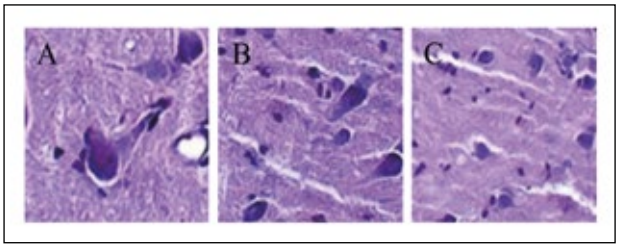

Figure 1: HE-stained images of rat spinal cord tissues in each group.
A: HE-stained image of rat spinal cord tissue from the sham operation group; B: HE-stained image of rat spinal cord tissue from the model group; C: HE-stained image of rat spinal cord tissue from the 3-MA group.

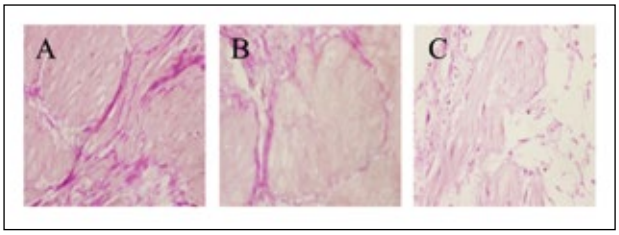

Figure 2: VG collagen-stained images of the detrusor muscle of rats in each group.
A: VG-stained image of bladder detrusor collagen in the sham operation group; B: VG-stained image of bladder detrusor collagen in the model group; C: VG-stained image of rat bladder detrusor muscle in the 3-MA group.

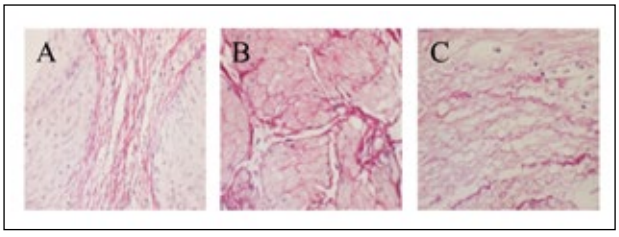

Figure 3: Sirius red-stained images of the urinary bladder detrusor muscles in each group.
A: Sirius red-stained image of urinary bladder detrusor muscle in the sham-operated group; B: Sirius red-stained image of the urinary bladder detrusor muscle in the model group; C: Detrusor muscular bitterness of the bladder in the 3-MA group, as demonstrated by a Sirius red-stained image

Comparison of the levels of the autophagy-related proteins LC3-II and MBP between groups

At each time point, the LC3-II levels of rats in the model group were significantly higher than those in the sham operation group, and the LC3-II levels of rats in the 3-MA group at each time point were significantly lower than those of the model group (P<0.05). In the model group, 3 and 18 d after the operation, the MBP levels were significantly lower than those in the sham operation group, and the MBP levels in the 3-MA group, 3 and 18 d after the operation, were significantly higher than those in the model group (P<0.05, Tables 2 and 3).

Group	LC3-II		
	3 d	9 d	18 d
The sham operation group	0.32±0.02	0.31±0.03	0.42±0.05
The model group	0.45±0.03[a]	0.49±0.02[a]	0.49±0.03[a]
3-MA group	0.23±0.02[ab]	0.17±0.03[ab]	0.33±0.02[ab]

Table 2: Comparison of the levels of the autophagy-related protein LC3-II among groups ($\bar{x}\pm s$).
Note: a is compared with the sham operation group, [a]P<0.05; b is compared with the sham operation group, [b]P<0.05.

Group	MBP		
	3 d	9 d	18 d
The sham operation group	1.01±0.03	1.03±0.05	1.03±0.06
The model group	0.85±0.02[a]	0.96±0.08	0.75±0.02[a]
3-MA group	0.94±0.02[ab]	1.01±0.08	0.92±0.05[ab]

Table 3: Comparison of the levels of the autophagy-related protein MBP among groups ($\bar{x}\pm s$).
Note: a is compared with the sham operation group, [a]P<0.05; b is compared with the sham operation group, [b]P<0.05.

Discussion

Axon demyelination is an important mechanism that leads to dysfunction after spinal cord injury. The apoptosis of neurons and oligodendrocytes can occur secondary to SCI. Oligodendrocyte apoptosis can impair the conduction capacity of the remaining axons and plays an important role in the demyelination of the axons in the spinal cord white matter[6]. Recent studies have shown that the apoptosis mechanism can occur through two primary methods: apoptotic death and autophagic death[7]. Autophagic death refers to programmed cell death in which autophagy serves as the initial factor, and is also referred to as type II programmed death[8-9]. Recent studies have found that the excessive activation of autophagy plays a negative role in the development of neurogenic bladder after SCI[10]. 3-MA can specifically block the activation of the PI3K/Akt pathway, which interferes with or block the formation of autophagosomes. 3-MA is

a commonly used autophagy inhibitor in biological experiments[11]. In the present experiment, the BBB scores of the rats in each group showed an increasing trend with increased postoperative time. The BBB scores for the model group and the 3-MA group, at each time point, were lower than those for the sham operation group, and the BBB scores of the 3-MA group, at all time points, were significantly higher than those in the model group ($P<0.05$), suggesting that 3-MA treatment was able to improve the hindlimb motor function of neurogenic bladder model rats after SCI. HE-stained spinal cord tissues, succinopicrin, and VG collagen-stained rat bladder detrusor muscles all showed that 3-MA treatment had a positive effect on the spinal cord tissue and smooth bladder muscles of neurogenic bladder model rats after SCI.

The LC3 protein is an important marker for the detection of autophagic activity. LC3 undergoes ubiquitination to form LC3-I and is then coupled with phosphatidylethanolamine to form LC3-II, which is stable on the inner membrane. Therefore, the level of LC3-II can reflect the level of autophagic activity[12, 13]. MBP is a major protein that is primarily found in the myelin sheath of the nervous system. After a central nervous system injury occurs, myelin sheath destruction or demyelination is induced, and MBP is released into the blood or cerebrospinal fluid[14].

Clinically, the occurrence of a central nervous system injury can be assessed indirectly by detecting MBP levels in the serum[15]. In this experiment, at each time point, the LC3-II levels of rats in the model group were significantly higher than those in the sham operation group, and the LC3-II levels of the 3-MA group, at each time point, were significantly lower than those of the model group ($P<0.05$). In the model group, 3 and 18 d after the operation, the MBP levels were significantly lower than those in the sham operation group, and the MBP levels in the 3-MA group, at 3 and 18 d after the operation, were significantly higher than those in the model group ($P<0.05$), indicating that 3-MA conveys a protective effect. These results suggested that neurogenic bladder after SCI may have a significant relationship with the inhibition of the autophagic response in bladder detrusor muscle cells.

In conclusion, an autophagy response occurs in the bladder detrusor myocytes in neurogenic bladder model rats after SCI. Treatment with 3-MA can reduce the loss of MBP and inhibit bladder detrusor dysfunction in rats by inhibiting the autophagy response in bladder detrusor muscle cells. The inhibition of collagen fiber expression in the bladder detrusor muscle interstitium of rats promotes the recovery of bladder function and plays a certain neuroprotective role.

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