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The Effects of 3-MA on Neurogenic Bladder after Spinal Cord Injury in Rats

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Abstract

To investigate the expression of autophagy in the neurogenic bladder cells after spinal cord injury in rats by the intervention of 3-MA of autophagy inhibitor. randomized, controlled trial. Second Hospital of Shandong University, Jinan, China. 27 Wistar rats were randomly divided into 3 groups: sham-operated group, control group, and the 3-Methyladenine (3-MA) injection group. Spinal cord injury was induced, using a modified Allen's method at T10-T11 level. Sham-operated group (Blank group) were subjected to laminectomy without SCI, while the control group and 3-MA group underwent laminectomy with SCI. Rats in 3-MA group were injected with 3-MA (2ml/kg body weight per day), while rats in blank and control group were injected with the same amount of saline per day. Outcome Measures: The Basso, Beattie, and Bresnahan (BBB) score, residual urine volume (RUV) and urinary bladder function score (UBFS) were estimated after 1, 4 and 14 days of surgery. Western blot, immunofluorescence staining and RT-PCR were used to measure the expression of microtubule associated protein 1 light chain 3 (LC3) and P62 in bladder tissue. Lower BBB score and UBFS, and higher RUV were found in the 3-MA groups compared to the control group ($P < 0.05$). In addition, at 1, 4 and 14-day post-surgery significantly lower LC3 expression was detected in the 3-MA group compared with the control group ($P<0.05$), while the P62 mRNA level was significantly increased at 4- and 14-days post-operation in the 3-MA group compared to control group ($P<0.05$); no significant differences in P62 mRNA levels were found between groups at day 1. 3-MA inhibits the expression of autophagy and prevents the recovery of bladder function and motor function of lower extremity.

Keywords: 3-MA, Autophagy, Neurogenic Bladder, Spinal Cord Injury

1. Introduction

Neurogenic bladder is a common complication associated with central nerve injury [1]. that emanates from internal and external trauma, injury or disease such as cerebrovascular accident, central nervous system tumor, spinal cord injury and Parkinson's disease [2]. During its early stage, it causes the obstruction of urination and the function of upper urinary tract, which leads to substantial decrease in quality of life [1]. In recent years, due to the social development and the improvement in living standards, the incidence of industrial injuries, traffic accidents and natural disasters have significantly increased. Consequently, the number of spinal cord injuries that cause neurogenic bladder which in turn brings great difficulty to the patients have been also on the rise [3].

At present, there is no effective treatment for renal deterioration after neurogenic bladder, and supportive therapy is the main treatment. Conventional methods such as routine catheterization can lead to multiple complications such as mechanical urethral injury and recurrent infection [4], while surgical treatments such as reconstructive surgery have been shown to be associated with stone formation, epithelial hyperplasia and malignant tumors [5].

During preliminary testing we found that autophagy was activated in the bladder detrusor after spinal cord injury, and that it lasted for a very long time. The expression of autophagy was consistent with the recovery of bladder function and motor function, which indicated that autophagy may participate in bladder detrusor self-repair that promote the recovery of bladder function and motor function after spinal cord injury [6].

3-Methyladenine (3-MA) can block the

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autophagy trough phosphoinositol 3 phosphokinase (PI3K) activation; PI3K controls the formation of nucleation and membrane pools of the autophagosome [7]. According to some studies, 3-MA has a promoting effect on bladder cancer cells apoptosis; nevertheless, the effect of 3-MA on the bladder detrusor muscle cells and bladder function after spinal cord injury remain unclear and need to be further investigated.

This study used rat models of neurogenic bladder after spinal cord injury intervened by the autophagy inhibitor 3-MA to investigate if 3-MA could inhibit the expression of autophagy and prevent the recovery of bladder function and motor function of lower extremity

2. Materials and Methods

2.1. Experimental Animals

Adult female Wistar rats (2-3 months, 260–280 g, n=27) were provided by the Shandong University experimental animal center. All animals were placeed in an environment with temperature of 22 ± 1 ºC, relative humidity of 50 ± 1% and a light/dark cycle of 12/12 hr, with free water and food. All the experimental procedures were in accordance with the Guidelines for Animal Science Procedures approved by the Second Hospital of Shandong University Experimental Aninmal Ethics Committee.

The rats were randomly divided into three groups (9 rats/group): sham-operated group (blank group), spinal cord injury group (control group), and the 3-MA injection group (3-MA group). Blank group was subjected to laminectomy without SCI, while the control group and 3-MA group underwent laminectomy with SCI. Consequently, the rats in 3-MA group were injected with 3-Methyladenine (2ml/kg body weight per day) for 14 days, while rats in blank and control group were injected with the same amount of saline per day

2.2. Laminectomy and Spinal Cord Injury

The rats were anesthetized by intraperitoneal injection with 10% chloral hydrate (3ml/kg body weight), and the rats' back were shaved, lying prone on the operating table. Located the T10-11 spinous process on the back of rats and expose the T10-11 spinal cord area. The spinal cord injury was then induced using the modified Allen's method [8]. Simply, 5g rod was dropped from a vertical distance of 20cm onto the T10 level where the spinal cord was exposed. During this process, the mice developed spastic spasms in their hind limbs and tails that lasted for several seconds. After surgery, the muscle, subcutaneous and skin are stitched in layers.

The temperature of rats was monitored by overheat pad during operation and was maintained at 37.0±0.5℃. To prevent the infection, all rats were intraperitoneally injected with normal saline (1mL) and antibiotics for 3 days immediately after the operation.

2.3. Evaluation of Hindlimb Locomotor Function

To assess bilateral hind limb motor function, we used Basso, Beattie and Bresnahan (BBB) scales in an open field [9]. The BBB score of 0 indicated no hindlimb movement, while score of 21 indicated normal hindlimb movement. The BBB scores of rats were performed on the smooth and open floor at 1, 3, 5, 7, and 14 days after surgery. All of the motor performance tests were recorded with a digital camera for at least four minutes and explained by two observers who knew the score details but were not involved in the study.

2.4. Evaluation of Bladder Function

In order to reduce the side effects and unnecessary interference to this experiment that can be caused when using invasive and common methods such as urodynamic testing, 2 noninvasive methods, i.e., measuring residual urine volume (RUV) and analyzing urinary bladder function score (UFBS) [10-11], were used to evaluate the bladder's function in rat. The first method was performed two times per day. Bladder emptying was accomplished by applying pressure to the lower and middle parts of the abdomen with the index and middle fingers. As urine is excreted, it was collected in a tube and the volume of urine was recorded.

UFBS was preformed according to Maria Kuricova's study by using four-point urinary bladder function score (UBFS) which was evaluated daily [11]. The standard for evaluation was: complete loss of function (manual expression of bladder 3×/day, the urine contains blood) = 0, partial recovery of UBF (manual expression 2×/day, urine may contain blood) = 1, partial recovery of UBF (partial/complete release of sphincter spasm, manual expression 1×/day, still blood in urine) = 2, advanced recovery (manual expression 1×/day, no blood in urine) = 3, physiological UBF= 4.

2.5. Western Blot

The rats were sacrificed at 1,4- and 14-days post-surgery. The bladders were homogenized and collected in RIPA lysis buffer and 1Mm PMSF (Beyotime Biotechnology, Shanghai, China), sonicated, centrifuged, and the supernatant was collected. Protein concentration was measured using a BCA-assay. The equivalent of 40ug of total

protein was loaded on 5%-12% SDS-PAGE and transferred to the PVDF membrane (Bio-Rad). The membrane was then blocked with 5% non-fat milk in Tris sodium buffer with 0.1%Tween 20 for 2 hours, and incubated with primary antibody solutions (servicebio,China) at 4℃ overnight, according to the manufacturer's recommendations. Consequently, the membranes were washed with TBST for 5 minutes three times and incubated with Horseradish Peroxidase conjugated secondary antibody solution (containing 5% non-fat milk, Tris sodium buffer (0.1 M Tris-HCl, pH-7.4, and 0.15 M NaCl) with 0.1% Tween 20 (TNT), and HRP-secondary antibody) (servicebio,China) for 1 hour, and washed with Tris sodium buffer(0.1 M Tris-HCl, pH-7.4, and 0.15 M NaCl) with 0.1% Tween 20 (TNT) for 7 minutes with four times. The Image J was used for quantification of band intensity of LC3 [12-13].

2.6. Immunohistochemistry

The rats in the SCI group and control group were sacrificed at each time point and their bladders were fixed in the 4% paraformaldehyde solution for > 24h. The bladder tissues were then embedded by paraffin wax and cut into 5μm pieces. Consequently, sections were deparaffinized and rehydrated, and incubated with 3% hydrogen peroxide for 10 minutes and antigen retrieval for additional 1 minute. Then, sections were blocked with 5% albumin from goat serum at 37°C for 1 hour, incubated with the primary antibodies at 4℃ overnight, and with the fluorescent Alexa488 goat anti-rabbit secondary antibody for 1 hour at room temperature. Samples were then incubated with DAPI for 10 minutes and finally washed in PBS and sealed with a coverslip. All images were captured on a Nikon ECLIPSE Ti microscope (Nikon, Japan) and analyzed by Image proplus [13].

2.7. Quantitative RT-PCR

Tissues were homogenized in ice-cold Trizol reagent (Takara). RNA was isolated by chloroform phase separation and alcohol precipitation, and its concentration was determined using the NanoDrop 2000 spectrophotometer (ThermoScientific, Maltham, MA, USA). cDNA synthesis was done using the PrimeScript™RT reagent Kit with gDNA Eraser (Takara,Japan). cDNA amplification was preformed using SYBR®Premix Ex Taq™ (Takara,Japan). The PCR primers (Takara,Japan) were the following: GAPDH forward: GGCACAGTCAAGGCTGAGAATG, reverse: ATGGTGGTGAAGACGCC-AGTA; BECN1 forward: GAAACTGGACACGAGCTTCAAGA, reverse: ACCATCCT-GGCGAGTTTCAATA; LC3 forward: CGAGAGCGAGAGAGATGAAGACGG, reverse: GGTAACGTCCCTTTTTGCCTTGGTA; P62 forward: AGAATGTGGGGGAGAGCGTGGC, reverse: GGGTGTCAGGCGGCTTCTCTT.

2.8. Statistical Analysis

All data were presented as the mean ± standard error of the mean (SEM) from three independent experiments. When there were two experimental groups, the student T test was used to test whether there were statistically significant differences. $P<0.05$ was considered statistically significant.

3. Results

3.1. Locomotor Function Assessment

The BBB rating scale was used to assess the locomotor functions of both hindlimbs in rats. The BBB scores for the experimental groups are summarized in Figure.1. After surgery, lost locomotor functions were observed in control and 3-MA group, while those functions gradually recovered after 1 day of surgery. The BBB rating increased over time. However, significantly lower BBB scores were found in the 3-MA groups compared to the control group ($P < 0.05$).

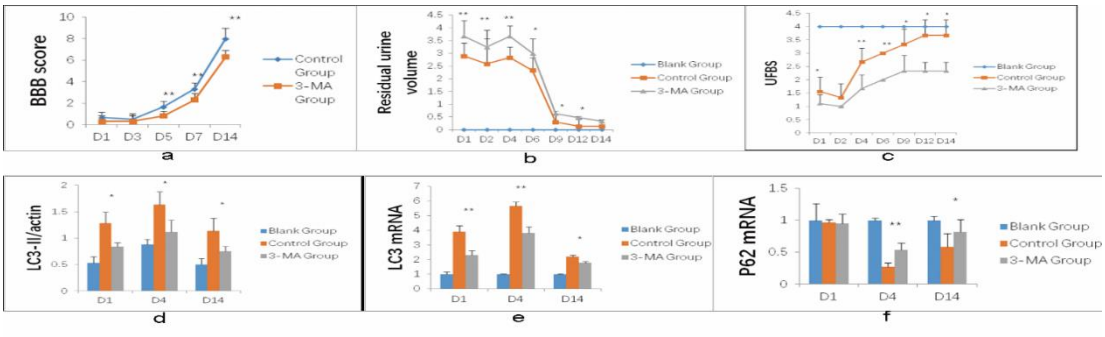

Figure 1. **Assessment of the motor function of hindlimbs, bladder function and autophagy related genes or proteins**

a. The rats' locomotor function evaluation according to the Basso Beattie Bresnahan (BBB) open field locomotor rating scale (X±s).
Mean values ± SD; compared to the control group
*P < 0.05, compared to the control group
** P < 0.01, compared to the control group.
b. The residual urine volume (X±s).
Mean values ± SD; compared to the control group
*P < 0.05, compared to the control group
** P < 0.01, compared to the control group.
c.The bladder function evaluation for the three groups of rats. (X±s).
Mean values ± SD; compared to the control group
*P < 0.05, compared to the control group
** P < 0.01, compared to the control group.
d. LC3II protein expression in the three rats group at 1, 4 and 14-day post- surgery, The gels have been run under the same experimental conditions (X±s).
*P < 0.05, compared to the control group
** P < 0.01, compared to the control group.
(e) The expression level of LC3mRNA in the bladder tissues of the rats at each time point (X±s).
*P < 0.05, compared to the control group
** P < 0.01, compared to the control group.
(f) The expression level of P62 mRNA in the bladder tissues of the rats at each time point (X±s).
Mean values ± SD; compared to the control group
*P < 0.05, compared to the control group.
** P < 0.01, compared to the control group.

3.2. Bladder Function Assessment

Bladder functions in rats were analyzed using RUV and UFBS. The RUV for all rats is shown in Figure.1. In control group and 3-MA group the RUV gradually reduced from 1 day to 14 days after surgery; the lowest volume was observed between 3 days and 9 days post-surgery. Also, the RUV were significantly increased in the 3-MA groups compared to the control group (P < 0.05).

The UBFS are summarized in Figure.1. After surgery, damaged urinary bladder function was observed in 3-MA group and control group; but was recovered gradually. In addition, UBFS were significantly lower in the 3-MA group compared to the control group at 1 day, 9 days, 12 days, 14 days (P<0.05), 4 days, 6 days (P<0.01) post-surgery.

3.3. Autophagy-Related Protein LC3 Expression in the Rats Bladder Tissues

It has been reported that autophagy is involved in the normal bladder. In this study we examined the expression of the autophagy-related protein LC3 in the bladder tissues at 1-, 4- and 14-days post-SCI using western blot and Immunofluorescence staining (Figure.1 and Figure.2).

Briefly, higher expression of LC3 was found in the 3-MA group and control group compared to blank group. In addition, significantly lower LC3 expression was detected in the 3-MA group compared with the control group (P<0.05). Furthermore, increased expression of P62 was observed in the 3-MA group compared to the control group at 4 days and 14 days (Figure.3).

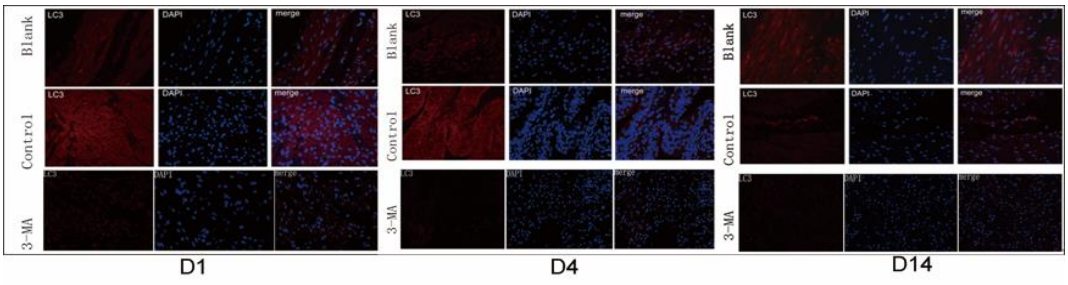

Figure 2. **LC3II expression at 1day, 4 days, 14 days post- SCI.**

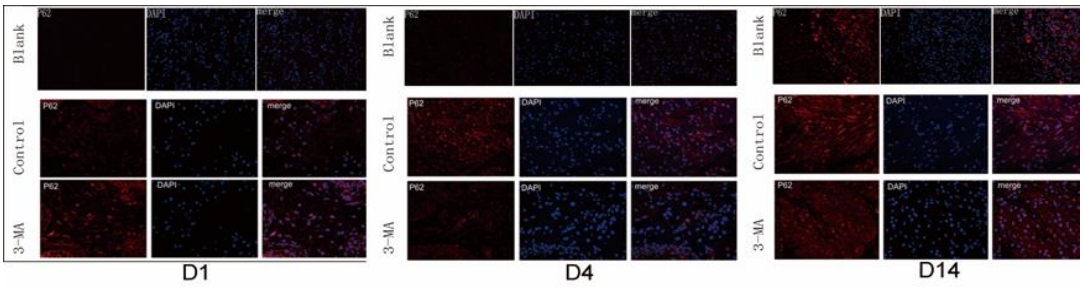

Figure 3. **P62 protein expression at 1 day, 4 days, 14 days post- SCI.**

Pictures of SCI rat bladder tissue immunofluorescence staining (DAPI for nucleus, LC3, P62 for green fluorescence, merge for fusion of the former two). Magnification×400.

3.4. LC3 mRNA Expression in the Rats Bladder Tissues

The RT-PCR analysis results showed that LC3 mRNA was significantly decreased in the 3-MA group compared to control group at 1 day, 4 days ($P<0.01$), and 14 days ($P<0.05$) after surgery (Figure.1). In addition, 1 day after surgery, no significant differences in P62 mRNA level were found between the three groups; while the P62 mRNA level was significantly decreased at 4 day, 14 days in the 3-MA group and control group compared to the blank group. However, at 4 days and 14 days, P62 mRNA level was significantly higher in the 3-MA group compared to the control group ($P<0.05$).

4. Conclusions

In this study, we found employing 3-MA could inhibit the expression of autophagy and prevent the recovery of bladder function and motor function of lower extremity

5. Discussion

Clinically the spinal cord injury above S1 level does not damage the S2-S4 level sympathetic and S1-S4 integrity; nonetheless, the pontine urination center does get destroyed when the cortical urinary reflex and detrusor-sphincter coordination disorders occur [14]. Furthermore, N2-S1 level of injury can cause involuntary urination, and S2-4 injury with the sphincter paralysis can lead to urinary barrier [15], decreased detrusor activity, detrusor without contraction and urethral sphincter relaxation which can lead to voiding of the bladder [16]. Cervical 2-sacral level of spinal cord injury can also cause detrusor-sphincter dyssynergia (DSD). Studies have found that the spinal cord injury at chest 9 level or above in patients with detrusor can lead to loss of normal contraction and normal urinary force. While the detrusor hyperresponsiveness and detrusor muscle - sphincter occur in the majority of patients, lumbar spine (including (49%), detrusor-sphincter dyssynchrony (33%), and detrusor contraction (39%) are absent in the patients with spinal cord injury [17-20].

Within a few minutes after the injury, injured areas are swollen, while other central gray matter may be bleeding and spreading to other parts of the spinal cord thus causing spinal cord injury, which is followed by function loss and spinal cord shock and needs to be further studied. The complete sacral spinal cord injury may last 6-12 weeks and may even reach 1-2 years [21]; regarding incomplete spinal cord injury, spinal cord shock period is short, and it usually lasts for several days [22]. During recovery of spinal cord injury, the recovery of skeletal muscle reflex often prompts the recovery of detrusor activity; the emergence of the ball cavernous reflex suggests rapid recovery of the spinal cord [23-24]. In this study, we modified the Allen's method for spinal cord injury, the modeling method to simulate the clinical common combat injury, consider the incomplete spinal cord injury.

Clinically, there are many drugs can affect the function of the urethra and bladder. Bladder detrusor contraction is mainly controlled by the muscarinic receptors. Anticholinergic drugs have shown the ability to treat spastic bladder-induced urinary incontinence, nonetheless the side effects and upper urinary tract changes need to be further elucidated [25-26]. The use of botulinum toxin treatment for detrusor hyperactivity and reduce urethral resistance, and achieved good results, the toxins by blocking synaptic membrane acetylcholine release caused by delayed paralysis play a role, but after a period of time nerve After the axonal regeneration of botulinum toxin was removed, need to re-inject, and other botulinum toxin will damage the detrusor of the detrusor, will affect the follow-up treatment options, so the standardized treatment dose and program need further study [27-28].

Autophagy has become a topic of interest in recent years. Autophagy is the process of degradation of cytoplasmic components (such as long-lived proteins, senescence or abnormal organelles, lipid droplets) within the lysosomes. When cell energy requirements increase, autophagy is activated to provide energy substrates [29-30], while autophagy over-activation can cause cell damage and cell death, also known as autophagy-related death [31]. Furthermore, after spinal cord injury autologous expression of spinal cord tissue increases which has suggested that autophagy might be involved in secondary mechanism of SCI [32-33]. Nevertheless, after the spinal cord injury, bladder function changes, bladder detrusor occurred pathological changes, autophagy in the bladder tissue expression changes no change. Studies have shown that in normal rat bladder tissue, autophagy has a low level of expression [34].

During preliminary testing, no damage in the case of rat spinal cord, bladder detrusor muscle also

exists a small amount of autophagy. Through autopsy, the autophagy in the bladder detrusor is activated and lasts for a very long time. The expression of autophagy is consistent with the recovery of bladder and motor function, i.e., spinal cord shock recovery. In addition, autophagy which occurs during the development of neurogenic bladder may participate in the bladder detrusor self-repair process. In order to further confirm the role of autophagy in the process of neurogenic bladder after spinal cord injury, the autophagy inhibitor 6-amino-3-methyl purine (3-MA) was used to examine the effects of autophagy on the posterior spinal cord injury, motor and bladder function, and the expression of autophagy in bladder detrusor.

3-MA is a selective PI3K inhibitor that acts on Vps34 and PI3Kγ. 3-MA binds with high affinity to type I PI3K and low affinity to type III PI3K, and in turn blocks the formation of autophagosomes. By preventing PI3K-III to bind with Beclin-1, it inhibits autophagic bubble formation [35-36]. In the present study, we used 3-MA to investigate the expression and mechanism of autophagy neurogenic bladder in spinal cord injury rats.

In this study, the BBB score was used to evaluate the motor function of the lower limbs in rats. The results showed that the lower extremity function of the rats after spinal cord injury was completely lost, but the function of the lower limbs gradually recovered after the spinal cord shock period, which was consistent with the previous studies. In addition, the BBB score in the 3-MA group was significantly lower compared to spinal cord injury group (control group) at 1 to 14 days post-surgery, which proved that the recovery of lower limb motor function was slower in rats with spinal cord injury.

Furthermore, RUV and UBFS were used to assess urinary function. Between day 1 and day 14 after operation, higher RUV and longer time of bladder detrusor weakness was observed in the 3-MA group compared to control group. At the same time, damaged urinary bladder function was observed in 3-MA group and control group, and then recovered gradually overtime; nevertheless, UBFS was significantly lower in MA group compared to control group, indicating that the group of rats with bladder function less intervention in rats to restore poor.

Briefly, significantly lower LC3 expression was detected in the 3-MA group compared to the control group ($P<0.05$). In addition, P62 mRNA level was significantly increased at 4 days and 14 days post- operation in the 3-MA group compared to control group ($P<0.05$), while no significant differences in P62 mRNA levels were found between groups at day 1. Moreover, Immunofluorescence staining results showed that autophagy occurred in the cytoplasm, around the nucleus. These data suggested that 3-MA inhibits the expression of LC3 protein and promotes the expression of P62 protein to regulate autophagy and affect the function of bladder detrusor.

To sum up, the autophagy of bladder detrusor cells increases after spinal cord injury, and the autophagy expression increases gradually with the recovery of bladder function. In addition, 3-MA inhibits the expression of autophagy, and prevents the recovery of bladder and motor function of lower extremity. These data further confirm that autophagy in the development of neurogenic bladder after spinal cord injury may have an important role in the development of bladder detrusor may be involved in self-repair, for bladder detrusor cells have a certain protective effect.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgement

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