

Protective effect of tauroursodeoxycholic acid on the autophagy of nerve cells in rats with acute spinal cord injury

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Abstract. – OBJECTIVE: To investigate the impact and protective effect of tauroursodeoxycholic acid (TUDCA) on the autophagy of nerve cells in rats with acute spinal cord injury.

MATERIALS AND METHODS: Seventy-two 6-8-week-old male Sprague-Dawley (SD) rats were selected and were randomly divided into a sham operation group, a saline control group and a TUDCA treatment group (high and low dose groups). The experimental animals were sacrificed at 24 hours, 5 days and 10 days after spinal cord injury. The Basso, Beattie, Bresnahan locomotor scale was used to assess the hind limb locomotor function after the rats were injured but before sudden death. Electron microscopy, hematoxylin and eosin (HE) staining, TUNEL assays and immunohistochemistry techniques were used to observe the autophagy of the cells. Western blotting was used to analyze the expression of the autophagy-related factor Beclin-1 and the apoptosis-related factor caspase-3, and reverse transcription polymerase chain reaction (RT-PCR) was used to analyze the mRNA expression levels of the above proteins.

RESULTS: The locomotor scores of the rats in the saline group were significantly reduced, their Beclin-1 expression levels in neurons were decreased, and caspase-3 expression was increased. The hind limb locomotor scores of rats in the TUDCA groups were decreased, with no difference between the high- and low-dose groups. Beclin-1 expression in their neurons was increased, and caspase-3 expression was decreased; there was a significant difference when compared with the control group, while there was no significant difference between the high- and low-dose groups.

CONCLUSIONS: TUDCA significantly activates the neuronal autophagic expression in rats with acute spinal cord injury to inhibit the apoptosis of nerve cells; therefore, it has a protective effect on neurons.

Key Words

Spinal cord injury, TUDCA, Autophagy, Neuroprotection.

Introduction

Spinal cord injury (SCI) refers to direct or indirect external adverse factors affecting the spinal cord, with various locomotor and sensory dysfunctions and changes in muscle strength and muscle tension appearing at the injury site or the segments below^{1,2}. Spinal cord injury is a severe trauma of the central nervous system, with high mortality and disability rates. At present, treatment is limited only to reducing the degree of early spinal cord injury³.

Tauroursodeoxycholic acid (TUDCA) is a type of steroid material found in the bile of bears. Its chemical name is 3 α , 7 β -dihydroxycholanyl-N-taurine, and it is a derivative of ursodeoxycholic acid. In recent years, TUDCA has been found to have an anti-neuronal apoptosis effect. Because the compound can easily pass through the blood-brain barrier, it has broad clinical application prospects, especially in nervous system protection⁴. Studies have shown that TUDCA can significantly reduce the degree of endoplasmic reticulum stress (ERS) and reduce the expression levels of caspase-12, caspase-9, and caspase-3 in a rat model of acute spinal cord injury, suggesting that TUDCA may reduce the degree of neuronal apoptosis through the ERS pathway⁵. Previous work in our lab has also confirmed that TUDCA can inhibit neuronal caspase-12 expression and reduce apoptosis after spinal cord injury in rats and that TUDCA has a protective effect on secondary nerve injury after SCI in rats⁶.

Autophagy is closely related to the secondary injury that occurs after spinal cord injury. In a rat spinal cord contusion model, cellular autophagy in the injured tissue was confirmed via ultrastructural transmission electron microscopy⁷. In this

study, we used a transmission electron microscopy technique to observe autophagosomes. With the regulation of the initiation and development of apoptosis by caspase-3 used as a control, we investigated the impact of TUDCA on nerve cell autophagy after spinal cord injury. We also investigated whether the autophagy of nerve cells had a positive or negative effect, providing experimental evidence of the effect of TUDCA intervention on the recovery level of nerve cells.

Materials and Methods

Experimental Animals

Seventy-two 6-8-week-old male Sprague-Dawley (SD) rats (body weight 250-300 g) were selected and were randomly divided into four groups according to the random number table method, with 6 rats in each group. The use of the SD rats in this study was reviewed and approved by the Animal Management Committee of General Hospital of Ningxia Medical University and was in line with animal experiment regulations.

Preparation of a Rat Model of Spinal Cord Injury

The rats were fasted for 8 h before surgery and were marked on their tails before the surgery. The Basso, Beattie, Bresnahan (BBB) method and scale were used to assess the postoperative locomotor ability and the efficacy scores of the rats. In a standard spinal cord injury rat model, congestion and edema can be observed at the spinal cord injury site. When the rat is hit by a free fall striker pin, swinging of the rat tail, retraction of the hind legs and deep breathing can be observed. After the rat is awakened from the anesthesia, the hind legs exhibit delayed paralysis. We made sure to generate an effect of incomplete spinal cord injury. For the control group, we only cut open the same spinal segment to expose the spinal cord, and the exposure time was the same as in the other groups. After surgery, the rat was confirmed not to have urinary retention, to have appropriate nutrition and not to have an infection of the skin wound.

Experimental Grouping and Extraction of the Spinal Cord

Seventy-two SD rats were divided into a sham operation group, a saline control group and high-dose and low-dose TUDCA groups. For the sham operation group, only the skin tissue was cut open and was then sutured. For the control group, the

same dose of saline as for the experimental group was injected intraperitoneally. For the high- and low-dose TUDCA groups, the doses of intraperitoneally injected TUDCA were 200 and 100 µg/kg body weight, respectively. The animals were sacrificed 24 hours, 5 days and 10 days after spinal cord injury. Fine microsurgical instruments were used to dissect the injured spinal cord segment, with a length of approximately 1-1.5 cm.

HE Staining

Each rat was anesthetized at the corresponding time point, intubation was placed via the left ventricle-ascending aorta, and the animal was quickly perfused with ice-cold saline. When the outflow liquid became clear, the animal was perfused with 4% paraformaldehyde phosphate buffer and was fixed for 45 min. The spinal cord was exposed by the original incision on the back, with the injury site as the center, and a 1.5-cm-long spinal cord segment was harvested, which was transferred to 4% paraformaldehyde phosphate buffer to incubate overnight, followed by gradient dehydration and paraffin embedding (2 tissue blocks were prepared for each animal). Consecutive sagittal sections were cut, with a thickness of 5 µm. For each tissue block, 5 consecutive sections were randomly selected to perform hematoxylin-eosin (HE) staining. After HE staining, with the severely injured spinal cord segment as the center to select the field, the morphology changes were observed under a light microscope.

TUNEL Staining

In situ apoptosis was performed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Merck DNA Fragmentation Detection Kit, Merck KGaA, Darmstadt, Germany) on paraffin sections according to the manufacturer's instructions. Positive nuclei that were within cells that were morphologically consistent with the cells were counted in 10 fields (×200).

Transmission Electron Microscopy Imaging

The tissue sections for transmission electron microscopy imaging were prepared. Certain issues required attention, described as follows. The procedure for the preparation of the injured anterior horn of the spinal cord should avoid damaging the anterior horn tissue as much as possible. The toluidine blue staining time should be monitored after epoxy resin embed-

ding. The thickness of the ultrathin section was controlled at 70 nm. The initial tissue fixation solution chosen was 4% paraformaldehyde + 2.5% glutaraldehyde. After rinsing with phosphate-buffered saline (PBS), 1% osmium acid was used as the fixation solution, and the fixation time was one hour.

RNA Isolation and Real-Time RT-PCR

RNA was isolated and purified using the RNeasy Mini kit (Qiagen, Hilden, Germany). For real-time RT-PCR analyses, 1g of DNase-treated total RNA was reverse transcribed. The amplification of the cDNA was accomplished using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) in the presence of the commercially available SYBR Green PCR Master Mix (Takara, Dalian, China) in a 40-cycle PCR. The primer sequences were as follows: β -actin (Forward, 5'-CCTAGACTTC-GAGCAAGAGA-3'; Reverse, 5'-GGAAG-GAAGGCTGGAAGA-3'); caspase-3 (Forward, 5'-CTGGACTGCGGTATTGAG -3'; Reverse, 5'-GGGTGCGGTAGAGTAAGC-3'); Beclin-1 (Forward, 5'-ACAAGCTCAAGAAAACCAA -3'; Reverse, 5'-TCCACTCCACAGGAACACT-3'). The denaturing, annealing, and extension conditions of each PCR cycle were 95°C for 5 seconds, 60°C for 20 seconds, and 72°C for 34 seconds, respectively. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. The mRNA levels of each target gene were normalized to the levels of β -actin and were represented as fold induction.

Western Blots

At appropriate time points, the remaining six rats in each group were given an overdose of anesthesia, and the spinal cords were exposed from the original incisions in the back. Centered at the injury site, a segment of the spinal cord of approximately 1.5 cm in length was dissected and stored in a -80°C refrigerator. The spinal cord tissue was removed, weighed, and ground, and the protein concentration was determined using the bicinchoninic acid (BCA) method in strict accordance with the instructions of the protein extraction kit and quantification kit. The protein samples were mixed with 5 × sodium dodecyl sulfate (SDS) gel loading buffer, denatured at 100°C for five minutes, electrophoresed, and transferred to a polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich, MS, USA) at 400 mA for 30 minutes. The membrane was

blocked in Tris-buffered saline-Tween (TBS-T) containing 5% skim milk at room temperature for one hour, and first antibody (1:1000) was added, followed by overnight incubation at 4°C and subsequent TBST washes. The secondary antibody (1:3000) was then added to the membrane for one hour at room temperature. The PVDF membrane treated with enhanced chemiluminescence (ECL) reagent (Sigma-Aldrich, St. Louis, MO, USA) was placed on a gel imager. The resulting images were then processed by GraphPad Prism 5 to obtain the gray values of each sample band, using β -actin as an internal reference.

Statistical Analysis

The SPSS18.0 statistical software package was used for the statistical analysis. The measurement data are presented as means $\pm$ standard deviations ($\bar{x} \pm s$). Analysis of variance (ANOVA) was used for the comparison of the measurement data between multiple groups. Repeated-measures ANOVA was used for the comparison of the measured data at different time points between various groups. When the difference of the comparison between groups was statistically significant, the Student-Newman-Keuls (SNK) method was used to perform pairwise comparisons between groups. The test level was $\alpha = 0.05$, and when $p < 0.05$, the result was considered statistically significant.

Results

Locomotor Function of Rats in Various Groups

The activities of the rats in the sham operation group were not limited. When the saline control group was compared with the TUDCA group, the BBB score value of the control group was significantly lower than that of the TUDCA group. Regarding the activities of rats, the activity of the control group was slightly worse than that of the TUDCA group. The effects of the high- and low-dose treatments in the TUDCA groups on rat activity were not significantly different (Table I).

Nerve Cell Autophagy in the Rats of the Various Groups

Twenty-four hours after the spinal cord injury, in the spinal cord cells and the surrounding myelin sheaths of the rats in the saline control group, rough endoplasmic reticulum was observed, mi-

Table I. The preoperative and postoperative BBB scores of various groups ($\bar{x} \pm s$).

Groups	N	24 hours	5 days	10 days
Sham operation group	6	21.762 $\pm$ 8.016	21.543 $\pm$ 7.936	20.678 $\pm$ 7.614
Saline control group*	6	0.330 $\pm$ 0.134	2.870 $\pm$ 0.133	3.899 $\pm$ 0.128
TUDCA high-dose group *	6	0.784 $\pm$ 0.151	6.270 $\pm$ 0.149	8.817 $\pm$ 0.143
TUDCA low-dose group*	6	0.679 $\pm$ 0.072	6.266 $\pm$ 0.071	8.312 $\pm$ 0.068

Note: Through repeated-measures ANOVA, $F_{\text{time}}=12208.14$; $p=0.000$; $F_{\text{group*time}}=2685.550$; $p=0.000$; $F_{\text{group}}=29.036$, $p=0.000$. The SNK method was used to perform pairwise comparisons between groups.

*Indicates a statistically significant difference from the sham operation group.

tochondria and other organelles had no visible injury and normal appearance, and small numbers of ribosomes were visible. In the low-dose TUDCA group, very few organelles were visible inside or outside of the cytoplasm, and apoptosis had occurred. In the high-dose TUDCA group, autophagosomes were present (Figure 1).

Five days after spinal cord injury, in the rats of the saline control group, the rough endoplasmic reticulum had expanded, and the ribosome num-

bers had decreased, suggesting that protein synthesis had decreased. In the low-dose TUDCA group, chromatin margination was not obvious in the cytoplasm, the number of lysosomes had increased dramatically, and nuclear pyknosis had occurred. In the high-dose TUDCA group, nuclear pyknosis had occurred, chromatin margination was visible, small numbers of organelles were scattered in the cytoplasm, some of the mitochondrial cristae had dissolved, fractures formed focal

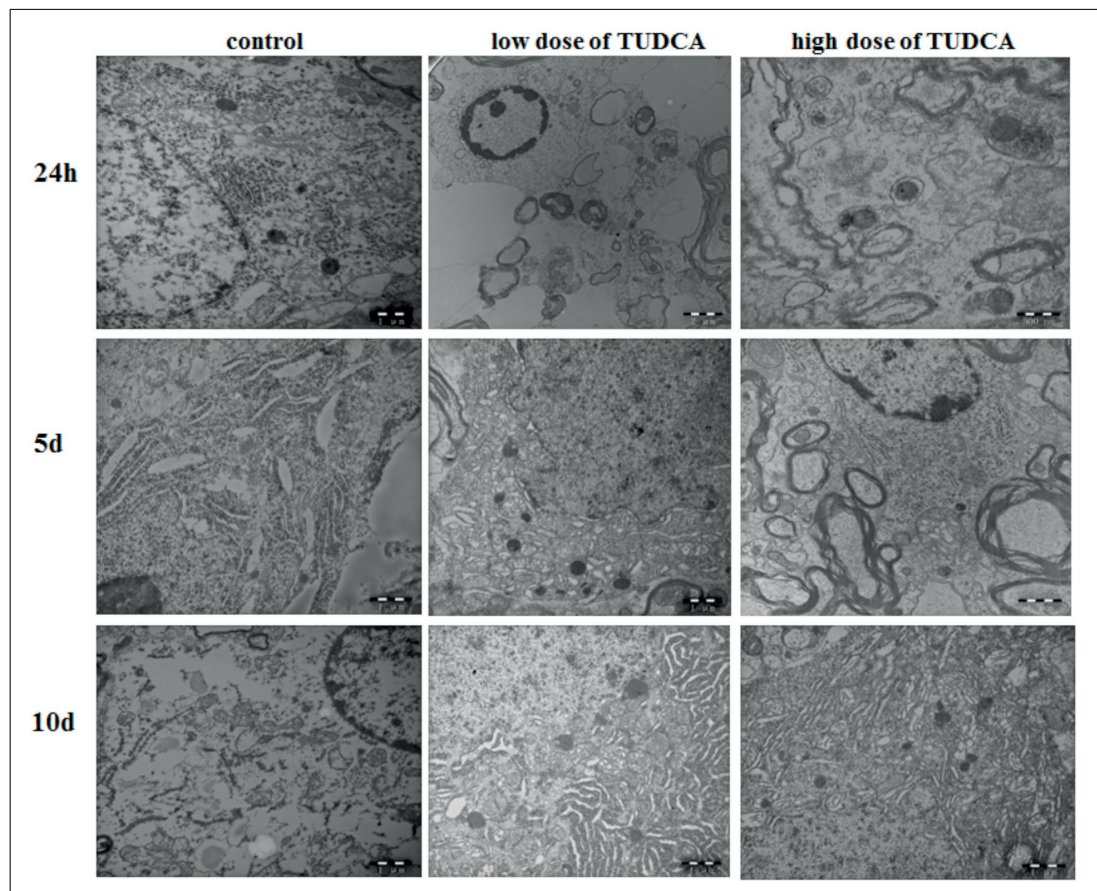
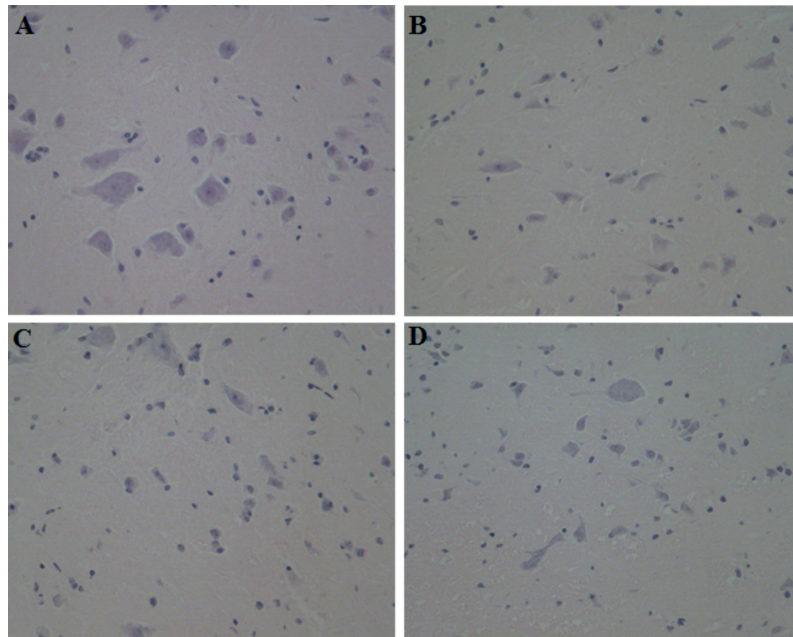

Figure 1. Transmission electron microscopy imaging for nerve cell autophagy in the rats of the various groups at different time points ($\times 20,000$).

Figure 2. HE staining for nerve cell autophagy in the rats of the various groups ($\times 200$). **A**, Sham operation group; **B**, control group; **C**, high-dose TUDCA group; **D**, low-dose TUDCA group.



vacuoles, and the surrounding myelin sheath had separated.

Ten days after the spinal injury, nuclear pyknosis had occurred in the saline control group, chromatin margination was visible, small numbers of damaged organelles were visible in the cytoplasm, and some damaged organelles were visible outside of the cytoplasm. In the low-dose TUDCA group, autophagosomes were visible. In the high-dose TUDCA group, the rough endoplasmic reticulum had expanded, mitochondrial cristae were broken, nuclear membrane pyknosis showed a serrated shape, and lysosomes had increased in number.

Pathological Changes of Neurons in Rats of Each Group

In the sham operation group, the spinal cord neurons and the glial cells were intact, and there were no obvious necrotic cells. In the spinal cord tissue at the injury site of the control group, the cells showed vacuole-like pathogenesis, there were large areas of hemorrhage and edema, cell and tissue structures were evidently loose, and the gray matter tissue was damaged, accompanied by the necrosis of large numbers of neurons (Figure 2). In the surviving neurons, nuclear pyknosis was visible in some neurons of the spinal cord tissue at the injured site. Compared with the control group, the main manifestations of the specimens of the high-and low-dose TUDCA groups similarly showed hemorrhage,

edema, and necrosis. However, the most obvious characteristics of the control group lay in the fact that the segment next to the injury center also showed signs of injury, the expansion of the injury was visible, the damage of the adjacent neurons was pronounced, and the morphology of some cells showed significant changes. In the TUDCA group, although the cell injury area was also larger than previous time points, compared with the control group, the involved area was significantly smaller, and the degree of damage to the adjacent cells was less severe (Figure 2).

Real-time PCR Analysis Results

Caspase-3 mRNA expression was not detected in the sham operation group. Compared with the saline control group, caspase-3 expression in the TUDCA groups was significantly reduced at the corresponding time point (Table II), and the difference between the two groups was significant ($p < 0.05$) (Figure 3). Beclin-1 mRNA expression was not detected in the sham operation group. Compared with the saline control group, Beclin-1 expression in the TUDCA groups was significantly reduced at the corresponding time point (Table III), and the difference between the two groups was significant ($p < 0.05$) (Figure 4).

Western Blot Analysis Results

Caspase-3 protein expression was not detected in the sham operation group. Compared with the saline control group, caspase-3 expression in the

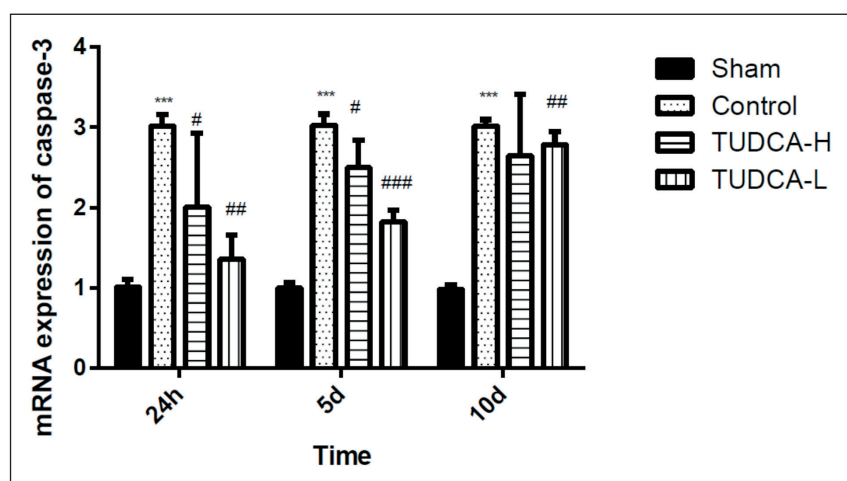


Figure 3. Caspase-3 mRNA expression in each group. #compared with the control group, $p < 0.05$; ##compared with the control group, $p < 0.01$; ###compared with the control group, $p < 0.001$; ***compared with the sham operation group, $p < 0.001$.

Table II. Relative quantification results of the real-time PCR detection of caspase-3 in various groups ($\bar{x} \pm s$).

Groups	24 hours	5 days	10 days
Sham operation group	1.015 ± 0.098	1.002 ± 0.071	0.980 ± 0.057
Saline control group	3.016 ± 0.142	3.029 ± 0.137	3.020 ± 0.080
TUDCA high-dose group	2.005 ± 0.921	2.498 ± 0.340	2.646 ± 0.765
TUDCA low-dose group	1.363 ± 0.299	1.823 ± 0.148	2.785 ± 0.161

Note: Through repeated-measures ANOVA, $F_{\text{time}} = 27.327$; $p = 0.000$; $F_{\text{group} \times \text{time}} = 13.430$; $p = 0.000$; $F_{\text{group}} = 41.287$, $p = 0.000$. The SNK method was used to perform pairwise comparisons between groups.

*Indicates a statistically significant difference from the sham operation group.

Table III. Relative quantification results of the real-time PCR detection of Beclin-1 in various groups ($\bar{x} \pm s$).

Groups	24 hours	5 days	10 days
Sham operation group	2.187 ± 0.806	2.165 ± 0.798	2.078 ± 0.765
Saline control group	2.530 ± 0.134	3.020 ± 0.133	3.199 ± 0.128
TUDCA high-dose group	3.984 ± 0.151	3.970 ± 0.149	3.917 ± 0.143
TUDCA low-dose group	4.179 ± 0.072	4.166 ± 0.071	4.112 ± 0.068

Note: Through repeated-measures ANOVA, $F_{\text{time}} = 772.234$; $p = 0.000$; $F_{\text{group} \times \text{time}} = 1802.400$; $p = 0.000$; $F_{\text{group}} = 31.660$, $p = 0.000$. The SNK method was used to perform pairwise comparisons between groups.

*Indicates a statistically significant difference from the sham operation group.

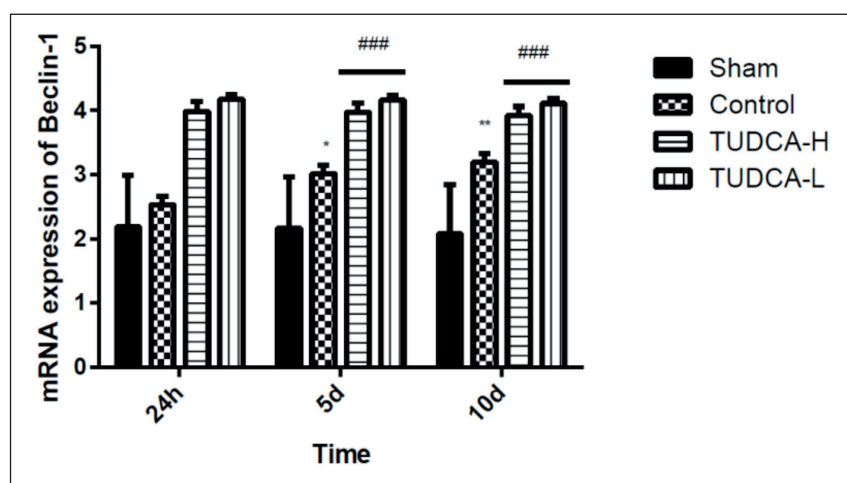


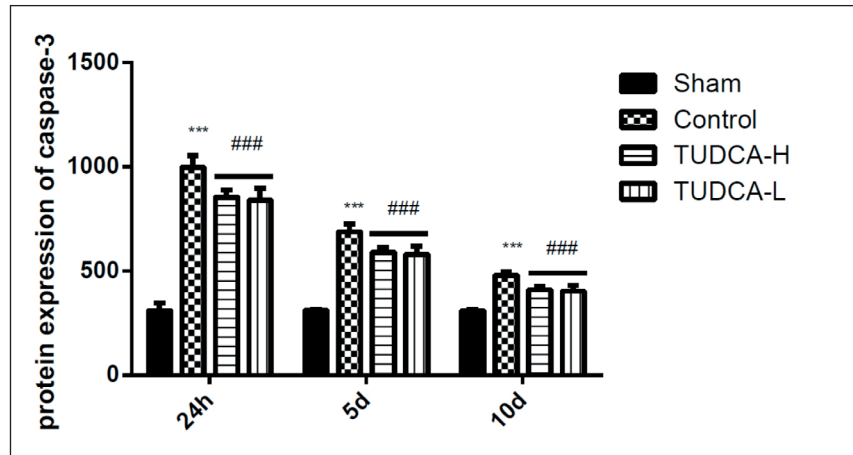
Figure 4. Beclin-1 mRNA expression in each group. *compared with the sham operation group, $p < 0.05$; **compared with the sham operation group, $p < 0.01$; ###compared with the control group, $p < 0.001$.

Table IV. Results of Western blot analysis of caspase-3 expression in each group ($\bar{x} \pm s$).

Group	24 hours	5 days	10 days
Sham operation group	309.334 $\pm$ 37.641	310.820 $\pm$ 6.572	309.632 $\pm$ 7.320
Saline control group	997.983 $\pm$ 56.246	688.608 $\pm$ 38.810	479.032 $\pm$ 26.998
TUDCA high-dose group	854.491 $\pm$ 33.586	589.599 $\pm$ 23.174	410.156 $\pm$ 16.121
TUDCA low-dose group	838.993 $\pm$ 58.253	578.905 $\pm$ 40.195	402.717 $\pm$ 27.961

Note: Through repeated-measures ANOVA, $F_{\text{time}}=2139.264$; $p=0.000$; $F_{\text{group*time}}=6.494$; $p=0.000$; $F_{\text{group}}=181.554$, $p=0.000$. The SNK method was used to perform pairwise comparisons between groups.

Figure 5. Caspase-3 protein expression in each group. *** compared with the sham operation group, $p<0.001$; ### compared with the control group, $p<0.001$.



TUDCA groups was significantly reduced at the corresponding time point (Table IV), and the difference between the two groups was significant ($p < 0.001$) (Figure 5). Beclin-1 protein expression was not detected in the sham operation group. Compared with the saline control group, Beclin-1 expression in the TUDCA groups was significantly reduced at the corresponding time point (Table V), and the difference between the two groups was significant ($p < 0.001$) (Figure 6).

Discussion

This study demonstrates that TUDCA can enhance neuronal autophagy and reduce apop-

toxis after spinal cord injury, thereby playing a role in protecting spinal cord neurons. After spinal cord injury⁸, most apoptotic cells are oligodendrocytes, which results in the demyelinating changes of axons. We also detected a large amount of demyelinating changes of axons via transmission electron microscopy, suggesting that autophagy is related to the demyelinating changes of axons in oligodendrocytes. Koike et al⁹ knocked out the caspase-3 gene in a neonatal brain hypoxic-ischemic mouse model and found that almost one-third of the neurons still survived, while the survival rate of mice in which the autophagy-related Beclin-1 gene was knocked out significantly exceeded those of apoptotic gene knockout mice. This finding raises the following

Table V. Results of Western blot analysis of Beclin-1 expression in each group ($\bar{x} \pm s$).

Group	24 hours	5 days	10 days
Sham operation group	242.187 $\pm$ 14.341	244.367 $\pm$ 14.470	241.969 $\pm$ 14.328
Saline control group	661.030 $\pm$ 30.368	542.045 $\pm$ 24.901	323.905 $\pm$ 14.880
TUDCA high dose group	704.500 $\pm$ 13.385	577.690 $\pm$ 10.976	345.205 $\pm$ 6.559
TUDCA low dose group	704.662 $\pm$ 25.667	577.823 $\pm$ 21.047	345.284 $\pm$ 12.577

Note: Through repeated-measures ANOVA, $F_{\text{time}}=7752.064$; $p=0.000$; $F_{\text{group*time}}=12.505$; $p=0.000$; $F_{\text{group}}=404.213$, $p=0.000$. The SNK method was used to perform pairwise comparisons between groups.

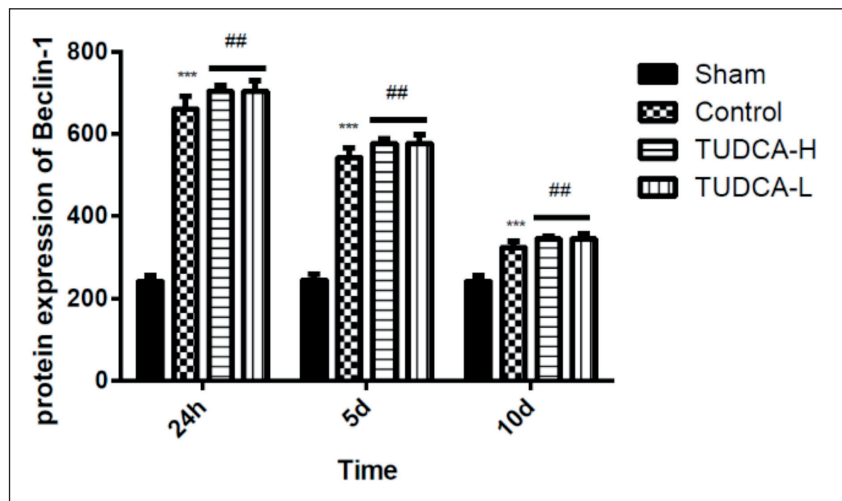


Figure 6. Beclin-1 protein expression in each group. ***compared with the sham operation group, $p < 0.001$; ##compared with the control group, $p < 0.01$.

question: what is the relationship between autophagy and apoptosis¹⁰? Beclin-1 is a key feedback molecule that regulates both autophagy and apoptosis¹¹. Haruo et al¹² found that Beclin-1 expression in neurons rapidly increased in rats in which half of the spinal cord was injured and that the expression reached peak values three days after the injury, though higher Beclin-1 expression could still be detected three weeks after the injury. They found that in the injured neurons, if Beclin-1-based autophagy was dominant, then the nucleus was round in shape after neuronal death; if caspase-3-based apoptosis was dominant, then the nucleus was pyknotic in shape and broken after neuronal death. Thus, it is not difficult to conclude that after nerve cell injury, when autophagy is dominant, it has a protective effect on neurons. Autophagy has been recognized as a mechanism of intracellular defense and stress regulation; however, it is not the case that the stronger the autophagy response, the greater the protective effect on nerve cells¹³.

Our experimental results show that after TUDCA treatment, nerve cells are protected, and apoptosis is inhibited. This effect is specifically reflected in the following aspects. (1) The hind leg locomotion of the rats in the control group was impaired during early spinal cord injury; after TUDCA administration, the hind leg locomotion of rats improved at every monitored time point, suggesting that TUDCA can significantly reduce the damage to spinal cord neurons and improve locomotor function. (2) Caspase-3 expression levels at various time points during early spinal cord injury in the control group were significantly higher than those of the sham operation group; after

TUDCA administration, caspase-3 expression was lower than that of the control group, while Beclin-1 expression was significantly higher than that of the control group after TUDCA administration. Therefore, we suggest that during early spinal cord injury, TUDCA can promote the repair of the injured neurons by enhancing neuronal autophagy and reducing apoptosis and has a protective effect on neurons. (3) During early spinal cord injury, after TUDCA administration, Beclin-1 expression in the spinal cord tissue of rats gradually increased over time, which enhanced autophagy. The enhancement of autophagy functions to remove damaged organelles in the cytoplasm of injured neurons and to degrade them, thereby, minimizing the effects of the damaged organelles in the cells¹⁴. Researchers have observed the positive effect of autophagy on cells, as manifested in tumors¹⁵, neurodegenerative lesions¹⁶ and infection¹⁷ and aging¹⁸ processes.

Conclusions

We provided experimental evidence of the neuroprotection provided by TUDCA administration. However, because the mechanism of spinal cord injury is complex, it is unrealistic to rely on a single drug to treat spinal cord injury. We must fully understand the various mechanisms that affect neuronal death after spinal cord injury, such as the mechanisms of autophagy and apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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