Int J Clin Exp Pathol 2020;13(9):2218-2227
www.ijcep.com /ISSN:1936-2625/IJCEP0116374

Original Article

Mechanism of tauroursodeoxycholic acid-mediated neuronal protection after acute spinal cord injury through AKT signaling pathway in rats

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Received June 16, 2020; Accepted July 18, 2020; Epub September 1, 2020; Published September 15, 2020

Abstract: Objective: *To explore themechanism of tauroursodeoxycholic acid- (TUDCA) mediated neuronal protection after acute spinal cord injury (ASCI) in rats. Methods: ASCI rat model was established following modified Allen's weight*-drop method and these rats were assigned to sham group (received sham operation), model group (ASCI rats), TUDCA group (ASCI rats received TUDCA treatment), MK2206 group (ASCI rats received AKT inhibitor MK2206 orally) and TUDCA + MK2206 group. Motor function of rats was evaluated using Basso Beattie Bresnahan (BBB) method. Hematoxylin-eosin (H&E) staining was used to detect histopathologic changes in the spinal cord and TUNEL fluorescence staining was used to check apoptosis. Real time fluorescence quantitative polymerase chain reaction (qRT-PCR) and western blot were employed to detect the production of AKT pathway related factors, apoptosis related factors (Bax, Bcl-2, caspase-3), autophagy related factor Beclin-1 and endoplasmic reticulum (ER) stress related factors (IRE1, Chop, ATF6) in spinal cord of rats. Results: Compared to the rats in the sham group, rats in ASCI group had decreased BBB scores ($P<0.05$), more significant tissue edema, structural cavity and apoptosis. Compared to rats in sham group, AKT pathway was inactivated in ASCI rats and was activated by TUDCA treatment ($P<0.05$). Compared to sham group, expressions of ER stress-related factors were increased, apoptosis was largely induced in other four groups, and expression of Beclin-1 was increased in the model group ($P<0.05$). TUDCA increased the expression of Beclin-1 and Bcl-2, and inhibited the expression of Bax, Caspase-3, and ER stress-related factors, thus suppressing apoptosis ($P<0.05$). Treatment by MK2206 had contrary effects and protective effects of TUDCA on ASCI rats could be counteracted by MK2206. Conclusion: TUDCA can significantly improve the neural damage, enhance neuron autophagy, alleviate ER stress, and inhibit apoptosis in ASCI rats, by activating the AKT signaling pathway.

Keywords: Tauroursodeoxycholic acid, AKT signaling pathway, endoplasmic reticulum stress, autophagy

Introduction

Acute spinal cord injury (ASCI) refers to spinal cord injury created by external factors directly or indirectly, mainly including sensory disorders, motor disorders of corresponding segments, abnormal muscle tension and pathophysiologic reflex. At present, ASCI is also one of the diseases with the highest disability rate in humans [1, 2].

Tauroursodeoxycholic acid (TUDCA) is the main bile acid in bear bile, and is also the conjugate derivative of ursodeoxycholic acid [3]. It can be taken orally, or injected intraperitoneally and intravenously. TUDCA has broad application prospects in clinic because it penetrates the blood-brain barrier and it has multiple routes of administration [4]. In recent years, TUDCA was also found to have important anti-apoptosis effects. For example, some studies have found that TUDCA can inhibit the inflammatory response of cells, and block oxidative stress and caspase-1 activation, thus inhibiting the apoptosis of hepatocytes and liver injury [5]. In addition, studies have confirmed that TUDCA has excellent effects on nervous system diseases by reducing the apoptosis of nerve cells [6, 7]. In our previous studies, we found that TUDCA elevated the expression of autophagy related factors in the spinal cord tissue of ASCI rats,

inhibited neuron apoptosis, and thus had a protective effect on neurons [8].

AKT signaling pathway is a classic anti-apoptosis pathway, that plays a regulatory role in various biologic effects, such as cell growth, apoptosis, and angiogenesis [9, 10]. Some studies have confirmed that Epimedium promoted neuron recovery and protected neuron survival in spinal cord injury by activating the PI3K/AKT signaling pathway, inhibiting endoplasmic reticulum stress and reducing spinal cord injury [11]. In addition, TUDCA has also been found to reduce early brain injury through activating the AKT pathway [12]. In this study, we built the ASCI model in rats and explored the effects of TUDCA in the progression of ASCI for the first time, hoping to further clarify the pathogenesis of ASCI and provide a relevant therapeutic schedule.

Materials and methods

Establishment of ASCI model in rats

Seventy 6-8 weeks old SD rats, weighing 260-280 g, were purchased from Guangdong Medical Experimental Animal Center of China. All rats were fed in the environment of 19-22°C, 50% humidity, alternate 12 hours of light and 12 hours of darkness every day, with free access to food and drink. Ten rats were used as sham group and 60 rats were used to establish ASCI model through modified Allen's weight-drop method [13]. All rats were fasted for 8 hours before modeling and 10 g/L pentobarbital sodium was used to anesthetize mice at a dose of 40 mg/kg. The rats were fixed onto the operating table in prone position, and the skin of the rats was cut in the middle position with the scalpel after disinfecting the chest and back of the rats to expose the T8-T10 vertebral plate. Then excise T9 vertebral plate completely, and fix T8-T10 spinous process together with clamp. The spinal cord injury was caused by Kirschner wire (weiging 10 g), which was dropped freely from a height of 25 mm, then the Kirschner wire was removed quickly and the skin of the rats was sutured. The standard of successful modeling is listed as following: the phenomenon of spinal cord hemorrhage could be observed by naked eyes, obvious tail swinging and body retraction were found, and the lower limbs of rats showed delayed paralysis after anesthesia. 49 rats were successfully modeled with a success rate of 81.67%. This study was approved by the animal Ethics Committee of General Hospital of Ningxia Medical University.

Experimental grouping and spinal cord tissue extraction

Seventy rats fell into the sham operation group (sham group: T9 vertebral plate was cut without spinal cord injury) and ASCI model group. Rats in ASCI model group were then fall into the following 4 groups randomly, with 12 rats in each group: Model group (ASCI rats), TUDCA group (ASCI rats received TUDCA treatment, TUDCA was purchased from Shanghai Yuanye Biotechnology Co., Ltd., China, product No. B20921), MK2206 group (ASCI rats received AKT inhibitor MK2206 orally, MK2206 was purchased from Selleck chemicals, USA, product No. S1078) and TUDCA + MK2206 group. One minute after spinal cord injury, TUDCA was injected into rats intraperitoneally at the dose of 200 mg/kg for 5 successive days. One minute after spinal cord injury, AKT inhibitor MK2206 was taken orally at the dose of 60 mg/kg for 5 successive days. After 5 days of modeling, rats in each group were euthanized by fast cervical dislocation under anesthesia after the motor function of each group was scored by two observers through the blind test according to Basso Beattie Bresnahan (BBB) scale [14]. The score ranged from 0 to 21, with 0 indicating complete hindlimb disability with no motor function, and 21 indicating normal motor function. Then, the spinal cord segment of the rat was dissected, the length of which was about 1-1.5 cm. The tissues were then fixed with formaldehyde and stored in paraffin for further analysis. The rats not included in the experiment and that failed in modeling were all euthanized by rapid cervical dislocation under anesthesia.

H&E staining

After cutting into 5 μm sections, deparaffinization and rehydration, nuclei were stained with hematoxylin, rinsed and differentiated in hydrochloric acid and alcohol. Then the sections were returned to blue with ammonia and rinsed with running water, and the cytoplasm was dyed with eosin dye solution. After dehydration, the sections were sealed with neutral gum. Three samples were taken from each group for

Table 1. Primer sequences

Name	Primer sequence (5'-3')
Bax	Forward: TCACATGGGAGACAACCC
	Reserve: CCTCTTAAGTGGGACGC
Bcl-2	Forward: ACCACCAACCAAGGGGAGAC
	Reserve: CGTGCCATGAGCATAGGGATAA
Caspase-3	Forward: CGTCAACTCGTGAGGTGGTTC
	Reserve: GGTATTCAGAGCACAGACTGAGA
Beclin-1	Forward: ACGCCTGAATCACCCAATACAT
	Reserve: GCCACTGCCTGAAGAGCTG
IRE1	Forward: CCCGCCACTCGAAGCATGA
	Reserve: TTCTCCCACCTGACAGCTACC
CHOP	Forward: ACGAGGCAAAAGGCTGAAGC
	Reserve: TGAGGTCAAGTTCGCTGATCTT
ATF6	Forward: GCCATATCACCGACTGCTC
	Reserve: ACGTAAGTTACAGGAGATAGGGC
GAPDH	Forward: GTACGCGCTATAACCCCTC
	Reserve: ACGAAGAATTATGAGCGGGAGTC

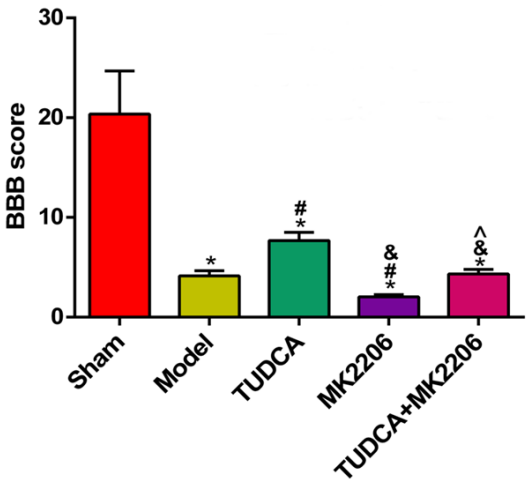

Figure 1. BBB scores of rats in each group (n=12). *P<0.05 compared with sham group; #P<0.05 compared with model group; &P<0.05 compared with TUDCA group; ^P<0.05 compared with MK2206 group. BBB: Basso Beattie Bresnahan; TUDCA: tauroursodeoxycholic acid.

observation under the microscope. We observed the clear and complete tissue structure, neuron degeneration, and other pathologic states.

TdT-mediated nick end labeling TUNEL

Cell apoptosis of rat spinal cord was detected following the instructions of the TUNEL kit (FA201-01, Beijing TransGen Biotechnology Co., Ltd., China). Three rats were selected from each group, and three sections from each rat were observed under the microscope. The number of positive cells was counted and corresponding apoptosis rate were calculated. Apoptosis rate = number of positive cells/total number of cells × 100%.

qRT-PCR

Total RNA in the spinal cord tissue was extracted using Trizol Kit (15596-026, Beijing Solabo Technology Co., Ltd., China) by one-step method. Then, cDNA was reverse transcribed from extracted RNA following the instructions of Takara reverse transcriptional Kit (RR037A, Takara, Japan), and was then diluted into the concentration of 50 ng/μL. 5 μL of cDNA product was taken and amplified by PCR with a reaction system of 25 μL as following: 6 μL of DNAase free water, 5 μL of reverse transcripts, 13 μL of 2× quantitative SYBR Green RT-PCR master mix, and 0.5 μL of upstream and downstream primers. The reaction procedure was as follows: 95°C 5 min, 95°C 20 s, 60°C 1 min, 72°C 30 s, 45 cycles in total. Expressions of genes were analyzed by $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an internal reference and the experiment was triplicated separately. The following primer sequences were designed and synthesized by Shanghai Jikai gene Chemistry Technology Co., Ltd. as listed in **Table 1**.

Western blot

Spinal cord tissues of each group were incubated with a proper amount of precooled tissue lysate for 60 min, and then were centrifuged at a speed of 2000 r/min for 30 min. We collect the supernatant and examine the protein concentration with BCA Kit (DQ111-01, Beijing TransGen Biotechnology Co., Ltd., China). The protein samples (20μg per hole) were separated through SDS-PAGE and were then transferred to PVDF membrane by wet transfer method. After blocking in 5% skimmed milk powder, the membranes were put in the following rabbit primary antibodies at 4°C overnight: anti-AKT (2 μg/mL, ab18785, Abcam, UK), anti-p-AKT (1/1,000, ab38449, Abcam, UK), anti-Bax (1/2,000, ab182733, Abcam, UK), anti-Bcl-2 (1/1,000, ab194583, Abcam, UK), anti-Caspase-3 (1/500, ab13847, Abcam, UK), anti-Beclin-1 (1/2,000, ab207612, Abcam, UK), anti-IRE1 (2 μg/mL, ab37073, Abcam, UK), anti-CHOP (1 mg/mL, YT664-CEV, Beijing Baileibo Technology Co., Ltd., China), anti-ATF6

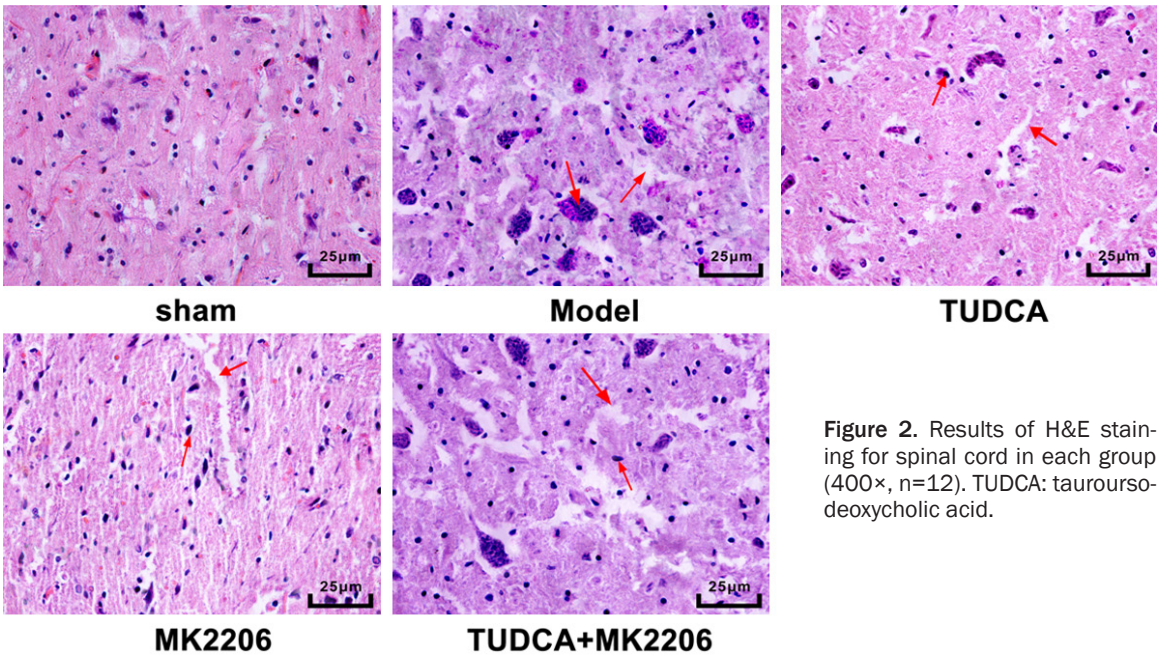

Figure 2. Results of H&E staining for spinal cord in each group (400×, n=12). TUDCA: tauroursodeoxycholic acid.

(1/1,000, ab203119, Abcam, UK). After rinsing with PBST, horseradish peroxidase labeled goat-anti-rabbit IgG (1/10,000, ab6721, Abcam, UK) was added on the membranes at room temperature for 1 hour. After rinsing with PBST, the membranes were exposed with ECL and were observed in dark. GAPDH was used as an internal parameter and Image J software was used to analyze relative expressions of proteins. Relative expression levels of proteins were calculated by the ratio of corresponding gray value of the target band to GAPDH. The experiment was triplicated separately.

Statistical analysis

SPSS 22.0 (SPSS Inc, Chicago, USA) was used for statistical analysis. Measurement data were shown as mean ± standard deviation ($\bar{\chi}$ ± sd). Comparison between groups was conducted by univariate analysis of variance combined with post-Bonferroni test. P<0.05 indicated a significant difference.

Results

TUDCA elevates BBB scores of ASCI rats, while AKT pathway inhibitor reduces BBB scores

Motor function of rats in each group was evaluated using the BBB scale. The results showed that compared to the sham group, BBB scores of the other four groups all decreased significantly, and MK2206 group decreased the most significantly. TUDCA treatment improved the BBB scores to a certain extent (P<0.05). However, no significant difference in BBB scores was detected between TUDCA + MK2206 group and model group, indicating that the improvement effect of BBB scores of ASCI rats by TUDCA was offset by AKT pathway inhibitors, **Figure 1**.

TUDCA improves the pathologic state of spinal cord in ASCI rats, while AKT pathway inhibitor aggravates spinal cord injury in ASCI rats

H&E staining was used to value the pathologic changes of the spinal cord, **Figure 2**. The results exhibited that the structure of spinal cord in the sham group was clear and complete, but neurons in other groups were significantly deformed and lost, some of them even formed vacuoles, and white matter was loose with a disordered structure. Compared to the model group, the pathologic changes of MK2206 group were aggravated, while the extent and range of spinal cord injury in the TUDCA group were alleviated. In addition, no significant difference was detected between the TUDCA + MK2206 group and model group.

TUDCA inhibits apoptosis of spinal cord cells in ASCI rats, while AKT pathway inhibitor promotes apoptosis of spinal cord cells

The apoptosis of spinal cord cells in each group was discovered through TUNEL staining and

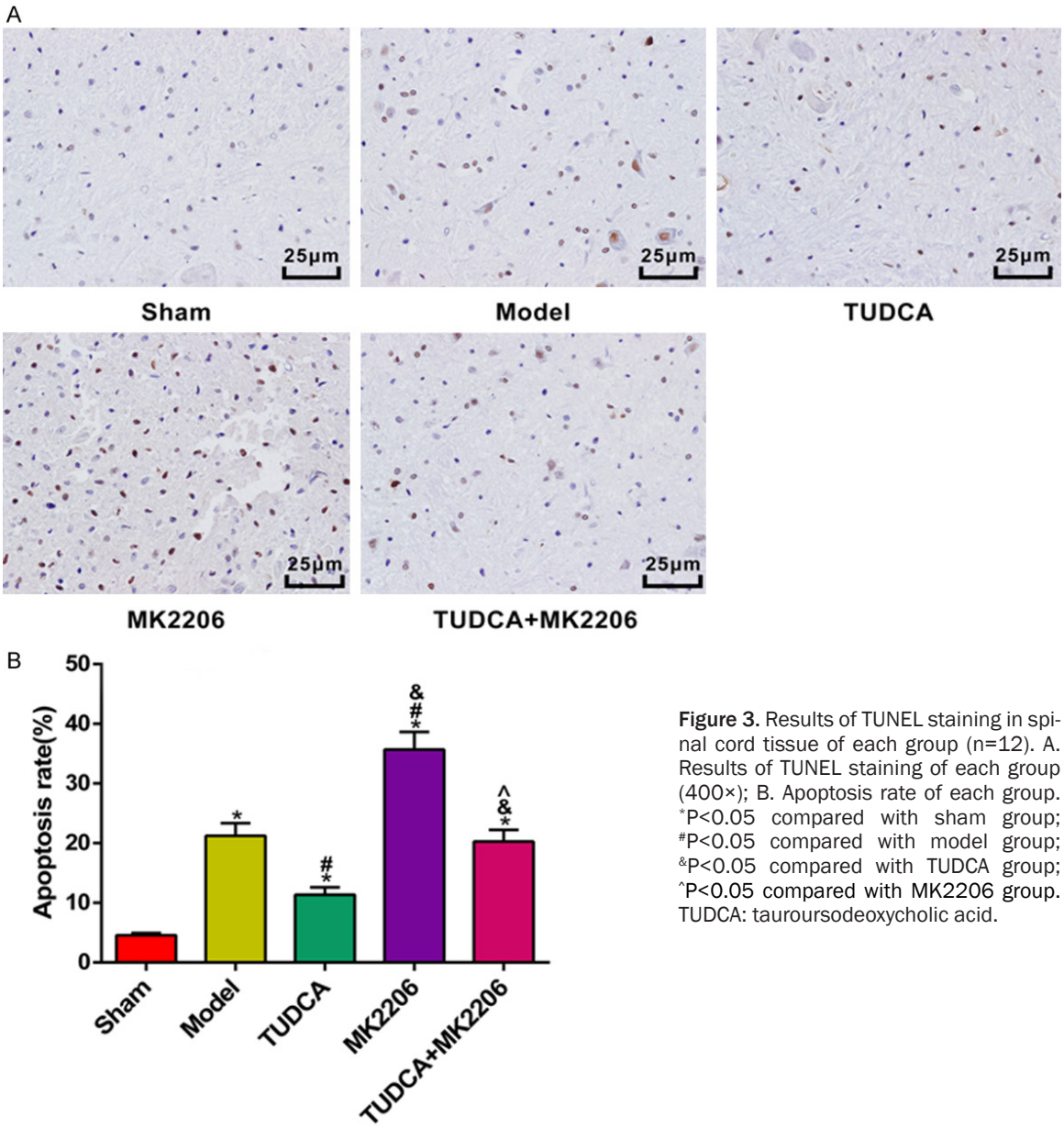

Figure 3. Results of TUNEL staining in spinal cord tissue of each group (n=12). A. Results of TUNEL staining of each group (400×); B. Apoptosis rate of each group. *P<0.05 compared with sham group; #P<0.05 compared with model group; &P<0.05 compared with TUDCA group; ^P<0.05 compared with MK2206 group. TUDCA: tauroursodeoxycholic acid.

the positive cells are shown in brown in **Figure 3**. The number of TUNEL positive cells in sham group was less than the other groups (P<0.05). Besides, the number of apoptotic cells was more in TUDCA group, and was less in the MK2206 group than model group (P<0.05), while no significant difference was detected between the model group and TUDCA + MK2206 group (P>0.05).

The expressions of AKT pathway related factors were elevated by TUDCA in the spinal cord tissues of ASCI rats

Expressions of AKT pathway related factors in the spinal cord tissues of ASCI rats were checked by western blot as shown in **Figure 4**. The results showed that the expression level of phosphorylated AKT proteins in the spinal cord of ASCI group was lower than the sham group (P<0.05). Expression of phosphorylated AKT was increased by TUDCA, but was sharply decreased by MK2206 (P<0.05).

TUDCA inhibits the expressions of Bax and Caspase-3, and elevates the expression of Bcl-2 and Beclin-1, but MK2206 has the opposite effect

Expression of apoptosis related factors Bax, Bcl-2, and caspase-3 and autophagy related Beclin-1 in spinal cord tissues of rats was

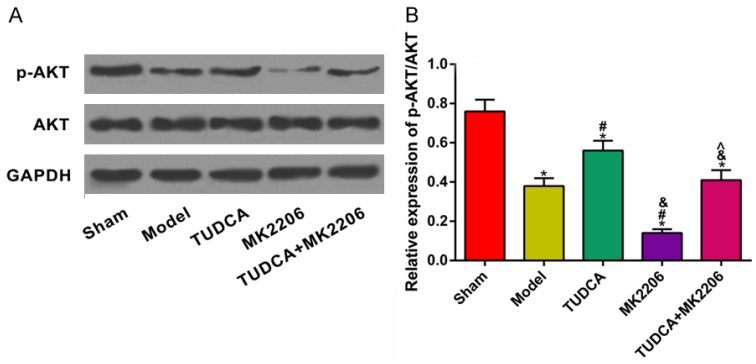

Figure 4. Expression of AKT pathway-related factors in spinal cord of each group (n=12). A: P-AKT and AKT protein bands in each group; B: Rate of p-AKT/AKT in each group. *P<0.05 compared with sham group; #P<0.05 compared with model group; &P<0.05 compared with TUDCA group; ^P<0.05 compared with MK2206 group. TUDCA: tauroursodeoxycholic acid.

detected as shown in **Figure 5**. Rats in ASCI group had higher expressions of Bax, and caspase-3 and lower expression of Bcl-2 in spinal cord than sham group (P<0.05). At the same time, TUDCA could significantly improve the expression of these above indicators, while MK2206 had the opposite effect (P<0.05). Compared to the sham group, expressions of Beclin-1 in model, TUDCA, TUDCA + MK2206 group were all enhanced and the change was most significant in TUDCA group, but was inhibited in MK2206 group (P<0.05).

Expression of ER stress-related factors in spinal cord of ASCI rats is suppressed by TUDCA, while elevated by MK2206

Expression of ER stress-related factors in the spinal cord of ASCI rats was checked through western blot, **Figure 6**. The results showed that sham group had the lowest expression of ER stress-related factors versus other groups (P<0.05). Compared to model group, these above factors were suppressed in TUDCA group and were elevated in the MK2206 group (P< 0.05). In addition, no significant difference in the expression of ER stress-related factors was found between the TUDCA + MK2206 group and model group (P>0.05).

Discussion

In this study, drug intervention and AKT pathway inhibitor intervention were carried out in ASCI rats. At the same time, oxidative stress and autophagy were analyzed in each group. The results showed that TUDCA could promote the recovery of neural injury, enhance autophagy, and inhibit the expression of oxidative stress-related factors.

Many studies have shown that TUDCA has a significant neuroprotective effect on nervous system diseases. For example, Yanguas-Casás et al. found that TUDCA had significant anti-inflammatory effect, and could effectively inhibit the inflammation of neuron and the over-activation of microglia in acute neuritis model of mice [15]. Besides, Dionísio et al. showed that TUDCA inhibited inflammation and over-deposition of β-amyloid protein in Alzheimer's disease, which is a promising strategy in Alzheimer's treatment [16]. In addition, in the study of spinal cord injury, Zhang et al. found that TUDCA could reduce the damage by inhibiting the up-regulation of CIBZ gene, suppressing oxidative stress and reducing neuronal apoptosis [17]. All the above studies have proven the excellent neuro-protective effect of TUDCA in spinal cord injury. Similarly, our study also confirmed that TUDCA could improve BBB scores and pathologic injury of spinal cord, and reduce apoptosis of spinal cord tissue. In addition, we found that expression of AKT pathway related factors was inhibited in the model group compared with the sham group, and was then rescued by TUDCA, suggesting that the protective effect of TUDCA in ASCI can be achieved by activating the AKT pathway.

The AKT signaling pathway is commonly found to promote cell growth, inhibit apoptosis, and promote tumor angiogenesis, which is abnormally activated in the progression of a variety of tumors [18-22]. In recent years, attention has been paid to the effect of the AKT signaling pathway on spinal cord injury. For example, expression of phosphorylated AKT in spinal cord tissue of rats was significantly inhibited after spinal cord injury compared with the sham group [23]. In addition, MANF has also been found to activate the expression of p-AKT to further reduce neuronal apoptosis and improve neural function [24]. In this study, we found that the BBB score was inhibited, histopathologic

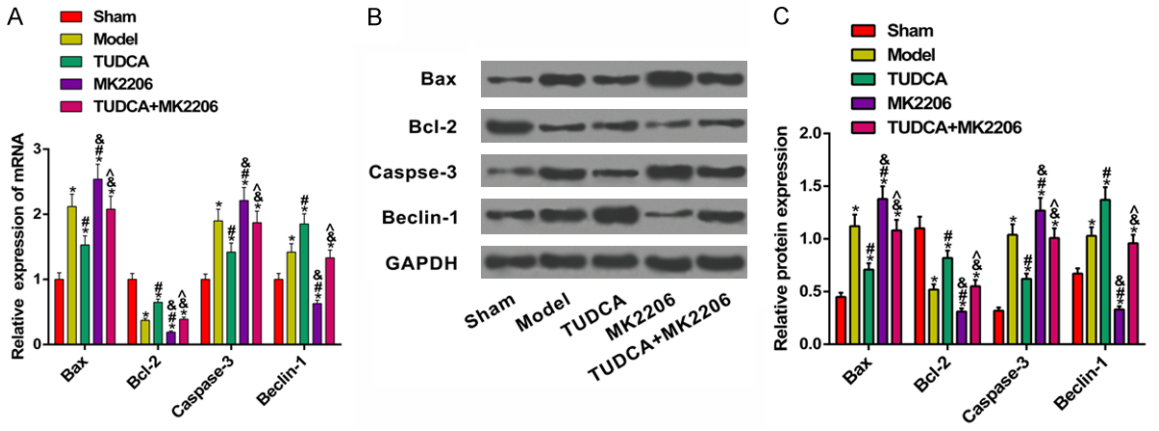

Figure 5. Expression of apoptosis and autophagy related factors in spinal cord tissues of each group (n=12). A. Relative expression of Bax, Bcl-2, Caspase-3, and Beclin-1 mRNA in the spinal cord of each group; B. Representative picture of protein bands in each group; C. Quantitative expression of proteins in each group. *P<0.05 compared with sham group; #P<0.05 compared with model group; &P<0.05 compared with TUDCA group; ^P<0.05 compared with MK2206 group. TUDCA: tauroursodeoxycholic acid.

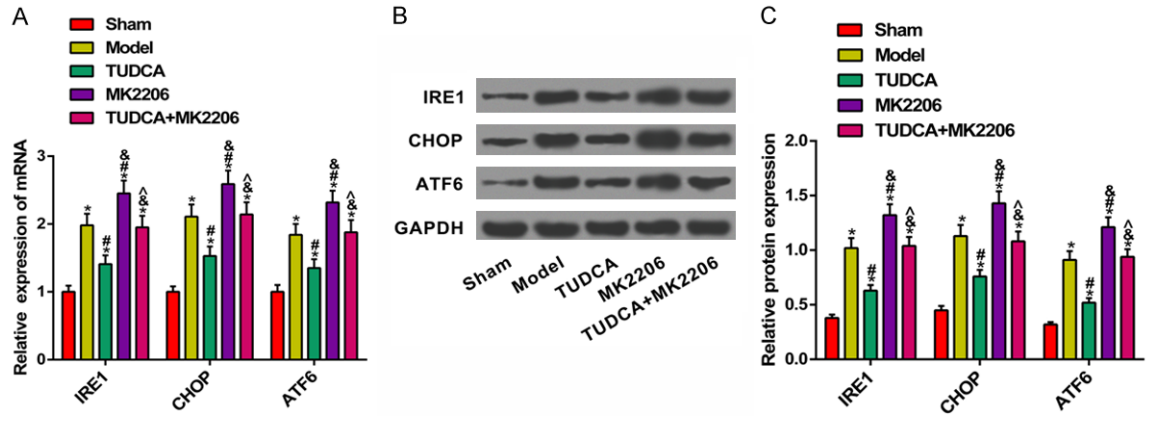

Figure 6. Expression of ER stress-related factors in each group (n=12). A. Relative expression of ER stress-related factors mRNA in each group; B. Representative picture of ER stress-related protein bands in each group; C. Quantitative expression of ER stress-related proteins of each group. *P<0.05 compared with sham group; #P<0.05 compared with model group; &P<0.05 compared with TUDCA group; ^P<0.05 compared with MK2206 group. TUDCA: tauroursodeoxycholic acid; ER: endoplasmic reticulum.

damage and apoptosis were aggravated, and the improvement effect of TUDCA on the indexes of ASCI rats could also be counteracted by AKT pathway inhibitors. This study for the first time confirmed that the protective effect of TUDCA in ASCI might be achieved by regulating the AKT signaling pathway. In addition, autophagy is another important pathologic mechanism after spinal cord injury. At present, the effect of autophagy in a variety of diseases is still controversial. Some studies believe that autophagy can induce cell death but other studies have found that autophagy has cytoprotective effects through reducing cell death [25-28]. Research on spinal cord injury has found that rapamycin treatment after spinal cord injury can significantly enhance the expression of Beclin-1 and inhibit apoptosis in spinal cord tissue, suggesting that enhanced autophagy can protect against neuronal injury after spinal cord injury [29]. We found that expression of autophagy-related factors in the model group was enhanced compared with the sham group, and treatment by TUDCA could further promote the expression of autophagy related factors, which further confirmed that autophagy had an appropriate protective effect in ASCI. Meanwhile, in the study of the relationship between miR-145 and myocardial infarction, Higashi et al. found that miR-145 could pro-

mote the recovery of infarcted myocardium by enhancing autophagy and the expression of phosphorylated AKT [30]. Our present study further found that TUDCA can affect autophagy by regulating AKT expression. In addition to autophagy, ER stress is also a common phenomenon in the process of ASCI [31-34]. Studies have already shown that ER stress can accelerate the apoptosis of spinal cord neurons, further aggravating the occurrence of injury and neurological dysfunction [35]. Tka et al. found that melatonin can reduce ER stress and activate the AKT signaling pathway after ischemia-reperfusion injury [36]. In our study, the expression of ER stress-related factors was also detected. The results showed that TUDCA inhibited the expression of ER stress-related factors and AKT pathway inhibitor played the opposite effect, which further explains the mechanism of TUDCA in ASCI.

This study also has some limitations. In addition to apoptosis and endoplasmic reticulum stress which affect the process of ASCI, inflammatory response and other processes are also important disease inducements; thus we will explore these further in our future research.

In conclusion, we suggest that TUDCA can affect AKT signaling pathway to regulate neural damage, promote autophagy, and inhibit oxidative stress and apoptosis in ASCI rats, which is of great significance to clarify the specific mechanism of TUDCA in ASCI.

Acknowledgements

This work was supported by the Natural Science Foundation of Ningxia for Study on the mechanism of tauroursodeoxycholic acid in autophagy of spinal cord injury (2019AAC03207).

Disclosure of conflict of interest

None.

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