

Original Article

Effects of tetrandrine pretreatment on spinal cord ischemia-reperfusion injury in rabbits

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Received June 16, 2020; Accepted July 19, 2020; Epub October 15, 2020; Published October 30, 2020

Abstract: Objective: Our study aimed to explore the effects of tetrandrine pretreatment on spinal cord ischemia-reperfusion injury in rabbits. Methods: Forty-eight New Zealand white rabbits were fall into three groups randomly according to the random number table as follows: Ischemia-reperfusion group (I/R group), sham operation group (sham group) and 22.5 mg/kg pretreatment group using 2 g/L tetrandrine solution (Tet+I/R group). After the model establishment and operation, the improved Tarlov score was valued, the permeability of blood spinal cord barrier (BSCB) (valued according to the content of Evans Blue (EB) in spinal cord tissue) was examined, the apoptosis staining of spinal cord cells and the content of apoptosis related protein Bcl-2 and Bax were detected through TUNEL. Results: In the sham group, the improved Tarlov score was 4 on average without dyskinesia. Besides, I/R group had evidently lower improved Tarlov score than sham group and Tet+I/R group ($P<0.01$). At the same time, Tarlov score of Tet+I/R group was markedly improved comparing to I/R group, but was still lower than that of sham group ($P<0.01$). In addition, the number of apoptotic cells, EB content, Bax expression, TNF- α and IL-6 expression in Tet+I/R group were all lower than those in I/R group. Tet+I/R group had much higher both expression of Bcl-2 and ratio of Bcl-2/Bax than I/R group with statistical difference ($P<0.05$). Conclusion: Tetrandrine maintains the integrity of blood-spinal cord barrier by reducing spinal cord ischemia-reperfusion injury and neuronal apoptosis. The protective effect may be related to the regulation of Bcl-2/Bax ratio and the reduction of pro-inflammatory factors.

Keywords: Tetrandrine, spinal cord ischemia-reperfusion injury, cell apoptosis, blood-spinal cord barrier

Introduction

Spinal cord ischemia-reperfusion injury (SCIRI) is a common complication in the operation of spinal cord and thoracoabdominal aorta. During the operation, ischemia and hypoxia occurred along with compressed spinal cord. In this state, oxidative stress and inflammatory reaction emerged in the nerve cells of the spinal cord, leading to structural disorder or dysfunction. More seriously, after the compression was relieved, the blood refluxing further damaged the nerve cells and induced the reperfusion injury of nerve cells [1, 2]. Many mechanisms are found to be associated with SCIRI, including inflammatory response, apoptosis, oxygen free radical mediated lipid peroxidation, intracellular calcium overload, leukocyte activation and so on. Among which, inflammatory response and neuronal apoptosis are two important pathophysiological mechanisms of SCIRI [3].

Tetrandrine (Tet) is a kind of natural selective calcium channel blocker, which has the pharmacological effects including heat dissipation, blood pressure reduction, anti-infection, anti-oxidation, anti-corrosion, anti-cytotoxin and so on. Tet is able to improve the state of local ischemia after spinal cord injury, prevent calcium overload, suppress the generation of free radicals and inflammatory response [4-8]. However, there are few researches on Tet in the field of SCIRI till now, and there are no relevant reports about whether Tet also has a protective effect on SCIRI. In this study, the effects of Tet pretreatment on expressions of Bcl-2, Bax protein and apoptosis gene, neural cell injury, water content of spinal cord, BSCB function and motor function of hind limbs were observed after SCIRI. We preliminarily observed the prospect of Tet in the treatment of SCIRI, aiming to provide experimental basis for the development of SCIRI treatment clinically.

Study on the effect of tetrandrine on reducing spinal cord re-injury

Materials and methods

Animal anesthesia group and administration

Forty-eight clean male New Zealand white rabbits, weighing 2.1-2.5 kg, were provided by the animal experimental center of Guizhou Medical University. Before the operation, the experimental rabbits were fed in separate cages in room temperature of 25°C with sufficient water and food. Then, no food and water was supplied for 8 h before the operation. SCIRI model was established according to improved Zivin method [9]. According to the body weight of the experimental rabbits, amylobarbitone (Shanghai Shangyao xinpressing Pharmaceutical Co., Ltd., China) was used for intraperitoneal injection anesthesia at a dose of 30 mg/kg. Then, 48 rabbits were fall into three groups randomly: Ischemia-reperfusion group (I/R group), sham operation group (sham group) and Tet+I/R group (Tet+I/R group) according to the random number table, 16 rabbits in each group. Tet (Jiangxi Pengze pharmaceutical factory, China) and normal saline (NS) were prepared into 2 g/L solution. After anesthesia, Tet was injected into the left ear marginal vein according to the weight of rabbits in the Tet+I/R experimental group at a dose of 22.5 mg/kg. After anesthesia, 0.9% NS of the same volume as Tet+I/R group was injected into I/R group and sham group.

Experimental method and process

(1) Sham group: One hour before the operation, the left ear marginal vein of experimental rabbits was injected with 0.9% normal saline about 24-28 ml (the volume of normal saline was the same as the Tet calculated according to the dose of Tet 22.5 mg/kg). After anesthesia, the rabbits in the sham group only underwent open surgery without clamping the abdominal aorta. 30 min after the operation, the wound was sutured.

(2) I/R group: One hour before the operation, the left ear marginal vein of experimental rabbits was injected with 0.9% normal saline about 24-28 ml (the volume of normal saline was the same as the Tet calculated according to the dose of Tet 22.5 mg/kg). After anesthesia, the experimental rabbits underwent open surgery and the abdominal aorta was closed with Scoville-Lewis artery clamp (Beijing granhui-kang Medical Instrument Co., Ltd., China). 30

min later, Scoville Lewis clamp was released to restore the blood supply of abdominal aorta, and the surgical wound was sutured.

(3) Tet+I/R group: One hour before the operation, the left ear marginal vein of experimental rabbits was injected with Tet about 24-28 ml (the volume was calculated according to the dose of Tet 22.5 mg/kg). After anesthesia, the experimental rabbits underwent open surgery and the abdominal aorta was closed with Scoville-Lewis artery clamp (Beijing granhui-kang Medical Instrument Co., Ltd., China). 30 min later, Scoville-Lewis clamp was released to restore the blood supply of abdominal aorta, and the surgical wound was sutured. The rabbits in these three groups were anesthetized with 30 mg/kg of amylobarbitone 24 h after the operation, and then were killed by rapid cervical dislocation.

Outcome measures

Motor function scores: The motor function of hind limbs of the experimental rabbits was scored at 24 h post the modeling and operation. The scoring standard was in accordance with the improved Tarlov scoring standard [10]. The total score of the movement ranges from 0 to 4. Higher score means better motor function of the hind limbs.

Permeability of BSCB

After the function scoring of hind limbs was completed within 24 h after the operation, 2% EB was injected into the ear vein of rabbit slowly at the dose of 10 mL/kg before the rabbits were killed. One hour later, under the anesthesia with amylobarbitone at the dose of 30 mg/kg, the chest of the experimental rabbit was opened to expose the heart, and 0.9% NS was used at the ascending aorta to perfuse at the dose of 500 mL/kg. The spinal cord tissue of the experimental rabbit was obtained, and the outer membrane of the spinal cord was stripped. Part of the tissues were fixed with 4% paraformaldehyde, then frozen and cut into 10 µm thin slices continuously. The distribution of EB was observed under green laser mode of fluorescence microscope, and the EB was red under the microscope. The other part was immersed in 4% formamide, and the EB content in each gram of spinal cord tissue was detected by enzyme detection instrument (Wuxi Huawei Delang Instrument Co., Ltd., China).

Study on the effect of tetrandrine on reducing spinal cord re-injury

Apoptosis staining of spinal cord cells by TUNEL

After the outer membrane of the spinal cord was stripped, part of the tissue was fixed with 4% paraformaldehyde and was made into frozen section. The section was completely covered with protease K working solution (Shanghai enzyme linked biology Co., Ltd., China) and the membrane breaking working fluid (Shanghai enzyme linked biology Co., Ltd., China) one by one. The slice tissue was then covered with mixed reagent solution in the TUNEL Kit (Shanghai enzyme linked biology Co., Ltd., China) according to the instructions. Then, the processed section was put into a wet box and was incubated with a proper amount of DAPI dye (Shanghai enzyme linked biology Co., Ltd., China). After these above steps were completed, the slices were washed, and were then incubated with anti-fluorescence quenching blocking agent (Shanghai enzyme linked biology Co., Ltd., China) for sealing. Observe the apoptotic nerve cells under fluorescence microscope and the number of apoptotic neurons in each group was recorded in 5 randomly selected fields.

Immunohistochemistry

After the spinal cord slices were fixed with formaldehyde solution, expression of Bcl-2 and Bax in the spinal cord was detected by streptavidin peroxidase (SP) method strictly using the kit (Shanghai enzyme linked biology Co., Ltd., China). The definition of Bcl-2 and Bax positive was determined by the percentage of positive cells and the degree of staining which was observed under 5 random high-power mirrors. The percentage $\leq 1\%$ scores 0, 2-10% scores 1, 11-50% scores 2, 51-80% scores 3 and 81-100% scores 4. The degree of staining: negative scores 0, weak positive scores 1, positive scores 2, strong positive scores 3. The product of the two was negative at 0, weak positive at 1-4, positive at 5-7 and strong positive at 8-12. Positive rate = (weak positive + positive + strong positive)/total cases * 100%.

qRT-PCR

Total RNA of spinal cord tissue was extracted using Trizol Kit (Molecular Research Center, USA), and the RNA was converted into cDNA by reverse transcription Kit (Fermentas, USA). Circulation system 25 μL : SYBR premix (2 \times) 12.5 μL . The upstream and downstream prim-

ers are designed and provided by Guangzhou Ruibo Biotechnology Co., Ltd. The primer sequences of Bcl-2 are as follows: upstream (5'-ATAGCTGTTGCCCACTCGAC-3') and downstream (5'-ATCTATGCCCAACAGGCCAC-3'). The primer sequences of Bax are as follows: upstream (5'-AGAGGGCCCATCACTGAGAA-3') and downstream (5'-CTCCGCCCATAGTTACTG-3'). The primer sequences of GAPDH are as follows: forward (5'-GTCGTAGCAAACCAACCAAGC-3') and reverse (5'-TGTGGGTGAGGAGCACATAG-3'). The reaction system is listed as follows: the 50 μL system includes 1 \times Taq man buffer, 3.5 mmol/L MgCl_2 , 200 $\mu\text{mol/L}$ Datp, dCTP and dGTP, 400 $\mu\text{mol/L}$ dUTP, 1.25 U AmpliTaq Gold, 0.5 U AmpErase UNG, 0.3 $\mu\text{mol/L}$ upstream and downstream primers of Bcl-2 respectively, 20 nmol/L fluorescent probe, the cDNA obtained by reverse transcription from 100 ng RNA or quantitative templates of different concentrations. Reaction conditions: Pre-denaturation for 4 min at 94°C, 40 s at 95°C, 30 s at 60°C, 30 s at 72°C, 35 cycles, prolongation for 1 min at 72°C. GAPDH mRNA was used as the internal control. Relative gene expression was quantified by $2^{-\Delta\Delta\text{Ct}}$ method.

Detection of related protein expression through western blotting

Total protein in the other part of the spinal cord tissues was extracted and protein concentration was valued using BCA protein concentration test kit (AmyJet scientific Co., Ltd., USA). 12% SDS-PAGE gel was prepared and samples were prepared for electrophoresis. Then the separated protein was transferred to PVDF membrane, and the membrane was sealed at room temperature for 1 h. Then discard the blocking solution and incubate the membrane with the following mouse anti human monoclonal antibody at 4°C overnight: anti-IgG (1:200, AmyJet scientific Co., Ltd., USA. Article No.: AAT-V100065), anti-TNF- α (1:500, Shanghai Xinyu Biotechnology Co., Ltd., China. Article No.: bs-0078R) and anti-IL-6 (1:500, Shanghai enzyme linked Biotechnology Co., Ltd., China. Article No.: ml027844). After incubated with HRP-labeled Goat anti rabbit IgG secondary antibody (1:10,000, AmyJet scientific Co., Ltd., USA. Article No.: A12004-1) at room temperature for 30 min. Then, fresh mixed ECL solution (Shanghai enzyme linked Biotechnology Co., Ltd., China) was added to the membrane for further exposure and development in the dark

Study on the effect of tetrandrine on reducing spinal cord re-injury

Table 1. Comparison of motor function scores of three groups of experimental rabbits ($\bar{x} \pm sd$)

Items	I/R group (n=16)	Sham group (n=16)	Tet+I/R group (n=16)	F	P
Tarlov rating (scores)	1.52±0.97	4.00±0.00 ^{***}	3.02±0.76 ^{**###}	49.320	<0.001

Note: Compared with I/R group, ^{**}P<0.01, ^{***}P<0.001; compared with Sham group, ^{###}P<0.001.

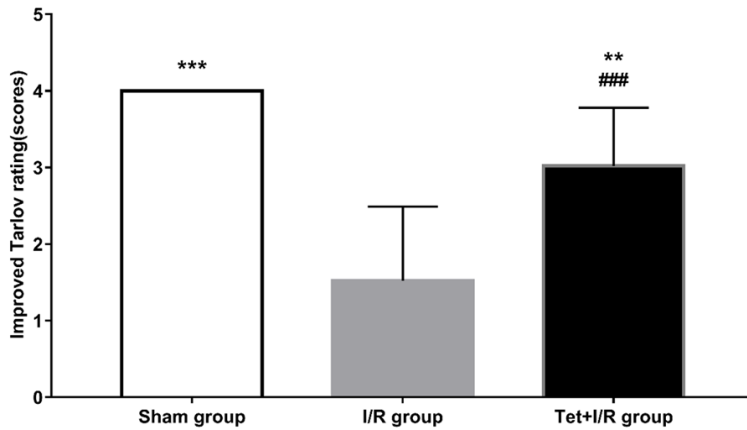


Figure 1. Comparison of motor function scores of three groups of experimental rabbits. Compared with I/R group, ^{**}P<0.01, ^{***}P<0.001; compared with sham group, ^{###}P<0.001.

room. Gray value of the target strip was analyzed using Alphaeasefc software processing system. Relative expression of the target protein was expressed as the ratio of the gray value of the target strip to GAPDH strip.

Statistical analysis

SPSS 17.0 statistical software was used for statistical analysis. Continuous variables are expressed as mean \pm standard deviation ($\bar{x} \pm sd$). One-way ANOVA and Turkey method were used for comparison between multiple groups and two groups, respectively. Enumeration data was expressed in the number of cases/percentage (n/%) and were further analyzed by Pearson chi square test and Fisher's exact probability method. P<0.05 was considered as statistically significant.

Results

Comparison of motor function scores of three groups of experimental rabbits

In the sham group, the improved Tarlov score was 4 on average, and there was no dyskinesia. The improved Tarlov score of I/R group was sharply decreased comparing to sham group and Tet+I/R group with statistical differences (P<0.01). Tarlov score of Tet+I/R group was

obviously higher than I/R group, but was still lower than sham group (P<0.01). As shown in **Table 1** and **Figure 1**.

Permeability of BSCB

Comparison about permeability of BSCB in three groups showed that sham group had the lowest content of EB (1.762±1.239) $\mu\text{g/g}$, while I/R group had much higher content of EB (16.023±2.614) $\mu\text{g/g}$ than sham group and Tet+I/R group with statistical differences (P<0.001). The EB content of Tet+I/R group (8.922±2.402) $\mu\text{g/g}$ was obviously improved comparing to that of I/R group, but was still higher than that of sham group (P<0.001). As shown in **Figure 2**.

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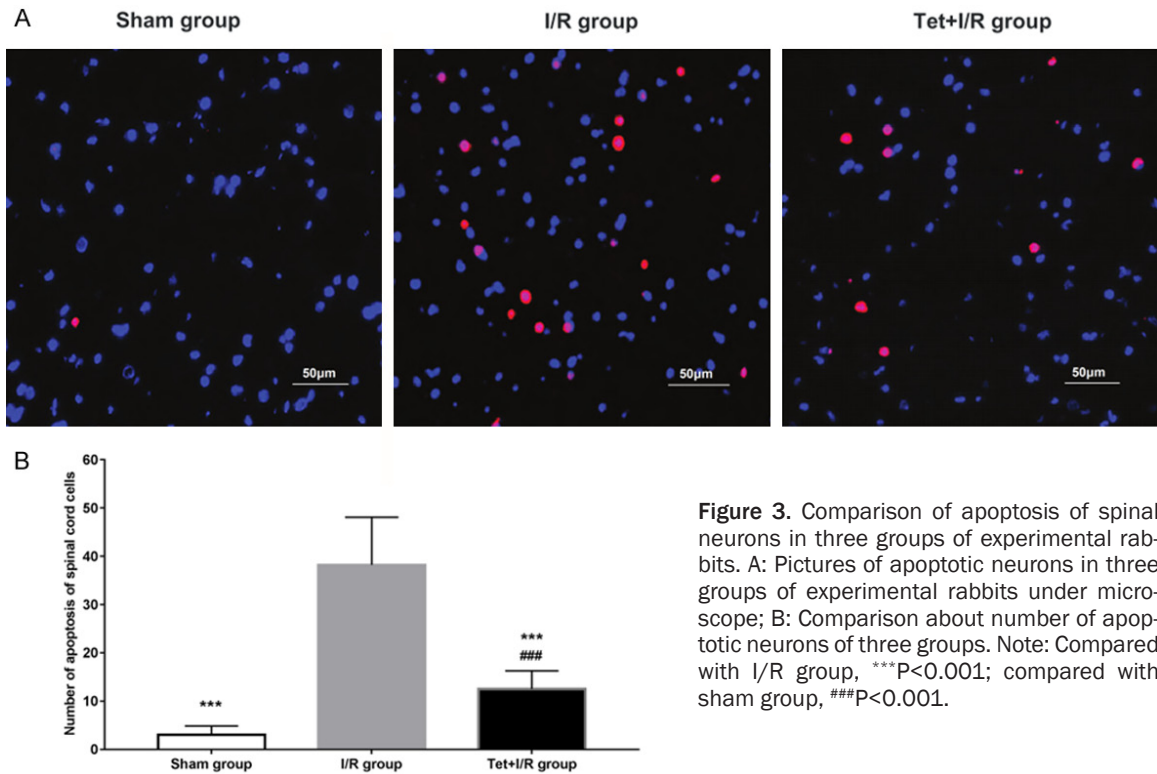
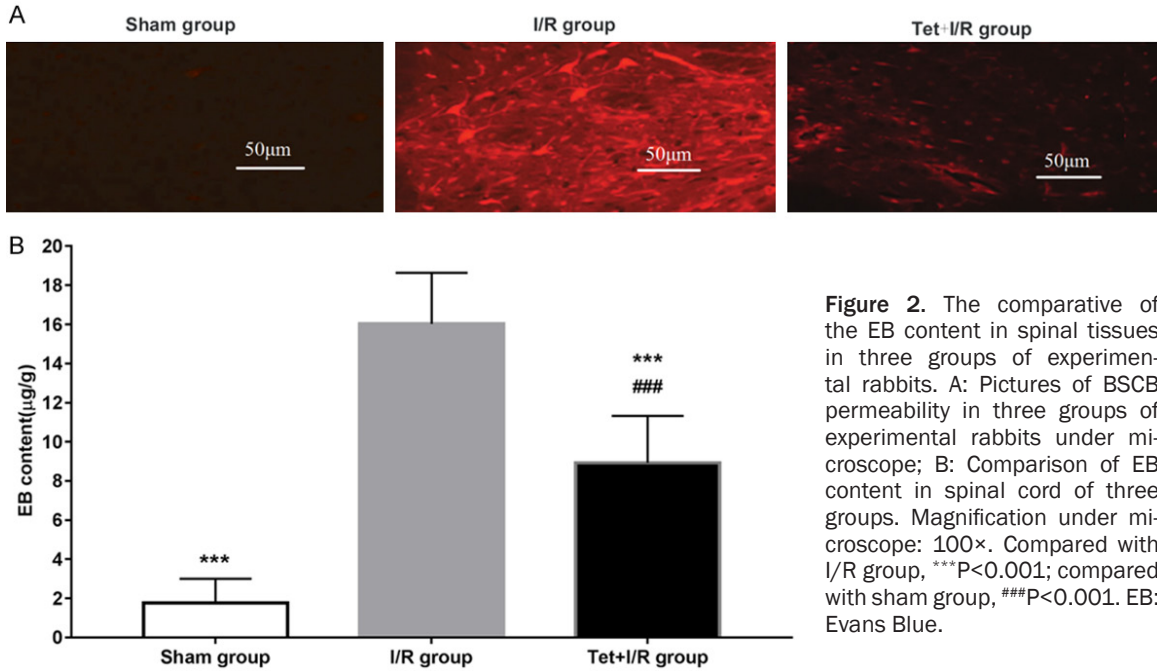
Apoptosis of neurons in spinal cord detected through TUNEL

Comparison of apoptosis of spinal neurons in three groups of experimental rabbits showed that sham group had the lowest number of apoptotic neurons (3.032±1.821). The number of apoptotic neurons in I/R group (38.232±9.872) was largely increased and was much higher than that of sham group and Tet+I/R group with statistical differences (P<0.001). The number of apoptotic neurons of Tet+I/R group (12.523±3.762) was largely improved comparing to that of I/R group, but was still much higher than that of sham group (P<0.001). As shown in **Figure 3**.

Comparison of immunohistochemistry and mRNA expression of Bcl-2 and Bax in different groups

The immunohistochemistry results of Bcl-2 and Bax in three groups showed that, compared with sham group and Tet+I/R group, I/R group had lower Bcl-2 and higher Bax expression. Besides, compared with sham group and Tet+

Study on the effect of tetrandrine on reducing spinal cord re-injury



I/R group, I/R group had lower rate of Bcl-2/Bax and lower expression of Bcl-2 mRNA, but higher expression of Bax mRNA, all with statistical differences (P<0.05). As shown in **Tables 2, 3** and **Figure 4**.

Comparison of relative expression of TNF- α and IL-6 proteins in different groups

The results showed that relative expression of TNF- α and IL-6 proteins in Tet+I/R group was

Study on the effect of tetrandrine on reducing spinal cord re-injury

Table 2. Comparison of relative mRNA expression of Bcl-2 and Bax in different groups ($\bar{x} \pm sd$)

Groups	Cases	Relative expression of Bcl-2 mRNA	Relative expression of Bax mRNA	Rate of Bcl-2/Bax
Sham group	16	0.42±0.09***	0.34±0.07***	1.24±0.05***
I/R group	16	0.25±0.08	0.52±0.09	0.49±0.04
Tet+I/R group	16	0.38±0.11*	0.39±0.08***	0.98±0.06***
F		14.260	21.360	16.654
P		<0.001	<0.001	<0.001

Note: Compared with I/R group, *P<0.05, ***P<0.001.

Table 3. Comparison of immunohistochemical positive rates of Bcl-2 and Bax in different groups (n, %)

Groups	Cases	Cases of Bcl-2 (-)	Cases of Bcl-2 (+)	Cases of Bax (-)	Cases of Bax (+)
Sham group	16	0	16***	16	0***
I/R group	16	8	8	6	10
Tet+I/R group	16	2	14**	14	2**
χ^2		13.137		18.667	
P		0.001		P<0.001	

Note: Compared with I/R group, **P<0.01, ***P<0.001.

much lower than that of I/R group with statistical differences (P<0.05). As shown in **Table 4** and **Figure 5**.

Discussion

Spinal cord ischemia-reperfusion injury (SCIRI) is commonly found in surgery clinically. Spinal cord injury may be accompanied by sensory disturbance and dyskinesia after, and it may also lead to quadriplegia, paraplegia, or even death in severe cases, seriously affecting the prognosis of patients and resulting in huge economic and social burden [11-13]. Previous studies have shown that tetrandrine (Tet) has protective effects on cerebral or myocardial ischemia [14, 15]. In this study, rabbits were used to make SCIRI models. The followed results showed that improved Tarlov scores of I/R group largely was significantly lower than both sham group and Tet+I/R group. It is suggested that pretreatment with Tet has a certain protective effect on motor function of the experimental rabbits.

Previous studies have pointed out that cell apoptosis and inflammation are crucial in the development of SCIRI [16]. Previous studies also found common neuronal cell loss during

the progress of SCIRI, but the associated mechanism is very complex. The reperfusion of blood flow after spinal cord ischemia leads to the generation of oxygen free radicals and inflammatory factors and the failure of mitochondria, which leads to further secondary injury of spinal cord and is also considered to be closely related to the loss of neurons [2]. In the process of secondary injury, the selective death of motoneuron cells induced by apoptosis promoting signals, together with the destruction of BSCB structure by inflammatory factors, eventually led

to irreversible spinal cord injury [17]. Therefore, it is very important to maintain the structural integrity of BSCB and reduce the damage to BSCB in the early stage of reperfusion [18]. It is found that Tet can inhibit the calcium channel, through which it can significantly reduce the calcium concentration and calmodulin activity in inflammatory cells, and further suppress the release of inflammatory mediators and associated inflammatory factors. Besides, Tet can also inhibit the production of oxygen free radicals but increase the release of SOD. The scavenging effect of SOD further reduced the content of oxygen free radicals and MDA in the injured spinal cord tissue, and finally maintained the stability of the biological membrane structure of the spinal cord [19, 20]. In this study, we found that the Tet pretreated I/R group had a much lower EB content of spinal cord tissue than I/R group, which indicated that Tet had a protective effect on the structural integrity of spinal cord barrier through early intervention. In the study of neuron apoptosis in spinal cord, we found that the number of apoptotic cells in Tet pretreated I/R group was much lower than I/R group. Thus, we speculated that Tet reduced the apoptosis of neural cells through exerting anti-inflammatory effect.

Study on the effect of tetrandrine on reducing spinal cord re-injury

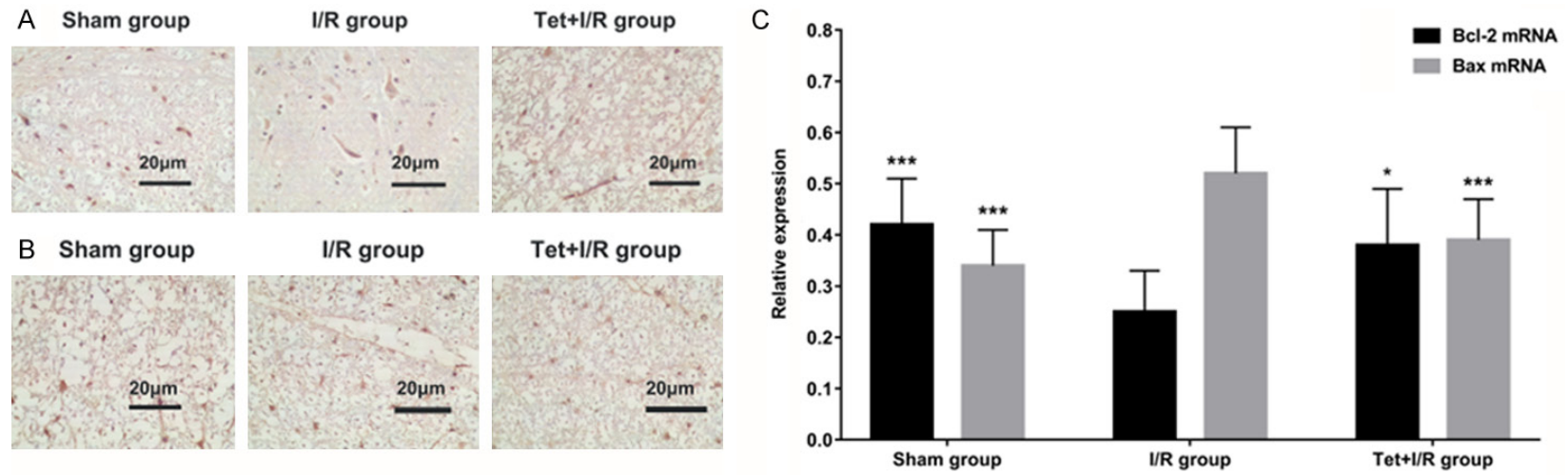


Figure 4. Comparison of Bcl-2 and Bax immunohistochemistry and relative mRNA expression in different groups. A: Pictures and semi quantitative results of representative Bcl-2 immunohistochemical staining in three groups of cells; B: Pictures and semi quantitative results of representative Bax immunohistochemical staining in three groups of cells; C: Comparison of relative expression of Bcl-2 and Bax mRNA in different groups. Compared with I/R group, *P<0.05, ***P<0.001.

Study on the effect of tetrandrine on reducing spinal cord re-injury

Table 4. Comparison of the relative expression of TNF- α and IL-6 proteins in different groups ($\bar{x} \pm sd$)

Groups	Cases	Relative expression of TNF- α protein	Relative expression of IL-6 protein
Sham group	16	0.09 \pm 0.01***	0.17 \pm 0.02***
I/R group	16	0.54 \pm 0.12	0.53 \pm 0.11
Tet+I/R group	16	0.22 \pm 0.09**	0.21 \pm 0.07**
F		113.123	107.414
P		<0.001	<0.001

Note: Compared with I/R group, **P<0.01, ***P<0.001.

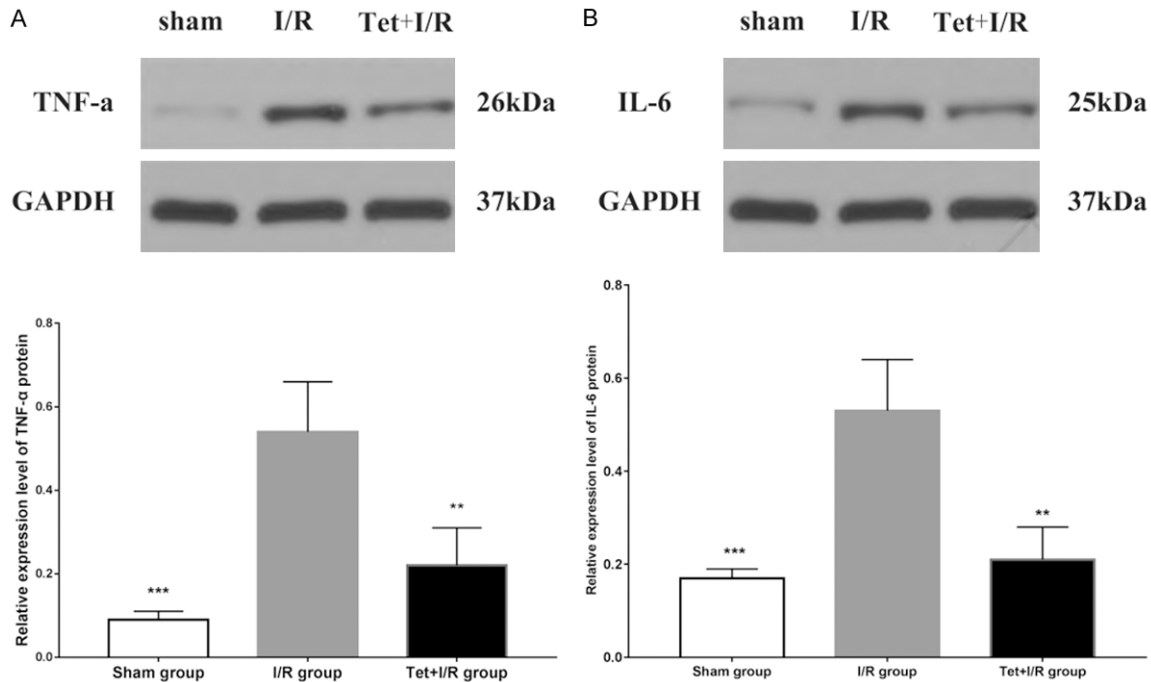


Figure 5. Comparison of the relative expression of TNF- α and IL-6 proteins in different groups. A: Pictures of western blot and comparison of relative expression of TNF- α proteins in different groups; B: Pictures of western blot and comparison of relative expression of IL-6 proteins in different groups. Compared with I/R group, **P<0.01, ***P<0.001.

Previous studies showed that Tet had a significant inhibitory effect on pro-inflammatory factors [12, 21]. In this study, Tet intervention is also found to reduce the level of pro-inflammatory factors TNF- α and IL-6 in SCIRI, thus reducing secondary injury in spinal cord.

It has been found that cell apoptosis is related to many mechanisms, such as free radical action and gene regulation, and it is a regulation process involving multiple genes. Among them, Bcl-2 family is considerable in the regulation of apoptosis [22]. Oxygen free radicals induce cell apoptosis through regulation of apoptosis related proteins such as Bcl-2 and Bax. Bcl-2 is a family of apoptosis related genes and has been widely studied recently. Bcl-2

and Bax are two important antagonistic apoptosis regulatory genes and they work together to regulate cell apoptosis through inhibiting cell apoptosis by Bcl-2 and promoting cell apoptosis by Bax. They form the positive and negative control of cell apoptosis, and determine whether the cell would go to apoptosis by the proportion of them [23]. Previous results showed that higher rate of Bcl-2/Bax had better inhibiting effect on cell apoptosis and the protective effect of highly expressed Bcl-2/Bax on myocardial ischemia-reperfusion injury has also been confirmed before [24, 25]. Our study showed that, compared with sham group and Tet+I/R group, I/R group had much lower rate of Bcl-2/Bax and lower expression of Bcl-2 mRNA, but had higher expression of Bax

Study on the effect of tetrandrine on reducing spinal cord re-injury

mRNA. These above results together suggest that Tet may protect the spinal cord and reduce reperfusion injury by elevating the rate of Bcl-2/Bax to reduce apoptosis and maintain the integrity of BSCB.

However, our present study did not carry out the intervention research of related signal pathway, and the observation time for the effect is not long enough. Besides, multi dose research has not been conducted, which is our next research plan.

In general, Tet can reduce the apoptosis of neurons and protect the integrity of BSCB, which may be related to the regulation of Bcl-2/Bax ratio and the reduction of pro-inflammatory factors.

Acknowledgements

This work was supported by the Science and Technology Project of Guizhou Health and Family Planning Commission (gzwjkj2016-1-029).

Disclosure of conflict of interest

None.

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Study on the effect of tetrandrine on reducing spinal cord re-injury

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