Effects of tetrandrine on neuronal apoptosis, bcl-2 and bax expressions following acute spinal cord injury***

In comparison with methylprednisolone

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Abstract

BACKGROUND: Studies have demonstrated that tetrandrine has protection on acute spinal cord injury, but the specific mechanism remains poorly understood.

OBJECTIVE: To study the protection of tetrandrine on rat acute spinal cord injury and to study its mechanism from apoptosis pathway.

METHODS: A total of 100 rats were randomly divided into 4 groups. All rats were prepared for spinal cord injury models using modified Allen method except that in the sham-surgery group. Methylprednisolone and tetrandrine was injected into rats in the methylprednisolone and tetrandrine groups by tail intravenous injection prior to and at 24, 48 hours after model preparation. The same volume of physiological saline was injected in the sham-surgery and model groups. Basso-Beattie-Bresnahan (BBB score) was recorded at 8 hours, 1, 3, 7 and 14 days after model preparation. The morphological changes of spinal cord injury sites were observed by hematoxylin-eosin staining and the expressions of bcl-2 and bax were determined by immunohistochemistry.

RESULTS AND CONCLUSION: The BBB score of methylprednisolone and tetrandrine groups were significantly higher than that model group at 7 and 14 days ($P < 0.05$), but there were no significant difference between the methylprednisolone group and tetrandrine group ($P > 0.05$). Hematoxylin-eosin staining showed that the spinal cord injured severely at 3-7 days, the injury degree in the methylprednisolone group and tetrandrine group was slighter than that of the model group, with smaller bax expression and greater bcl-2 expression ($P < 0.01$). The findings demonstrated that, tetrandrine is able to protect neurons from apoptosis and promote the nerve function recovery by inhibiting the expression of Bax and promoting the expression of Bcl-2. Its effect is not inferior to methylprednisolone.

INTRODUCTION

Acute spinal cord injury is a common reason for paraplegia, which lead to high disability rate and has not effective treating methods. How to promote nerve function recovery following acute spinal cord injury puzzles spinal surgeons. Plenty of experiments demonstrated that neuronal necrosis is the main appearance at the injury region and adjacent area at the early stage after spinal cord injury, but presented with inducing neuronal necrosis at subacute phase[1]. Tetrandrine, isolated from the root of Stephania tetrandra, with molecular formula of $C_{33}H_{42}N_2O_6$, is a natural selective calcium-channel blocker, has roles on dissipate heat, antihypertension, anti-infection, antioxygen, as well as anticytotoxin[2-3]. However, the specific mechanism remains unclear. In the experiment, based on rat spinal cord injury models, we aimed to study the protection of tetrandrine on structural recovery and the restoration of motor function after spinal cord injury in rats and to study its mechanism, in addition, compare the effects with methylprednisolone.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

The experiment was performed at the Center Laboratory of People's Hospital of Guizhou Province from March 2007 to January 2008.

Materials

Totally 100 healthy adult Sprague Dawley rats, irrespective of genders, weighted (250±50) g, were provided by experimental animal center of Guiyang Medical College. All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals* formulated by the Ministry of Science and Technology of the People's Republic of China[4].

The reagents and equipment used are as follows:

Reagent and equipment	Source
Tetrandrine (Purity > 95%)	Jiangxi Pengze Pharmaceutical Factory
Rabbit anti rat bcl-2/bax monoclonal antibody, SABC kit	Wuhan Boster Biological Technology, Co., Ltd.
Biomias2001 image analysis system	Institute of Image and Graphics, Sichuan University

Methods

Grouping, model preparation and intervention

A total of 100 rats were randomly divided into 4 groups: sham-surgery group (n=10), model group (n=30), methylprednisolone group (n=30), and tetrandrine group (n=30). Following intraperitoneal anesthesia with4% napental (40 mg/kg), a 2-cm median incision was cut at the thoracic back, the thoracic vertebrae T_{8-9} was exposed, and a 0.4 cm× 0.7 cm vertebral plate defect was prepared. Rats were prepared for spinal cord injury models using modified Allen method with wounding energy of 100 g•cm, 10-g load and 10-cm height. Hematomyelia and hind limb pamplegia was

considered successful models[5]. No wound was performed on rats in the sham-surgery group.

Methylprednisolone (90 mg/kg) and 1% tetrandrine (22.5 mg/kg) was tail intravenous injected into rats in the methylprednisolone and tetrandrine groups prior to and 24, 48 hours after model preparation. The same volume of physiological saline was injected in the sham-surgery and model groups. Penicillin (8×10^6 U/d) was injected into animals *via* muscle. Rats were housed at 22−26 ℃, and forced urination twice per day by extruding bladder.

Basso-Beattie-Bresnahan (BBB) score

BBB score was recorded at 8 hours, 1, 3, 7 and 14 days after model preparation[6]. The scoring was performed at 19:00 by well-trained people using blind method.

Sample preparation

Rats in the sham-surgery group were sacrificed at 14 days, and 6 animals in other groups were sacrificed at 8 hours, 1, 3, 7, and 14 days after BBB scoring. The injured spinal cord was exposed, and a 3-mm long segment was harvested around injury site, fixed with 40 g/L paraform overnight, dehydrated, embed, prepared for serial, transverse, 4-μm thick sections, followed by hematoxylin-eosin staining and immunohistochemistry.

Hematoxylin-eosin staining

A total of 6 sections of each rat was performed hematoxylin-eosin staining at different time points. The sections were dewaxed, washed with distilled water, stained by hematoxylin for 10 minutes, color separated by 1% chlorhydric acid for 20 minutes, washed by running water, followed by eosin staining for 10 minutes, desiccated by ethanol, and sealed. Five non-overlapping fields were selected from each slice, and the morphological changes of spinal cord were observed by Biomias2001 image analysis system.

Immunohistochemistry

According to SABC kit instructions, 6 sections of each rat were performed bcl-2 and bax immunohistochemistry at different time points. The paraffin sections were deparaffinized, repaired antigen by microwave, and incubated 10 minutes at room temperature followed by incubated 2 hours at 37 ℃ with bcl-2 or bax monoclonal antibody (1:100), washed by PBS, and than the sections were incubated with biotinylated goat anti-rabbit IgG at 37 ℃ for 20 minutes, washed by PBS, and visualized by DAB. The visualization was terminated by distilled water and restained by eosin. PBS instead of firstly antibody served as negative control. A total of 5 non-overlapping fields (×400) were selected from each slice for calculation positive cells. The expression of bcl-2 and bax was observed in kytoplasm of spinal cord anterior horn motor neurons.

Main outcome measures

The motor function, pathological changes after spinal cord injury, as well as the bcl-2 and bax expressions.

Design, enforcement and evaluation

The experiment was designed by the first author, performed and evaluated by all authors.

Statistical analysis

The data were expressed as Mean±SD and analyzed by SPSS13.0 software (SPSS, Chicago, IL, USA). Intergroup differences were compared using independent sample *t* test. A *P* value < 0.05 was considered significant.

RESULTS

Quantitative analysis of experimental animals

A total of 100 rats were included in the final analysis.

Results of BBB score

The BBB score was dramatically decreased after spinal cord injury ($P < 0.05$). There were no significant differences between the model, methylprednisolone and tetrandrine groups at 8 hours, 1 and 3 days after model preparation ($P > 0.05$). However, the BBB score of the methylprednisolone and tetrandrine groups were obviously greater than that of the model group at 7 and 14 days after model preparation ($P < 0.05$), the difference between the methylprednisolone group and tetrandrine group had no significant ($P > 0.05$, Table 1).

Table 1 BBB result of rats in each group at different time points ($\bar{x}\pm s$, n=6)

Group	Time post-surgery		
	8 h	1 d	3 d
Sham-surgery	20.24±1.24	20.41±1.59	21.16±1.16
Model	3.63±1.37[a]	4.28±1.28[a]	5.01±2.01[a]
Tetrandrine	4.52±1.48[a]	4.93±2.07[a]	5.72±1.72[a]
Methylprednisolone	4.35±1.35[a]	5.29±1.71[a]	5.99±2.01[a]

Group	Time post-surgery	
	7 d	14 d
Sham-surgery	20.86±1.14	20.58±1.42
Model	5.85±1.15[a]	7.66±2.34[a]
Tetrandrine	8.29±1.71[ab]	10.91±1.91[ab]
Methylprednisolone	8.54±1.54[ab]	12.01±2.01[ab]

[a]$P < 0.01$, *vs*. sham-surgery group; [b]$P < 0.05$, *vs*. model group

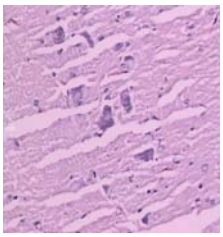
Pathological changes of spinal cord tissues

Sham-surgery group: The spinal cord tissue was normal, no hemorrhage, necrosis or tissue edema.

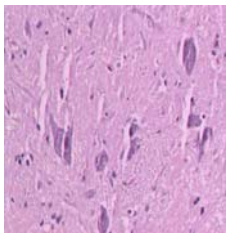
Model group: Regional hemorrhage appeared at 8 hours after spinal cord injury, accompanied by edema. At 1 day, severe peritumoral edema and neuronal degeneration could be found. At 7 days, there were plenty of neuronal degeneration in the injured spinal cord tissue, and tiny cavitation zone formed in grey matter. At 14 days, necrosis appeared in grey matter of injured spinal cord tissue, formed capsular space, only few residual neurons (Figure 1 a).

Tetrandrine group: There were slightly edema and mild hemorrhage at 8 hours after spinal cord injury. At 1 day, the neurons were mild/moderate swelled, and few neurons denaturated. At 7 days, the swelling began to regress, only scattered spotty focal necrosis was remained; At 14 days, the swelling basically disappeared, few necrosis formed tiny

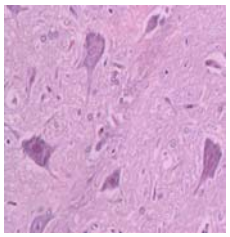
cavitation, and plenty of neurons remained (Figure 1 b). Methylprednisolone group: Slightly edema and mild denaturation could be seen at 8 hours after spinal cord injury. At 1, 3 and 7 days, the tissue swelling and necrosis were slighter than that of the tetrandrine group. At 14 days, compared with the tetrandrine group, there were fewer tiny cavitation in the methylprednisolone group, accompanied by larger amount of residual neurons (Figure 1 c).



a: Model group



b: Tetrandrine group

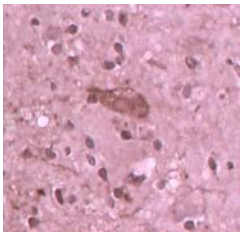


c: Methylprednisolone group

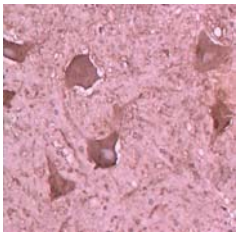
Figure 1 Pathological change of spinal cord tissues at 14 d after injury (Hematoxylin-eosin staining, ×200)

Expressions of bcl-2 and bax in spinal cord tissues

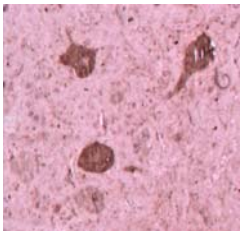
The bcl-2 and bax was expressed at 1 day after spinal cord injury, reached a peak at 7 days, and then gradually decreased. Compared with the model group, the bcl-2 expression was increased, but bax expression was decreased in the methylprednisolone and tetrandrine groups ($P < 0.01$), however, there was no significant difference between methylprednisolone and tetrandrine groups ($P > 0.05$, Figures 2, 3 and Tables 2, 3).



a: Model group

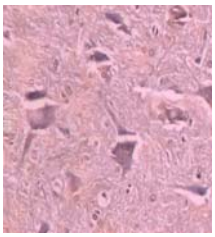


b: Tetrandrine group

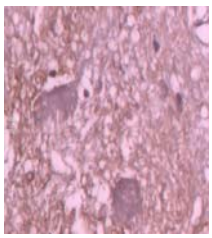


c: Methylprednisolone group

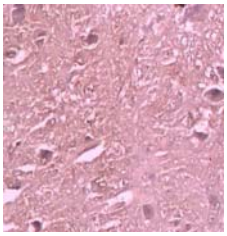
Figure 2 bcl-2 expression of spinal cord tissue at 7 d after injury (Immunohistochemistry, ×400)



a: Model group



b: Tetrandrine group



c: Methylprednisolone group

Figure 3 bax expression of spinal cord tissue at 7 d after injury (Immunohistochemistry, ×400)

Table 2 bcl-2 positive cells in the injury tissues at different time points ($\bar{x}\pm s$, n=6, n/400-fold visual field)

Time post-surgery	Model group	Tetrandrine group	Methylprednisolone group
8 h	15.60±1.78	27.21±1.89[ab]	28.10±2.51[ab]
1 d	23.10±2.74	31.00±3.02[ab]	34.10±3.46[ab]
3 d	26.10±2.13	38.10±2.73[ab]	40.60±3.23[ab]
7 d	30.10±2.57	42.00±3.23[ab]	44.66±2.91[ab]
14 d	21.30±2.42	33.80±2.24[ab]	35.23±2.34[ab]

The bcl-2 positive cells in sham-surgery group were (3.20±1.40)/400-fold visual field; [a]$P < 0.01$, *vs*. sham-surgery group; [b]$P < 0.01$, *vs*. model group

Table 3 bax positive cells in the injury tissue at different time points ($\bar{x}\pm s$, n=6, n/400-fold visual field)

Time post-surgery	Model group	Tetrandrine group	Methylprednisolone group
8 h	28.20±2.45	20.21±2.24[ab]	18.94±2.19[ab]
1 d	32.10±2.21	27.60±2.45[ab]	26.70±2.92[ab]
3 d	46.72±3.13	35.24±2.34[ab]	34.20±2.73[ab]
7 d	49.40±4.24	40.30±2.56[ab]	38.53±3.31[ab]
14 d	38.72±2.32	29.70±2.64[ab]	27.58±2.38[ab]

The bax positive cells in sham-surgery group were (3.40±0.65)/400-fold visual field; [a]$P < 0.01$, *vs*. sham-surgery group; [b]$P < 0.01$, *vs*. model group

DISCUSSION

The secondary injury following acute spinal cord injury comprises cell necrosis and apoptosis. Studies have demonstrated that, apoptosis dependent on active protein synthesis contributes to the neuronal and glial cell death, as well as to the neurological dysfunction, induced by mild-to-moderate severity traumatic insults to the rat spinal cord[7]. The key of drug treatment is how to reduce the neural

cells apoptosis following acute spinal cord injury and prevent secondary injury.

Li *et al*[8] firstly reported that there were a great amount expression of neuronal apoptosis related gene and apoptosis protein in rat spinal cord injury models in 1996. bcl-2 is a plasmosin, which was highly expressed in the development of central nervous system and maintained at low level in mature nervous system[9]. It is also thought to be involved in resistance to apoptosis, which can inhibit various apoptosis pathways following spinal cord injury[10]. The mechanism may be related to suppressing free radical production and maintaining membranous structure. Studies demonstrated that, bcl-2 is an inducible gene, the overexpression of bcl-2 can mitigate nerve injury, elevate anti-surgery function of tissues, promote axonal outgrowth and repair of injured central nervous system, as well as inhibit neural cells apoptosis[11-13]. Bax locates in cytoplasm, which precipitates neural cells apoptosis via altering permeability of mitochondrial membrane after activated by receiving information convection of apoptosis[14]. Studiesfound that, the oligodendrocyte apoptosis obvious decreased and the axonal demyelination mitigated when the bax gene was removed after mice spinal cord injury[15]. Scorrano *et al*[16] found that bcl-2 and bax can alter cell permeability through regulating calcium ion flow. Fan *et al*[17] demonstrated that bcl-2 and bax expression affect the spinal cord neuronal apoptosis directly or indirectly, and the apoptosis extent is closely associate with its ratio.

Methylprednisolone has received acceptable therapeutic effect in the treatment of spinal cord injury. Tetrandrine can prevent body from acute spinal cord injury by ameliorating microcirculation, inhibiting free radical production, preventing calcium overload, as well as improving tissue metabolism[2-3, 18]. The experiment found that, compared with the sham-surgery group, the expression of bcl-2 and bax were increased after spinal cord injury, which suggested that there were apoptosis. The bcl-2 expression were increased in the methylprednisolone and tetrandrine groups, but bax expression were decreased, the bax expression was highest in the model group, all of these demonstrated that methylprednisolone and tetrandrine play an important role in interfere in expression of apoptotic effector, that is, suppress apoptosis by subliming bcl-2 and depressing bax expression. The expression of bcl-2 and bax reached a peak at 3−7 days after spinal cord injury, and then gradually decreased, which showed that, the apoptosis peaked at 3−7 days, at this time, part of spinal cord function suffered sub-damage still can be retrieved if interfere in time. This was confirmed by the experiment, that the BBB score of the methylprednisolone and tetrandrine groups were greater than the model group at 7 and 14 days. Previous research verified that, hypoxic ischemia, over production of oxygen free radical, as well as peroxidation, lead to membrane permeability increase, calcium overload, and activate endonuclease, finally, induce apoptosis[19]. However, tetrandrine can improve microcirculation, suppress free radical production, and prevent calcium overload[2-3], accordingly, it is supposed to inhibit apoptosis and exhibit protection role in spinal cord injury.

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汉防己甲素干预急性损伤脊髓神经元凋亡及 bcl-2 和 bax 表达：与甲基强的松龙的比较***

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摘要

背景：研究证实汉防己甲素对急性脊髓损伤有保护作用，但其具体机制尚不清楚。

目的：观察汉防己甲素对急性脊髓损伤大鼠的神经保护作用，并从细胞凋亡通路探讨其作用机制。

方法：将 100 只成年大鼠随机分为 4 组。采用加速压迫型 Allen’s 打击法制备脊髓损伤模型。甲强龙组和汉防己甲素组分别于造模前和造模后 24，48 h 经尾静脉注射甲基强的松龙和汉防己甲素。假手术组与模型组注射等量生理盐水。造模后 8 h，1，3，7，14 d 采用 BBB 评分评估大鼠的运动功能，苏木精-伊红染色观察损伤脊髓组织的形态改变，免疫组织化学染色检测 bcl-2 和 bax 的表达。

结果与结论：伤后 7，14 d，甲强龙组和汉防己甲素组大鼠的 BBB 评分显著高于模型组($P < 0.05$)，各时间点甲强龙组和汉防己甲素组间 BBB 评分差异无显著性意义($P > 0.05$)。伤后 3~7 d，脊髓组织损伤最为严重，甲强龙组和汉防己甲素组的损伤程度较模型组轻，同时 bax 表达较模型组少，而 bcl-2 表达较模型组多($P < 0.01$)。说明汉防己甲素可通过增加 bcl-2 表达、降低 bax 表达，抑制急性脊髓损伤大鼠神经细胞凋亡，促进大鼠运动功能恢复，其作用不逊于甲基强的松龙。

关键词：汉防己甲素；急性脊髓损伤；凋亡；bcl-2；bax；甲基强的松龙
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利益冲突：课题未涉及任何厂家及相关雇主或其他经济组织直接或间接的经济或利益的赞助。

课题的创新点：已有研究证实汉防己甲素能改善微循环、抑制自由基的生成、防止钙超载，从而推测其可能通过这一药理作用抑制细胞凋亡，对脊髓损伤有一定的保护作用，国内外尚未见汉防己甲素应用于脊髓损伤后凋亡的研究。

课题评估的“金标准”：脊髓损伤的治疗目前在医学上是一个难题，甲基强的松龙是目前公认的治疗急性脊髓损伤的“金标准”，已在临床广泛使用，实验采用甲基强的松龙作为对照来研究汉防己甲素对急性脊髓损伤的保护作用。

设计或课题的偏倚与不足：实验将应用更准确的定量方法从多角度验证结果。

提供临床借鉴的价值：实验为临床上应用汉防己甲素治疗急性脊髓损伤提供了实验依据。

CRTER 杂志“软组织工程”栏目关于“神经组织工程”研究的组稿内容

○组织工程周围神经修复坐骨神经缺损
○神经组织工程生物支架材料生物相容性
○组织工程神经中雪旺细胞的形态
○去细胞同种异体移植面神经材料的制备
○外周神经缺损的组织工程修复方法研究
○神经组织工程修复脊髓损伤
○雪旺细胞复合 PLGA 修复兔面神经缺损
○人胚雪旺细胞组织工程神经修复坐骨神经缺损
○骨髓神经组织定向干细胞性组织工程化神经的构建
○去细胞同种异体神经支架的制备及种植雪旺细胞
○复合转化生长因子-β 的组织工程化周围神经修复坐骨神经缺损
○大鼠坐骨神经 Wallerian 变性规律及其 S-100 蛋白表达和雪旺细胞增殖研究
○组织工程人工周围神经的研制
○人工组织神经移植物引导大鼠再生坐骨神经通过 10mm 缺损早期的形态学观察
○自体成肌细胞修复周围神经缺损
○BrdU 标记冷冻胚胎雪旺细胞构建人工神经
○猕猴组织工程化周围神经移植物的研究
○成年兔雪旺细胞体外培养增殖和纯化的研究
○透明质酸基中枢神经组织工程框架材料的研究
○犬自体骨髓间充质干细胞组织工程化神经荧光金逆行示踪标记实验