Original Article
Tanshinone IIA inhibits cell apoptosis in the compressed spinal cord by activating the PI3K/AKT signaling pathway

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Abstract: Objective: To study the effect and molecular mechanisms of tanshinone IIA on neuronal apoptosis in the compressed spinal cord. Method: SD rats were selected as experimental animals and randomly divided into a sham-operated group (Sham group), a spinal cord compression injury group (SCI group), a tanshinone IIA group (TAN group), and an LY2904002 group (LY group). The spinal cord compression model was established by clamping the spinal cords with an arterial clamp. Tanshinone IIA was given in the TAN group, and tanshinone IIA combined with LY2904002 was given in the LY group. On the third day after the intervention, the rats’ motor functions were evaluated, and the expressions of the apoptotic gene and the PI3K/AKT signal molecules in the compressed spinal cord were measured. Results: The BBB scores, the ramp angles, and the expressions of GSK-3β, Cyclin D1, Bcl-2, p-PI3K, and p-AKT in the compressed spinal cords of the SCI group were lower than they were in the Sham group, but the expressions of Caspase-9 and Caspase-3 in the compressed spinal cords were higher than they were in the Sham group; the BBB scores, the ramp angles, and the expressions of GSK-3β, Cyclin D1, Bcl-2, p-PI3K, and p-AKT in the compressed spinal cords of the TAN group were higher than they were in the Sham group, but the expressions of Caspase-9 and Caspase-3 in the compressed spinal cords were lower than they were in the SCI group; the BBB scores, the ramp angles, and the expressions of GSK-3β, Cyclin D1, Bcl-2, p-PI3K, and p-AKT in the compressed spinal cords of the LY group were lower than they were in the TAN group, but the expressions of Caspase-9 and Caspase-3 in the compressed spinal cords were higher than they were in the TAN group. Conclusion: Tanshinone IIA can inhibit cell apoptosis in compressed spinal cords by activating the PI3K/AKT signaling pathway and can alleviate the degree of spinal cord injury in rats with spinal cord compression.

Keywords: Spinal cord compression injury, tanshinone IIA, phosphatidylinositol 3 kinase/protein kinase B, apoptosis

Introduction
Spinal cord compression injury (SCI) is a common type of spinal cord injury seen in the clinic, and it is often secondary to lumbar disc protrusion, lumbar fracture, and other diseases. Spontaneous axonal sprouting and the formation of spontaneous neurons can be produced after a spinal cord injury, has and they have a certain repair effect on the injured spinal cord function [1, 2].

However, the regeneration ability of the neurons and axons in spinal cord tissue is weak, and the recovery of spinal cord function after compressive injury is difficult. In clinical practice, functional exercise, neurotrophic drugs, and hyperbaric oxygen are commonly used rehabilitation methods after spinal cord injury, but the effect is not ideal [3, 4]. Tanshinone IIA is the main active ingredient in Salvia miltiorrhiza, which has antioxidant and anti-apoptotic biological activities and has been proved to be able to alleviate spinal cord ischemia-reperfusion injury [5].

In the following studies, in order to clarify the repairing effect of tanshinone IIA on spinal cord injury, and taking the rat model of spinal cord compression as the research object, the effect of tanshinone IIA on neuronal apoptosis in compressed spinal cord tissue was analyzed, and the specific molecular mechanism was explored.
Materials and methods

Experimental materials

Male SD rats (Shanghai Animal Center, Chinese Academy of Sciences) weighing 250-300 g; tanshinone IIA and the PI3K selective inhibitor LY294002 (Sigma Company); GSK-3β, Cyclin D1, Bcl-2, Caspase-9, Caspase-3, p-PI3K, p-AKT first antibody, and HRP-labeled second antibody (Santa Cruz Company); BCA protein quantitative kit (Shanghai Biyuntian Company).

Experimental method

Animal grouping and modeling method: 60 SD rats were randomly divided into a sham-operated group (Sham group), a spinal cord compression injury group (SCI group), a tanshinone IIA group (TAN group), and an LY294002 group (LY group). Every group had 15 rats. The SCI group, the TAN group, and the LY group were given intraperitoneal injections of 30 mg/kg pentobarbital sodium, placed in the prone position after anesthesia, and given a posterior median incision that exposed the eighth to tenth thoracic vertebrae. At the ninth thoracic vertebral segment, the vertebral plate was removed, and the vertebral canal was opened. After exposing the dorsal side of the spinal cord, the spinal cord was clamped with an arterial clamp. The intensity was 30 g, and the time was 2 minutes. The incision was sutured after unclipping the artery clamp. Rats in the Sham group were anesthetized with the same method and underwent the surgical incision and the exposure of spinal cord, but they were sutured directly without any clipping. Every procedure was approved by the Animal Care and Use Committee of the Affiliated Hospital of Hebei University and was in conformity with the guidelines of the National Institute of Health.

Drug administration method: After the successful establishment of the model, the drug was administered as follows: (1) Sham group: intraperitoneal injection of normal saline at the same dose once a day; (2) SCI group: intraperitoneal injection of normal saline once a day; (3) TAN group: intraperitoneal injection of 30 mg/kg tanshinone IIA once a day; (4) LY group: intraperitoneal injection of 0.3 mg/kg LY294002, intraperitoneal injection of 30 mg/kg tanshinone IIA 5 minutes later, once a day.

Motor function evaluation method: On the third day after the intervention, the BBB motor function scores and inclined plate functions were measured in the three groups respectively. The methods were as follows: (1) BBB motor function score: the rats were placed in the open field, and two observers stood on opposite sides and observed the coordination of buttocks, knees, ankles, and trunk as the rats walked for 5 minutes. Score maps were drawn, and the average values were calculated. (2) Inclined plate function determination: the rats were placed in the Rivlin inclined plate with head-to-left and transversely, and the inclined angle was gradually increased from the horizontal position. The inclined plate angle was measured according to the standard that the rats stayed on the inclined plate for 5 seconds without falling.

Protein expression detection method: On the third day after the intervention, the rats were sacrificed after the motor function evaluation, and the spinal cord tissues at the compression injury sites were dissected. The spinal cord tissue was homogenized after adding a protein lysate. The homogenate was centrifuged for 15 minutes at 12000 r/min in a 4°C centrifuge. The supernatant was taken and the total protein was quantified using a BCA kit. The 8 μg protein sample was added into SDS-polyacrylamide gel, and then vertical electrophoresis was carried out at 100 V for 90 minutes. Then the protein samples from SDS-polyacrylamide gel were transferred to a PVDF membrane, and then the PVDF membrane was taken and sealed in 2% skim milk for 2 hours, The GSK-3β (1:1000), Cyclin D1 (1:1000), Bcl-2, (1:1000) Caspase-9 (1:1000), Caspase-3 (1:1000), Caspase-9 (1:1000), p-PI3K (1:1000), and p-AKT (1:1000) first antibodies were incubated respectively and stored overnight at 4°C. The next day, after being washed 3 times with a PBST solution, the second antibody (1:5000) labeled with HRP was added, incubated at 37°C for 2 hours. The protein samples were washed three times with a PBST solution and then added to the developer to develop the protein bands. Image J software was used to calculate the gray value of the protein bands. The protein expressions in the other groups of spinal cord tissues were calculated by taking β-actin as an internal parameter and the Sham group protein expression as 1.
Statistical method: SPSS 20.0 software was used to input and analyze the data. After the homogeneity of the variance test of the measurement data was determined to be consistent with the homogeneity of the variance, a variance analysis was used among the three groups, an LSD-<i>t</i> test was used for comparison between the two groups, and a <i>t</i> test was used for the analysis between the two groups. When <i>P</i> < 0.05, there was considered to be a significant difference in the test results.

Results

The effects of tanshinone IIA combined with pi3k inhibitor on motor function in rats with spinal cord compression

On the third day after the intervention, the BBB motor function scores and the inclined plate functions were measured in the four groups (Figure 1). The BBB scores in the SCI group were significantly lower than they were in the Sham group (P < 0.05). The inclined plate angle degrees were significantly lower than they were in the Sham group (P < 0.05). The BBB scores of the TAN group were significantly higher than they were in the SCI group. The inclined plate angle degrees were significantly higher than they were in the SCI group degree (P < 0.05). The BBB scores in the LY group was significantly lower than they were in the TAN group (P < 0.05), and the inclined angle degrees were significantly lower than they were in the TAN group (P < 0.05).

The effects of tanshinone IIA combined with pi3k inhibitor on apoptotic gene expression in spinal cord tissue

On the third day after the intervention, the spinal cord tissues of the compression sites were taken from the three groups of rats and the expressions of the apoptotic genes GSK-3β, Cyclin D1, Bcl-2, Caspase-9, and Caspase-3 were measured (Figure 2).

The expressions of GSK-3β, Cyclin D1 and Bcl-2 in the compressed spinal cord tissues of SCI group were significantly lower than they were in the Sham group (P < 0.05). The expressions of GSK-3β, Cyclin D1, and Bcl-2 in the compressed spinal cord tissues in the TAN group were significantly higher than they were in the SCI group. The expressions of Caspase-9 and Caspase-3 in the compressed spinal cord tissues in the TAN group were significantly lower than they were in of SCI group (P < 0.05). The effects of tanshinone IIA combined with the PI3K inhibitor on the expression of the apoptotic genes GSK-3β, Cyclin D1, Bcl-2, Caspase-9, and Caspase-3 in the spinal cord tissues are shown in Figure 2. The expressions of GSK-3β, Cyclin D1, and Bcl-2 in the spinal cord tissues of LY rats were significantly lower than they were in the TAN group. The expressions of Caspase-9 and Caspase-3 in the compressed spinal cord tissues in the LY group were significantly higher than they were in the TAN group (P < 0.05).

The effect of tanshinone IIA on PI3K/AKT signaling molecule expression in spinal cord tissue

On the third day after the intervention, the spinal cord tissues of the compression sites were taken from the three groups of rats, and the expressions of the PI3K/AKT signal molecules p-PI3K and p-AKT were measured (Figure 3).

The expressions of p-PI3K and p-AKT in the compressed spinal cords in the SCI group were significantly lower than they were in the Sham group (P < 0.05). The expressions of p-PI3K and p-AKT in the compressed spinal cords in the TAN group were significantly lower than they were in the SCI group (P < 0.05).
Spinal cord injury can cause sensory and motor dysfunction in the dominant area and then causes corresponding clinical symptoms. In this study, T9 segment spinal cord clamping was used to establish a model of spinal cord injury. By analyzing the motor function to reflect the degree of spinal cord injury, the BBB scores and inclined plate angles of the SCI group rats were lower than those of the Sham group. This indicates that spinal cord function damage in the animal model of spinal cord compression was significant, which is manifested by motor function degeneration. Spinal cord compression injury caused by lumbar disc protrusion and spinal fracture is a common type of spinal cord injury seen in the clinic. Rehabilitation exercises and drug treatment after injury are the main means to promote the recovery and reconstruction of spinal cord function. As the regeneration ability of neurons in spinal cord tissue is weak, the speed of functional recovery after spinal cord injury is slow, and the effect of functional reconstruction is not ideal.

Figure 2. The expressions of the apoptotic genes GSK-3β, Cyclin D1, Bcl-2, Caspase-9, and Caspase-3 in the compressed spinal cord tissues after tanshinone IIA intervention. (A) GSK-3β expression, (B) Cyclin D1 expression, (C) Bcl-2 expression, (D) Caspase-9 expression, (E) Caspase-3 expression. *: SCI group compared with the Sham group, P < 0.05; #: TAN group compared with the SCI group, P < 0.05. &: LY group compared with the TAN group, P < 0.05.

Figure 3. The expressions of p-PI3K and p-AKT in the compressed spinal cord tissues after tanshinone IIA intervention. A. p-PI3K expression; B. p-AKT expression. *: SCI group compared with the Sham group, P < 0.05; #: TAN group compared with the SCI group, P < 0.05. &: LY group compared with the TAN group, P < 0.05.
Tanshinone IIA and neuronal apoptosis

Tanshinone IIA is an active ingredient in *Salvia miltiorrhiza*, which has antioxidant and anti-apoptotic activities. Intervention with tanshinone IIA for spinal cord ischemia-reperfusion can reduce spinal cord injury and promote neuron regeneration [6-8]. To clarify the effect of tanshinone IIA on spinal cord compression injury, we further analyzed the changes of spinal cord injury after tanshinone IIA intervention. The results showed that the BBB scores and the inclined angle of rats in the TAN group were higher than those in the SCI group. It indicated that tanshinone IIA can improve the motor function in a spinal cord compression animal model and can reduce the degree of spinal cord injury.

Excessive apoptosis is an important pathological change in local tissues during spinal cord compression injury, and the expression of many apoptotic genes is significantly abnormal. Cyclin D1 is a cyclin protein, and it can form complexes with CDK4 and CDK6 and accelerate the cell cycle process [9]. GSK-3β is a catalysis regulating the degradation of Cyclin D1, and it can inhibit the degradation of Cyclin D1 and make it continue to play a positive regulatory role in the cell cycle and then promote cell proliferation [10, 11]. Bcl-2 is a regulator of mitochondrial pathway apoptosis, which can prevent cytochrome C release into the cytoplasm of mitochondria, thereby inhibiting caspase-3 and caspase-9 cascade activation downstream and hindering cell apoptosis [12, 13]. By analyzing the changes of the above apoptotic gene expressions in the compressed spinal cord tissues of rats with spinal cord compression, the expressions of GSK-3β, Cyclin D1, and Bcl-2 in the compressed spinal cord tissues of the SCI group were lower than they were in the Sham group, but the expressions of Caspase-9 and Caspase-3 were higher than those in the SCI group, and the expressions of Caspase-9 and Caspase-3 were lower than they were in the SCI group. It is suggested that tanshinone IIA can regulate the expression of apoptotic genes during spinal cord compression injury, increase the expressions of promoting proliferation and inhibiting apoptosis genes, reduce the expressions of promoting proliferation and inhibiting apoptosis genes, and inhibit the apoptosis of compressed spinal cord tissues, thereby alleviating spinal cord injury.

The phosphorylation activation of GSK-3β during the cell cycle and the expression of Bcl-2 in the mitochondrial apoptotic pathway were regulated by the PI3K/AKT signaling pathway [14-16]. PI3K activation can phosphorylate PIP2 into PIP3 on the inner surface of cell membranes, and PIP3 can bind to the threonine T308 and serine S473 sites of AKT and phosphorylate AKT [17, 18]. On the one hand, phosphorylated AKT can induce the phosphorylation of GSK-3β and increase the content of Cyclin D1; on the other hand, it can directly regulate the expression of Bcl-2 [19, 20]. In order to clarify the molecular mechanisms of excessive cell apoptosis and tanshinone IIA alleviating the apoptosis of compressed spinal cord tissues during spinal cord compression injury, we analyzed the changes of the above-mentioned signaling pathways in compressed spinal cord tissues. The results showed that the expressions of p-PI3K and p-AKT in the compressed spinal cord tissues of the SCI group were lower than they were in the Sham group, and the expressions of p-PI3K and p-AKT in the compressed spinal cord tissues of the TAN group were higher than they were in the SCI group. It is suggested that the activation of the PI3K/AKT signaling pathway is inhibited during spinal cord compression injury. Tanshinone IIA intervention can reverse the inhibition of spinal cord compression on the PI3K/AKT signaling pathway. It was suggested that the abnormal activation of the PI3K/AKT signaling pathway is related to the occurrence of spinal cord compression injury. Inhibiting the PI3K/AKT signaling pathway in compressed spinal cord tissue can induce cell apoptosis and tissue injury by affecting the expressions of downstream apoptotic genes. Tanshinone IIA can promote the PI3K/AKT signaling pathway in compressed spinal cord tissue, inhibit cell apoptosis, and reduce tissue damage.
Tanshinone IIA and neuronal apoptosis

After clarifying that tanshinone IIA can regulate the expressions of apoptotic genes and the activation of the PI3K/AKT signaling pathway in compressed spinal cord tissues, in order to confirm whether tanshinone IIA can directly regulate the expression of apoptotic genes in compressed spinal cord tissues through the PI3K/AKT signaling pathway and alleviate the degree of spinal cord injury, LY294002, an inhibitor of this signaling pathway, was combined with tanshinone IIA to intervene in the spinal cord compression model rats. By comparing the differences in motor functions and apoptotic gene expressions between the TAN and LY groups, the BBB scores, inclined plate angles, and the expressions of GSK-3β, Cyclin D1, and Bcl-2 in the spinal cord tissue of the LY group were lower than they were in the TAN group, and the expressions of Caspase-9 and Caspase-3 in the spinal cord tissue of the LY group were higher than they were in the TAN group. This suggests that the PI3K/AKT signaling pathway inhibitors can attenuate the effect of tanshinone IIA on improving motor function and cell apoptosis in spinal cord compression injury animal models. It also confirmed that tanshinone IIA can improve spinal cord function, alleviate spinal cord injury and regulate apoptotic gene expressions in compressed spinal cord tissue by activating the PI3K/AKT signaling pathway.

In conclusion, we believe that during spinal cord compression, the expressions of the promoting proliferation and inhibiting apoptosis genes are down-regulated, and the expressions of the promoting apoptosis genes are up-regulated, and the PI3K/AKT signaling pathway is inhibited. Tanshinone IIA can regulate the expressions of the apoptotic genes in compressed spinal cords by activating the PI3K/AKT signaling pathway, thereby inhibiting apoptosis, and reducing the degree of spinal cord injury in rats with spinal cord compression.

Disclosure of conflict of interest

None.

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References

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