Ginkgo lactone B's protective effects on spinal cord injury and its relationship with STAT1 signaling.

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Abstract

Spinal cord injury is a common form of trauma. Ginkgolide B can affect the function of the nervous system. This research established a rat spinal cord injury model to investigate the impact of ginkgolide B on spinal cord injury and its relationship with STAT1 signaling pathway. Spinal cord injury model was applied on rats according to the reference. The rats were randomly divided into sham operation group, spinal cord injury group, and ginkgolide B group. Inclined plate test and Basso Beattie Bresnahan (BBB) score were adopted for evaluation. Cell apoptosis was evaluated by TUNEL assay. Bcl-2, Bax, and p-STAT1 expressions were measured by Western blot. Ginkgolide B significantly reduced cell apoptosis induced by spinal cord injury at 12 h, 24 h, and 48 h (P<0.05). Bcl-2 increased, while Bax declined in spinal cord injury rat. Ginkgolide B treatment markedly decreased Bcl-2 level and elevated Bax expression (P<0.05). Cell number in the spinal gray matter obviously increased after ginkgolide B treatment for 12 h, 24 h, and 48 h compared with spinal cord injury group (P<0.05). Spinal cord injury enhanced STAT1 phosphorylation, whereas ginkgolide B declined STAT1 phosphorylation at 12 h, 24 h, and 48 h (P<0.05). Ginkgolide B significantly increased rat inclined plate test results and BBB score (P<0.05). Ginkgolide B showed protective role in spinal cord injury by affecting STAT1 signaling pathway.

Keywords: Ginkgolide B, Spinal cord injury, STAT1, Mechanism.
Reagents
DMEM medium, penicillin-streptomycin, and fetal calf serum were from Gibco. Mouse anti human Bcl-2, Bax, and p-STAT1 monoclonal antibodies and rabbit anti mouse secondary antibody were from ZSbio. Benchtop was from Formal. Inverted microscope was from Olympus. Incubator was from Thermo. CO2 incubator was from SANYO. Low temperature high speed centrifuge was from Beckman. -80°C refrigerator was from SANYO.

Animal grouping
The rats were randomly divided into sham operation group, spinal cord injury group, and ginkgolide B group. There were 48 rats in each group that were randomly divided into different time point subgroups (12 h, 24 h, and 72 h).

Experimental methods
Acute spinal cord injury model construction and ginkgolide B treatment: After anesthesia, the rat was incised on back midline. T10 spinous process was treated as the central, and the spinous process and spinous layer of T9-T11 were also exposed [15,16]. At 30 min after surgery, the rats in ginkgolide B group received ginkgolide B intraperitoneal injection at 4 mg/kg daily for 14 continuous days.

Spinal tissue collection
The rat was anesthetized at different time points to open the thorax. Left ventricular and aorta were perfused by normal saline. T10 segment was collected and fixed by 4% paraformaldehyde for paraffin section. The left spinal tissue was stored in liquid nitrogen.

Spinal tissue section
The spinal tissue was dewaxed by xylene and dehydrated by ethanol. After washed by distilled water, the tissue was stained by hematoxylin for 10 min. Next, the tissue was immersed in 1% ethanol for layering. Then the slice was incubated in alkaline phosphatase solution for staining. After washed by distilled water, the slice was stained by eosin for 5-10 min. At last, after dehydration, hyalinization, and sealing, the slice was observed under microscope.

TUNEL assay
The slice was stained by TUNEL reagent according to the manual (Beyotime). Under the microscope, 4-6 visual fields were randomly selected for cell counting. Apoptotic index=(TUNEL positive cell/total cell) × 100%.

Western blot
Spinal tissue protein was separated by SDS-PAGE and transferred to PVDF membrane. After blocked by 5% skim milk, the membrane was incubated in phosphor-STAT1, Bcl-2, and Bax antibodies. Total STAT1 and GAPDH were selected as internal reference. The membrane was detected by chemiluminescence and the image was disposed using Image J.

Spinal function evaluation
Inclined plate test and Basso Beattie Bresnahan (BBB) score were adopted at 22:00 every day to evaluate spinal function [17,18]. For inclined plate test, the angle between inclined
plate and horizontal plate was gradually increased until the rat can stay on plate for at least 5 s. The angle was defined as inclined plate angle. For BBB score, the rat walked in specific time point and the posterior limbs motion was observed. The rat posterior limbs motor nerve function was scored according to BBB.

**Statistical analysis**

All the data analysis was performed on SPSS17.0 software. The data depicted as mean ± standard deviation. T test and ANOVA were applied for data comparison. P<0.05 was considered as statistical significance.

**Results**

**Ginkgolide B suppressed spinal cell apoptosis**

It was showed that ginkgolide B obviously alleviated spinal cord induced cell apoptosis at 12 h, 24 h, and 72 h (Figure 1, P<0.05).

**Ginkgolide B increased cell number in spinal gray matter after spinal cord injury**

We collected spinal tissue for section and HE staining. The results demonstrated that compared with spinal cord injury group, the cell number in spinal gray matter markedly elevated in ginkgolide B group at 12 h, 24 h, and 48 h (Figure 3, P<0.05).

**Ginkgolide B affected Bcl-2 and Bax expression**

Western blot results revealed that Bcl-2 significantly elevated in spinal cord injury group, while ginkgolide B treatment markedly downregulated its level after 72 h. Bax declined in spinal cord group, whereas obviously upregulated after ginkgolide B treatment (Figure 2, P<0.05).

**Ginkgolide B affected p-STAT1 expression**

*a*P*<*0.05, compared with spinal cord injury group.  
**Ginkgolide B affected spinal cord injury through inhibiting STAT1 phosphorylation**

STAT1 phosphorylation significantly increased in spinal cord injury group compared with sham group. Ginkgolide B treatment obviously declined STAT1 phosphorylation at 12 h, 24 h, and 48 h (Figures 4, P<0.05).

**Ginkgolide B improved neural functional recovery**

Inclined plate test and BBB score were applied to evaluate neural functional recovery at different time points. Inclined plate angle and BBB score gradually increased in ginkgolide B group following time extension (Figure 5, P<0.05).

**Discussion**

In this study, we successfully established rat spinal cord injury model and used the ginkgolide B for treatment. Our results indicated that ginkgolide B can inhibit cell apoptosis in spinal cord injury.
Bcl-2 family is mainly composed of anti-apoptotic genes Bcl-2, Bcl-XL, and Mcl-1, and pro-apoptosis gene Bax and Bik [19]. Bcl-2 and Bax are the most representativeness among these genes, which plays apoptosis inhibition and promotion role, respectively. Current research suggested that the ratio of Bcl-2 and Bax may affect cell apoptosis. Its elevation inhibited cell apoptosis, while downregulation promoted cell apoptosis [20]. In this study, our results showed that the ratio of Bcl-2 and Bax obviously decreased in spinal cord injury group, while it apparently upregulated after ginkgolide B treatment, suggesting that ginkgolide B played the curative and protective effect on spinal cord injury model through affecting the ratio of Bcl-2 and Bax.

We further analyzed the protective effect of ginkgolide B on spinal cord injury phenotype. The results presented that cell number obviously increased in spinal cord injury after ginkgolide B treatment. We investigated the related signaling pathways based on this. Previous studies considered that JAK/STAT signaling pathway may be involved in the protective effect of ginkgolide B on spinal cord injury [21]. It was thought that STAT1 activation may affect cell apoptosis in pathologic state [22]. Moreover, STAT1 could be found activated in neurons in the case of ischemic brain injury. Studies found that free radical scavenger and antioxidant can inhibit STAT1 phosphorylation to affect ischemic brain injury [23]. In addition, the mouse with STAT1 defect showed alleviated symptom than wild type mouse in cerebral infarction we proposed that ginkgolide B protect spinal cord injury by inhibiting STAT1 phosphorylation. Our investigation further found that ginkgolide B can improve neural functional recovery after spinal cord injury in long term by using inclined plate test and BBB score. To sum up, our results showed that ginkgolide B can improve spinal cord injury phenotype by inhibiting cell apoptosis in spinal cord tissue. Ginkgolide B may protect spinal cord injury by affecting STAT1 phosphorylation.

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References

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