

Original Article

Mechanism of JAK-STAT signaling pathway inhibitor in post-spinal cord injury

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Abstract: Spinal cord injury (SCI) severely threatened public health. JAK-STAT signaling pathway has been suggested to be involved in the pathogenesis of multiple diseases. The participation of JAK-STAT in SCI, however, requires further studies. We thus investigated the mechanism of JAK-STAT after the occurrence of SCI. A total of 30 SD rats were generated for SCI model, and were randomly divided into group A and group B (N=15 each). Group A rats received the injection of JAK-STAT signaling pathway inhibitor after SCI, while group B received saline injection instead. Motor dyskinesia assay was then performed, followed by HE staining on spinal cord tissues. Ki67 immunohistochemistry was used to detect cell proliferation while immunofluorescent assay detected nuclear status. Cell apoptosis was measured by flow cytometry. Motor dyskinesia assay showed significant retard onset of motor disorder in animals with JAK-STAT inhibitor injection (Group A). Group A also had regular cell morphology with less SCI, and less proliferative cells compared to group B cells ($P<0.05$). Flow cytometry assay showed larger number of apoptotic cells in JAK-STAT inhibitor treated tissues compared to control cells ($P<0.05$). JAK-STAT signaling pathway inhibitor may slow the development of SCI via decreasing cell proliferation and enhancing apoptosis.

Keywords: JAK-STAT, signaling pathway, spinal cord injury

Introduction

Spinal cord injury (SCI) is caused by physical or physiological injury of spinal cord tissues. It can severely impair motor function of patients and decrease life quality. Even with major development of modern medicine technique, lots of SCI patients were still incurable [1, 2]. SCI can be further divided into primary injury and secondary injury. Primary injury is caused by physical damage within a short time and lead to irreversible neural damage. Secondary injury, however, consists of a series of chronic pathological alternations such as focal ischemia, tissue edema and inflammation [3-5].

Intracellular signaling transduction plays a crucial role during SCI, as it can form a huge regulatory network for gene regulation. Recent studies about SCI focus on MAPK, PI3K/AKT, GSK-3 β and JAK/STAT pathways, most of which are related with inflammation [6, 7]. JAK/STAT is one signaling transduction pathway that is recently found to be initiated by cytokines, and plays important roles during multiple biological

processes including cell proliferation, differentiation, apoptosis and immune modulation [8, 9]. JAK/STAT pathway mainly consists of three components: tyrosine kinase related receptor, tyrosine kinase JAK and transcriptional factor STAT [10]. Tyrosine kinase related receptor includes macrophage colony stimulating factor and epidermal growth factor. These cytokines and stimulating factors were all expressed at cell surface by relevant receptor to form signal transduction. Tyrosine kinase JAK consists of four members: JAK1, JAK2, JAK3 and Tyk2, all of which share similar structural domain. Transcriptional factor STAT consists of six important members and directs the transcription process in which mRNA is synthesized [11-13]. Previous report mentioned the effect of JAK/STAT on the progression of colorectal carcinoma via mediating cellular proliferation and apoptosis, as it can activate cytokine CCL5 expression [14]. Current study of JAK/STAT in SCI, however, limits on the study of signal modulation, but lacks the regulatory role of JAK/STAT on SCI. We thus investigated the regula-

tion of JAK/STAT on cell proliferation and apoptosis after SCI.

Materials and methods

SCI model animals and reagents

Rats were provided by Shantou University Medical College, A total of 30 SD rats were divided into group A and B (7 males and 8 females in each group). After anesthesia, an area of 9 mm diameter around T10 spine was exposed. The spine was clipped for 3 seconds to generate SCI. Successful SCI model was deduced as the occurrence of spastic tail movement, retraction of hind limb and trunk, and paralysis of forelimbs [15]. Group A rats received injection of JAK-STAT inhibitor (KB-2454, Sigma, US) while group B received saline injection. Other chemical reagents were purchased locally.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the 2nd Hospital, Shantou University Medical College.

Motor dyskinesia grading

The bladder was emptied before testing. After 5-10 min acclimation, rats were allowed to move freely in the test field. Those with normal body movement in stable had 9 points. Dysfunctions in the position of hind limb during movement, or dis-coordination between forelimbs and hind limbs were deduced as 5-8 points. Inability for hind limbs to stretch the trunk or forward movement was interpreted as 3-4 points. Leaving only ankle movement made the total score between 0 and 2 points.

BBB score

Forced movement was performed on 2200 each night for 15-30 min. The movement trace was recorded and analyzed for the movement pattern. No major abnormality was deduced as >25 points. Those animals with irregular movement had 15-25 points. Animals having movement disorder had 5-15 points. Those animals with difficulty in movement had less than 5 points.

HE staining

Tissue samples were fixed in formalin overnight, and were dehydrated in 70%, 80%, 95% and 100% ethanol (3 h, 3 h, 2 h, and 1.5 h × 2

times). Xylene was used to treat tissues (1.5 h × 2 times), followed by paraffin immersion at 60°C (1 h + 2 h). Paraffin blocks were sectioned into 3 μm slices, which were de-waxed using routine methods (xylene, absolute ethanol, 95%, 90%, 85% and 80% ethanol). Tissues slides were then stained in hematoxylin for 1 min, and were rinsed in tap water. Eosin was then added to stain tissues for 10 sec, followed by washing under tap water. The slice was dried and mounted with coverslips. Under the microscope, 20 × filed in the middle was captured. Three independent pathologists performed the diagnosis of tissue injury.

Immunohistochemistry (IHC) staining

All tissue samples were fixed in formalin overnight, and were dehydrated in 70%, 80%, 95% and 100% ethanol (3 h, 3 h, 2 h, and 1.5 h × 2 times). Xylene was used to treat tissues (1.5 h × 2 times), followed by paraffin immersion at 60°C (1 h + 2 h). Paraffin blocks were sectioned into 3 μm slices, which were de-waxed using routine methods (xylene, absolute ethanol, 95%, 90%, 85% and 80% ethanol). After washing in PBS for 3 times (5 min each), antigen retrieval was performed using citric acid buffer (pH=6.0) for 5 min, followed by PBS washing (5 min × 3 times). Blocking was performed using 10% bovine serum albumin (BSA) for 30-min incubation. Primary antibody (mouse anti-human Ki67 monoclonal antibody, 1:100, Santa Cruz) was added for overnight incubation. On the next day, tissue slides were washed in PBS (5 min × 3 changes), and were mixed with anti-mouse antibody (1:100, Santa Cruz) at 37°C for 1 hour. The slices were washed again in PBS and were developed by DAB. After counter-staining by hematoxylin, and processing in tap water. Under the microscope, 20 × filed in the middle was captured. Three independent pathologists performed the deduction of staining patters. DAB chromogenic substrate and EDTA antigen retrieval reagent were produced by Zhongshan (Beijing, China) in working concentration. Tissue samples were fixed in 10% neutral buffered formalin. PBS was used in negative control in parallel.

Immunofluorescence

After fixation, tissues were dehydrated in gradient sucrose, and were sectioned into 6 μm slices. Antigen retrieval was performed by 5-min heating. After cooling down, tissue slices were

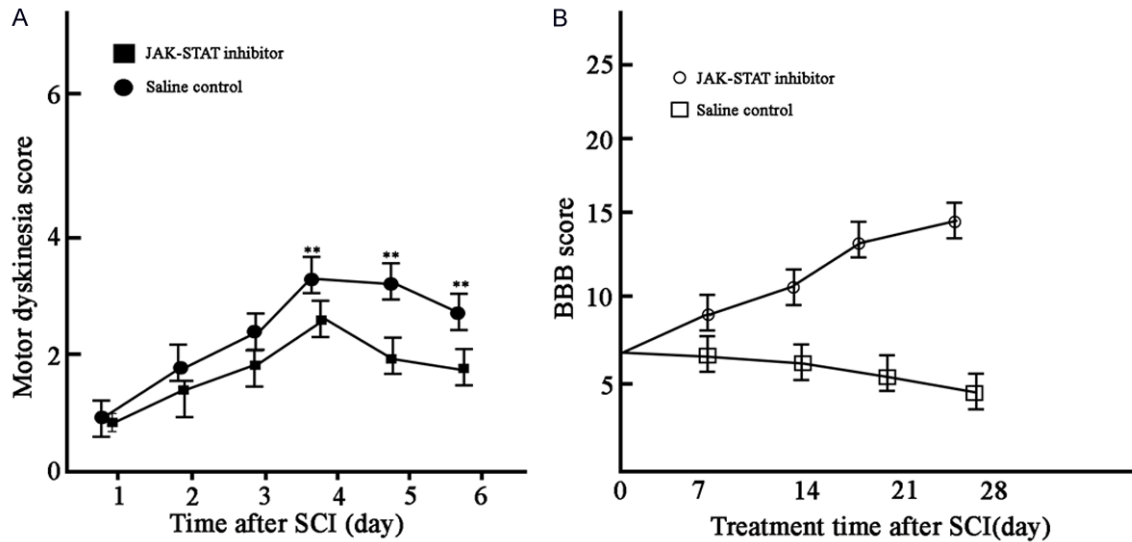


Figure 1. Motor function of rats. A. Motor dyskinesia score; B. BBB score.

rinsed in PBS (5 min × 3 changes) and were blocked in 10% BSA for 50 min. DAPI dye (1:100, Cell signaling technology, US) was added for staining, followed by observation under a fluorescent microscope.

Cell apoptosis assay

Cells were discarded for culture medium, and were digested in trypsin. After rinsing in culture medium and centrifugation at 800 rpm for 10 min, supernatants were discarded. Cells were then fixed at -20°C overnight. On the next day, PBS was used to wash and re-suspend cells, which were added with 100 µL Annexin-V-Fluos, 10 µL PI and 10 µL Annexin within 150 µL buffer. After incubation at room temperature for 7 min, cells were collected for flow cytometry assay.

Statistical analysis

SPSS 11.0 software was used to process all data. Student t-test was employed to compare means, in addition to Pearson correlation analysis, and chi-square test. A statistical significance was defined when P<0.05. *, ** and *** represented P<0.05, P<0.01 and P<0.001. Each experiment was carried out in at least triplicates. The number of positive cells in IHC was calculated by ImageJ software.

Results

SCI model and motor scores

Group A rats received injection of JAK-STAT inhibitor (KB2454, Sigma, US) while group B

received saline injection at 28 days after surgery. Motor behaviors of two groups were observed, plus the grading by motor dyskinesia score. Results showed significantly decreased motor dyskinesia in group A, which received inhibitor of JAK-STAT signal pathway (P<0.01, **Figure 1A**). BBB score showed better motor function of group A rats compared to group B, which showed remarkable motor disorders (P<0.01, **Figure 1B**).

Decreased cell proliferation after JAK-STAT inhibitor treatment

After successful generation of SCI model, we collected spinal cord tissues in two groups for HE staining. Results showed relatively milder tissue injury in group A rats, which received JAK-STAT inhibitor treatment (**Figure 2A**). Whilst in group B rats, which received saline injection, severe neuronal damage were observed (**Figure 2A**). Further IHC staining for Ki67 expression showed significantly more positive cells in group B compared to group A (P=0.0041, **Figure 2B**).

Elevated apoptotic cell number after JAK-STAT inhibitor treatment

Immunofluorescent staining showed nuclear condensation, which is the signature of cell apoptosis in JAK-STAT inhibitor treated group but not in saline control group (**Figure 3A**). Further quantitative assay for apoptotic cell numbers using flow cytometry revealed significantly elevated apoptotic cell number in JAK-

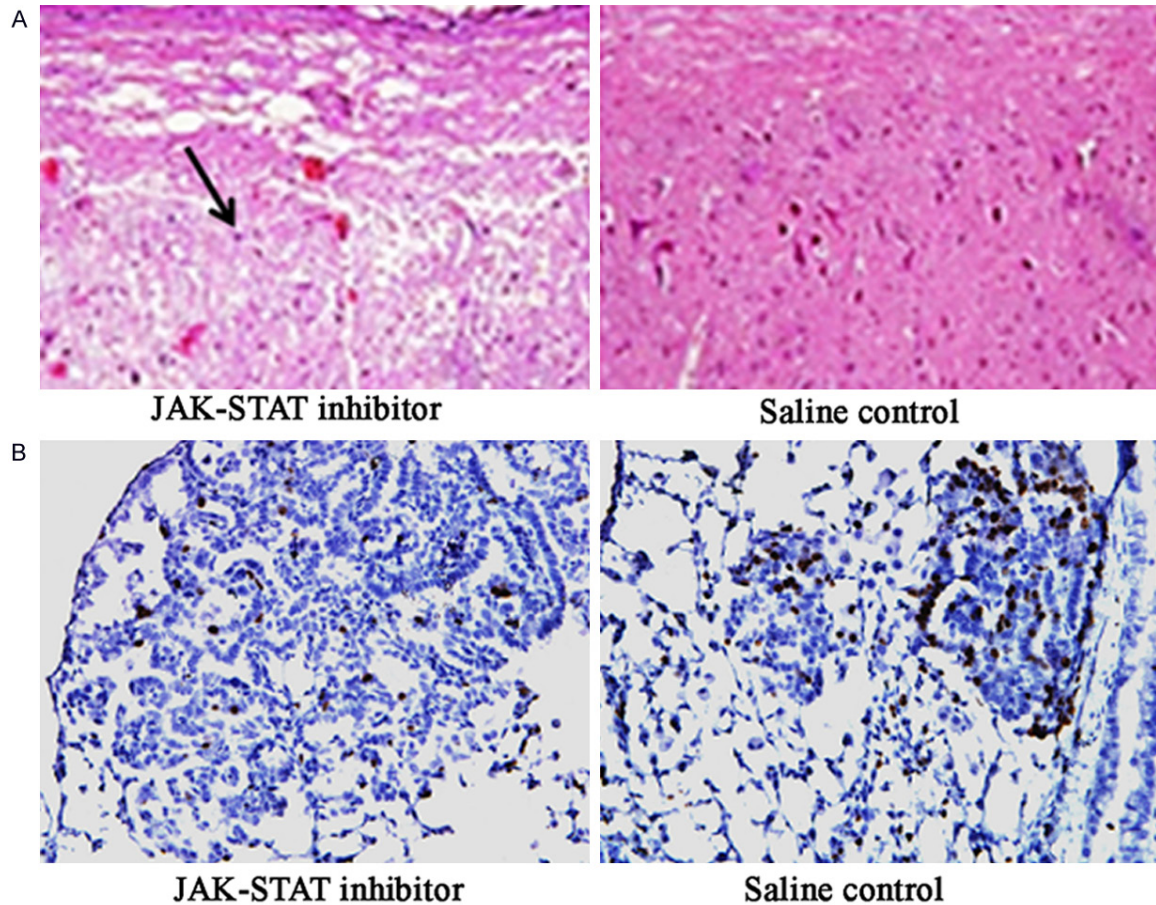


Figure 2. Tissue injury and cell proliferation after JAK-STAT inhibitor treatment. A. Neuronal injury after JAK-STAT inhibitor treatment. Saline control group had severer tissue damage compared to JAK-STAT inhibitor group. B. Ki67 proliferation. Saline group had more positive cells than inhibitor group.

STAT inhibitor treated group compared to saline group ($P=0.0024$, **Figure 3B**).

Discussion

SCI is caused by physical injury including traffic accidents, exercise injury and other trauma. Consequent injuries of neuropathology and neural function disorders also occur after primary injury. Physiological injury including tissue damage and cell hyper-proliferation may cause insufficient nutrients in tissues. The inflammation response after SCI is severer than brain damages. Although various trials have been performed such as the manipulation of inflammatory cell composition during secondary injury in mice models, but obtained unsatisfactory results mainly due to the difficulty in separating inflammatory cells [16].

Previous study has shown the immediate activation of JAK/STAT signal, along with alterna-

tion of cell morphology and cell apoptosis [17]. However, no clear explanation has been made regarding the mechanism underlying such apoptosis. Other scholars have reported the activation of STAT1 in focal ischemia of brain and coagulated brain damage, as potent anti-oxidation reagent may prevent the nuclear entry of cells by inhibiting STAT1 phosphorylation, thus protecting cardiomyocytes or brain [18]. Some studies have suggested the protection against neural damage of astrocytes by inhibiting JAK/STAT signaling pathway, with the participation of inflammatory cytokines including IL-6 and TNF- α [19]. Our study obtained similar results, to demonstrate the effective retard of SCI progression by inhibitor of inflammatory related signaling pathway JAK/STAT.

The mechanism of JAK/STAT in SCI, however, has not been fully illustrated. We thus hypothesized if JAK/STAT might affect cell apoptosis

JAK-STAT in SCI

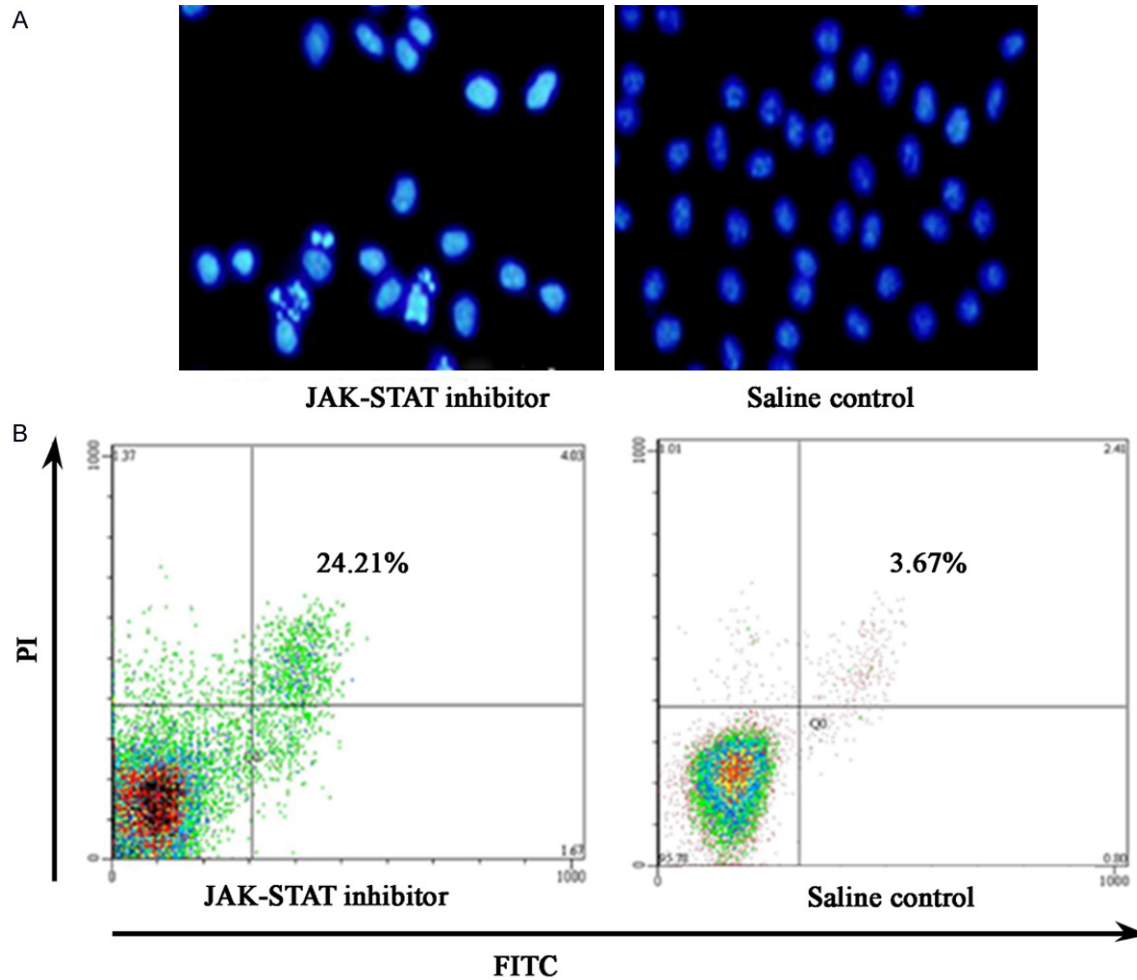


Figure 3. Elevated number of apoptotic cells after JAK-STAT inhibitor treatment. A. Immunofluorescent staining showing growth status of nucleus. B. Cell apoptosis assayed by flow cytometry. JAK-STAT inhibitor treatment significantly increased the number of apoptotic cells.

or proliferation to protect spinal cord tissues. Such hypothesis is based on the significant alternation of cell proliferation and/or apoptosis by key transcription factors such as STAT1, TGF- β and P65, with STAT1 as one common changing transcription factor [20]. We thus believed if STAT1 had similar patterns in SCI. In this study, SCI model rats were generated, followed by treatment of JAK/STAT inhibitor or saline. Motor ability assay was performed to deduce the generation of model. In spinal cord tissues, we found significantly increased number of neurons in JAK/STAT1 inhibitor group without vacuoles. In saline group, however, small numbers of neurons were observable, with many tissue vacuoles. Therefore, JAK/STAT inhibitor can effectively alleviate SCI. Further test on tissue proliferation and apopto-

sis in two models strengthened the idea that JAK/STAT inhibitor may reduce SCI via decreasing cell proliferation and increasing cell apoptosis.

In summary, our study demonstrated that JAK/STAT inhibitor could facilitate the functional recovery of spinal cord after SCI, along with the potential mechanism for the first time. Our results provide further evidences for protecting against SCI in clinics.

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Disclosure of conflict of interest

None.

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