

Effects of STAT3 inhibitors on neural functional recovery after spinal cord injury in rats

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Summary Spinal cord injuries (SCIs) can induce primary and secondary injury, resulting in severe neurological damage and dysfunction in patients. Studies have reported that signal transducer and activator of transcription 3 (STAT3) plays an important role in the inflammatory immune response and neural stem cell differentiation. In order to examine whether a STAT3 inhibitor can prevent worsening of an SCI and promote neural stem cell differentiation, a rat model of surgically induced SCI was created and rats were treated with the STAT3 inhibitor S31-201. Tissue from the injured region was harvested and fixed in formalin and paraffin. H&E staining was used to look for morphological changes. The Basso, Beattie, and Bresnahan locomotor scale (BBB score), somatosensory evoked potentials (SEP), and motor evoked potentials (MEP) were examined. Western blotting was used to detect the expression of β -tubulin III, vimentin, GFAP, NF-200, and OX-42 protein. Results indicated that the STAT3 inhibitor S31-201 reduces the extent of SCI and it promotes neural stem cell differentiation.

Keywords: Signal transducer and activator of transcription 3 (STAT3), spinal cord injury, STAT3 inhibitor

1. Introduction

Spinal cord injuries (SCIs) are a problem worldwide. An SCI is known to cause direct mechanical damage and subsequent secondary injury cascades. An inflammatory immune response starts with an immediate influx of inflammatory cells into the injured spinal cord, and cytokines and neurotrophic factors hamper spinal cord regeneration (1,2). Other factors that affect regeneration of axons are reactive hyperplasia of astrocytes, microglia, and oligodendrocytes and the formation of a glial scar after spinal cord injury (1,2). Therefore, preventing the inflammatory process, decreasing secondary injury, and promoting neuron regeneration after an SCI are key areas in which to treat an SCI in its early stages (3). Although current clinical approaches to treating an SCI,

including the use of high doses of methylprednisolone, surgery, intensive multisystem medical management, and rehabilitative care, provide some benefits, novel approaches to treating SCIs need to be developed. Over the past few years, researchers have begun to use endogenous neural stem cells (NSCs) to treat SCIs. Recently, a study reported generating autologous pluripotent stem cells from skin fibroblasts, thus avoiding ethical problems and the problem of immune rejection (4). However, few studies have reported on regulation of the differentiation of NSCs.

In the early stage of an SCI, interleukin-6 (IL-6) rises rapidly, promoting and regulating the inflammatory response, inducing NSCs to selectively undergo astrocytic differentiation, and aggravating a secondary SCI (5,6). Signal transducer and activator of transcription 3 (STAT3) plays a crucial role in the inflammatory response of IL-6 (7). STAT3 inhibits the expression of Bim1 and Oct4 downstream of STAT3 and it promotes NSC differentiation (8).

Based on these previous findings, a murine model of SCI was used to investigate whether inhibiting STAT3 would modify the acute inflammatory response after an SCI and promote NSC differentiation.

Released online in J-STAGE as advance publication December 21, 2016.

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2. Materials and Methods

2.1. Animals and groups

One hundred and twenty Sprague-Dawley rats (weight: 180-200 g) were purchased from the Laboratory Animal Center of the Military Academy of Medical Sciences (license: SCXK - (People's Liberation Army of China), 2012-0004) and were housed in individual cages in the Animal Facility of Tianjin Medical University with the approval of the Institutional Animal Care and Use Committee. These rats were randomly divided into three groups: a sham-operated (SO) group, an SCI group, and an SCI and treatment with the STAT3 inhibitor S31-201 (SCI+S31-201) group (40 rats in each group). Rats in each group were randomly sacrificed at five different points in time. At each time point, 8 rats from each group were sacrificed, and 3 outliers were eliminated. All rats were handled in accordance with the recommendations of the Guidelines for the welfare and use of animals in research.

2.2. Creation of a model of SCI

Rats in the SCI group were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium (50 mg/kg). Using the T8 spinous process as the center, a median skin incision was made on the back and paravertebral muscle at T7-T9 was dissected under sterile surgical conditions. A laminectomy of the Th8 vertebra was performed. A model of acute SCI was produced with improved Allen's devices – a 30-g weight was dropped from a height of 5 cm onto the exposed dura of the spinal cord. An SCI was induced at T8. The skin and musculature were then sutured closed. Whether an SCI was successfully induced was determined by symptoms such as tail twitches, spinal cord bleeding and edema, contraction-like twitching of both hind limbs and the body, and delayed paralysis of both hind limbs after waking. After surgery, 1 million IU of penicillin was given daily *via* subcutaneous injection to prevent infections. The bladder was squeezed twice a day to assist voiding until the bladder reflex returned. All rats were housed in warm, separate cages. The rats were monitored daily. A laminectomy was performed on the SO group but an SCI was not induced.

2.3. Treatment with a specific STAT3 inhibitor

S31-201, a specific STAT3 inhibitor, was dissolved in dimethyl sulphoxide (DMSO) and injected into the abdominal cavity at 5 mg/Kg in rats in the SCI+S31-201 group. The other two groups were injected with DMSO as controls. All of the rats were injected once a day for a week.

2.4. Hematoxylin and eosin (H&E) staining

Specimens of spinal cord tissue were observed on day 14 and day 28 after surgically induced SCI. Tissue was fixed in 10% paraformaldehyde for 24 h, embedded in paraffin, and cut into sections 5 μ m in thickness. Hematoxylin and eosin (H&E) staining was performed to observe morphological changes.

2.5. Somatosensory evoked potentials (SEP) and motor evoked potentials (MEP)

Somatosensory evoked potentials (SEP) and motor evoked potentials (MEP) were examined on days 1, 7, 14, and 28 postoperatively.

2.6. Behavioral testing

Neurological function in the hind limbs was evaluated on days 1, 3, 7, 14, and 28 postoperatively using the Basso, Beattie, and Bresnahan locomotor scale (BBB score). Six rats per group were randomly selected for functional testing. Each rat was placed in an open field and was scored by two blind observers after an observation period of 5 min.

2.7. Western blotting

Spinal cord tissue was harvested on days 1, 3, 7, 14, and 28 postoperatively. One hundred mg of tissue was treated with 400 μ L of RIPA lysate containing PMSF and 1% protease inhibitor and placed on ice for 30 min. Produced proteins were stored at -80°C.

Proteins were transferred onto nitrocellulose membranes for 10% SDS-PAGE gel electrophoresis. Blots were blocked and incubated with a primary antibody. Western Lightning[®]-ECL, Enhanced Chemiluminescence Substrate (Perkin Elmer, NEL100001EA) was used to measure protein expression, with GAPDH serving as the internal control. Bands were imaged and analyzed using LABWORKS 4.0.

2.8. Statistical analysis

Data were collected and 3 outliers for each group were eliminated. Data were analyzed with SPSS.18 and are presented as the mean \pm standard deviation (M \pm S.D.). One-way analysis of variance (ANOVA) followed by the least significance difference (LSD)-multiple range test were used to analyze the differences between groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Morphological changes

Macroscopic observation: In group 2 and group 3 with

a surgically induced SCI (SCI group and SCI+S31-201 group), diffuse hyperemia and edema in the dorsolateral spinal cord were observed. On day 14 and day 28 postoperatively, the formation of an epidural scar and epidural adhesions were evident. In group 1 (SO group), epidural scar formation and epidural adhesions were noted but hyperemia and edema were not noted.

Microscopic observation: On day 14 postoperatively, the structure of the spinal cord and nerve cells in the SCI group had been destroyed, liquefactive necrosis in damaged areas had resolved and was followed by the formation of a cystic space, and swollen axons and disordered nerve fibers were noted in white matter (Figure 1A). On day 28 postoperatively, a long, oval cavity had formed where the spinal cord was severed and the vertical axis of the cavity ran parallel to the long axis of the spinal cord. There are many foamy macrophages and glial scar in the cavity (Figure 1B). In the SCI+S31-201 group, the inflammatory response diminished and the cavity where the spinal cord was severed had shrunk; on day 28 postoperatively, the cavity where the spinal cord was severed and the glial

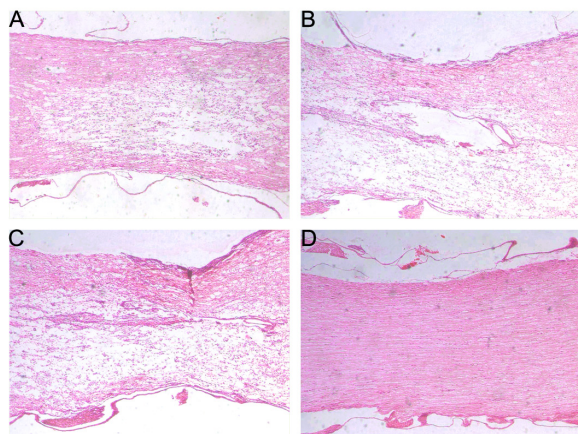


Figure 1. Microscopic observation. (A), Two weeks after surgically induced SCI, pathological changes indicative of liquefactive necrosis occurred in the area near the injured spinal cord, and the border of the cavity where the spinal cord was severed was not clear (SCI group); (B), Four weeks after surgically induced SCI, the border of the cavity where the spinal cord was severed was demarcated and the vertical axis of the cavity was consistent with the long axis of the spinal cord in the SCI group; (C), Four weeks after surgically induced SCI, demarcation was less evident in the SCI+S31-201 group than that in the SCI group; (D), After four weeks, there were no obvious changes in the structure of spinal cord in the SO group.

scar had shrunk (Figure 1C). In the SO group, the structure of the spinal cord was normal and there was no cavity in the spinal cord (Figure 1D).

3.2. BBB score

As Figure 2 and Table 1 show, the BBB scores for the SCI group and the SCI+S31-201 group were lower than that for the SO group. However, the BBB score for rats treated with S31-201 was higher than that for untreated rats with surgically induced SCI. Starting on day 14 postoperatively, the BBB scores for the SCI group and the SCI+S31-201 group differed significantly ($p < 0.001$).

3.3. SEP and MEP evaluation

As Figure 3 shows, both the MEP (Table 2) and SEP (Table 3) scores in the SCI group and the SCI+S31-201 group were significantly higher than those in the SO group. However, the MEP and SEP scores for rats treated with S31-201 were lower than those for untreated rats with surgically induced SCI. Starting on day 14 postoperatively, MEP and SEP scores for the SCI group and the SCI+S31-201 group differed significantly ($p < 0.001$).

3.4. Expression of proteins of interest

OX-42 was used to detect changes in morphology and the number of microglia. As Figure 4A and Table 4

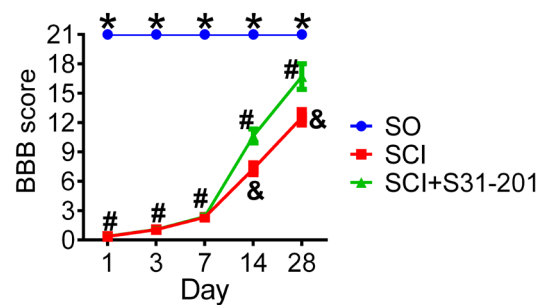


Figure 2. BBB scores for individual groups. The score for the SO group was significantly higher than that for the other two groups. * $p < 0.001$, vs. SCI groups. # $p < 0.001$, vs. SO group. & $p < 0.001$, vs. SCI+S31-201 groups ($n = 5$ points for each group; $n = 15$ points for 3 groups).

Table 1. BBB Score

day	group 1	group 2	group 3	F value	p	LSD		LSD		LSD	
						group 1 vs. group 2	p	group 1 vs. group 3	p	group 2 vs. group 3	p
1	21 ± 0	0.36 ± 0.03	0.37 ± 0.02	1940061.82	*	t = 2955.71	*	t = 2953.70	*	t = 2.01	0.052
3	21 ± 0	1.05 ± 0.03	1.07 ± 0.05	535833.03	*	t = 1553.60	*	t = 1552.04	*	t = 1.56	0.128
7	21 ± 0	2.32 ± 0.09	2.39 ± 0.14	60142.17	*	t = 521.20	*	t = 519.25	*	t = 1.95	0.058
14	21 ± 0	7.28 ± 0.61	10.62 ± 0.71	789.31	*	t = 65.98	*	t = 49.92	*	t = 16.06	*
28	21 ± 0	12.53 ± 0.78	16.70 ± 1.30	105.83	*	t = 25.20	*	t = 12.79	*	t = 12.41	*

$n = 5$ points for each group; $n = 15$ points for 3 groups, * $p < 0.001$.

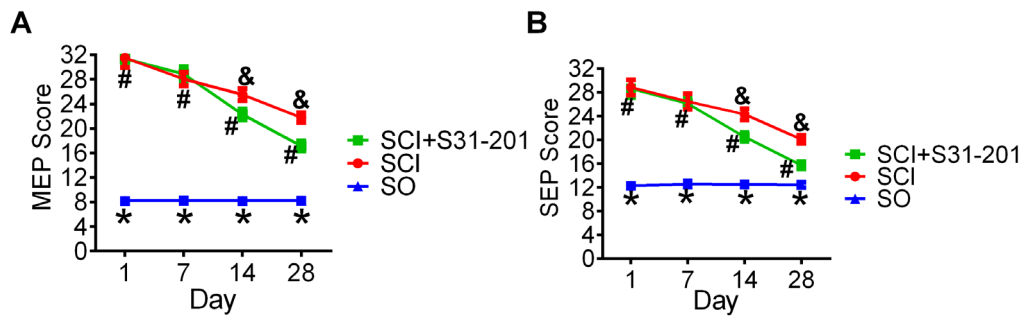


Figure 3. MEP (A) and SEP (B) were measured in rats on days 1, 7, 14, and 28 postoperatively. * $p < 0.001$, vs. SCI group. # $p < 0.001$, vs. SO group. * $p < 0.001$, vs. SCI+S31-201 group ($n = 5$ points for each group; $n = 15$ points for 3 groups).

Table 2. MEP

day	group 1	group 2	group 3	F value	p	LSD		LSD		LSD	
						group 1 vs. group 2	p	group 1 vs. group 3	p	group 2 vs. group 3	p
1	8.23 ± 0.35	31.51 ± 1.61	31.35 ± 1.00	656.70	*	$t = 54.55$	*	$t = 54.17$	*	$t = 0.38$	0.710
7	8.28 ± 0.40	28.06 ± 1.12	28.88 ± 1.16	671.08	*	$t = 53.80$	*	$t = 56.04$	*	$t = 2.23$	0.032
14	8.21 ± 0.32	25.52 ± 0.97	22.29 ± 0.87	639.32	*	$t = 58.24$	*	$t = 47.37$	*	$t = 10.87$	*
28	8.25 ± 0.37	21.77 ± 0.76	17.15 ± 0.82	459.17	*	$t = 51.63$	*	$t = 33.99$	*	$t = 17.64$	*

$n = 5$ points for each group; $n = 15$ points for 3 groups; group 1: SO group; group 2: SCI group; and group 3: SCI+S31-201 group; * $p < 0.001$.

Table 3. SEP

day	group 1	group 2	group 3	F value	p	LSD		LSD		LSD	
						group 1 vs. group 2	p	group 1 vs. group 3	p	group 2 vs. group 3	p
1	12.27 ± 0.40	28.85 ± 1.30	28.63 ± 1.52	295.97	*	$t = 36.74$	*	$t = 36.25$	*	$t = 0.49$	0.629
7	12.53 ± 0.53	26.46 ± 1.30	26.12 ± 0.91	306.75	*	$t = 37.60$	*	$t = 36.69$	*	$t = 0.92$	0.364
14	12.5 ± 0.48	24.33 ± 0.97	20.51 ± 0.78	277.90	*	$t = 40.01$	*	$t = 27.09$	*	$t = 12.92$	*
28	12.42 ± 0.43	20.09 ± 0.64	15.74 ± 0.61	205.13	*	$t = 34.98$	*	$t = 15.14$	*	$t = 19.84$	*

$n = 5$ points for each group; $n = 15$ points for 3 groups; group 1: SO group; group 2: SCI group; group 3: SCI+S31-201 group; * $p < 0.001$.

show, the level of OX-42 protein expression increased significantly as a result of SCI. S31-201 treatment decreased the level of OX-42 expression. The grayscale intensity of expression differed significantly ($p < 0.001$, Figure 4D).

Glial fibrillary acidic protein (GFAP) is a component of the cytoskeleton of astrocytes and vimentin is closely related to gliosis and glial recovery after brain injury. Western blotting indicated that on day 14 and day 28 postoperatively levels of both vimentin and GFAP expression were significantly higher in the SCI group and the SCI+S31-201 group. Moreover, S31-201 treatment significantly decreased the level of GFAP expression on day 28 postoperatively. The significant difference in vimentin expression caused by S31-201 treatment started on day 14 postoperatively (Figure 4B, $p < 0.001$).

Western blotting indicated that expression of the early neuronal specific marker β -tubulin III was downregulated as a result of surgically induced SCI and that S31-201 treatment upregulated the expression of β -tubulin III. The grayscale intensity of blots differed significantly (Figure 4E, $p < 0.001$).

The level of neurofilament-200 (NF-200) expression

was lower in the SCI group than in the SO group. The STAT3 inhibitor significantly upregulated the expression of NF-200 starting on day 14 postoperatively (Figures 4C and 4F, $p < 0.001$).

4. Discussion

SCI often results in devastating permanent neurological deficits. Glial scar tissue is considered to be a physical barrier that prevents axonal regeneration by producing axonal growth inhibitors (9). Pro-inflammatory cytokines such as IL-6, IL-1b, and tumor necrosis factor- α (TNF- α) initiate production of a number of specific molecules and contribute to secondary damage (10). Expression of IL-6 mRNA has also been observed in motoneurons 24-72 h after injury, indicating that these inflammatory cytokines may be involved in promoting axonal sprouting in the process of SCI. Studies have reported that STAT3 is a signal transducer of various cytokines and growth factors and that it plays an important role in IL-6 intercellular signaling after central nervous system (CNS) injury (which includes an SCI) (11-13). Activation of the JAK-STAT pathway has previously been reported in CNS injury and is directly associated with neurogenesis and

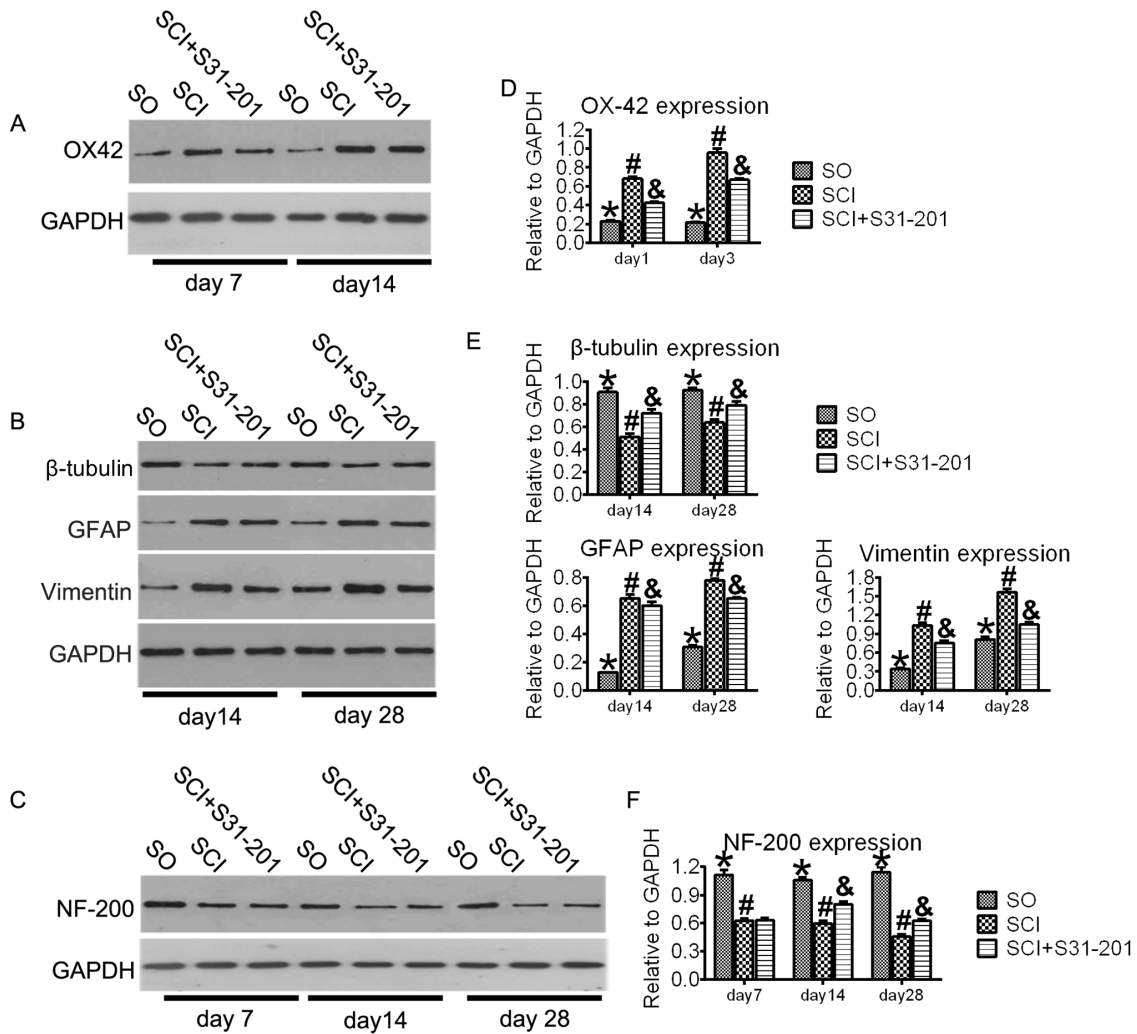


Figure 4. Western blotting and grayscale values for blots. **p* < 0.001, vs. SCI+S31-201 group. #*p* < 0.001, vs. SO group. &*p* < 0.001, vs. SCI group. (*n* = 5 points for each group; *n* = 15 points for 3 groups).

Table 4. Grayscale values for Western blots (relative to GAPDH)

indices	day	group 1	group 2	group 3	<i>F</i> value	<i>p</i>	LSD		LSD		LSD	
							group 1 vs. group 2	<i>P</i>	group 1 vs. group 3	<i>P</i>	group 2 vs. group 3	<i>P</i>
OX-42	1	0.23 ± 0.01	0.68 ± 0.02	0.43 ± 0.01	548.29	*	<i>t</i> = 57.19	*	<i>t</i> = 24.85	*	<i>t</i> = 32.34	*
	3	0.21 ± 0.00	0.96 ± 0.04	0.67 ± 0.02	490.39	*	<i>t</i> = 53.80	*	<i>t</i> = 32.92	*	<i>t</i> = 20.88	*
NF200	7	1.11 ± 0.05	0.63 ± 0.02	0.63 ± 0.03	148.31	*	<i>t</i> = 25.91	*	<i>t</i> = 25.76	*	<i>t</i> = 0.15	0882
	14	1.14 ± 0.05	0.46 ± 0.02	0.63 ± 0.02	290.80	*	<i>t</i> = 40.02	*	<i>t</i> = 30.38	*	<i>t</i> = 9.64	*
β-tubulin	14	0.91 ± 0.04	0.51 ± 0.02	0.72 ± 0.04	92.29	*	<i>t</i> = 23.52	*	<i>t</i> = 11.21	*	<i>t</i> = 12.31	*
	28	0.92 ± 0.02	0.64 ± 0.02	0.79 ± 0.03	77.56	*	<i>t</i> = 21.56	*	<i>t</i> = 10.10	*	<i>t</i> = 11.46	*
GFAP	14	0.13 ± 0.00	0.65 ± 0.03	0.60 ± 0.03	366.59	*	<i>t</i> = 42.45	*	<i>t</i> = 38.49	*	<i>t</i> = 3.96	*
	28	0.31 ± 0.01	0.78 ± 0.01	0.65 ± 0.01	1265.13	*	<i>t</i> = 84.30	*	<i>t</i> = 61.22	*	<i>t</i> = 23.08	*
Vimentin	14	0.34 ± 0.02	1.04 ± 0.04	0.76 ± 0.03	317.19	*	<i>t</i> = 43.35	*	<i>t</i> = 25.91	*	<i>t</i> = 17.44	*
	28	0.81 ± 0.04	1.56 ± 0.05	1.06 ± 0.03	206.24	*	<i>t</i> = 34.47	*	<i>t</i> = 11.18	*	<i>t</i> = 23.30	*

n = 5 points for each group; *n* = 15 points for 3 groups; **p* < 0.001.

glia scar formation in the injured region (14,15). The roles of STAT3 in SCI are not yet clear (16). Many pieces of evidence suggest that this family of cytokines plays important roles in regulating the immune response,

inflammation, and the CNS.

The current study investigated the effects of the STAT3 inhibitor S31-201 on the process of SCI. Results indicated that S31-201 reduced the inflammatory

response, it reduced secondary injury, and it promoted regeneration. Glial scar formation is considered to be major cause of poor regeneration of the adult CNS after injury (17). According to both macroscopic observation and microscopic examination, surgically induced SCI damaged the structure of the spinal cord, an epidural scar had formed, and there was a long, oval cavity where the spinal cord was severed. However, these morphological changes improved significantly as a result of S31-201 treatment. The extent of the decrease in the BBB score and the increase in SEP and MEP scores decreased as a result of treatment with the STAT3 inhibitor S31-201.

Western blotting indicated that S31-201 treatment decreased GFAP, vimentin, and OX-42 expression. The STAT3 inhibitor significantly upregulated the expression of NF-200 and β -tubulin III. A study has reported that IL-6 and the activity of its signaling partner establish a positive feedback mechanism with PAR1 that may promote IL-6-STAT3 signaling to levels sufficient to elicit fulminant astrogliosis, including increases in levels of the astroglial intermediate filament proteins GFAP and vimentin (18,19). The current results indicated that the STAT3 inhibitor S31-201 decreased the expression of GFAP and vimentin. In addition, S31-201 reduced the extent of the increase in OX-42. Thus, the STAT3 inhibitor S31-201 presumably attenuates the inflammatory response after SCI.

NF-200 is an indicator of neuronal differentiation and β -tubulin III indicates neural stem cell differentiation after an SCI in rats (5,20). Western blotting indicated that the STAT3 inhibitor S31-201 promoted the expression of NF-200 and β -tubulin III protein. The current study indicated that S31-201 stimulates neurogenesis in the region of an SCI.

In contrast to the current study, other studies have reported that STAT3 promotes motor neuron differentiation by collaborating with a motor neuron-specific LIM complex (21). STAT3 performs different roles during neural stem cell maintenance and motor neuron differentiation. In summary, the current mouse model of SCI suggests that early treatment with the STAT3 inhibitor S31-201 significantly improves functional recovery and reduces the posttraumatic inflammatory response and glial scar formation. The STAT3 inhibitor S31-201 inhibits the inflammatory action of IL-6 and neural stem cell differentiation, so S31-201 might be a potential treatment for SCI.

Acknowledgements

This study was supported by the Tianjin Natural Science Foundation (grant no. 16JCYBJC24900).

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(Received August 17, 2016; Revised October 12, 2016; Accepted October 25, 2016)