Original Article
Effects of Oncostatin M on the neurological function of rats with spinal cord injury through STAT3 signal pathway

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Abstract: Objective: To investigate the effects and mechanisms of Oncostatin M (OSM) on spinal cord ischemia and reperfusion injury in rat model. Methods: Thirty healthy male Sprague-Dawley (SD) rats were randomly divided into two groups in the first part of the experiment: spinal cord ischemia and reperfusion group (I/R group): 24 rats underwent abdominal aorta occlusion for 30 min, then 6 in the 3 h post reperfusion, 6 in the 6 h post reperfusion, 6 in the 24 h post reperfusion and 6 in the 72 h post reperfusion, and 6 rats were in the sham-operated control group (Control group). The neurological function was evaluated by Modified Tarlov score. Myeloperoxidase (MPO) activation of the spinal tissue in each group was detected. The expression of OSM was measured by real-time PCR after reperfusion. Twenty-four rats were divided into 2 groups in the second experiment, 12 rats were injected with OSM (I/R+OSM group) and 12 rats were injected with PBS (I/R+PBS group), The neurological function and MPO activation were evaluated after 24 h; the activation of STAT3 protein was detected by Western Blot. Results: After spinal cord ischemia and reperfusion, the neurological function injury was serious and the expression of MPO increased gradually in the spinal tissue; OSM expression increased significantly 6 h after reperfusion; 24 h after local injection of OSM to the rats with spinal cord ischemia and reperfusion injury, the expression of p-STAT3 protein increased, and the neurological function of rats significantly improved, while MPO activation decreased. Conclusions: The OSM/STAT3 signal pathway plays a protective role in the spinal cord ischemia and reperfusion injury.

Keywords: Spinal cord injury, OSM, STAT3, ischemia and reperfusion, MPO

Introduction

Ischemic spinal cord injury is a common complication of thoracoabdominal aortic surgery. In clinical treatments, the neurological function has not been improved after the recovery of blood supply to the spinal cord, but its injury will further aggravate, which is spinal cord ischemia and reperfusion injury (SCIRI). And it can lead to acute and delayed paraplegia with its incidence rate up to 3% to 18% [1]. The mechanism of SCIRI is still not very clear, but the present studies have shown that the main mechanism of SCRI damage includes inflammatory mechanism, microcirculation disorder mechanism, apoptosis mechanism, cell autophagy, etc. [2-6]. Especially, inflammatory mechanism is a vital pathophysiologic basis of SCIRI [7]. Therefore, the control of spinal cord inflammatory response plays an essential role in the treatment of SCRI.

The spinal cord injury can induce the expression of various inflammatory factors. And OSM, as a member of the interleukin-6 (IL-6) family, can exert a variety of biological functions by activating its receptors [8]. Many studies have found that OSM is associated with the growth and differentiation of tumor cells [9-11]. In addition, OSM plays a significant role in diverse kinds of diseases and animal models [12-14]. The IL-6 family can up-regulate the expression of suppressors-of-cytokine-signaling 3 (SOCS3) by activating the signal transducer and activator of transcription-3 (STAT3) [15]. It has been reported that SOCS3 gene deficiency can reduce neuronal necrosis and demyelination induced by spinal cord injury, and it possibly played a protective role by activating STAT3 protein on spinal cord injury in mice [16, 17]. In tumor cells, OSM can also activate the STAT3 protein to have a tumor suppressive effect by activating tyrosine protein kinases Jak1, Jak2

and Tyk2 [18]. The role of OSM in SCIRI is unclear, but it is possible that OSM can protect spinal cord against ischemia and reperfusion injury by activating STAT3 protein.

The purpose of this study is through establishing a rat ischemia and reperfusion (I/R) model, to study the role of OSM and STAT3 signal pathway in SCIRI, and to provide new ideas for spinal cord injury.

Materials and methods

Experimental animals and major reagents

Eight-week-old healthy male Sprague-Dawley (SD) rats, with a weight of 220-270 g, were provided by Beijing Charles River Laboratories Co., Ltd. Purity of OSM was more than 97% (Sigma, USA); RNeasy Mini Plus Kit (Qiagen, the Netherlands); PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Japan); STAT3/p-STAT3 protein specific antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Preparation of animal model with spinal cord ischemia and reperfusion

The rat spinal cord ischemia and reperfusion model was made according to Zivin method [19]. Rats were anesthetized by intraperitoneal injection with 2.5% pentobarbital sodium (40 mg/kg body weight), and were placed in a supine position. The abdominal area of mice was sheared and disinfected, and then followed with abdominal median incision. The abdominal aorta was gently exposed and clamped with non-invasive artery clamps in the distal part of the left renal artery; 30 minutes later, the artery clamps were released to restore blood flow and then the abdomen was closed. During the surgery, maintain the room temperature at 22°C and keep the ventilation well. The rats were anesthetized again 3 h, 6 h, 24 h and 72 h after reperfusion. And then skins and muscle of backswere separated. Spine canal was dissected by the rongeur to expose the spinal cord. The spinal tissue below L4 segment was rapidly frozen in liquid nitrogen for examination. In I/R+OSM group, intraperitoneal injection of OSM (60 ug/kg/d) started one week before the operation. Rats in I/R+PBS group were injected with equivalent PBS in the same way until the scheduled test time.

Testing items

Postoperative neurological function evaluation: The hind-limb motion of rats in each group was observed, recorded and determined 24 h after reperfusion according to the modified Tarlov score: 0 point, no activity in the hind-limbs and could not bear weight; 1 point, the hind-limbs could move but could not bear the weight; 2 points, the hind-limbs' activity was frequent or hind-limbs were powerful but could not load weight; 3 points, the hind-limbs could support the rats' own weight and walk for 1 to 2 steps; 4 points, the rats could walk with mild obstacles; 5 points, the rats could walk normally.

Detection of MPO activation in spinal cord tissue: The samples were put on ice to melt before being weighted and were homogenized in PBS (PH 6.0) at 4°C with a weight/volume ratio of 1:20 (W/V). Supernatants were discarded after centrifuged at 5,000 rpm for 10 min. And the sediments were washed again according to the methods above. PBS (PH 6.0, 1:10 W/V) which contained 0.5% hexadecyl trimethyl ammonium bromide (HTAB) was added to the sediments. After sonicated (10 sec*3 times), it was incubated at 4°C for 20 min and was centrifuged at 15,000 rpm/min for 20 min. Add 0.1 ml supernatant to 2.9 ml reaction solution (50 mM, PH 6.0 and containing 0.167 mg/ml of o-dianisidine and 0.0005% of H_2O_2), and the absorbance change was observed immediately at 460 nm for 3 minutes (25°C).

Expression of OSM in the tissues was detected after reperfusion by real-time quantitative PCR: Take out 30 mg spinal cord tissue from the liquid nitrogen to extract total RNA according to the instruction of the kit. CDNA was obtained by using the reverse transcription kit, and thin-walled PCR tubes were taken and numbered respectively. The reaction agents were added to each tube which contained: 1.0 ul (cDNA)/ ddH_2O, 0.5 ul primers (F/R, 10 uM), 12.5 ul 2×qPCR Mix, and 11.0 ul ddH_2O. All the samples were taken quantification test by ABI PRISM 7500 FQ-PCR (Applied Biosystems, USA). The sequences of specific primers were as follows: OSM (5'->3') forward primer: CTG-CTCAGTTTGACCCTCAGT; reverse primer: ATTC-TGTGTTCCCCGTGAGG; β-actin, forward primer: AGTGTGACGTTGACATCCGT; reverse primer: GC-AGCTCAGTAACAGTCCGC. Amplification conditions were as follows: pre-denaturation at 95°C

Table 1. Tarlov scores of the groups (mean ± sd)

Group	Before surgery	I/R 3 h	I/R 6 h	I/R 24 h	I/R 72 h
Control	5±0	5±0	5±0	5±0	5±0
I/R	5±0	0.17±0.41***	0.5±0.55***	0.83±0.57***	1.67±0.82***

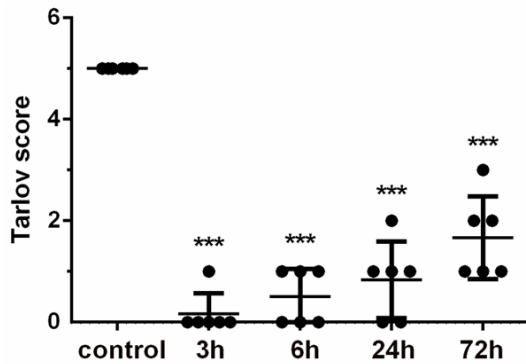Note: Compared with control groups, ***P<0.001.

Figure 1. Tarlov scores of the SCIRI rats in each group. Compared with control groups, ***P<0.001.
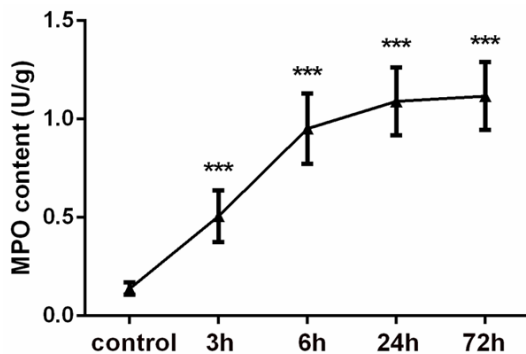

Figure 2. MPO content of the SCIRI rats in each group. Compared with control groups, ***P<0.001.

for 20 s followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. Ct value was calculated for each sample. Compared with the Ct value of the internal reference gene β-actin with that of samples, $2^{-\Delta\Delta Ct}$ was used to account the relative quantification.

The expression of STAT3/p-STAT3 protein was detected by Western Blot: Six rats in each group were anesthetized at the scheduled time. The spinal cord was rapidly removed from rats and then was homogenized. All the samples were processed with tissue lysis buffer and the supernatant was collected. The protein concentration was determined; 50 μg of protein mixture was added to each electrophoresis channel and was separated by polyacrylamide gel electrophoresis, then proteins were transferred onto membrane. Hybridization was made by adding first-antibody (p-STAT3, total STAT3, β-actin, rabbit polyclonal antibody, dilution 1:500) and secondary antibody (goat anti-rabbit monoclonal antibody, dilution 1:5,000). Western Blot luminescence films were scanned with a gel imaging system (UVP Company).

Statistical analysis

The measurement data were expressed as mean ± standard deviation ($\overline{X}$±S), and the difference between groups was analyzed by SPSS17.0 statistical software. Nonparametric ranksum test (Kruskal-Wallis) was used to evaluate the neurological function score and one-way ANOVA was used to compare the two groups. P<0.05 was considered statistically significant.

Results

Evaluation for neurological function after spinal cord injury

The neurological function of rats with spinal cord injury was detected before operation and 3 h, 6 h, 24 h and 72 h after reperfusion. The neurological function was normal for all rats before operation and their Tarlov scores were 5 points. After the surgery, the Tarlov scores were significantly lower in I/R group than that in control group. The difference had statistical significance (P<0.001) (**Table 1**; **Figure 1**).

Detection of MPO activation after spinal cord injury

MPO activation of rats increased gradually from 3 h to 72 h after SCIRI, which was obviously higher than that in control group. The difference reached statistical significance (P< 0.001, **Figure 2**).

Expressional changes of OSM in spinal cord of SCIRI rats

The expression of OSM in the spinal cord tissue increased 3 h after SCIRI (P<0.05) and reached its peak at 6 h, which was significantly higher than that in control group (P<0.001); it decreased at 24 h and 72 h and there was no statistical difference in control group (**Figure 3**).
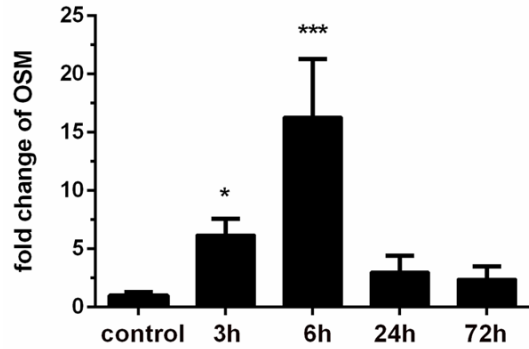
Figure 3. OSM expression of the SCIRI rats in each group. Compared with control groups, *P<0.05, ***P<0.001.
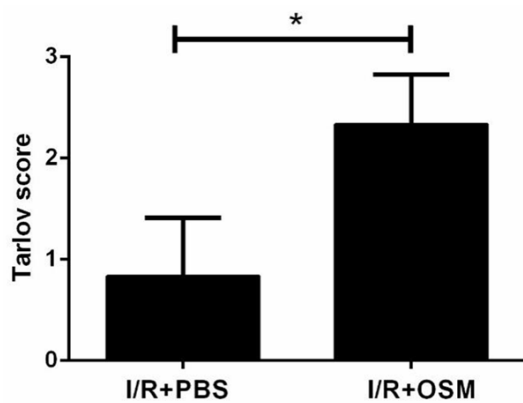

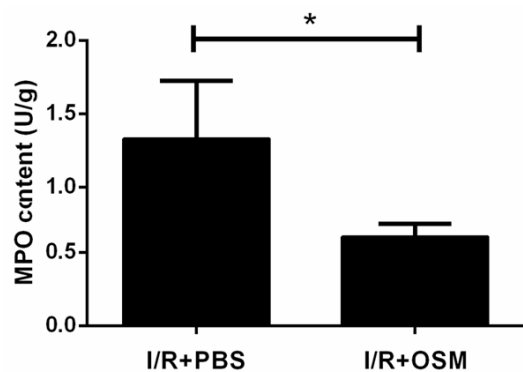**Figure 4.** OSM application improved Tarlov score of SCIRI rats, *P<0.05.

Figure 5. OSM application inhibited MPO content of SCIRI rats, *P<0.05.

Effect of OSM on neurological function, MPO and STAT3/p-STAT3 protein expression for rats with spinal cord injury

Compared with I/R+PBS group, the Tarlov scores of rats in I/R+OSM group was significantly higher 24 h after reperfusion, and there
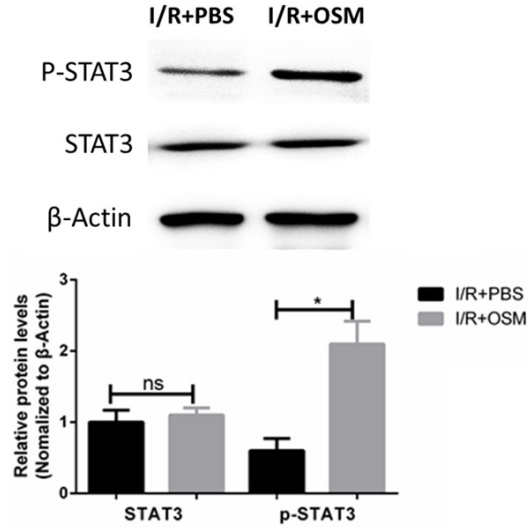

Figure 6. OSM application increased expression of p-STAT3.

was significant difference (P<0.05, **Figure 4**). After the application of OSM, MPO activation in the spinal tissue of the SCIRI rats was significantly lower than that in I/R+PBS group, and there was significant difference (P<0.05, **Figure 5**). The expression of STAT3/p-STAT3 protein in the spinal tissue was determined by Western Blot, and the expression of STAT3 protein was not significantly different between I/R+OSM group and the I/R+PBS group, but the expression of p-STAT3 protein significantly increased (**Figure 6**), indicating that STAT3 protein activation increased.

Discussion

SCIRI, as a major complication of thoracoabdominal aortic surgery, was great harm to patients. According to the cause and mechanism of the SCIRI, a series of treatments have been applied as the intervene therapies, including the ischemic preconditioning, shortening the ischemic time [20], ensuring perfusion of spinal cord blood flow [21], cryogenic techniques [22] and drug protection of ischemic preconditioning (e.g. free-radicals scavengers, calcium channel blockers, leukocyte adhesion inhibitors, neuroprotective factors, heat shock proteins, traditional Chinese medicine preparations, cytokines) and inhibitors of inflammatory cytokines, etc. [23]. Although there are many treatments, most of curative effects are still not satisfying. The expression of cytokines and adhesion molecules increased in the early stage of SCIRI, which accelerated the change

from ischemic and hypoxic injury to inflammatory injury. A lot of cytokines, such as TNF-α, IL-6, IL-8, IL-1, PAF and so on, play an important role in the process of pathological changes of spinal cord injury. They can not only directly damage the central nervous system in ischemia and reperfusion injury, but also synergize, induce a variety of inflammatory mediators and cytokines, which further aggravate the secondary injury.

Among all sorts of inflammatory factors, the IL-6 family is essential and has various effects. IL-6 is known to be a very important proinflammatory factor, which can aggravate spinal cord inflammatory injury. leukemia inhibitory factor (LIF) was found that it had a protective effect on neurons and oligodendrocytes [24, 25]. In addition, OSM can also activate the LIF receptor. Studies have confirmed that OSM can promote wound healing and reduce inflammation in early stage of trauma [26]. In this study, OSM expression increased significantly after several hours of SCIRI, indicating that it was also involved in the development of SCIRI. Further studies have shown that injecting OSM before the surgery may have significant protective effect on spinal cord injury. Infiltration of polymorphonuclear (PMN), which can lead to inflammatory injury, is a key factor of SCIRI. In this study, MPO activation that induced by PMN increased after ischemia and reperfusion injury, indicating that PMN infiltration increased after spinal cord injury which induced the overexpression of inflammatory factors and caused serious damage to the spinal cord. However, the application of OSM before the operation can reduce the activity of MPO after ischemia and reperfusion injury, alleviate the damage of spinal cord, and improve the neurological function of rats. STAT3 protein can play a role in SCIRI after being activated in a variety of signal pathways. For example, in macrophages, IL-10 can exert its anti-inflammatory effect through STAT3 protein; in the process of granulocyte formation, G-CSF receptor signaling pathway can significantly promote STAT3 protein activity, causing cell proliferation. STAT3 protein has also been found play an important role in spinal cord injury repair [27]. In the mechanism research of our study, we found that the exogenous injection of OSM could up-regulate the activation of STAT3 in spinal cord injury rats, which suggested that OSM could protect SCIRI by activating STAT3 protein. Additionally, the

role of LIF in central nervous system injury suggests that the protective effect of OSM on spinal cord injury may also be produced by activating LIF receptors to regulate inflammatory cytokines.

The pathophysiological process of SCIRI is very complex, so it is extremely significant to apply all kinds of prevention and treatment measures effectively according to the pathogenesis. This study shows that OSM can protect SCIRI from further damages, which has important clinical significance. One characteristic of ischemia and reperfusion injury is retardance, which also provides time window for the prevention and treatment of further injury of the spinal cord. To limit the damage factors of spinal cord injury, avoid progressive spinal cord injury and recover the damaged spinal neurological function, there still needs more experimental researches and explorations.

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Disclosure of conflict of interest

None.

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