

Inflammation Level after Decompression Surgery for a Rat Model of Chronic Severe Spinal Cord Compression and Effects on Ischemia-Reperfusion Injury

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Abstract

Delayed neurological deterioration in the absence of direct spinal cord insult following surgical decompression is a severe postoperative complication in patients with chronic severe spinal cord compression (SCC). The spinal cord ischemia-reperfusion injury (IRI) has been verified as a potential etiology of the complication. However, the exact pathophysiologic mechanisms of the decompression-related IRI remain to be defined. In this study, we developed a practical rat model of chronic severe SCC. To explore the underlying role of inflammation in decompression-related IRI, immunoreactivity of pro-inflammatory cytokines including tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) before and after decompression were measured. In addition, expression level of TNF- α and IL-1 β was examined with Western blot. Immunohistochemical staining showed negative result in gray matters in the sham group and sham-decompression group. In the severe compression group, strong positive staining of TNF- α and IL-1 β were found, suggesting a dramatic infiltration of inflammatory cells in gray matters. Furthermore, the severe compression group showed a significant increase in expression level of TNF- α and IL-1 β as compared with the sham group ($p < 0.05$). In the severe compression-decompression group, both immunostaining and Western blot showed significant increase of TNF- α and IL-1 β levels in the spinal cord compared with the severe compression group ($p < 0.05$). The results demonstrated that surgical decompression plays a stimulative role in inflammation through increasing the expression of inflammatory cytokines in the rat model of chronic severe SCC injury. Inflammation may be one of the important pathological mechanisms of decompression-related IRI of chronic ischemia.

Key words: rat model, chronic severe spinal cord compression, inflammation, decompression surgery, ischemia-reperfusion injury.

Introduction

Chronic spinal cord compression (SCC) is commonly caused by intraspinal tumors, spondylosis, and disc herniation.^{1–6)} Although the condition of most patients with these lesions has improved after surgery, decompression of a previously severely compressed region of the spinal cord may lead to delayed neurological deterioration during the early postoperative period.^{2–8)} The most widely accepted

pathological mechanism of the complication is spinal cord ischemia-reperfusion injury (IRI).^{3,6,7)}

According to the pathophysiologic features, spinal cord injury (SCI) is mainly categorized into primary injury and secondary injury.⁹⁾ IRI is one of the most frequent types of secondary SCI, which is defined as follows: after removing the factors that cause spinal cord ischemia and the recovery of cord blood supply, neuronal function cannot be improved and its injury is more intense than its original level, or even present in the irreversible tardive neurons death.¹⁰⁾ Although the exact biomechanism of spinal cord IRI is not

fully understood, inflammation is known to play an important role after ischemia and contributes to SCI, especially in the reperfusion period.^{11–13)}

Tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) are the key inflammatory mediators and play a major role in the pathological mechanisms of primary ischemic injury and secondary injury.^{14–16)} Recent researches have also shown that TNF- α and IL-1 β levels increased significantly in reperfusion period and remained at high levels after reperfusion.^{11–13,17,18)}

In a previous study, we verified that IRI occur after decompression surgery for a rat model of chronic severe SCC. However, the exact pathophysiologic mechanisms of the decompression-related IRI, especially the role of inflammation, remain to be defined. Therefore, the aim of our study was to investigate the role of inflammation in decompression-related IRI of chronic ischemia. In this study, we continued to establish the rat model of chronic severe SCC by using water-absorbing materials, which was similar to the literature.^{19,20)} The levels of TNF- α and IL-1 β before and after decompression were measured and analyzed, to further test the hypothesis that inflammation may be one of the important pathological mechanisms of decompression-related IRI.

Methods

Sprague–Dawley (SD) male rats, 9–10 weeks of age (280–320 g), from the Experimental Animal Facilities of the hospital were used in the study. Food and water were provided *ad libitum* before and after the experiments. The rats were housed in a temperature-controlled environment under a light-dark cycle condition with free access to food. The study was approved by the ethnic committee of Beijing Tiantan Hospital, Capital Medical University, and performed in accordance with the policies of Chinese animal research committees and guidelines from United States National Institute of Health (NIH publication No. 96-23, revised 1996). Every effort was made to minimize the suffering and the number of animals.

I. Compression material

The compression sheet is made of a water-absorbing material. The sample is a penetrating polymer network hydrogel composed of polyvinyl alcohol and polyacrylamide, the ratio of which is 1:1. After absorbing water, it demonstrates long-lasting water retaining capability and durability. The expansion rate can gradually reach a maximum of three times its original thickness. In preliminary experiments, the material was implanted subcutaneously in the abdomen of the rats. Over the period of 12 weeks, no obvious tissue reaction was observed.

II. Rat model of chronic severe SCC

Before surgery, the rats were kept in their cages for 1 week for adaptation to the environment. The rats were anesthetized with an intraperitoneal injection of trichloroacetaldehyde (300 mg/kg, i.p. Qingdao Yulong Algae Co., Ltd., Shandong, China, No. H37022673). A longitudinal incision was made on the back, centered on the spinous processes of the T7–10. The paravertebral muscles were stripped from the spinous processes and laminae. After the yellow ligament was removed, the compression sheet (original size: 2.5 × 2.0 × 0.8 mm³; expanded size: 3.5 × 3.0 × 2.4 mm³) was inserted between the T8–9 laminae and dura. Afterwards, muscle and skin were sutured with 6-0 Vicryl (Vicryl, Ethicon, Johnson & Johnson International, Lenneke Marelaan, Belgium). Following the surgical procedure, temperatures were strictly maintained and all the rats were housed individually with free access to food. Ampicillin liquid formulation (Suzhou Two Leaves Pharmaceuticals Inc., Ltd., China, Batch No. H32021320) was injected into the back exterior muscles once per day for 3 days to prevent infection. Padding in each cage was changed every day to keep it dry. Bladders were manually emptied daily until animals regained voiding function. The rat models were elevated in the 12 weeks post-surgery and effect of the spinal cord self-repair was very limited.

III. Experimental groups

Twenty four rats were randomly allocated into four groups, six rats each: in the sham group (n = 6), rats underwent the surgical procedure but without implantation of the compression material and sacrificed the 12 weeks post-surgery. In the severe compression (SC) group (n = 6), rats underwent polymer sheet implantation, and were sacrificed the 12 weeks post-surgery. In the severe compression-decompression (SC-d) group (n = 6), rats underwent laminectomy and the removal of the expanded materials. The rats were kept alive for 24 hours after decompression, and then sacrifice took place. In the sham-decompression (sham-d) group (n = 6), rats underwent laminectomy and the postoperative protocol was the same as the SC-d group. Spinal cord samples (15 mm) were obtained from the compressed area and divided into two equal parts. Cranial parts were obtained for immunostaining; caudal parts were stored in a –20°C freezer for Western blot.

IV. Radiographic imaging

All magnetic resonance imaging (MRI) were conducted with a 3 Tesla MRI (Magnetom Trio, Siemens Medical Solutions, Erlangen, Germany). T₂-weighted images (echo delay time = 92 ms; repetition time = 3,620

ms; flip angle $\alpha = 120^\circ$; slice thickness: 2 mm; field of view = 80 mm) were obtained at the 12th week following the operation to evaluate the degree of SCC. The cross-sectional area was measured using the Siemens Workstation software (NUMARIS/4).

V. Immunohistochemistry

Hydrogen streptavidin (HRP) labeled Streptavidin (S/P) kit was used for immunostaining TNF- α and IL-1 β . After deparaffinization, endogenous peroxidase was quenched with 0.3% hydrogen peroxide in 60% methanol for 30 minutes. The sections were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 20 minutes. Nonspecific adsorption was minimized by incubating the sections in 2% normal goat serum in PBS for 20 minutes. Endogenous biotin-binding or avidin-binding sites were blocked by sequential incubation for 15 minutes with biotin and avidin, to avoid nonspecific staining. The following primary antibodies were used in experiments: rabbit-anti TNF- α (1: 200) and rabbit-anti IL-1 β (1: 200). Horseradish peroxidase (HRP) conjugated goat-anti rabbit secondary antibody (Santa Cruz, California, USA) was used for 3, 3'-diaminobenzidine (DAB) staining. All primary antibodies and kits were obtained from Zhongshan Jinqiao Biotechnology (Beijing, China). The number of positive cells of high power fields (original magnification $\times 40$) in four groups was counted.

VI. Western blot

The expression level of pro-inflammatory cytokines including TNF- α and IL-1 β were measured. For Western blot, tissue proteins were extracted with RIPA Lysis buffer kit (Bio-Tek, California, USA), centrifuged at 10,000 g, and aliquoted after quantitation. Protein levels were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the concentration of SDS-PAGE was set to 12% and 10% for TNF- α and IL-1 β , respectively. The following primary antibodies were used for Western blot: rabbit anti-TNF- α (1: 500), rabbit anti-IL-1 β (1: 500), and rabbit anti-actin (1: 400, Santa Cruz, California, USA). HRP conjugated goat-anti rabbit secondary antibody (Santa Cruz, California, USA) was used for enhanced chemiluminescence. The membrane was subsequently exposed to X-ray film. Western blot results were quantified by the analysis of X-ray films using Image J software. All primary antibodies and kits were bought from Zhongshan Jinqiao Biotechnology (Beijing, China). Beta-actin was used as an internal control.

VII. Statistical analysis

Data were analyzed by the Statistical Package for the Social Sciences 16.0 software (SPSS, Chicago,

Illinois, USA) and represented as mean \pm standard deviation. Samples from each group were compared with independent t-test. A *p* value less than 0.05 was considered statistical significance.

Results

I. Neuroradiological observations

Sagittal and axial projections of the thoracic spine were obtained to ascertain the location of the compression sheet and to evaluate the degree of SCC. The expanded materials compressed the spinal cord severely and the average invasion ratio of the spinal canal was over 70%, which was measured on axial T₂-weighted images (Fig. 1). The cross-sectional area in the sham group was larger than that in the compressed groups (*p* < 0.05) (sham: 16.20 \pm 1.34 mm²; SC: 4.52 \pm 1.42 mm²).

II. Immunohistochemical staining

Immunohistochemical staining of TNF- α and IL-1 β were displayed in Figs. 2 and 3. In the sham group and sham-d group, the negative result in the gray matters suggesting neither obvious infiltration of inflammatory cells nor obvious expression of these factors in the neurons in this area (TNF- α cells: sham 1.01 \pm 0.57, sham-d 1.03 \pm 0.69; IL-1 β cells: sham 1.03 \pm 0.48, sham-d 1.04 \pm 0.55). In the SC group, strong positive staining of TNF- α and IL-1 β were found, suggesting a dramatic infiltration of inflammatory cells in gray matters compared with the sham group (*p* < 0.05) (TNF- α cells: 10.32 \pm 0.96; IL-1 β cells: 11.12 \pm 0.82). After surgical decompression, the degree of positive staining for TNF- α and IL-1 β

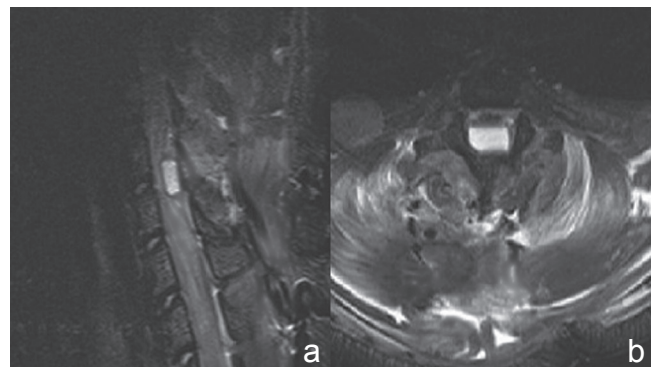


Fig. 1 Magnetic resonance imaging after chronic severe spinal cord compression. **a:** The severe compression material was located dorsally within the spinal canal at the T8-9 levels on sagittal T₂-weighted images. **b:** The sheet compressed the spinal cord severely and the invasion ratio of the spinal canal was over 70%, which was measured on axial T₂-weighted images.

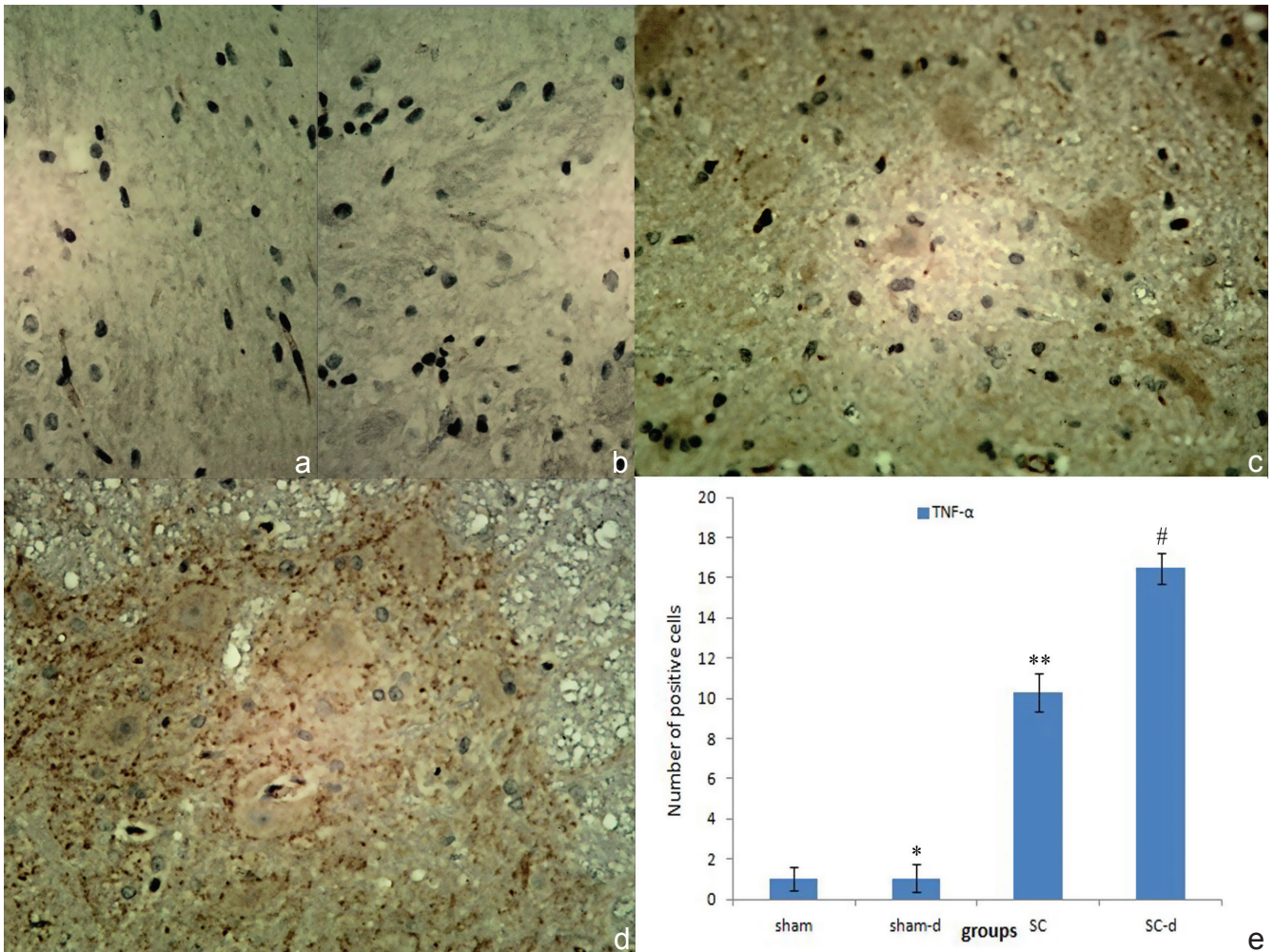


Fig. 2 Immunohistochemical localizations of tumor necrosis factor-alpha (TNF- α) in spinal cord in sham group (a), sham-decompression (sham-d) group (b), severe compression (SC) group (c), and severe compression-decompression (SC-d) (d) (original magnification $\times 40$). No obvious positive staining of TNF- α was observed in sham group and sham-d group ($*p > 0.05$); a substantial increase in the release of TNF- α was found in various cells in the SC group compared with the sham group ($**p < 0.05$); whereas TNF- α production increased significantly in the SC-d group compared with the SC group ($\#p < 0.05$) (e).

increased significantly and infiltration of inflammatory cells aggravated compared with the SC group ($p < 0.05$) (TNF- α cells: 16.47 ± 0.74 ; IL-1 β cells: 16.88 ± 0.78).

III. Levels of inflammatory molecules

The results of Western blot for TNF- α (17 kD) and IL-1 β (30 kD) are displayed in Fig. 4. The expression levels of the proteins were normalized with sham group and presented as percentages (relative density: 1). In the sham-d group, the two proteins expressed at low levels without difference from those in the sham group. The SC group showed a significant increase in expression level of TNF- α and IL-1 β as compared with the sham group ($p < 0.05$)

(TNF- α relative density: 2.412 ± 0.124 ; IL-1 β relative density: 3.866 ± 0.172). Furthermore, SCI-induced increase of the immunoreactivity was stimulated in the SC-d group as shown by significant increase in expressions of inflammatory cytokines ($p < 0.05$) (TNF- α relative density: 3.352 ± 0.083 ; IL-1 β relative density: 4.884 ± 0.079). This result indicated that surgical decompression can significantly increase the immunoreactivity in the rat model of chronic severe SCC.

Discussion

Although adequate surgical decompression has been the treatment choice for chronic severe SCC with

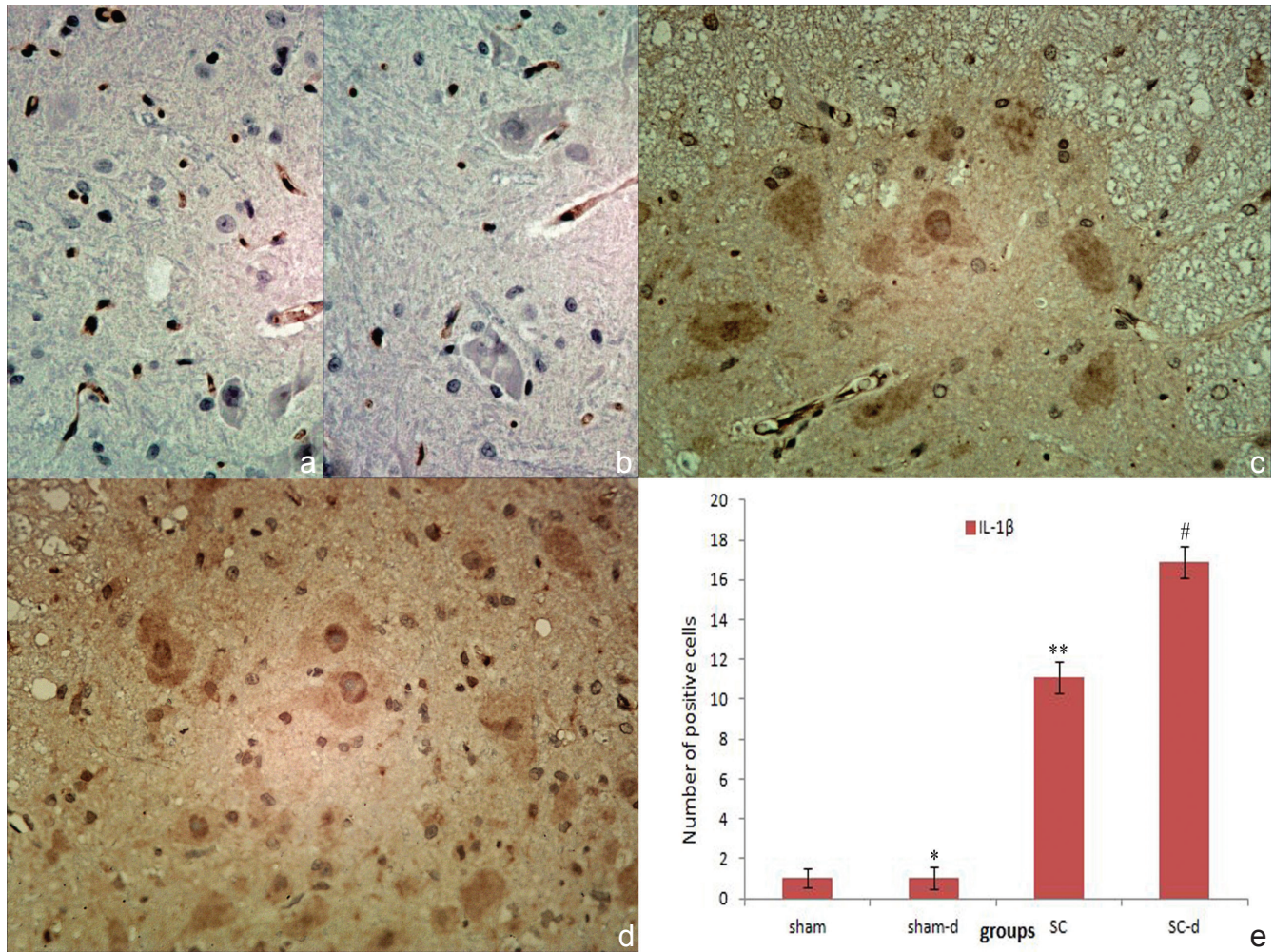


Fig. 3 Immunohistochemical localizations of interleukin-1 β (IL-1 β) in spinal cord in sham group (a), sham-decompression (sham-d) group (b), severe compression (SC) group (c), and severe compression-decompression (SC-d) (d) (original magnification $\times 40$). No obvious positive staining of IL-1 β was observed in sham group and sham-d group ($*p > 0.05$); a substantial increase in the release of IL-1 β was found in various cells in the SC group compared with the sham group ($**p < 0.05$); whereas IL-1 β production increased significantly in the SC-d group compared with the SC group ($\#p < 0.05$) (e).

favorable surgical outcomes, delayed neurological deterioration is still an unpredictable and disastrous postoperative complication.²⁻⁸⁾ The reported incidences of the disorder vary from 3.5% to 14.5%.^{2,4,5,8)} In general, postoperative neurological deficit is most often due to iatrogenic spinal cord insult.^{2,21,22)} However, the patients in previous studies could move their extremities after the surgery.²⁻⁸⁾ Moreover, no postoperative hematomas or any compressive lesions were found.²⁻⁸⁾ All the evidences make alternative underlying mechanisms more likely than direct spinal cord trauma. Some studies have shown that the blood flow in the compressed segment significantly reduced compared with the rostral segment, which demonstrated spinal cord ischemia caused by severe

mechanical compression.²³⁾ In our previous study, blood vessels in the gray and white matter were dilated remarkably after decompression, which suggested that blood supply of compressed segment get partially restored. The most widely accepted pathogenesis is that the sudden cord expansion and reperfusion after surgical decompression may have led to disruption in the blood brain barrier and triggered a cascade of reperfusion injury resulting in neurologic dysfunction.^{3,4,7,8)} In our previous study, the incidence of delayed neurological deterioration was very low. Nonetheless, lipid peroxidation got worse after decompression, signifying ischemia-reperfusion did occur. Although we verified that IRI occur after decompression and may be a potential

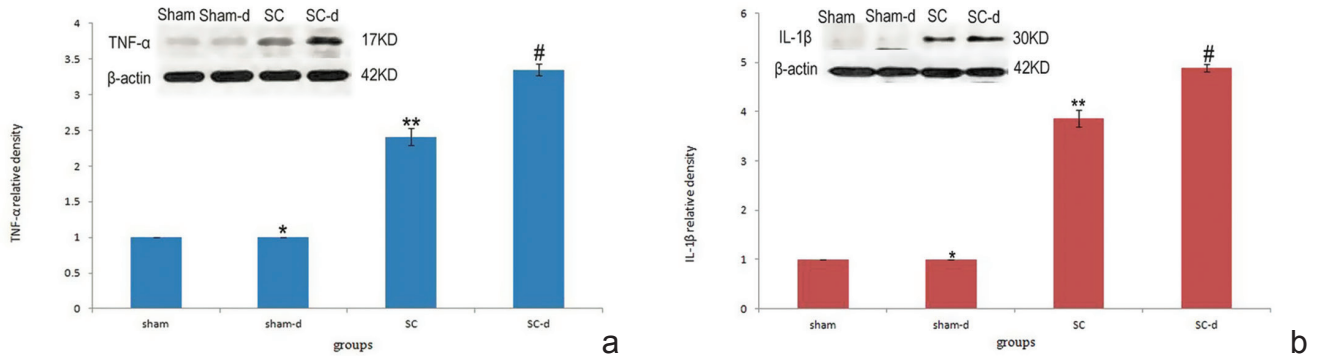


Fig. 4 Expression levels of pro-inflammatory cytokines in the four groups. No difference was observed between the sham and sham-decompression groups ($*p > 0.05$) (a). Severe compression resulted in higher level of tumor necrosis factor- α (TNF- α) in the spinal cord compared with the sham group ($**p < 0.05$). Meanwhile, decompression surgery substantially elevated TNF- α levels in the decompression group compared with the compression group ($\#p < 0.05$). The levels of TNF- α were measured by Western blot analysis using actin as a standard control. No difference was observed between the sham and sham-decompression groups ($*p > 0.05$) (b). Severe compression resulted in higher level of interleukin-1 β (IL-1 β) levels compared with the sham group ($**p < 0.05$). Meanwhile, decompression surgery substantially elevated IL-1 β levels in the decompression group compared with the compression group ($\#p < 0.05$). The levels of IL-1 β were measured by Western blot analysis using actin as a standard control.

etiology of delayed neurological deterioration, the precise pathophysiologic mechanisms of the decompression-related IRI remain unclear.

Spinal cord IRI includes primary ischemic injury and delayed reperfusion injury.^{24,25} Recent studies have indicated that inflammatory cells play a crucial role in the pathological mechanisms of primary ischemic injury and the delayed reperfusion injury in the spinal cord.^{11,12,14,20} Inflammatory cells produce a number of pro-inflammatory cytokines, which recruit peripheral inflammatory cells into the spinal cord and cause delayed neuron injury.²⁶ Thus, this study was undertaken to measure and analyze inflammatory cytokines levels before and after decompression surgery to identify the underlying role of inflammation in decompression-related IRI. The rat model of chronic SCC induced by water-absorbing materials is a well-characterized animal model, which is used to study histopathologic and neurological changes of chronic compressive SCI.^{19,20} This rat model reproduced the characteristic course and features of chronic severe SCC injury; reduced activity of surviving neurons, neuronal degeneration, and demyelination of axons, observed as the decrease in the capacity of locomotion.^{19,20,27} The mean spinal cord narrowing rate in our SC models was over 70%. We insure that the degree of compression is consistent with the criteria on the chronic severe SCC.^{1,27}

TNF- α and IL-1 β are the key inflammatory mediators and play a major role in the inflammatory response to central nervous system injury.^{11,28} They share the

same signaling molecules as nuclear factor (NF)- κ B and have similar biological effects.^{11,29} Microglia are the primary sources of the two pro-inflammatory cytokines.³⁰ TNF- α and IL-1 β increase neutrophil accumulation by increasing vascular endothelial permeability and upregulating the expression of endothelial leukocyte adhesion molecules.³¹ They control the production of IL-6 which stimulates the growth of mature B cells and is responsible for the respiratory burst of neutrophils and release of free radicals.^{11,32} Additionally, they have toxic effects on oligodendrocytes and promote necrosis and apoptosis in neurons.^{33,34}

As the most important pro-inflammatory cytokines, there is growing amount of information implicating a possible responsibility of TNF- α and IL-1 β in the pathogenesis of SCI. Elevated expression of TNF- α and IL-1 β have been demonstrated in animal models of traumatic SCI.^{27,35,36} Yang et al. revealed that immunoreactivity of TNF- α and IL-1 β increased in human neurons at both early and late phases after SCI.³⁵ Researchers reported that levels of TNF- α and IL-1 β in the spinal cord increased significantly in animal models of SCI.³⁶ Similarly, Inukai et al. found overexpression of TNF- α in the spinal cord of *twy/twy* mice sustaining chronic severe compression.²⁷

Recently, researches have indicated that TNF- α and IL-1 β levels increased significantly in reperfusion period and remained at high levels after reperfusion in animals suffered from spinal cord IRI. For example, Huang et al. have found significant increase of TNF- α

level in serum and spinal cord 24 hours after IRI.¹²⁾ Fan et al. reported that spinal cord sections from the 48 hours reperfusion group exhibited a strong positive staining for TNF- α and IL-1 β , mainly localized in the gray matter.³⁷⁾ Hasturk et al. showed that TNF- α and IL-1 β levels were significantly higher in 24 hours IRI group than those in the sham group.¹¹⁾ Furthermore, there were no significant changes for TNF- α and IL-1 β levels between the 24 hours and 48 hours after IRI.¹¹⁾ There are some other reports supporting that increase in inflammatory cytokines release is correlated to spinal cord IRI. Lu et al. reported that administration of U0126 (a specific inhibitor of MAPK/ERK kinases 1/2) significantly increased neuronal survival by reducing microglia accumulation and IL-1 β expression.¹³⁾ Similarly, interleukine-18 binding protein plays anti-inflammatory roles in spinal cord IRI by reducing TNF- α expression.²⁵⁾ Moreover, tetramethylpyrazine might also exert neuroprotective effects on spinal cord IRI by reducing the expressions of TNF- α and IL-1 β through inhibition of NF- κ B activity.^{9,37)} Hydrogen gas is a new popular therapeutic agent for IRI. In a rabbit model of spinal cord IRI, the beneficial effects of hydrogen gas treatment on IRI were associated with reduce of TNF- α in serum and spinal cord.¹²⁾ The above data confirm that inflammation induced by TNF- α and IL-1 β , is an important pathological mechanism of injury caused by blood reperfusion of ischemic spinal cord.^{12,35–37)}

In the present study, both immunostaining and Western blot showed low level of TNF- α and IL-1 β in the spinal cord in the sham-d group without difference from those in the sham group. It suggests that decompression surgery has no direct spinal cord insult. Additionally, we found significant increase of TNF- α and IL-1 β after chronic severe SCC. Briefly, in the SC group, the expression of the two pro-inflammatory cytokines in the spinal cord reached significantly higher levels as compared with the sham group. The results indicate that infiltration of neutrophil and increase of pro-inflammatory cytokines are involved in chronic severe compressive SCI. These observations complied with the data revealed by compressive SCI studies in the literature.³⁷⁾ Furthermore, these findings were more prominent in the SC-d group. We observed that the degree of positive staining for TNF- α and IL-1 β increased significantly and infiltration of inflammatory cells aggravated in the SC-d group compared with the SC group. Western blot also showed significant increase of TNF- α and IL-1 β in the spinal cord 24 hours after surgical decompression. These results suggest that surgical decompression play a stimulative role in inflammatory cascade in chronic severe SCC model. Our study

supported the hypothesis that inflammation may be one of the important pathological mechanisms of decompression-related IRI of chronic ischemia. Such knowledge will facilitate the development of anti-inflammation approaches for prevention and/or treatment of this severe complication.

Conclusion

Decompression surgery plays a stimulative role in inflammatory processes by increasing the expression levels of inflammatory cytokines in the chronic severe compressive SCC model. Inflammation may be one of the important pathological mechanisms of decompression-related IRI. Such knowledge will facilitate the development of anti-inflammation approaches for prevention and/or treatment of this severe complication.

Ethical Standards

This study was approved by Institutional Review Board of Beijing Tiantan Hospital, Capital Medical University.

Acknowledgment

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Conflicts of Interest Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings described in this article. No benefits in any form have been or will be received from any commercial party related directly or indirectly to the subject of this manuscript.

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