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ORIGINAL ARTICLE

# PMX53 protects spinal cord from ischemia-reperfusion injury in rats in the short term

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**Study design:** Experimental study.
**Objectives:** To investigate the effect of pre-treatment with PMX53, a C5aR antagonist, on spinal cord ischemia-reperfusion injury (IRI) in rat.
**Setting:** Department of Neurosurgery, Second Affiliated Hospital, Xi'an Jiaotong University School of Medicine, Xi'an, Shaanxi Province, China.
**Methods:** IRI was induced in the lumbar spinal cord by applying a mini aneurysm clamp to the abdominal aorta for 60 min in adult Sprague–Dawley rats. PMX53 (1 mg $kg^{-1}$) was administered through femoral vein injection 30 min before ischemia on the rats in the PMX53 group ($n=18$). The saline group ($n=18$) was given saline at the same volume through femoral vein injection. The neurologic outcome of the posterior limbs was assessed by the Basso-Beattie-Bresnahan (BBB) score at 1, 6, 12, 24 and 48 h after reperfusion. Histologic changes of the spinal cord were detected with hematoxylin–eosin (H–E) staining. Enzyme-linked immunosorbent assay (ELISA) was used to detect myeloperoxidase (MPO) activity in the spinal cord. Immunohistochemistry was used to investigate the quantity of activated astrocytes and microglia.
**Results:** After pre-treatment with PMX53, neurologic function improved gradually after 6, 12, 24 and 48 h reperfusion. The BBB score of the PMX53 group increased significantly ($P<0.05$) compared with the saline group. H–E staining showed that pathologic damage in the PMX53 group was reduced. Moreover, administration of PMX53 significantly inhibited neutrophil infiltration in the spinal cord. Levels of MPO activity in the spinal cord were remarkably lower in the PMX53 group ($P<0.05$). There were also more activated microglia and astrocytes in the spinal cord of the PMX53 group than in the saline group ($P<0.05$).
**Conclusion:** PMX53 delivered 30 min prior to ischemic injury protects the spinal cord from IRI, probably via the inhibition of neutrophil activity, increased activated microglia and astrocyte.
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## INTRODUCTION

Spinal cord ischemia-reperfusion injury (SIRI) mainly occurs after operations on the descending thoracic or thoracoabdominal aorta, such as thoracoabdominal aneurysm.[1] SIRI causes various complications, including paraparesis and paraplegia.[2] These complications have been attributed to temporary or permanent ischemia of the spinal cord caused by interruption of the blood supply during aortic cross-clamping. The incidence of paraplegia has been correlated with dissection, rupture and prolonged clamp times.[3]

Systemic hypothermia, spinal fluid drainage and preconditioning ischemia were considered to prevent paraplegia,[4] but the effect was limited. Recent studies indicated that pathophysiological mechanisms underlying SIRI are complicated, including the release of inflammatory factors,[5] calcium overload,[6] oxidative stress,[7] excitotoxicity[8] and neuronal apoptosis.[9,10] In addition, there are more mechanisms that remain unclear. It has been found that the inflammatory cascade is activated due to SIRI, including neutrophil and neuroglia mobilization and infiltration, as well as elevated levels of inflammatory cytokines, such as tumor necrosis factor-α and interleukin-6.[11] The complement system, a major component of the innate immune system, is constituted by a series of complement components and involved in synapse elimination,[12] development[13] and maturation of neural systems.[14] A range of pathologies in the brain or the spinal cord, including ischemia-reperfusion injury (IRI),[15] stroke, traumatic brain injury and spinal cord injury,[16] potently trigger complement activation following the disruption of neuronal homeostasis.

Complement activation produced large amounts of anaphylatoxin C5a, which regulates immune activation and takes part in various pathophysiological progresses via binding with C5a receptor (C5aR).[17] Recent studies have shown that the C5a-C5aR signaling pathway activated in cerebral ischemia reperfusion after stroke and C5aR antagonist (PMX53) protects the brain from IRI in rats.[18] The same phenomenon was also documented in amyotrophic lateral sclerosis.[19] Besides, it has been demonstrated that PMX53 protected IRI in the heart, liver, kidneys, limb and small intestine.[20] However, the role of PMX53 in SIRI remains unexplored. Therefore, we systematically investigated neurologic function, histology of the spinal cord, myeloperoxidase activity, neuroglia mobilization and infiltration activation after pre-treatment with PMX53 or saline treatment when SIRI was induced in rats. Our results indicated that pre-treatment with

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PMX53 protected SIRI possibly via the inhibition of the C5a-C5aR signaling pathway.

## MATERIALS AND METHODS

### Experimental animals and surgical procedure

With approval from the animal ethics committee of the Second Affiliated Hospital, Xi'an Jiaotong University School of Medicine for the protocol (permit number: Radiol-11-2012), 54 male Sprague–Dawley (SD) rats weighed from 250 to 300 g were used in this study. The rats were purchased from the Animal Center of Xi'an Jiaotong University School of Medicine, Shaanxi, China. The SIRI model was established according to Zivin's process[21] and previously published articles.[9,10,22] Ischemia of the lumbar spinal cord was induced by clamping the abdominal aorta with a mini aneurysm clamp for 60 min followed by reperfusion for 48 h. The occlusion sites were ~0.5 cm below the left renal artery and just proximal to the aortic bifurcation. Rats in the sham operation group underwent the same operative procedure apart from aortic occlusion.

### Experimental design

SD rats were randomly divided into three groups, sham operation group (Sham operation, $n=18$), saline group (Saline, $n=18$) and PMX53 group (PMX53, $n=18$). The PMX53 group received 1 mg kg$^{-1}$ of PMX53 (Gill, Shanghai, China; 1 mg ml$^{-1}$) via the femoral vein 30 min prior to ischemia. An equal volume of saline was given to the saline group via femoral vein at the same time points. Following Basso-Beattie-Bresnahan (BBB) scale assessment, rats were anesthetized (amobarbitol sodium, 0.75%, 4 ml kg$^{-1}$, i.p.) and killed. Plasma and L4–L5 spinal cord were collected for future analysis.

### Evaluation of neurologic function

To assess the neurologic function of each rat after SIRI, 18 rats from each group were investigated at 1, 6, 12, 24 and 48 h after reperfusion by experienced investigators who were blinded to the treatment group. Locomotor function of the hind limbs was graded using the BBB hind limb locomotor rating scale, which is a 21-point system on the basis of operationally defined behavioral features.[23] The BBB rating scale follows the recovery progression from complete paralysis to normal locomotion. In the present study, after recovery from surgery, the rats typically began to show slight but definite, isolated movements of one or two hind limb joints.

### Assay of myeloperoxidase activity

The spinal cord was collected and homogenized, followed by centrifugation. MPO levels in ischemic spinal cord tissues were measured with a rat MPO ELISA assay kit (Beijing Dakewei Bioengineering Institute, Beijing, China) according to the manufacturer's instructions. Optical density was read at 450 nm using a microtiter plate reader within 15 min. MPO activities in spinal cord tissues were calculated by using a standard curve generated with human MPO and expressed in units per gram weight (u g$^{-1}$) of tissue.

### Immunohistochemistry

The L4–L5 segment of the spinal cord was paraffin embedded and sectioned at a thickness of 4 μm. Six sections from each rat, at least 200 μm apart, were processed in a blinded manner. The staining was carried out as previously described.[9,10] Antibodies used were as follows: anti-GFAP polyclonal antibody (1:1000 diluted, Abcam, Cambridge, MA, USA), anti-Iba1 polyclonal antibody (1:1000 diluted, Abcam) and biotinylated goat secondary antibody (1:100 diluted, Santa Cruz Biotechnology, Dallas, TX, USA). Five different vision fields of each section from different groups were analyzed by an image analysis collection system (Q553Cw; Leica, Wetzlar, Germany), and average optical densities (gray scale) were measured.

### Sample size calculation and statistical analysis

A power calculation was carried out using SPSS Sample Power software (SPSS, Chicago, IL, USA). Eighteen animals were estimated to be sufficient to demonstrate 1 point on the BBB scale in the primary outcome groups, given an alpha level of 0.05 and a power of 0.80. The data were analyzed with SPSS18.0 software (SPSS). Continuous normally distributed variables were described as mean ± s.d. One-way analysis of variance (ANOVA) was conducted to analyze comparison between groups. Statistical significance was accepted for $P<0.05$.

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

## RESULTS

### Pre-treatment with PMX53 ameliorated neurologic dysfunctions

Hind limb function was evaluated using the BBB score.[23] Prior to the establishment of spinal cord IRI, the baseline BBB score was 21 points. After sham operation, rats showed normal function. In addition, BBB scores were significantly lower in the saline group and the PMX53 group when compared with the sham operation group. The BBB scores for the saline group at 1, 6, 12, 24 and 48 h were 0, 4 ± 1.08, 4.77 ± 0.81, 4.44 ± 0.62 and 4.22 ± 1.11, respectively. The rats in the PMX53 group demonstrated a more rapid recovery in locomotor function than did the saline group, with the BBB scores being 5.05 ± 0.80, 7.61 ± 0.98, 11.39 ± 0.61 and 13.94 ± 0.73 at 6, 12, 24 and 48 h, respectively, after reperfusion. The saline group demonstrated a significant movement dysfunction compared with the sham operation group ($P<0.05$), and PMX53 significantly attenuated the decrease in the BBB score of the PMX53 group ($P<0.05$; Figure 1).

### Pre-treatment with PMX53 reduced pathologic damage

There were significantly more normal neurons in the anterior horn of spinal cord in the sham operation group, where multipolar structure of neuron appeared normal. Axons, dendrites and nucleus of neurons were clear, and the nucleolus was located centrally (Figure 2a). However, in the anterior horn of spinal cord in the saline group, extensive vacuolation, necrotic changes and pyknotic nuclei of neurons were noticed (Figure 2b). In contrast, slighter histologic changes were observed in the spinal cords of rats in the PMX53 group, and the intact motor neurons were preserved to a much greater extent (Figure 2c). The number of motor neurons with normal morphology in the anterior horn of spinal cord at 48 h after reperfusion was significantly reduced in the saline group (2.00 ± 0.89) than in the sham operation group (13.83 ± 3.19) and the PMX53 group (7.00 ± 1.41; Figure 2d).

### Pre-treatment with PMX53 inhibited neutrophil infiltration

The saline group showed strong MPO activity in the ischemic region compared with the sham operation group ($P<0.05$), and PMX53 significantly attenuated the increase in MPO activity ($P<0.05$; Figure 3). This indicated obvious activation and infiltration of

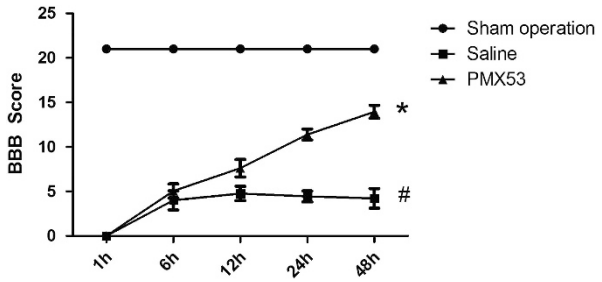


**Figure 1** Evaluation of neurologic function using the BBB scores after transient ischemia of the spinal cord. *$P<0.05$ vs the sham operation group at every time point. #$P<0.05$ vs the saline group at the time point of 6, 12, 24 and 48 h. Data are presented as mean ± s.d.; $n=18$ per group.

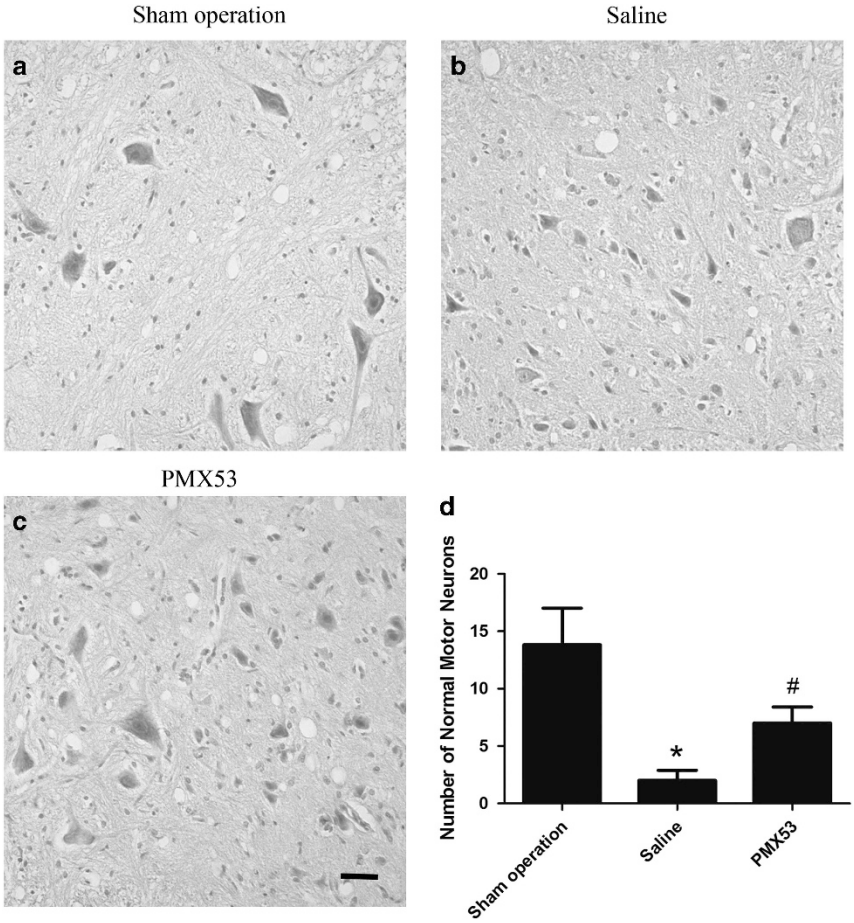


**Figure 2** H–E staining of the spinal cord in the sham operation group (Sham operation), the saline group (Saline) and the PMX53 group (PMX53). The intact neurons of the gray matter were observed in the sham operation group (**a**), whereas the saline group only showed a large population of swollen neurons with vacuolated cytoplasm and disintegrated nucleus (**b**). The number of normal neurons was significantly less in the saline group than in the sham operation group and the PMX53 group (**a**, **b**, **c** and **d**). Treatment with PMX53 markedly reduced cellular damages in the saline group. $^{*}P<0.05$ vs the sham operation group, $^{\#}P<0.05$ vs the saline group. Scale bar, 25 μm; $n=6$ per group. A full color version of this figure is available at the *Spinal Cord* journal online.

inflammatory cells into ischemic spinal cord, which could be reduced by pre-treatment with PMX53.

**Pre-treatment with PMX53 suppressed glial activation**

In the event of infection, inflammation, trauma, ischemia and neurodegeneration, microglia quickly respond and can undergo morphologic transformation from a resting state referred to as 'ramified' to an active 'amoeboid' state.[24] In the present study, the number of activated microglia in the saline group increased in the ischemic region compared with the sham operation group ($P<0.05$), but PMX53 significantly attenuated mobilization of microglia ($P<0.05$; Figure 4a, c and g). In addition, compared with the sham operation group, the saline group showed more activated astrocytes ($P<0.05$), which are larger and possess more neuritis. However, in the PMX53 group, there was a significant reduction in astrocyte activation ($P<0.05$; Figure 4h), and the shape of astrocytes was similar to the sham operation group ($P<0.05$; Figure 4d,e and f).

## DISSUSSION

In this study, we found that pre-treatment with PMX53, a C5aR antagonist, could protect spinal cord from IRI possibly via the inhibition of neutrophil activity, decreasing activated microglia and

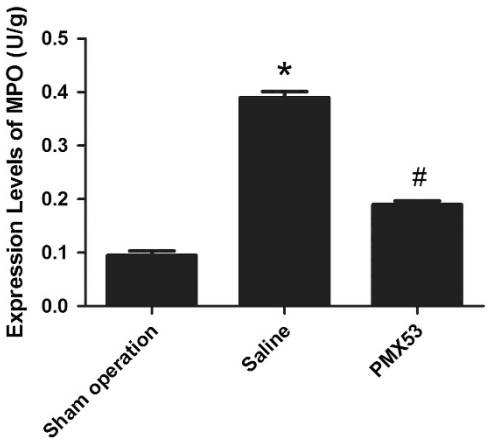


**Figure 3** Myeloperoxidase (MPO) activities in spinal cord of the three groups. The tissue homogenates were obtained from the ischemic spinal cord, and the expression level of MPO was measured 48 h after reperfusion. $^{*}P<0.05$ vs the sham operation group, $^{\#}P<0.05$ vs the saline group. $n=6$ per group.

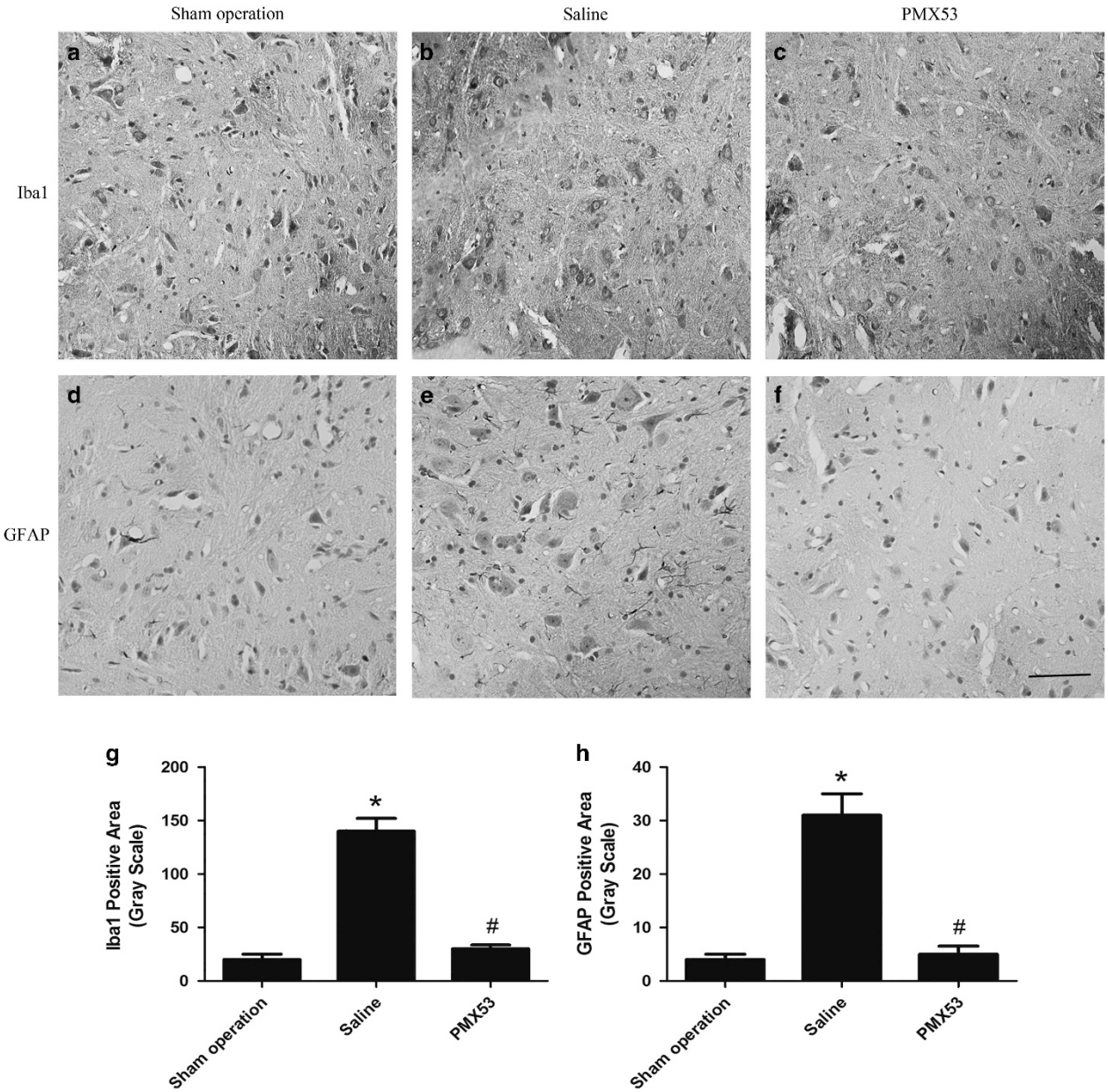


**Figure 4** Immunohistochemical stainings of Iba1 and GFAP in the spinal cord 48 h after reperfusion in the sham operation group (Sham operation), the saline group (Saline) and the PMX53 group (PMX53). Sections were immunolabeled with Iba-1and GFAP to identify macrophages/microglia and astrocytes, respectively. The number of both macrophages/microglia and astrocytes was considerably increased in the spinal cord from the saline group (**b** and **e**) compared with the sham operation group (**a** and **d**). Rats pretreated with PMX53 showed a reduction in macrophages/microglia (**c**) and astrocytes (**f**). The gray scale of Iba1 and GFAP was quantified using image analysis collection system over 30 individual vision fields (**g** and **h**), which showed significantly reduced expression levels of Iba1 and GFAP in PMX53-treated rats. $^{*}P<0.05$ vs the sham operation group, $^{\#}P<0.05$ vs the saline group. Scale bar, 25 μm; $n=6$ per group. A full color version of this figure is available at the *Spinal Cord* journal online.

astrocytes. In other words, the blockage of C5aR may promote the recovery of spinal cord after SIRI.

C5aR is the specific receptor of C5a, an anaphylatoxin, which has various roles in inflammatory cell chemotaxis, increasing vascular permeability and so on. C5aR is widely expressed on various cells in central nervous system[17] and involved in many physiological and pathological processes. The C5a-C5aR signal pathway has been proved to participate in many central nervous system diseases, including infection,[25] hypoxic ischemia disease[26] and neurodegeneration.[27] PMX53 is a specific C5aR antagonist with a molecular formula as AcF-[OP(D-Cha)WR]. It has been found that C5aR inhibition played a protective role in cerebral ischemia reperfusion.[17] The protective role of PMX53 has also been found in IRI of multiple organs.[28–31] The mechanisms are mostly related to inhibiting inflammation, including attenuation of TNF-α expression in tissue or serum, decreasing myeloperoxidase activity and reducing the number of infiltrating neutrophils and neutrophilia.[20] However, the role of PMX53 in SIRI has yet to be reported.

It has been demonstrated that neutrophils activation has a key role in the development of SIRI. The infiltration of neutrophils into the spinal cord can be examined by measuring MPO activity of the tissue.[32] Tissue MPO activity increased significantly at 24 h after

reperfusion in SIRI.[33] In the present study, MPO activity in the spinal cord increased significantly after SIRI but was markedly inhibited by the administration of pre-treatment with PMX53. It suggests that PMX53 protected the spinal cord from decreasing the infiltration of neutrophilic granulocytes during SIRI. The same protective mechanisms of C5aR antagonist can be found in traumatic brain cryoinjury and IRI in other organs.[20]

Astrocytes and microglia are regarded as contributors to the pathology observed in central nervous system inflammation diseases.[25] A recent study showed that proliferation and activation of microglia resulted in excitotoxicity[34] and took part in the development of SIRI. It was also noticed that astrocytes are one of the major components of the blood-brain barrier and BSB, the dysfunction of which contributed to SIRI and the following neurological outcomes.[35] It has been proposed that the protective function of C5aR antagonism is due to the reduction in C5a-mediated activation of astrocytes in a rat model of amyotrophic lateral sclerosis.[18] In support of this hypothesis, the present study showed that the activation of astrocytes and microglia in the spinal cord tissue increased significantly after SIRI but was markedly inhibited by PMX53 administration. Our data suggest that PMX53 reduces C5a-mediated pathology in SIRI models probably via inhibiting the astrocytic and microglia activation.

Our results reveal that blocking C5aR with PMX53 alleviated the adverse effect of SIRI. PMX53 may serve as a therapeutical choice in the future. However, given the nature of pre-administration of PMX53, unless post-ischemia treatment is investigated, the pharmaceutical value would be limited for clinics. Moreover, we only examined the effect of PMX53 in short term after injury. Further long-term studies are required on the possible benefits, or otherwise, of PMX53 pre-treatment.

## CONCLUSIONS

This study showed that the C5a-C5aR signal pathway was involved in the development of SIRI, and the blockage of C5aR could protect the spinal cord from IRI in short term, which probably through the blockage of neutrophil activity, reducing the activated microglia and astrocyte.

## DATA ARCHIVING

There were no data to deposit.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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