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Autophagy exerts a protective role in cervical spinal cord injury by microglia inhibition through the nuclear factor kappa-B pathway

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Background: *Spinal cord injury (SCI) is a serious trauma to the central nervous system. M1/M2 microglial polarization as well as the following neuroinflammatory response are crucial factors in SCI. Autophagy plays an important role in SCI, but its neuroprotective or neurodegenerative role remains controversial.*

Materials and methods: *Here, we majorly examined the properties of autophagy in SCI and uncovered the regulatory relationship between autophagy and microglial polarization in SCI.*

Results: *In our study, the Basso-Beattie-Bresnahan (BBB) score was declined in SCI. The cervical contusion SCI stimulated a sustaining neuropathic pain-linked phenotype characterized by thermal hyperalgesia as well as mechanical allodynia. It was revealed the structural damage to the spinal cord in SCI. Besides, the expression of microglia markers as well as inflammatory factor were promoted in SCI. Cervical contusion SCI induced autophagy inhibition and nuclear factor kappa-B (NF-κB) activation in mice. More importantly, enhanced autophagy induced by rapamycin suppressed the NF-κB pathway and alleviated cervical contusion SCI-induced neurological function damage in mice. Additionally, rapamycin promoted microglia M2 polarization and improved microglia-mediated inflammatory response.*

Conclusions: *In conclusion, our study demonstrated that autophagy played a protective role in cervical SCI by promoting microglia polarization toward M2 through the NF-κB pathway. Our study may provide a novel sight for SCI treatment.* (Folia Morphol 2024; 83, 1: 113–124)

Keywords: spinal cord injury, microglia activation, NF-κB, autophagy

INTRODUCTION

Spinal cord injury (SCI) belongs to a serious disease resulting in specific neurological symptoms depending on the degree of injury, with high morbidity and mortality [4]. About 60% of SCI involves the cervical spinal cord, resulting in complete or incomplete quadriplegia, and the mortality rate is higher than that of thoracolumbar injuries [10]. Primary injury of the spinal cord is linked to the destruction of axons along with neurons, whereas secondary injury is resulted by neuroinflammation and can result in morphologic oedema, cavitation, as well as reactive gliosis [17]. Up to now, long-term treatment mainly targets the symptoms of secondary complications containing

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severe neuroinflammation as well as poor adaptive plasticity after secondary injury [27]. Nevertheless, due to the existence of blood-brain barrier, few therapeutic drugs or other interventions have been proven to suppress the development of secondary injury after SCI and effectively facilitate functional recovery [34].

To the best of our knowledge, SCI induces inflammatory responses that include the release of cytokines and the activation of microglia [7]. Microglia belongs to a main resident cell in the central nervous system, and activates and modulates neuroinflammation after SCI [39]. Microglia are activated into two polarization states: the pro-inflammatory phenotype (M1) as well as the anti-inflammatory phenotype (M2) [25]. Microglia plays dual roles in neuroinflammation together with neurogenesis, which depends on its polarization: the classic M1 phenotype secrets proinflammatory cytokines that are detrimental to neurogenesis. The alternative type M2 secretes anti-inflammatory cytokines and is beneficial to neurogenesis [26]. Therefore, in the treatment of SCI, efforts should be made to explore therapeutic methods to convert microglia from M1 to M2 type and to inhibit harmful excessive neuroinflammation.

The neuroinflammatory responses stimulated by activation of microglia through the nuclear factor kappa-B (NF-κB) pathway is a key factor in SCI [2]. After necrotic or damaged cells are injured, the NF-κB signalling pathway is released, which activates microglia to secrete inflammatory cytokines [8]. NF-κB activation is started by IκB kinase, degrading IκB protein in the cytoplasm and causing release and nuclear translocation of NF-κB [29].

Autophagy, a catabolic process that protects cells from various stresses by degrading dysfunctional organelles and proteins, has been reported to be involved in SCI recovery [36]. Increasing evidence has suggested that autophagy exerts neuroprotection in SCI [30]. According to the location as well as severity of SCI, autophagic flow may increase or decrease. Thus, it remains unclear whether autophagy is beneficial or detrimental after injury [28]. However, restoring and increasing autophagic flow can improve functional recovery after injury by enhancing cell survival, which mirrored that autophagy is a possible therapeutic target for SCI treatment [44].

In this research, a mouse model of cervical spinal cord injury was established to explore the role of autophagy in cervical spinal cord injury and the relationship between autophagy and microglia activation.

MATERIALS AND METHODS

Establishment of the mouse model of cervical SCI

Animal procedures were approved by The First Affiliated Hospital of Hebei North University and this study was approved by the Ethics Committee of The First Affiliated Hospital of Hebei North University. To probe the property of autophagy in SCI and uncover the regulatory relationship between autophagy and microglial polarization in SCI, 10 male C57BL/6 mice (26–30 g) frequently used in the construction of SCI models [11, 14, 21] were anesthetized with 1% isoflurane. As described before, contusion SCI was performed (n = 5) [35]. C5/C6 right spinal cord contusion was generated using an Infinite Horizons impactor with 0.7 mm impactor tip, 40 nephron force, and 2-second dwell time. The sham group (n = 5), which underwent laminectomy only, underwent the same procedure but did not develop contusion. To explore the regulatory mechanism between autophagy and the polarization of microglia in SCI, 10 male C57BL/6 mice (26–30 g) were subjected to either rapamycin (RAP) administration, mice were intraperitoneally injected with RAP (1.5 mg/kg every day) after injury (SCI+RAP, n = 5) or sham surgery (sham+RAP, n = 5) [24]. All the mice were administrated by RAP for 6 weeks.

Behavioural testing

The recovery of general motor function was assessed by the Basso-Beattie-Bresnahan (BBB) scale, in accordance with the previous reports [40]. BBB scores ranged from 0 to 21. A total score of 0 suggested a serious neurological deficit and a total score of 21 represented normal function.

Assessment of mechanical allodynia

The von Frey filament test was implemented to measure mechanical allodynia [3]. Mice were kept in transparent boxes on a raised platform of barbed wire. The tactile stimulation device with a thin wire was placed below the midplantar surface of the left hind paw. With an automatic increase in force, the filaments are lifted to the plantar surface. Maximal force at which the animal retracted its paw was recorded. The 5 g dominant force within 20 s was used as the cut-off point.

Assessment of thermal hyperalgesia

The Hargreaves test (Ugo Basile, Italy) was implemented to measure thermal hyperalgesia [38]. Mice were permitted to acclimate in a transparent box

placed on a raised glass platform. A mobile infrared heat source was placed below the midsurface of the left hind foot of the mice. The time for mice to retract the paw against the heat source was recorded. The cutoff point was set to 20 s.

Tissue processing and haematoxylin and eosin staining

Six weeks after SCI, mice were sacrificed by given an overdose of ketamine (100 mg/kg) together with xylazine (5 mg/kg). 0.9% saline was then transcardially perfused, followed by 4% paraformaldehyde. The spinal cord was dissected to a thickness of 30 μm. Tissue was fixed and dehydration, and then embedded in paraffin wax. Finally, slices were cut to obtain paraffin sections (thickness: 4 μm). The paraffin sections were stained with haematoxylin (Solarbio, Beijing, China) solution for 5 min, and then dyed with Eosin (Solarbio, Beijing, China) for another 2 min. An optical microscope was utilized to observe the changes at the injury epicentre.

RT-qPCR

To evaluate the expression levels of pro-inflammatory cytokines including tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6 and microglia markers including Iba-1, CD16 (M1 markers) and CD206 (M2 marker) in the SCI, total RNA from tissues was extracted with TRIzol reagent (Ambion, USA), Then, total RNA was implemented for reverse transcription to synthesize cDNA (Promega, USA), followed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) using SYBR Green (Promega, USA). Gene expression was normalized to β-actin. Each sample was measured in triplicate using the $2^{-\Delta\Delta Ct}$ method. The following primers were used as follows:

— Iba-1: forward, 5′-ATGAGCCAGAGCAAGGATT-3′ and reverse, 5′-GCATTCGCTTCAAGGACA-3′;
— CD16: forward, 5′-CCACGGATGACCTGTGCTC-3′ and reverse, 5′-TTTATGGTCCTTCCAGTCTCTTG-3′;
— CD206: forward, 5′-CCACGGATGACCTGTGCTC-3′ and reverse, 5′-CCACGGATGACCTGTGCTC-3′;
— TNF-α: forward, 5′-ATGAGCCAGAGCAAGGATT-3′ and reverse, 5′-GCATTCGCTTCAAGGACA-3′;
— IL-6: forward, 5′-TGCCTTCTTGGGACTGAT-3′ and reverse, 5′-TTGCCATTGCACAACTCT-3′;
— IL-1β: forward, 5′-TGTGATGTTCCCATTAGAC-3′ and reverse, 5′-AATACCACTTGTTGGCTTA-3′;
— β-actin: forward, 5′-GTGACGTTGACATCCG-TAAAGA-3′ and reverse, 5′-GCCGGACTCATCG-TACTCC-3′.

Western blot

Proteins were extracted from spinal cords tissues using the lysis buffer (Beyotime, Shanghai). The samples were separated using 10% SDS-PAGE and transferred onto nitrocellulose membranes (Life sciences, USA). The membranes were incubated with different primary antibodies for overnight at 4°C after blocking in 5% skim milk. Primary antibodies included Iba-1 (ab178846, 1/500), CD16 (ab246222, 1/1000), CD206 (ab252921, 1/1000), TNF-α (ab183218, 1/1000), IL-6 (ab233706, 1/1000), IL-1β (ab254360, 1/1000), LC3 (ab192890, 1/2000), Beclin-1 (ab207612, 1/2000), p62 (ab109012, 1/10000), p65 (ab32536, 1/1000), IKB-α (ab32518, 1/1000), p50 (ab32360, 1/1000), and β-actin (ab8227, 1/1000) were provided by Abcam. After washing, the blots were then treated with the secondary antibodies (Abcam, ab6728, 1/2000), followed by detection using the ECL detection kit (Bio-Rad, USA).

Immunofluorescence staining

Embedded sections (4-μm-thick) were deparaffinized with xylene and rehydrated in a graded series of alcohol before antigen repair. The sections were then hatched overnight at 4°C with primary antibody anti-LC3B (Abcam, ab63817, 1 μg/mL), followed by treating with secondary antibodies (Abcam, ab150077, 1:200) after washing. Next, the sections were labelled with Alexa Red fluorescent dye for 1 h, and then dyed with a fluorescent dye of DAPI to evidence the nucleus, followed by visualization under a fluorescence microscope.

NF-κB DNA-binding activity assay

NF-κB p65 DNA-binding activity was tested by a transcription factor binding assay colorimetric ELISA kit (Cayman Chemical, USA). The absorbance at 450 nm was determined by a microplate reader.

Ethics approval and consent to participate

Animal procedures were approved by the First Affiliated Hospital of Hebei North University and this study was approved by the Ethics Committee of the First Affiliated Hospital of Hebei North University.

Statistical analysis

The data was analysed with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Comparisons were assessed by the unpaired Student's t test or one-way ANOVA. $P < 0.05$ was statistically significant.

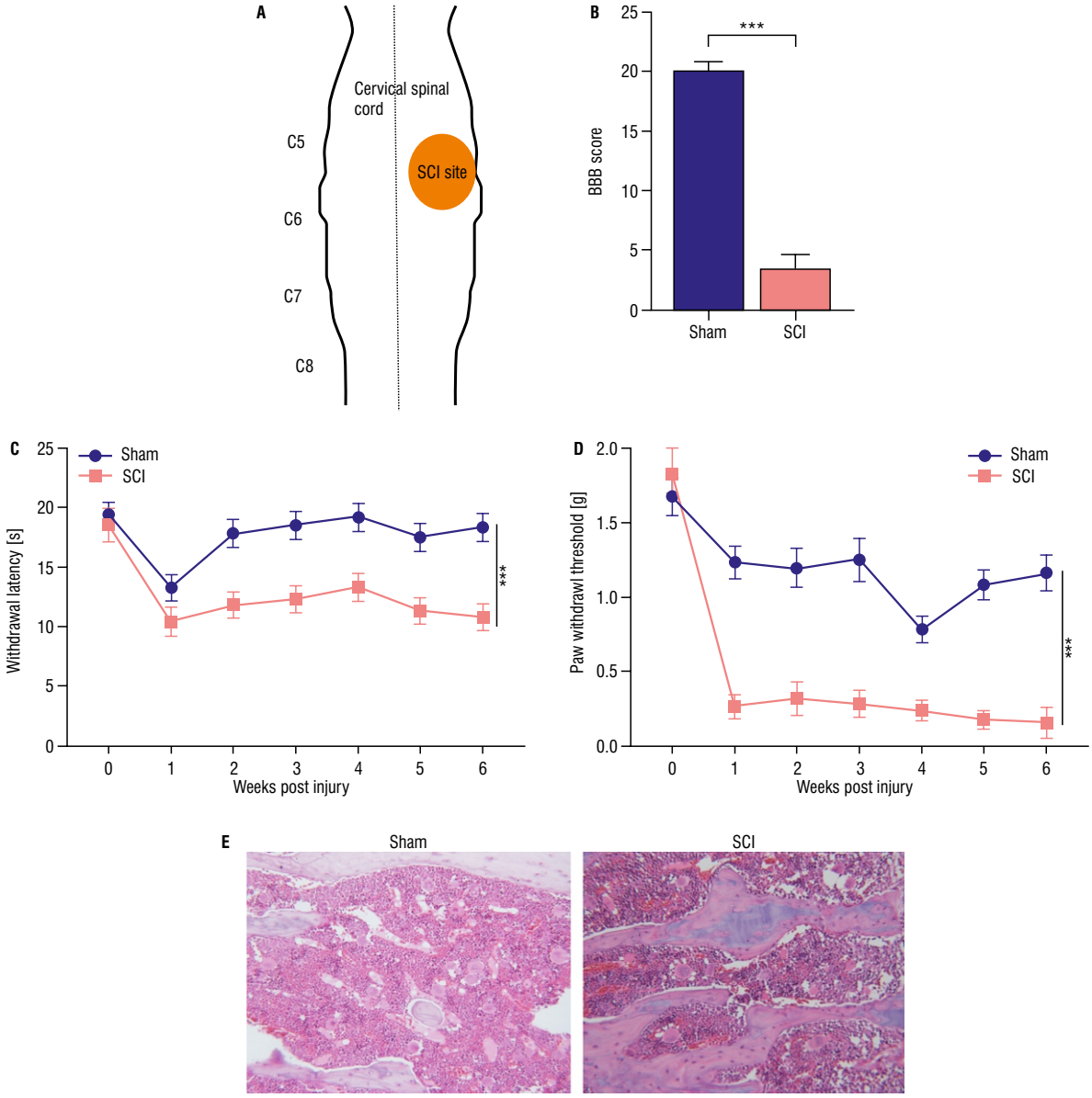

Figure 1. Successful establishment of the mouse model of cervical spinal cord injury (SCI); **A.** A cross-sectional view of the spinal cord in the region of analysis; **B.** Basso-Beattie-Bresnahan (BBB) score of mice in the sham group and SCI group, respectively; **C, D.** Thermal hyperalgesia and mechanical allodynia in the plantar surface of each forepaw in the sham group and SCI group were measured by Hargreaves test and von Frey filament test, respectively; **E.** Haematoxylin and eosin staining detected the histological changes at the injury epicentre in the sham and SCI groups; ***$p < 0.001$.

RESULTS

Successful establishment of the mouse model of cervical SCI

For the quantitative histological analysis carried out in the cervical spinal cord, the tissue sections caudal to the epicentre of the C5/C6 contusion were adopted. This area for histological assessment was accordance with the C6/C7 spinal cord, which was the site of the central projections of primary afferent sensory neurons innervating the plantar surface of the forepaw (Fig. 1A). The BBB score was implemented to evaluate the recovery of general motor function after SCI. The mice in the sham obtained the maximum BBB score (21 points). However, the BBB score was declined in SCI groups, indicating the neurological function of mice was severely impaired immediately after the SCI (Fig. 1B). Through Hargreaves test and von Frey filament test to assess thermal sensitivity and mechanical sensitivity in the plantar surface of each forepaw, respectively, we discovered that cervical contusion SCI at the C5/C6 spinal cord level stimulated a sustaining neuropathic pain-linked phenotype characterized by

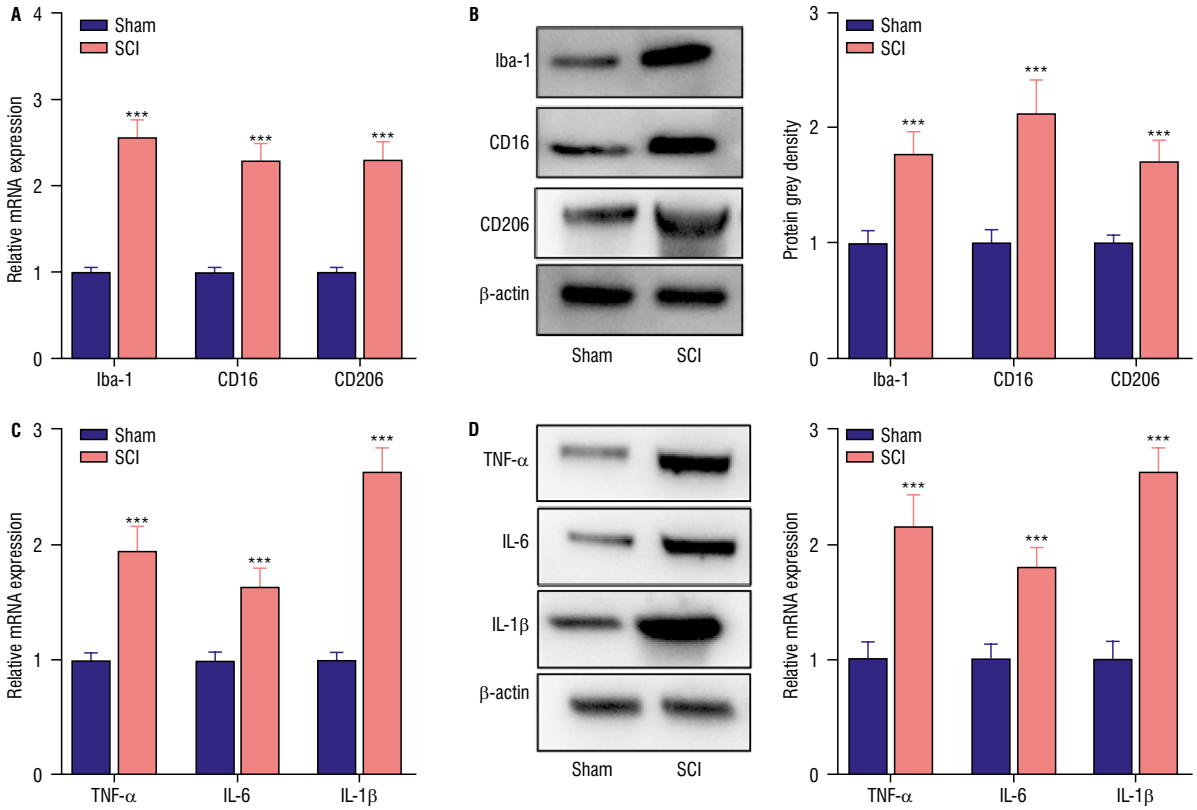

Figure 2. Microglia activation and inflammatory response in spinal cord of mice with cervical contusion spinal cord injury (SCI); **A, B.** Expression of Iba-1, CD16 and CD206 in the sham and SCI groups was detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot; **C, D.** Expression of tumour necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-1β in the sham and SCI groups was detected by RT-qPCR and western blot; ***$p < 0.001$.

thermal hyperalgesia (Fig. 1C) and mechanical allodynia (Fig. 1D). Haematoxylin and eosin staining demonstrated histological changes at the injury epicentre. The spinal cord was intact in the sham group. The SCI group showed the structural damage to the spinal cord, including neuronal nuclear fragmentation, pyknosis, neurocilia destruction, extracellular matrix degradation, interstitial oedema, cytoplasm reduction, as well as cavity formation (Fig. 1E).

Microglia activation and inflammatory response in spinal cord of mice with cervical contusion SCI

Based on qRT-PCR and western blot analysis, we observed that relative to the sham group, the mRNA and protein levels of microglia markers (Iba-1, CD16 and CD206), as well as inflammatory factor (TNF-α, IL-6 and IL-1β) were increased in the SCI group (Fig. 2A–D).

Cervical contusion SCI induces autophagy inhibition and NF-κB activation in mice

Consistently, in our study, the immunofluorescence staining results for LC3 expression showed that the number of LC3 puncta was deceased in the SCI group compared with the sham group (Fig. 3A). Besides, western blot analysis revealed that LC3 II and Beclin-1 protein levels were declined, whereas p62 protein level was elevated in the SCI group relative to the sham group (Fig. 3B). Herein, we found that the activity of NF-κB p65 DNA-binding was enhanced in the SCI group relative to the sham group (Fig. 3C). At the same time, western blot analysis revealed that p65, IKB-α and p50 protein levels were significantly elevated in the SCI group relative to the sham group (Fig. 3D), suggesting that cervical contusion SCI could activate the NF-κB pathway.

Enhanced autophagy suppresses the NF-κB pathway in mice with cervical contusion SCI

Based on above results, we concluded that cervical contusion SCI induced autophagy inhibition and NF-κB activation in mice. Thus, a hypothesis that autophagy activation could regulate the NF-κB pathway in mice with cervical contusion SCI of our study was made. To verify our hypothesis, rapamycin (RAP) was

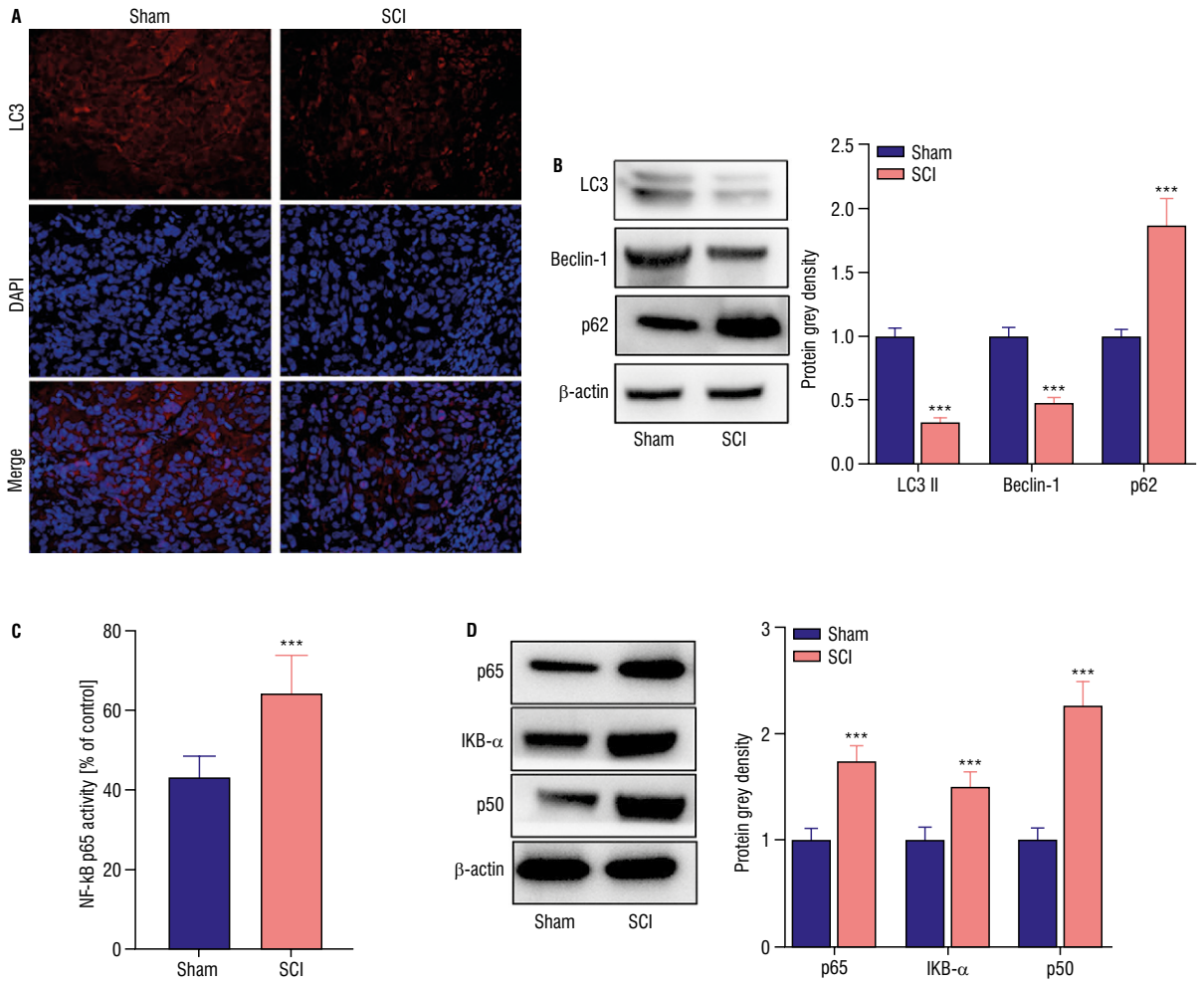

Figure 3. Cervical contusion spinal cord injury (SCI) induces autophagy inhibition and nuclear factor kappa-B (NF-κB) activation in mice; **A.** Immunofluorescence staining results for LC3 expression in the sham and SCI groups; **B.** Protein levels of LC3, Beclin-1 and p62 in the sham and SCI groups were examined by western blot; **C.** A transcription factor binding assay colorimetric ELISA kit was used to detect NF-κB p65 DNA-binding activity in the sham and SCI groups; **D.** Protein levels of p65, IKB-α and p50 in the sham and SCI groups were tested by western blot; ***$p < 0.001$.

firstly used to intraperitoneally inject into mice for 6 weeks after SCI. The outcomes displayed that RAP could obviously enhance the number of LC3 puncta in both sham and SCI groups, and the number of LC3 puncta in the SCI+RAP group was less than that in the sham+RAP group (Fig. 4A). Then, we detected the impacts of RAP on the transcriptional activity of NF-κB. We observed that the enhanced activity of NF-κB p65 DNA-binding caused by SCI was abolished after RAP treatment (Fig. 4B). Similarly, the elevated protein levels of p65, IKB-α and p50 in the SCI group were lessened after injection of RAP (Fig. 4C), which implied that activated autophagy could suppress the NF-κB pathway in mice with cervical contusion SCI.

RAP alleviates cervical contusion SCI-induced neurological function damage in mice

The effects of autophagy on cervical contusion SCI-induced neurological function damage in mice were further investigated. As shown in Figure 5A, the reduced BBB score in the SCI group was partly enhanced after RAP treatment. Besides, we found that SCI-caused the obvious decrease in both mechanical withdrawal thresholds as well as thermal withdrawal latencies of paw was partially reversed after RAP induction (Fig. 5B, C). Moreover, haematoxylin and eosin staining results demonstrated the structural damage to the spinal cord in the SCI group was partly improved after RAP treatment (Fig. 5D).

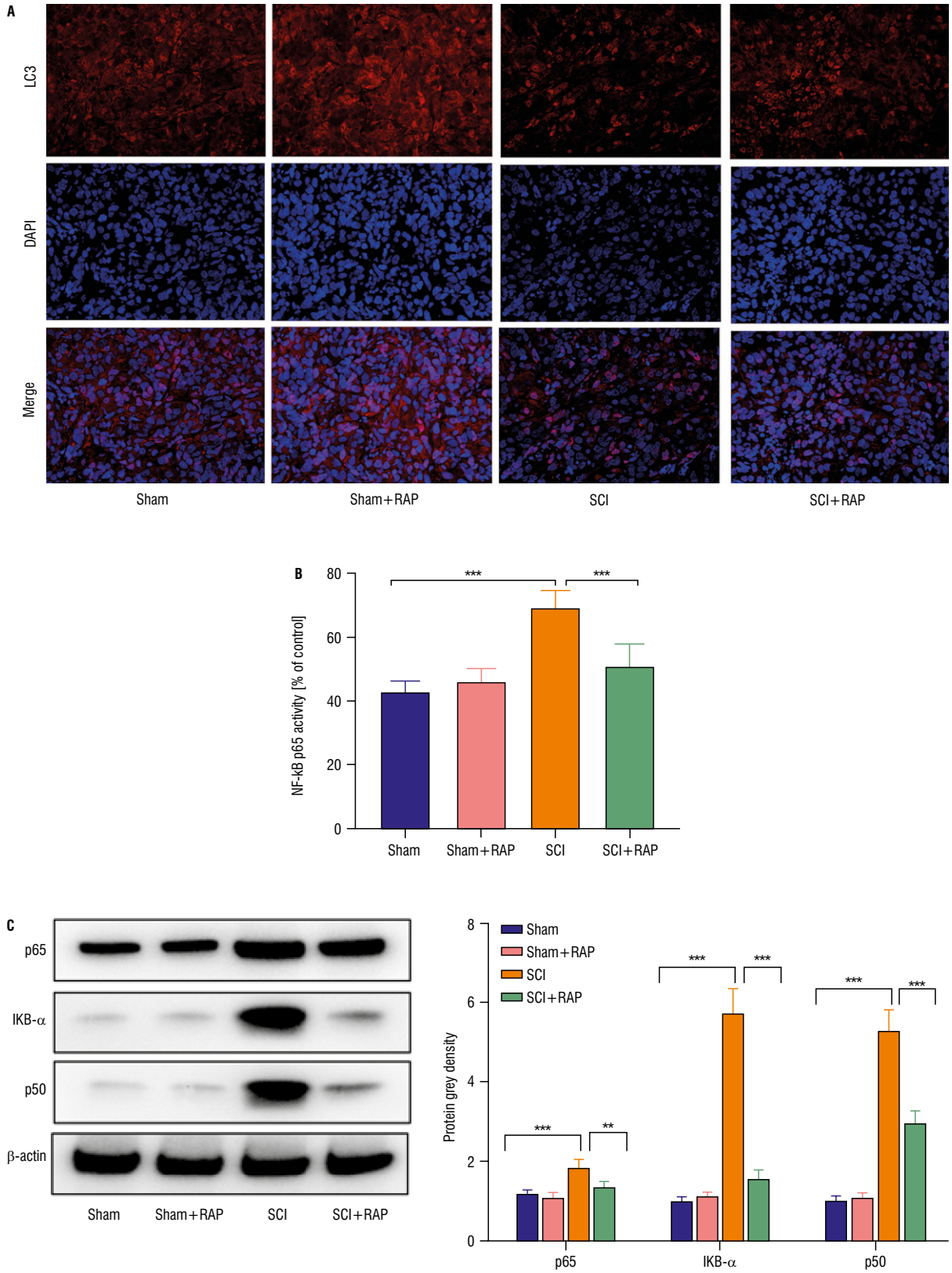

Figure 4. Enhanced autophagy suppresses the nuclear factor kappa-B (NF-κB) pathway in mice with cervical contusion spinal cord injury (SCI); **A.** Immunofluorescence staining results for LC3 expression in the sham, sham+rapamycin (RAP), SCI and SCI+RAP groups; **B.** A transcription factor binding assay colorimetric ELISA kit was used to detect NF-κB p65 DNA-binding activity in the sham, sham+RAP, SCI and SCI+RAP groups; **C.** Protein levels of p65, IKB-α and p50 in the sham, sham+RAP, SCI and SCI+RAP groups were tested by western blot; ***$p < 0.001$.

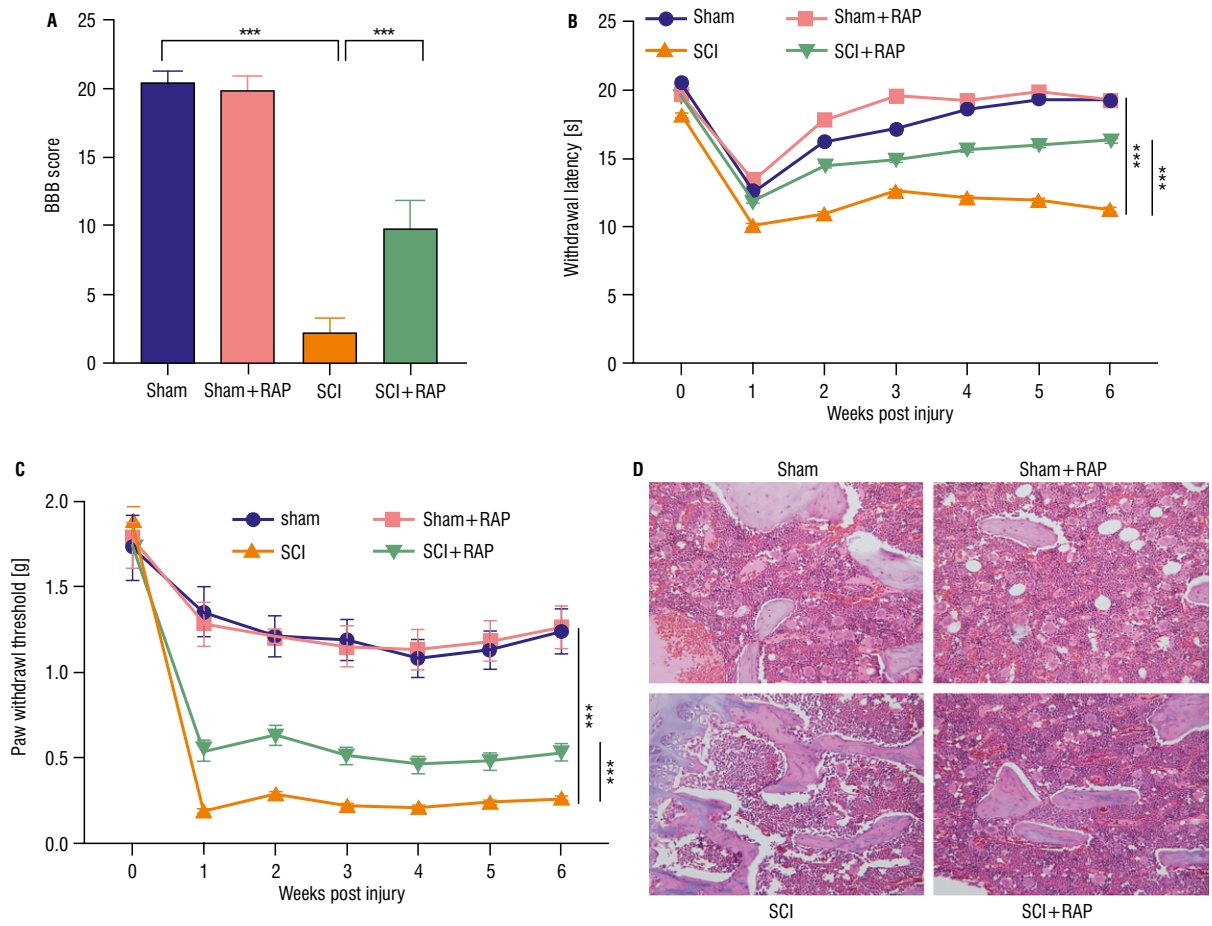

Figure 5. Rapamycin (RAP) alleviates cervical contusion spinal cord injury (SCI)-induced neurological function damage in mice; **A.** Basso-Beattie-Bresnahan (BBB) score of mice in the sham, sham+RAP, SCI and SCI+RAP groups, respectively; **B, C.** Thermal hyperalgesia and mechanical allodynia in the plantar surface of each forepaw in the sham, sham+RAP, SCI and SCI+RAP groups were measured by Hargreaves test and von Frey filament test, respectively; **D.** Haematoxylin and eosin staining detected the histological changes at the injury epicentre in the sham, sham+RAP, SCI and SCI+RAP groups; ***$p < 0.001$.

RAP facilitates microglia M2 polarization and improves microglia-mediated inflammatory reaction

Here, the role of autophagy in microglia polarization and inflammatory response was assessed. Based on western blot analysis, we discovered that the elevated protein levels of M1 markers (Iba-1 and CD16) caused by SCI were offset after RAP treatment. However, the increased protein level of M2 marker (CD206) caused by SCI was further elevated after RAP treatment (Fig. 6A), indicating that RAP promoted microglia polarization toward M2. Accordantly, we discovered that the increased protein levels of TNF-α, IL-6 as well as IL-1β in the SCI group were counteracted after RAP treatment (Fig. 6B).

DISCUSSION

Spinal cord injury is a global problem and a heavy burden for society and families. In addition, the treatment of SCI has always been a challenge [1]. Many biochemical events happen after SCI-mediated secondary injury, and microglia infiltration plays an important role in this process. The proinflammatory and anti-inflammatory potentials of microglia play a key role throughout the process of secondary injury [19]. Kwiecien et al. [16] elucidated a number of fundamental mechanisms in pathogenesis of SCI, and they confirmed the increased levels of TNF-α, IL-1β, interferon-gamma and other proinflammatory cytokines, chemokines and proteases decrease and anti-inflammatory cytokines increase in the late stage of SCI. Therefore, our study established the mouse model of cervical SCI and explored the influences of SCI on microglia activation as well as inflammatory factors. The results demonstrated the activated microglia and increased inflammatory response in spinal cord of mice with cervical contusion SCI, which was consistent with previous literatures [20].

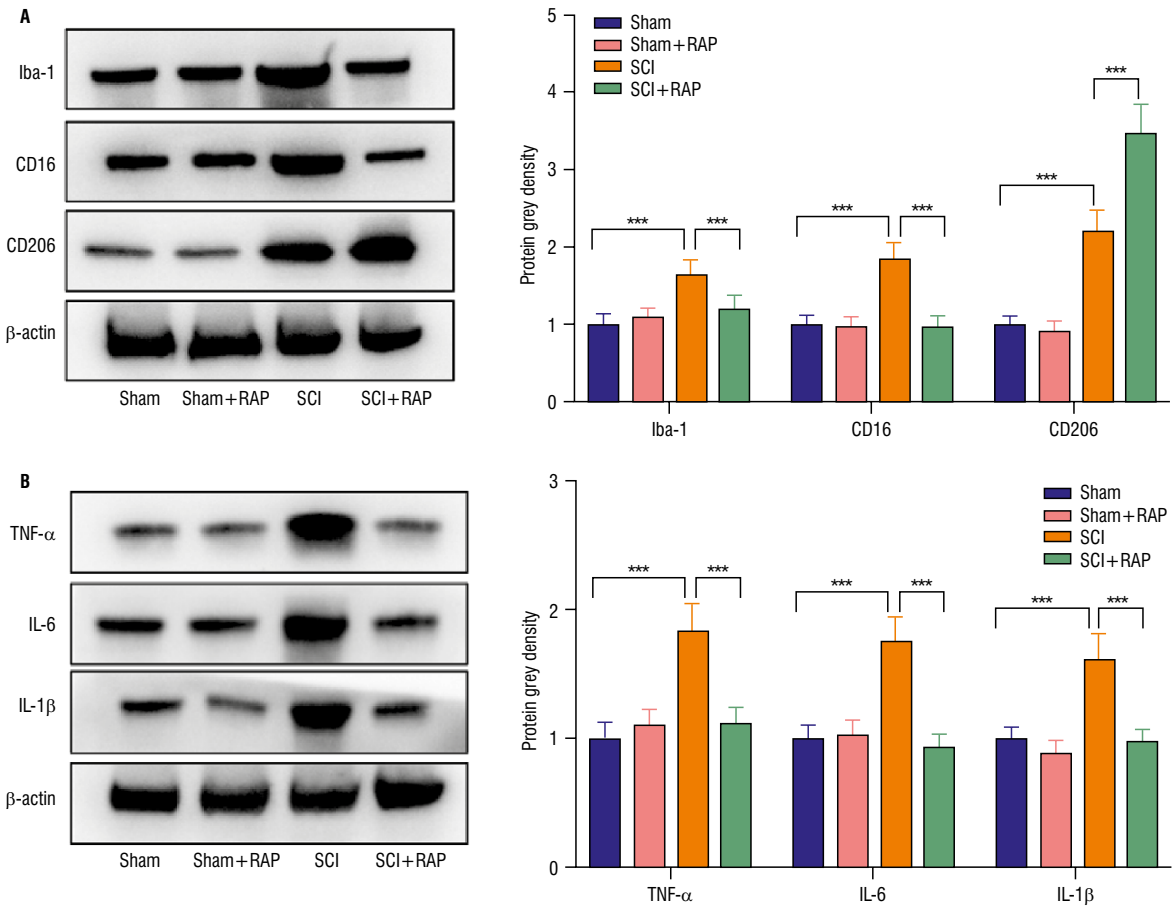

Figure 6. Rapamycin (RAP) promotes microglia polarization toward M2 and alleviates microglia-mediated inflammatory response; **A.** Expression of Iba-1, CD16 and CD206 in the sham, sham+RAP, spinal cord injury (SCI) and SCI+RAP groups was detected by western blot; **B.** Expression of tumour necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-1β in the sham, sham+RAP, SCI and SCI+RAP groups was detected by western blot; ***$p < 0.001$.

Autophagy is a lysosomal-dependent degradation pathway of intracellular proteins, which has a crucial part in human diseases [18]. Pathological situations or cellular stress can stimulate autophagy to be an adaptive as well as protective mechanism [15]. Reports have proved autophagy can mitigate cell damage in rat models of traumatic brain injury [41]. Furthermore, autophagy has been suggested to have a protective role in traumatic SCI [43]. Autophagy is a conserved activity controlling protein degradation and the clearance of damaged organelles. Regarding the autophagy-related signalling, LC3 is the marker for the formation of autophagosome, and the level of p62 protein reflects the activity of autophagic flux [13]. Beclin-1 is also a critical molecular participating in autophagy [42]. In our study, we discovered that the number of LC3 puncta was deceased in SCI. Moreover, western blot analysis demonstrated the protein levels of LC3 II and Beclin-1 were declined, whereas p62 protein level was elevated in SCI. All these findings in our study supported the protective role of autophagy in SCI, which was in accordance with previous reports [32].

NF-κB is a core transcription factor of inflammatory response, and exerts a crucial potential in microglial activation [22]. Additionally, NF-κB signalling is implicated in the inflammatory response during SCI [23]. Former studies have also verified a modulatory cross-talk between autophagy and NF-κB signalling pathway in SCI, which demonstrates that activation of autophagy can hinder the NF-κB signalling pathway [9]. The most abundant form of NF-κB is a heterodimer of p50 and p65 subunits [31]. Consistent with the above studies, our research showed that the activity of NF-κB p65 DNA-binding was enhanced in SCI. Meanwhile, western blot analysis showed the protein levels of p65, IKB-α (NF-κB inhibitor alpha) and p50 were elevated in SCI, suggesting that cervical contusion SCI could activate the NF-κB pathway. More importantly, our

study proved that activated autophagy by RAP, a well-known autophagy activator [6], could repress the NF-κB signalling and alleviate cervical contusion SCI-induced neurological function damage in mice, which implied that autophagy is conductive to the context of SCI.

Increasing evidence has manifested that microglial activation in the central nervous system can be categorized into M1 phenotype and M2 phenotype [37]. Microglia M2 polarization is conducive to local anti-inflammatory response after SCI [5]. Besides, recent researches have shown that autophagy modulates microglia polarization to affect neurological diseases [12]. As mentioned by Shi et al. [33], granule protein precursor has an anti-inflammatory role by enhancing autophagy and inducing M2 microglial polarization, which relieves neurological function after acute SCI. In line with these evidences, our study indicated that activated autophagy by RAP promoted microglia M2 polarization toward and mitigated microglia-mediated inflammatory response. However, there are still some limitations in the current research. For example, we should conduct sufficient clinical observations to further consolidate the clinical significance of the article. In addition, the number of mice constructing SCI models is relatively small, and there are fewer independent duplicate data. In future research, we will further address these issues, making the data more sufficient and the results more reliable.

CONCLUSIONS

In conclusion, our study demonstrated that autophagy played a protective role in cervical SCI by promoting microglia M2 polarization through the NF-κB pathway. Our study may be provided a novel sight for SCI treatment.

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Conflict of interest: None declared

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