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ORIGINAL PAPER

Angiopoietin-1 Protects Spinal Cord Ischemia and Reperfusion Injury by Inhibiting Autophagy in Rats

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Abstract

Spinal cord ischemia and reperfusion (SCIR) injury can induce autophagy, which is involved in the survival of neurons. However, whether autophagy plays a neuroprotective or a detrimental role in SCIR injury remains controversial. Angiopoietin-1 (Ang-1), an endothelial growth factor, has been shown to have neuroprotective effects. The present study aimed to explore the neuroprotective mechanisms of Ang-1 in neuronal cells in a rat model of SCIR injury in vivo. Ang-1 protein and rapamycin were injected intrathecally. Basso Beattie Bresnahan (BBB) scoring and hematoxylin and eosin staining were used to assess the degree of SCIR injury. Proteins that reflected the level of autophagy expression, such as Beclin-1 and LC3, were evaluated by western blotting. The results indicated that SCIR injury resulted in loss in lower limb motor function. Ang-1 protein inhibited the expression of Beclin-1 and LC3, which improved the BBB score and alleviated spinal cord injury. In contrast, rapamycin, an autophagy activator, caused the opposite effect. This study provides evidence that Ang-1 plays a neuroprotective role by inhibiting of autophagy expression in SCIR injury. Overall, findings could be useful for the treatment of SCIR injury.

Keywords Spinal cord · Ischemia · Reperfusion · Angiopoietin-1 · Autophagy

Introduction

Spinal cord ischemia and reperfusion (SCIR) injury is a pathogenic mechanism whereby the ischemic spinal cord is further aggravated after blood reperfusion [1, 2], and is common in thoracic and spinal surgery, often leading to paraplegia [3]. Despite considerable laboratory and clinical studies to decrease SCIR injury, such as ischemic and chemical preconditioning [4, 5] and hypothermia [6], the incidence of paraplegia remains high [7, 8], bringing with it a great psychological and economic burden to patients. Furthermore, the pathophysiological molecular mechanism of SCIR injury is yet unclear. Therefore, effective treatments or drugs to protect the spinal cord against SCIR injury needs further investigation.

In the nervous system, ischemia and reperfusion injury triggers a complex series of pathophysiological events leading to neuron injury and/or death [3, 9]. Autophagy, a new type of programmed cell death, is a unique intracellular bulk degradation mechanism of eukaryotic cells that involves the degrading and recycling of long-lived proteins, damaged organelles and exogenous substances [10, 11]. Usually, autophagy occurs at a basal level, but can be markedly induced by ischemia, hypoxia, or nutrition deprivation [12]. Rapamycin (Rap) is an autophagy activator that inhibits the mammalian target of rapamycin (mTOR) [13]. Previously, SCIR injury was shown to upregulate the expression of autophagy marker proteins microtubule-associated protein 1 light chain 3 (LC3) and Beclin-1 [7]. To a certain extent, physiological level autophagy ensures the stabilization of the

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intracellular environment, which is beneficial to cell survival [14, 15]. However, excessive activation of autophagy can eventually lead to cell death [16]. Interestingly, the functions of autophagy were found to be contradictory. Several studies showed that autophagy contributed to cytoprotection in SCIR injury [17], yet others indicated that autophagy caused cell death, which is known as autophagic neuronal cell [18]. The role of autophagy in SCIR injury is still controversial and remains to be elucidated.

The angiopoietin-Tie signaling pathway includes angiopoietin-1 (Ang-1), Ang-2, and Ang-4 (the human orthologue of mouse Ang-3). Although Ang-1 is recognized as an endothelial growth factor [19], several studies have reported the neuroprotective effects of Ang-1 in nervous system injury because of its angiogenesis effect and stabilization ability of the blood-spinal cord barrier [20, 21]. We reviewed its neuroprotective and Neurotrophic effects [22]. Valable et al. [23] confirmed that neurons express Ang-1 and possess the functional Ang-receptor Tie-2, which is phosphorylated in the presence of Ang-1. Therefore, Ang-1 not only acts on vascular endothelial cells but also directly acts on neurons. However, the regulatory function of Ang-1 in SCIR injury is not yet clarified. Emerging studies have shown that Ang proteins are involved in the regulation of autophagy [24]. These findings led us to speculate that Ang-1 could prevent SCIR injury by inhibiting autophagic neuronal death. In this study, we successfully established a rat model of SCIR injury in vivo. We explored the effects of Ang-1 on the recovery of lower limb motor function and used hematoxylin and eosin (H&E) staining to evaluate the severity of spinal cord injury. In addition, we analyzed the role of autophagy in SCIR injury by using rapamycin to induce autophagy. These findings might provide a novel therapeutic target with respect to autophagic cell death in SCIR injury.

Materials and Methods

Establishment of SCIR Injury Model

Eight-week-old male Sprague-Dawley rats (240–320 g) were obtained from the Animal Center of Nanjing Medical University (Nanjing, China). All rats were maintained in individual cages with ad libitum access to water and food in an air-conditioned room with a 12-h light/dark cycle, at 22 °C to 24 °C and 60% relative humidity. All animals were housed separately after surgery. Animal experiments were approved by the Animal Care Committee of Nanjing Medical University (Nanjing, China). The SCIR injury model was performed with a modification of a method described previously [3]. Ang-1 protein (Sigma-Aldrich; SRP3007) was dissolved in distilled water (DW) and injected intrathecally. Rapamycin (Sigma-Aldrich; 553210) was dissolved in DMSO (Sigma-Aldrich; D2650) and administered intrathecally. Briefly, after anesthetization with 2.5% pentobarbital (P-010; Sigma-Aldrich, USA) at 60 mg/kg via an intraperitoneal injection, the rat abdominal aorta was exposed via a median abdominal incision approach. After the separation of abdominal blood vessels, the abdominal aorta was blocked at 0.5 cm under left renal artery using a 50 g artery clip for 60 min (Fig. 1a, b).

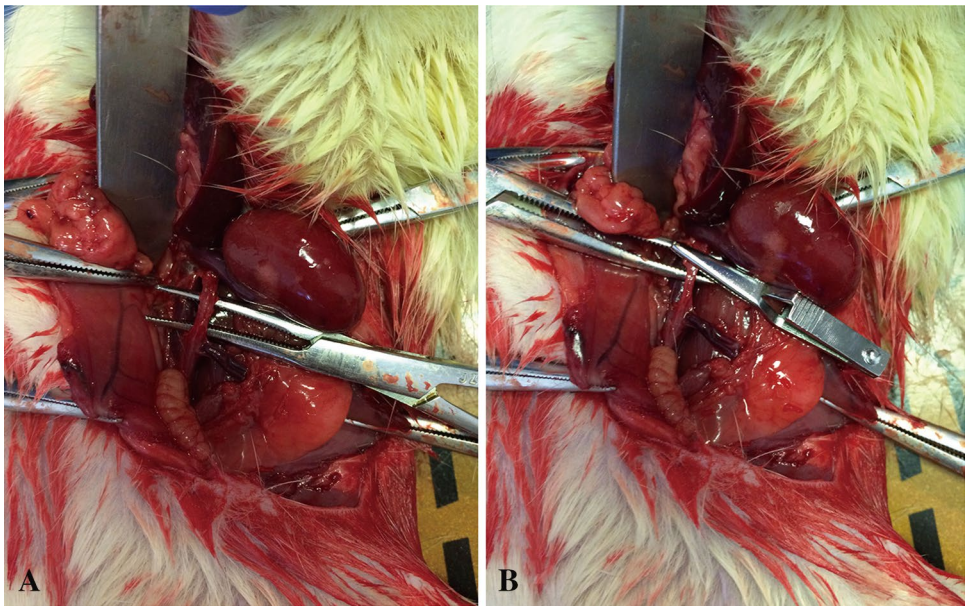

Fig. 1 Establishment of SCIR injury model. **a** The abdominal aorta was dissected alone. **b** The abdominal aorta was blocked at 0.5 cm under the left renal artery using a 50 g artery clip for 60 min

Drug Administration

The sham group (n = 4) underwent surgery but without aortic clamping. The SCIR group (n = 4) underwent abdominal aorta cross-clamping for 60 min, and then in both groups received intrathecal injection of 100 µl of DW or DMSO, respectively. The SCIR group (n = 40) received intrathecal injection of different concentrations of Ang-1 or rapamycin (dissolved in 100 µl of DW or DMSO respectively) and the appropriate concentrations were chose to use in the following model. The sham group (n = 4) received intrathecal injection of appropriate concentration of Ang-1 (800 ng/kg), rapamycin (50 ng/kg) or Ang-1 (800 ng/kg) + rapamycin (50 ng/kg) to assess background effects. The SCIR + Ang-1 group (Ang-1 group, n = 8), SCIR + Rap group (Rap group, n = 8) and SCIR + Ang-1 + Rap group (Ang-1 + Rap group, n = 8) also underwent the same surgical procedure as the SCIR group, but were treated with Ang-1 (800 ng/kg), rapamycin (50 ng/kg) or Ang-1 (800 ng/kg) + rapamycin (50 ng/kg) [25], respectively, immediately after blood reperfusion (Table 1).

Neurological Function Assessment by BBB Scoring Assay

Locomotor recovery of the rat lower limb after SCIR was assessed by the Basso, Beattie, and Bresnahan (BBB) open-field locomotor scale [26] ranging from 0 (complete paralysis) to 21 (normal locomotion). The BBB scores were evaluated at 36 h after the establishment of the model in "Drug Administration" section and at 0, 12, 24, 36, 48, and 72 h after reperfusion in "Neurological Function Assessment by BBB Scoring Assay" section by three experienced investigators who were blinded to the experiment.

Histopathological Analysis

Four rats were sacrificed at 36 h after the establishment of the model in each group for histological staining (n = 4). The spinal cord of the lumbar 4–5 segments of the rat was taken for use in subsequent experiments. Alternate perfusion of normal saline and paraformaldehyde (P0099; Beyotime, China) from the aortic arch of the heart was performed before surgical removal of the spinal cords. The samples were fixed in 4% paraformaldehyde, embedded in paraffin wax and sliced into 5 µm-thick sections. Subsequently, H&E staining was performed for the histological assessment of the spinal cord injury. The gray and white matter of the spinal cord was generally observed and the numbers of the nucleus of neuron under a magnification of 400 times were counted and take the average by three experienced investigators who were blinded to the experiment.

Western Blot Assay

The expressions of Beclin-1 and LC3-II/I in spinal cord tissues of rats (n = 4) at 36 h after reperfusion were detected by western blotting. The samples were homogenized and the total proteins were extracted from samples with tissue protein extraction kit (KGBSP002; KeyGEN BioTech, China). Aliquots of proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinyldifluoride membranes (C3117; Millipore, USA). Subsequently, the membrane was blocked with 5% nonfat dry milk for 1 h at room temperature and then incubated with primary antibody anti-Beclin-1 (ab62557; Abcam, USA) and anti-LC3 (Abcam; ab128025) overnight at 4 °C. All three primary antibodies were diluted 1,000 times with primary antibody diluent (P0256; Beyotime, China). The next day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody

Table 1 Experimental grouping and animals number (N)

Group	Description	N
Sham	Underwent surgery but without aortic clamping	4
SCIR	Underwent abdominal aorta cross-clamping for 60 min	4
Sham + DW	The sham group received intrathecal injection of 100 µl of DW	4
SCIR + DMSO	The SCIR group received intrathecal injection of 100 µl of DW	4
Sham + DMSO	The sham group received intrathecal injection of 100 µl of DMSO	4
SCIR + DMSO	The SCIR group received intrathecal injection of 100 µl of DMSO	4
Sham + Ang-1	The sham group received intrathecal injection of Ang-1	4
Sham + Rap	The sham group received intrathecal injection of Rap	4
Sham + Ang-1 + Rap	The sham group received intrathecal injection of Ang-1 + Rap	4
Ang-1	The SCIR group received intrathecal injection of Ang-1	28
Rap	The SCIR group received intrathecal injection of rapamycin	28
Ang-1 + Rap	The SCIR group received intrathecal injection of Ang-1 and rapamycin	8

DW distilled water, *Rap* rapamycin

(Abcam; ab205718 and ab205719) for 1 h at room temperature. The secondary antibody was diluted 200 times by the secondary antibody diluent (Beyotime; P0258). Reactive bands were visualized using an enhanced chemiluminescence (ECL) kit (WBLS0500; Millipore, USA). The protein expression levels in different samples were analyzed using Quantity One software (Bio-Rad, USA).

Statistical Analysis

Each experiment was repeated at least three times. All data are present as mean ± SD. Statistical significance was analyzed by the Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test using SPSS version 17.0 statistical software (Chicago, USA). P values < 0.05 were considered to be statistically significant.

Result

Selecting Appropriate Concentrations of Ang-1 and Rapamycin by BBB Scores of Rats

BBB scores, based on the motor function of the lower limb, were evaluated at 36 h after the establishment of the model in each group in "Drug Administration" section, respectively. The results suggested that intrathecal injection of 100 μl of DW or DMSO has no effect on lower limb motor function in the sham group (Fig. 2a) or the SCIR group (Fig. 2b) ($P > 0.05$). Intrathecal injection of rapamycin at a concentration of 50 ng/kg had no significant effect on BBB score in rats ($P > 0.05$). However, Intrathecal injection of rapamycin at a concentration of 200 ng/kg or 500 ng/kg can significantly improve the BBB score of rats ($F = 5.655$, $P < 0.001$; $F = 1.984$, $P < 0.001$) (Fig. 2c). Moreover, Ang-1 at a concentration of 100 ng/kg, 400 ng/kg or 800 ng/kg can significantly improve the BBB score of rats ($F = 6.902$, $P < 0.001$; $F = 4.296$, $P < 0.001$; $F = 7.275$, $P < 0.001$) (Fig. 2d). In the following experiment, the selected concentration of rapamycin was 50 ng/kg and the concentration of Ang-1 was 800 ng/kg. This concentration of rapamycin had no effect on the motor function of spinal cord in sham group and SCIR group. In addition, administration of Ang-1 and rapamycin alone or in combination had no significant effect on BBB score in sham group (Fig. 2e) ($P > 0.05$).

Ang-1 Protects Against SCIR Injury-Induced Loss of Lower Limb Motor Function

BBB scores were evaluated at 0, 12, 24, 36, 48, and 72 h in the model in "Drug Administration", respectively (Fig. 3). The results showed that the lower limb motor function scores were significantly decreased in the SCIR group, the SCIR + Ang-1 group, the SCIR + Rap group and the SCIR + Ang-1 + Rap group at each point after reperfusion compared with the sham group ($P < 0.05$). However, the BBB scores of the SCIR + Ang-1 group were significantly higher than those of the SCIR group, the SCIR + Rap group and the SCIR + Ang-1 + Rap group at 12, 24, 36, 48, and 72 h after SCIR injury; however, the scores were significantly lower than the sham group at each point in time ($P < 0.05$). There was no significant difference between the SCIR group, the SCIR + Rap group and the SCIR + Ang-1 + Rap group ($P > 0.05$). These results suggested that Ang-1 protected against SCIR injury-induced loss of lower limb motor function and that rapamycin counteracted this protective effect.

Ang-1 Enhances the Survival Rate of Neurons After SCIR Injury

To evaluate the effect of Ang-1 on spinal cord damage caused by SCIR, H&E staining was used to analyze the survival rate of neurons at 36 h after reperfusion. The boundary between the gray matter and the white matter in the sham group, the sham + Ang-1 group and the sham + Rap group were clearly evident and the neurons were normal in shape and arranged in a regular manner (Fig. 4a–c). In contrast, in the SCIR group, the SCIR + Rap group and the SCIR + Ang-1 + Rap group, the nerve fibers were disordered and the morphology of neurons became deformed. Neurite atrophy and vacuolation were also observed in the white matter (Fig. 4d, f, g). However, in the SCIR + Ang-1 group, the boundary between the gray matter and the white matter was evident and the morphology of the neurons was only slightly altered. In addition, the neurites were arranged neatly and the vacuolation was greatly reduced (Fig. 4e). As expected, in the SCIR + Ang-1 + Rap group, the white matter was visibly vacuolated and the morphology of neurons and neurites were obviously altered (Fig. 4g). Although the number of neurons in SCIR + Ang-1 group was significantly less than that in the sham group ($F = 2.694$, $P = 0.014$), this number was significantly higher than that in the SCIR group ($F = 5.018$, $P < 0.001$), the SCIR + Rap group ($F = 4.532$, $P < 0.001$) and the SCIR + Ang-1 + Rap group ($F = 17.286$, $P < 0.001$). Rapamycin at the concentration of 50 ng/kg had no effect on the morphology of spinal cord and the number of nerve cells in the sham group and the SCIR group ($P > 0.05$). These findings indicated that Ang-1 effectively attenuated spinal cord damage after SCIR injury and rapamycin counteracted this protective effect.

Ang-1 Inhibits Autophagy in Neurons After SCIR Injury

To verify the changes in autophagy intensity, western blotting was performed to detect LC3-II/I, and Beclin-1

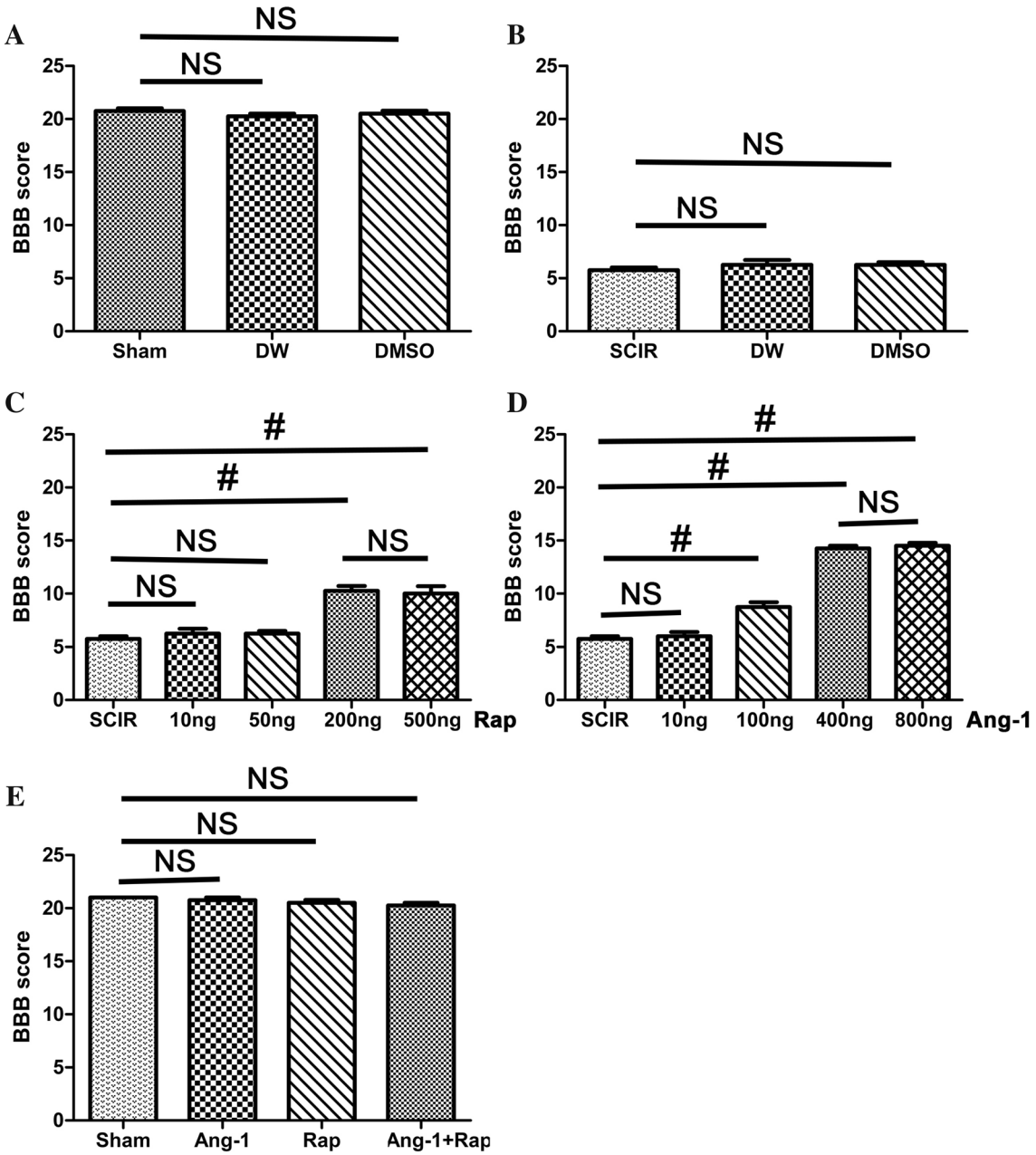

Fig. 2 BBB scores for drug testing. **a**, **b** Intrathecal injection of 100 µl of distilled water (DW) or DMSO, respectively, in the sham group and the SCIR group (n = 4). **c**, **d** Intrathecal injection of different concentrations of rapamycin and Ang-1 in the SCIR group, respectively (n = 4). **e** Intrathecal injection of Ang-1 and rapamycin alone or in combination in the sham group (n = 4). Data represent the mean ± SD of three independent experiments. $^{\#}P < 0.05$; *NS* no significance

expression levels in each group at 36 h after reperfusion (Fig. 5). The results showed a significant increase in LC3-II/I and Beclin-1 protein expression protein in the SCIR group in comparison to the sham group ($P < 0.05$). However, in the SCIR + Ang-1 (400 ng/kg and 800 ng/kg, respectively) group, the expression of LC3-II/I and Beclin-1 protein was significantly lower than that of the SCIR group but significantly higher than that of the sham group ($P < 0.05$). There was no significant change in LC3-II/I and Beclin-1 levels between the SCIR group and the SCIR + Ang-1 + Rap group, but LC3-II/I and Beclin-1 levels in the SCIR + Ang-1 (400 ng/kg and 800 ng/kg, respectively) + Rap group were significantly higher than those in the sham group and the SCIR + Ang-1 (400 ng/kg and 800 ng/kg, respectively) group ($P < 0.05$). The expression of LC3-II/I and Beclin-1 protein in the SCIR + Rap group was significantly higher than SCIR group. There was no significant change in LC3-II/I and Beclin-1 expression between different concentrations of Ang-1

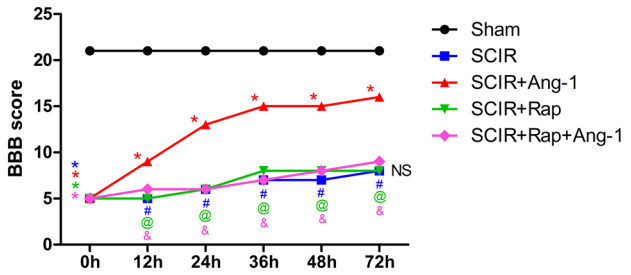

Fig. 3 BBB scores after drug administration. The BBB scores were evaluated at 0, 12, 24, 36, 48, and 72 h after reperfusion (n = 8). Data represent the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus the sham group; $^{\#}P < 0.05$ SCIR group versus the SCIR + Ang-1 group; $^{@}P < 0.05$ SCIR + Rap group versus the SCIR + Ang-1 group; $^{\&}P < 0.05$ SCIR + Ang-1 + Rap group versus the SCIR + Ang-1 group; *NS* no significance

in the SCIR + Ang-1 group and SCIR + Ang-1 + Rap group ($P > 0.05$). The results indicated that intrathecal injection of rapamycin with 50 ng/kg had no effect on the morphology and motor function of spinal cord in the sham group and the SCIR group, but it could significantly enhance the autophagy level after SCIR. Ang-1 with 400 ng/kg and 800 ng/kg had the same regulatory effect on autophagy, which was consistent with BBB score. Based on these results, Ang-1 alleviates spinal cord injury after SCIR by inhibiting autophagy.

Discussion

SCIR injury refers to secondary damage after reperfusion [27] whereby the restored blood flow may further aggravate the initial injury [28]. More specifically, after a specific period of ischemia, the neural function of spinal cord does not improve after reperfusion, but actually causes neurological dysfunction, which may led to paralysis [5, 29]. Despite many efforts by researchers to explore methods for understanding and mitigating SCIR injury, the molecular biological mechanisms that result in SCIR injury have yet to be clearly elucidated.

The autophagy–lysosomal pathway and the ubiquitin–proteasome pathway are the two main pathways that maintain the physiological metabolism of eukaryotic cells [30]. As a material recycling machine, autophagy maintains the balance between protein synthesis and degradation. The ratio of LC3-II/LC3-I and expression of Beclin-1 are both considered as representations of autophagy level. However, autophagy is thought to have seemingly contradictory roles. Specifically, autophagy can either have a cytoprotective or cytodestructive role depending on specific pathological events [7]. At physiological levels, autophagy has been shown to be protective, but after certain events, autophagy levels increase beyond the physiological range and become cytodestructive. Autophagy has been reported to contribute to induce cytoprotective effects in neurodegenerative disease [31]. Conversely, recent reports indicated that autophagy can lead to autophagic cell death in cerebral ischemia and reperfusion injury [32]. Recent studies have also reported that autophagy plays major roles in the central nervous system under stress conditions, especially in spinal cord injury [33, 34]. Therefore, inhibition of autophagy in delayed neuronal death deserves focused attention for the treatment of SCIR injury.

Ang-1, a member of the Ang growth factor, has been well documented for its role in angiogenesis following SCIR injury [5] and cerebral ischemia and reperfusion injury [35]; however, whether Ang-1 directly protects neurons is well studied. Valable et al. [23] showed that Ang-1 protected neurons by inhibiting neuronal apoptosis. This is the first direct evidence in the world that Ang-1 acts directly on nerve cells. The authors confirmed that primary cultured mouse cortical neurons expressed functional Tie-2. Moreover, Ang-1 promoted neurite outgrowth from Tie2-expressing dorsal root ganglion cells [36]. Additionally, another study showed that adipocyte-derived Ang-1 supported neurite outgrowth and synaptogenesis of sensory neurons [37]. Based on these studies, we postulated that Ang-1 could prevent SCIR injury by acting not only as an angiogenic factor but also as a direct neuroprotective factor.

We found that after 60 min of abdominal aorta occlusion, paralysis appeared in the lower limbs of rats. Rapamycin at the concentration of 50 ng/kg had no effect on SCIR, however, Ang-1 at the concentration of 800 ng/kg could significantly improve the BBB score. The results revealed that with the increase of autophagy levels after SCIR injury, the BBB score decreased, the quantity of neurons decreased and the white matter of the spinal cord was vacuolated. On the contrary, Ang-1 inhibited the expression of autophagy after SCIR injury and alleviated paralysis of the lower extremities by ameliorating spinal cord injury. When Rap was used to promote autophagy, however, the protective effect of Ang-1 was counteracted and the symptoms of spinal cord injury were aggravated. These results indicated that autophagy exerted a destructive role in SCIR injury and that Ang-1 significantly reduced autophagic cell death and promoted neurological and motor functions.

These results provide novel evidence that Ang-1 likely exerts a neuroprotective effect after SCIR injury by alleviating autophagic cell death, thus elucidating a mechanism of SCIR injury. Moreover, our study encourages further investigation into whether Ang-1 inhibits autophagic cell death in SCIR injury via the AKT-mTOR and PI3K/AKT pathways, as both are involved in the angiopoietin-Tie pathway and autophagy.

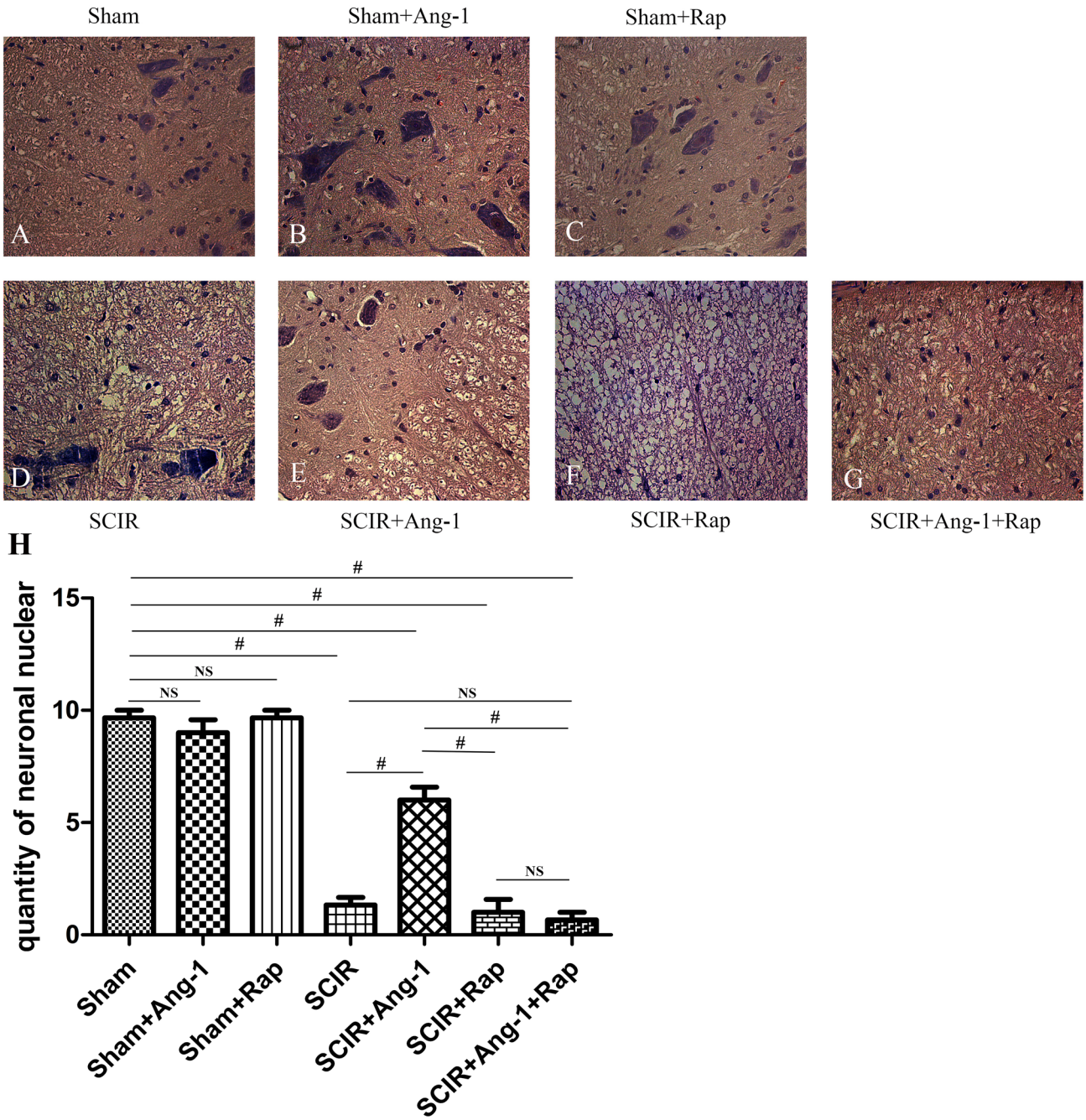

Fig. 4 H&E staining of the spinal cord tissue after 36 h of establishment of the model and quantification of neuronal nuclei. **a** The sham group. **b** The sham + Ang-1 group. **c** The sham + Rap group. **d** SCIR group. **e** SCIR + Ang-1 group. **f** SCIR + Rap group. **g** SCIR + Ang-1 + Rap group. Magnification × 400. **h** Quantification of neuronal nuclei in each group (n = 4). Data represent the mean ± SD of three independent experiments. $^{\#}P < 0.05$, *NS* no significance

In conclusion, our study demonstrated that Ang-1 exerts a neuroprotective effect after SCIR injury by reducing autophagy. The current findings suggest potential applications of Ang-1 in SCIR treatment, thereby facilitating novel drug development.

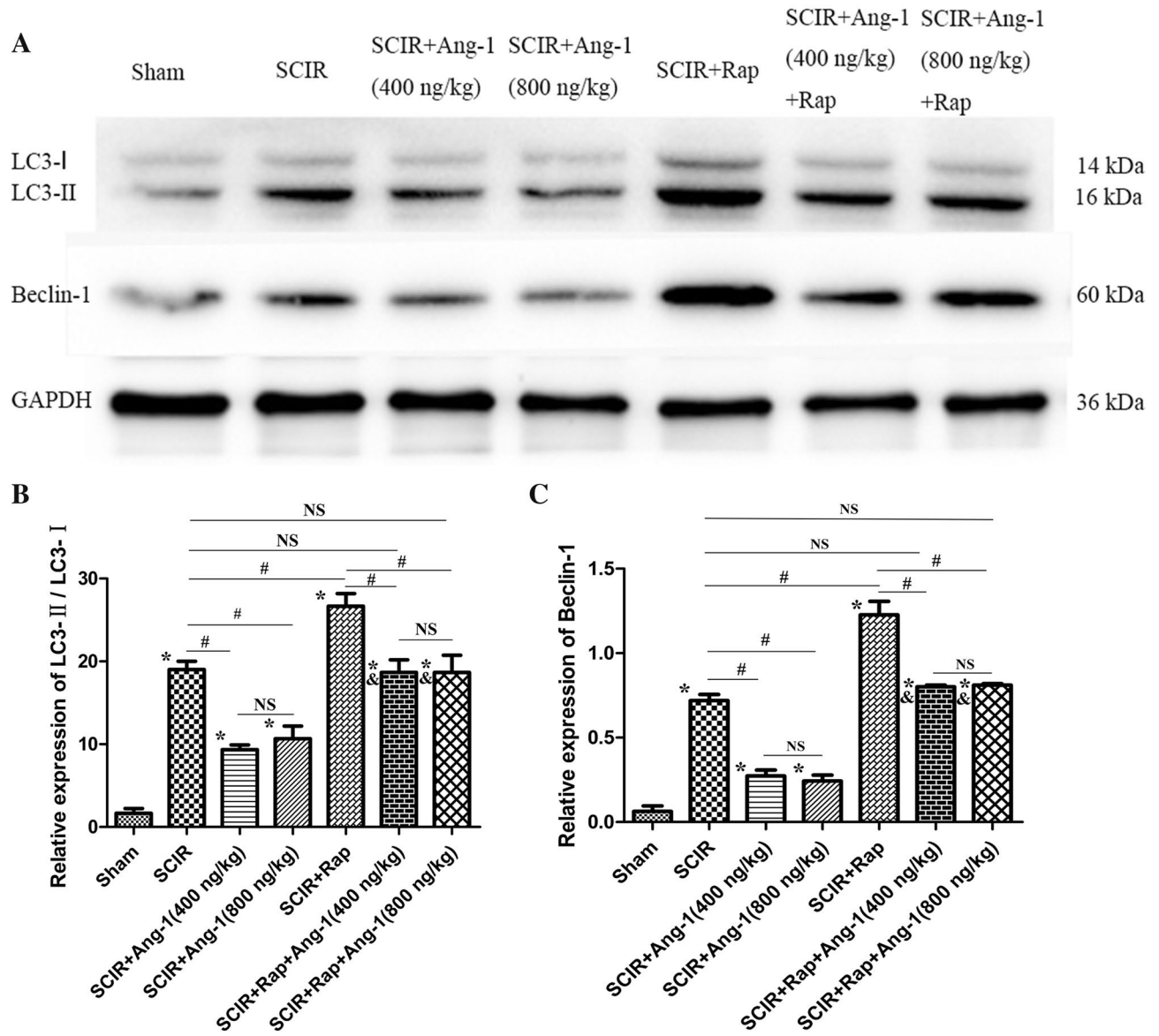

Fig. 5 Levels of autophagy detected by western blotting. **a** Western blot analysis was used to detect the expression of autophagy-related proteins in the spinal cord tissue of each group after 36 h of establishment of the model (n = 4). **b**, **c** Quantitative analysis of protein levels were conducted. Data represents mean ± SD of three independent experiments. $^{\#}P < 0.05$; $^{*}P < 0.05$ versus the Sham group; $^{\&}P < 0.05$ SCIR + Rap + Ang-1 group versus the SCIR + Ang-1 group; *NS* no significance; (n = 4)

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Compliance with Ethical Standards

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical Approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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