Activation of SIRT1 by Hyperbaric Oxygenation Promotes Recovery of Motor Dysfunction in Spinal Cord Injury Rats

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Research

Keywords: HBO, SIRT1, motor dysfunction, inflammation, autophagy, apoptosis

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Abstract

Background: Emerging evidence demonstrated that hyperbaric oxygenation (HBO) therapy improved the locomotor dysfunction following spinal cord injury (SCI). Sirtuin1(SIRT1) has been characterized as neuroprotection in nerve system. However, whether SIRT1 is involved in alleviation of locomotor function by HBO therapy is unclear.

Methods: The Basso, Beattie Bresnahan (BBB) locomotor rating scale was used to evaluate the open-field locomotor function. Western blot, real-time quantitative reverse transcription polymerase chain reaction, SIRT1 activity assay and enzyme-linked immunosorbent assays were performed to explore the molecular mechanisms in adult Sprague-Dawley rats.

Results: We found that series HBO therapy significantly improved the locomotor dysfunction and ameliorated the decrease mRNA, protein and activity of spinal cord SIRT1 induced by traumatic SCI injury in rats. In addition, intraperitoneal injection SIRT1 antagonist EX-527 abolished the beneficial effects of series HBO treatment on locomotor deficits and SIRT1 activity loss caused by traumatic SCI injury. However, the rats undergone both series HBO therapy and SIRT1 agonist SRT1720 got the higher BBB score than that undergone series HBO treatment only. Importantly, series HBO treatment following the traumatic SCI injury inhibited the inflammatory cascade and apoptosis-related protein, which was retained by EX-527 and enhanced by SRT1720. Furthermore, EX-527 blocked the enhanced induction of autophagy series with HBO application.

Conclusion: These findings demonstrated a new mechanism for series HBO therapy involving activation of SIRT1 and subsequent modulation of inflammatory cascade, apoptosis and autophagy, which contributed to the recovery of motor dysfunction.

Key words: HBO, SIRT1, motor dysfunction, inflammation, autophagy, apoptosis

1. Introduction

Traumatic spinal cord injury (SCI), a catastrophic and disabling disease, mainly results from external physical impacts such as traffic accidents, sports, falls and accident at work (primary injury), followed with the secondary injury[1]. The primary injury is usually temporary and irreversible[2] while the secondary injury causes the permanent neurological deficits primarily including motor and sensory dysfunction[1, 3]. The direct damage to spinal cord cells initiates the complicated and devastating secondary injury cascade which may contribute to the permanent function impairment[4] and psychological debilitation[5]. In the onset of secondary injury cascades, the death of neurons and glia and inflammation caused by primary damage is cyclical[4]. More importantly, the uncontrolled posttraumatic inflammation and apoptosis of neurons aggravate the motor dysfunction[6]. Therefore, controlling the posttraumatic inflammation cascade and apoptosis-related protein have great benefits for recovery of motor dysfunction.

Hyperbaric oxygenation (HBO) refers that 100% oxygen is administered at a pressure between one and three times that of atmospheric pressure. HBO therapy has been used clinically and experimentally to improve the neurological recovery in brain injuries[7, 8]and cerebral ischemia[9, 10]. These years, the HBO therapy has been advocated to apply to cure SCI for its protection for neurons[11]. For example, the HBO therapy improved the locomotor recovery via regulating the mRNA and protein levels of monocyte chemoattractant protein-1(MCP-1)[12], which is a pivotal inflammatory cytokine. Meanwhile, apoptosis in injured spinal cord tissue is significantly reduced after HBO therapy application[13]. Although couples of studies reported HBO therapy had effectiveness on recovery of locomotor dysfunction induced by spinal cord injury[14–16], the mechanism of effect of HBO therapy on traumatic spinal cord injury is still largely unknown.

Sirtuin1(SIRT1) protein belongs to a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase, which is highly conserved from bacteria to human[17]. It has well established that SIRT1 modulates the deacetylation of histones[18] and other substrates, such as p53[19], nuclear factor kappa B (NF-κB)[20], peroxisome proliferator-activated receptor γ (PPARγ)[20] and others. By regulation of these protein, SIRT1 is implicated many cellular processes, such as energy metabolic supply, cell survival, autophagy, inflammation and apoptosis[20, 21]. Emerging evidence demonstrated that SIRT1 acted as a protective role in Alzheimer's[22] and Parkinson's[23], which may be closely related to its function in anti-inflammation[19], anti-apoptosis[24] and genomic stability[17]. However, to our knowledge, whether HBO therapy improves locomotor dysfunction induced by traumatic SCI by regulation of SIRT1 is not studied. Therefore, in the present study, we hypothesize that HBO modulates SIRT1 and subsequently ameliorates the motor dysfunction induced by traumatic SCI.

2. Materials And Methods

2.1 Animals

Adult female Sprague-Dawley rats, weighing 260 to 320 g, were obtained from The Medical Experimental Animal Center of Guangdong Province (Guangzhou, China). The animals were housed under a 12-h light/dark cycle with free access to food and water at a constant room temperature of 26 °C ± 1 °C. The rats were randomly separated into different group. All experimental protocols were approved and conducted in accordance with the guidelines of the General Hospital of Southern Theater Command Animal Ethic Committee in accordance with the National Institutes of Health on animal care and the ethical guidelines.

2.2 Surgical and HBO treatment

The traumatic SCI model was established according to previous study[15] with minor change. Briefly, the surgical site was shaved and swabbed with an iodine solution and then with 75% alcohol after animals were anesthetized intraperitoneally with 50 mg/kg sodium pentobarbital. The laminectomy was conducted to expose the T9–11 spinal cord without rupturing the dura mater after a midline skin incision

was made to expose the T9–11 spinal column. Then, traumatic spinal cord injury was induced with an impacting stick (10 g) from a height of 10 mm (10-g/cm force) onto the exposed spinal cord. After the incision was sutured, 5 mL of saline was immediately administered via intraperitoneal injection to replace the blood lost. In addition, a heater was used to monitor and maintain body temperature of the rats at 37 ℃ for 24 h to reduce postoperative mortality. The rats were assisted in urinating twice daily until the micturition reflex has recovered. For HBO treatment, the chamber was flushed with pure oxygen for 10 min, and then the pressure was increased at a rate of 0.2 MPa (2 ATA) per 20 min. The oxygen concentration in the chamber was maintained at 90%. After continuous oxygen treatment for 60 minutes, decompression was performed at a uniform rate within 20 min. HBO therapy was conducted once daily for 10 consecutive days. The time point of administration of series HBO treatment for rats is at posttraumatic 8 h.

2.3 Drugs Administration

The SIRT1 agonist SRT1720 and antagonist EX-527 was purchased from Selleck Chemical (Houston, TX) and dissolved in 1% dimethyl sulfoxide (DMSO). All of the drugs were diluted with 0.9% saline to the appropriate concentration. After induced traumatic SCI, SRT1720 and EX-527 was intraperitoneally injected (i.p.) at concentration of 150 mg/kg and 100 mg/kg respectively once daily for 7 day.

2.4 Behavior Test

The Basso, Beattie Bresnahan (BBB) locomotor rating scale was used to evaluate the open-field locomotor function as previously described[15]. In brief, rats were exposed to the behavioral testing facility daily for 1 week to acclimate them to open-field exploration before the SCI surgery was performed. Then the BBB scores was assessed according to the BBB locomotor rating scale. The examiners were blinded to the type of treatment each animal received.

2.5 Tissue collection

After animals were anesthetized with 5% pentobarbital sodium (50 mg/kg), the spinal cord(T9-T11) tissues were removed and frozen in liquid nitrogen for the western blot and RT-PCR analysis.

2.6 Quantitative RT-PCR

Total RNA extractions frozen tissue specimens were conducted with TRIzol® reagent (Invitrogen). The Takara Reverse Transcription System Kit (Takara Biotechnology Co. Ltd, Japan) were used to synthesized cDNA. The quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed using the SYBR green premix kit (BioRad, Hercules, CA, USA). GAPDH was used as internal controls. The primer sequences were as the following. SIRT1 Forward: ACCTCCTCATTGTTATTGGGTCTTC, Reverse: GGCATACTCGCCACCTAACC.

2.7 Western Blot

Western blot was performed as the previous study. General speaking, spinal cord tissues was homogenized in 15 mmol/l Tris containing a cocktail of proteinase inhibitors and phosphatase inhibitors.

The extracted protein after boiled for 6 min was separated by 10% SDS-PAGE (BioRad, Germany) and then transferred onto a PVDF membrane (Millipore, USA). Then the PVDF membrane is blocked with 5% fat-free milk for 60 min. Primary antibodies were incubated at 4 °C overnight. In the following day, the TBST is used to wash out the primary antibodies and the membranes are incubated with secondary antibodies. The primary antibodies used in the experiment were as follows: rabbit SIRT1(1:1000, Cell Signaling Technology#2496), rabbit NF-κB(1:2000, Abcam#ab16502), rabbit NF-κB(Acetyl K310)(1:2000, Abcam#ab16502), mouse p62(1:1000, Cell Signaling Technology#88588), rabbit anti-ATG-5(1:1000, Cell Signaling Technology#12994), rabbit anti-Beclin-1(1:1500, Abcam#ab207612), rabbit anti-LC3B (1:1000, Abcam#ab48394),mouse anti-GAPDH (1:1500, Abcam#ab8245)

2.8 Enzyme-linked immunosorbent (Elisa) assay

The tissue sample was collected and immediately homogenized in extraction buffer with protease inhibitor. The inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-10) and caspase (caspase1, caspase3, caspase 8 and caspase 12) were detected as manufactures instruction.

2.9 SIRT1 activity assay

SIRT1 enzymatic activity was measured using a fluorescent assay kit (Cayman) according to the manufacturer's instructions. Briefly, tissue samples were sonicated in RIPA buffer (0.7% Nadeoxycholate, 0.5 M LiCl, 50 mM HEPES-KOH, pH 7.6, 1% NP-40, 1 mM EDTA) and lysates were mixed with assay buffer and substrate and incubated for 45 min at room temperature with shaking. Then, developing solutionwas added and incubated for 30 min. Fluorescence was measured with a microplate reader (excitation:360 nm, emission: 465 nm; SaFire 2, Tecan).

2.10 Data and analysis

Data were presented as the mean values ± SEM and analyzed with GraphPad Prism™, version 6.00 software (GraphPad, La Jolla, CA, USA). The Student's t-test or one-way ANOVA was used to determine statistical significance of differences between two groups or among variant groups, respectively. A p value□0.05 was considered statistically significant.

3. Results

3.1 The reduced expression and activity of SIRT1 induced by traumatic SCI was significant inhibited following the HBO treatment

In consistent with previous study[15], we found that application of series HBO treatment began at 8 h following the SCI injury in rats and lasted for 7 continuous days which significantly alleviated the SCI-induced injury locomotor dysfunction (Fig. 1A). As show in Fig. 1B, a decrease protein level of spinal cord SIRT1 was induced by SCI injury in rats since the 8 h (1/3 day) which lasted at least at day 14 postinjury. To further evaluate the role of SIRT1 in effects of HBO on SCI-induced locomotor dysfunction, we observed that the reduced expression of mRNA and protein of spinal cord SIRT1 in SCI rats on day 7

postinjury was dramatically upregulated by HBO treatment (Fig. 1C and Fig. 1.D). In addition, SIRT1 activity assay revealed that series HBO administration overturned the decrease of SIRT1 activity on day 7 postinjury (Fig. 1.E).

3.2 SIRT1 was implicated in the beneficial of series HBO treatment on traumatic SCI-induced locomotor dysfunction in rats

Given the modulation of series HBO treatment on SIRT1, we next determined to assess whether the SIRT1 function was involved in the effects of HBO treatment on locomotor deficit.We found that the series HBO treatment together with SIRT1 antagonist EX-527 treatment for consecutive 7 days almost blocked the effects of HBO treatment on alleviation of locomotor deficit (Fig. 2.A). However, the recovery effect of series HBO treatment on locomotor dysfunction was boosted after application of SIRT1 agonist SRT1720 for continuous 7 days (Fig. 2.B). Furthermore, SIRT1 antagonist EX-527 impeded the enhanced activity of SIRT1 following series HBO treatment in SCI rats (Fig. 2C), while SIRT1 agonist SRT1720 for consecutive 7 days and series HBO treatment had synergistic effects on activity of SIRT1 (Fig. 2D).

3.3 Series HBO treatment regulated the inflammatory cascade depending the function of SIRT1

Considering that the SIRT1 can directly deacetylate and subsequently inactivate the important inflammatory transcription factor NF-κB[20], which inhibit the exposure of the inflammatory cascade. The western blots showed that the acetylation of spinal cord NF-κB was significantly enhanced following the traumatic SCI injury on day 7 postinjury, while the series HBO treatment stopped the process of acetylation of NF-κB (Fig. 3A). Next, we performed a series of Elisa assays to elucidate the role of SIRT1 in HBO treatment on inflammatory cascade. Firstly, we detected three important pro-inflammatory cytokines(TNF-α,IL-6 ,IL-1β) and a well-known anti-inflammatory cytokines(IL-10) in spinal cord of rats on day 7 postinjury and following the series HBO treatment. As shown in the Fig. 3B, the enhanced expression of TNF-α、IL-6 and IL-1β in spinal cord of SCI rats was ameliorated by HBO therapy on day 7, which was retarded by the administration of SIRT1 antagonist EX-527 treatment for consecutive 7 days(Fig. 3C). In addition, the spinal cord of SCI injury rats that undergone the series HBO treatment together with SIRT1 agonist SRT1720 treatment for consecutive 7 days showed a decrease of TNF-α、IL-1β but not IL-6 and abundance of IL-10 compared with that of series HBO treatment together with vehicle(Fig. 4C).

3.4 SIRT1 was involved in the effects of series HBO treatment on regulation of caspase family.

It has been well established caspase family play a key role in the apoptosis pathway[25] which mediated the process of secondary injury following the SCI[26]. SIRT1 has been characterized as inhibiting the apoptosis. Based on the above evidence, we conducted Elisa assay to detect the expression of caspase family. As shown in the Fig. 4A, series HBO treatment led to a reduction of the increased expression of caspase 1, caspase 3, caspase 8 but not caspase 12 of spinal cord in SCI rats on day 7 postinjury. To further evaluate the SIRT1 function in effects of series HBO treatment on caspase family, both intraperitoneal injection SIRT1 antagonist for continuous 7 days abolished the HBO effects on caspase 1, caspase 3 and caspase 8 at day 7 postinjury (Fig. 4B). Furthermore, the SIRT1 agonist enhanced the effects of HBO therapy on caspase 3 and caspase 8 but not caspase1 and caspase12(Fig. 4C).

3.5 Series HBO treatment boosted the induction of autophagy postinjury in rats

Emerging evidences demonstrated that activation of SIRT1 promoted the cell survival by induction of autophagy[27]. Additionally, SCI injury induced the autophagy to protect the damage of neuron in spinal cord. To assess whether series HBO treatment have effects on autophagy, we performed western blotting assay to detect the protein level of autophagy-related protein. As shown in Fig. 5A-Fig. 5C, the expression of ATG-5, Beclin-1 and LC3-II which played a central role in the process of autophagy formation was enhanced at day 7 postinjury following the traumatic SCI injury and the series HBO treatment boosted the enhanced the effects. However, the decreased p62 implicated in the termination of autophagy in rats after SCI injury was upregulated by HBO therapy at day 7 postinjury (Fig. 5D).

3.6 The enhanced induction of autophagy following the series HBO therapy in rats subjected to SCI was diminished by EX-527

Recently a study reported that SIRT1 improved motor nerve regeneration after peripheral nerve injury through activation of autophagy[28]. These findings instigated us to further explore whether SIRT1 was participated in the central nerve injury following the series HBO therapy. The immunoblot showed that the series HBO treatment promoted the increased pro-autophagy formation protein (ATG5, Beclin-1 and LC3-II), which was suppressed by administration of SIRT1 antagonist for continuous 7 days (Fig. 6A-Fig. 6C), while expression of the reduced anti-autophagy formation protein (p62) was increased after application of SIRT1 antagonist for continuous 7 days (Fig. 6.D). These results may indicate that series HBO promoted autophagy via promotion the activation of activity of SIRT1 in rats subjected to SCI injury.

4. Discussion

In the present study, we first found that series HBO therapy impeded the reduction of expression and activity of spinal cord SIRT1 induced by traumatic SCI. EX-527, a SIRT1 selective antagonist, abolished the beneficial effects of series HBO therapy on SCI-induced locomotor deficits by inhibiting the activity of

SIRT1. However, the intraperitoneal injection of SIRT1 agonist SRT1720 boosted series HBO function outcome on locomotor dysfunction. In addition, we observed that series HBO treatment enhanced the activity of SIRT1 and subsequent suppressed the inflammatory cascade, apoptosis-related proteins. Furthermore, SIRT1 enhanced postinjury autophagy through upregulation the autophagy formation proteins. Taken together, above results suggested a new mechanism by which series HBO therapy regulated the expression and activity of SIRT1 and subsequently modulated the inflammatory cascade, apoptosis, and autophagy, which improved the motor dysfunction recovery.

4.1 The mechanism that series HBO mediated the inflammation cascade in a SIRT1 dependent manner.

The traumatic injury exposed the spinal cord to an infiltration of inflammatory cells and induced the overwhelming inflammatory response[6]. Furthermore, the neuron and glia of spinal cord around the injury site could propagate the inflammatory response[1, 29]. The inflammatory cascade has been well known for its role in the process from primary to secondary damage following the SCI. Therefore, controlling the inflammatory reaction has been thought to be a versatile choice for this intractable problem. Methylprednisolone is the typical drug to control inflammatory cascade after SCI, but its side effect may restrain its function outcome[30]. Emerging evidence suggested that HBO treatment reduced the inflammatory response which may have fewer side effects[31]. However, the precise mechanism underlying the anti-inflammation function of HBO deserved further investigated. Couple of studies revealed that SIRT1 can directly deacetylated histone and pivotal transcript factor such as NF-κB, resulting in the repression the inflammatory cytokines expression[20]. In the present study, we firstly found that the enhanced acetylation of NF-κB after SCI was reduced by series HBO therapy. Series HBO therapy reduced the expression of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) and increased that of anti-inflammatory cytokine (IL-10) by activation of SIRT1. So, it is possible that series HBO therapy activated the SIRT1 and subsequently deacetylated the NF-κB which resulted in suppressing of inflammatory response induced by SCI and improving the locomotor dysfunction. In addition, application of SIRT1 agonist together with HBO therapy enhanced the effects of HBO on anti-inflammation and locomotor deficits, which may present a new potential strategy to alleviate the SCI-induced locomotor dysfunction.

4.2 The mechanism that series HBO mediated apoptosis and autophagy

Activation of pro-apoptosis signaling played a central role in the process of cell death that survived from primary damage and thus controlling the apoptosis pathway can be an attractive intervention for the neuroprotection[32]. A vast array of protein was involved in the process of apoptosis. Among them, the caspase family, TNF-α and IL-1β acted as pivotal role in pro-apoptosis. In our study, we revealed that series HBO treatment suppressed the enhanced expression of caspase 1, caspase 3, caspase 8, TNF-α and IL-1β but not caspase 12 in spinal cord of rats subjected to traumatic SCI via regulation of SIRT1. TNF-α following binding with TNFR mediates the recruitment and activation caspase 8 to amplify the apoptosis reaction. The activated caspase 1 participates in the cleavage and maturation of IL-1β. The

above evidence indicated that series HBO repressed TNFR/caspase 8 and caspase 1/ IL-1β pro-apoptosis signaling pathway through regulation of SIRT1. Recently, autophagy has been reported to promote neuronal survive via protecting motoneurons from apoptosis[28]. Importantly, SIRT1 positively regulated the autophagy in different cells[33, 34]. In the present study, we found that series HBO therapy enhanced the induced autophagy-related proteins through activation of SIRT1 which suggested that modulation of SIRT1 by series HBO may serve as neuroprotection for the survival neurons from the primary injury through enhancing the autophagy.

Conclusion

Taken together, we demonstrated that series HBO therapy regulated the SIRT1 and improved the locomotor dysfunction in rats subjected to traumatic SCI. Series HBO treatment modulated the inflammation cascade, apoptosis and autophagy in a SIRT1 dependent manner. Herein, our study opened an avenue to combination of HBO and SIRT agonist for alleviation motor dysfunction after SCI.

Abbreviations

HBO: hyperbaric oxygenation; SCI: spinal cord injury; SIRT1: sirtuin1; BBB: Basso, Beattie Bresnahan; MCP-1: monocyte chemoattractant protein-1; NAD: nicotinamide adenine dinucleotide; NF-κB: nuclear factor kappa B; PPARγ: peroxisome proliferator-activated receptor γ; ATG-5: autophagy related protein-5; LC-3: microtubule associated protein light chain-3; IL-6: interleukin-6; IL-1β: interleukin-1β; IL-10: interleukin-10; TNF-α: tumor necrosis factor-α.

Declarations

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Huiqiang Chen and Huai Huang designed the study and Huiqiang Chen, Mengyu Yao, Zhibo Li, and Ranran Xing performed the experiments. Huiqiang Chen, Cheng Zhang, Yong Dai, Xinwei Yin and Jiewen

Tang analyzed the data and Huai Huang wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval

The experiments performed in accordance with the guidelines of the General Hospital of Southern Theater Command Animal Ethic Committee. The protocol number is SYXK (Yue) 2019-0100.

Consent for publication

All authors were consent for publication.

Conflict of interests

There are no conflicts to declare.

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Figures

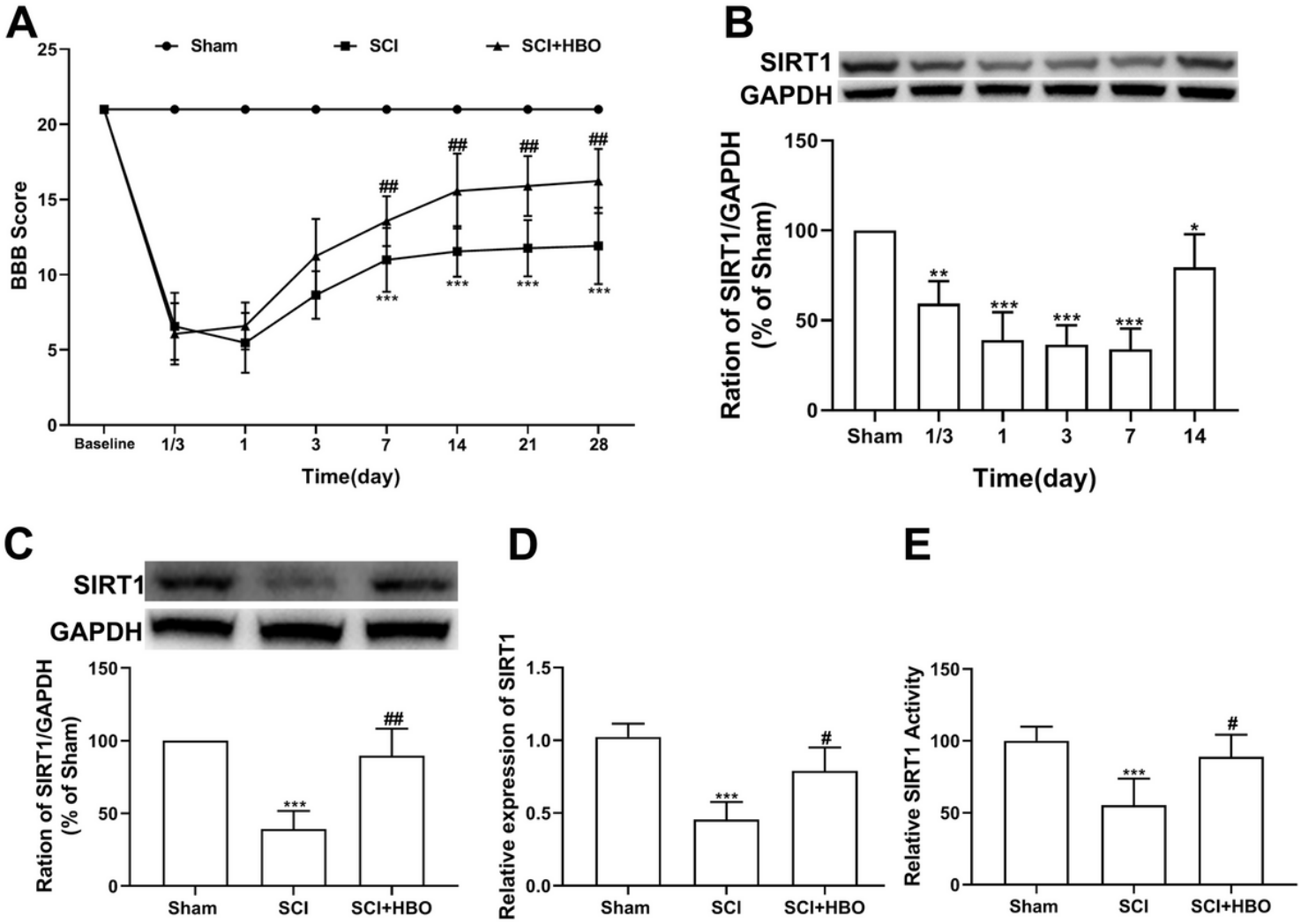

Figure 2

HBO therapy enhanced the decreased expression and activity of spinal cord SIRT1 induced by traumatic SCI. (A) The locomotor dysfunction was significant improved after traumatic SCI rats were applicated with series HBO therapy (n=12/per group). (B) The protein level of spinal cord SIRT1 began to reduce at 8 hour (1/3 day) and lasted at least at day 14 after the SCI injury established (n=4/per group). (C-E) HBO treatment for consecutive 7 days significantly enhanced the decreased protein, mRNA and activity of SIRT1 in spinal cord (n=5/per group). *p□0.05, **p□0.01, ***p□0.001 compared with sham group, #p□0.05, ##p□0.01 compared with SCI group.

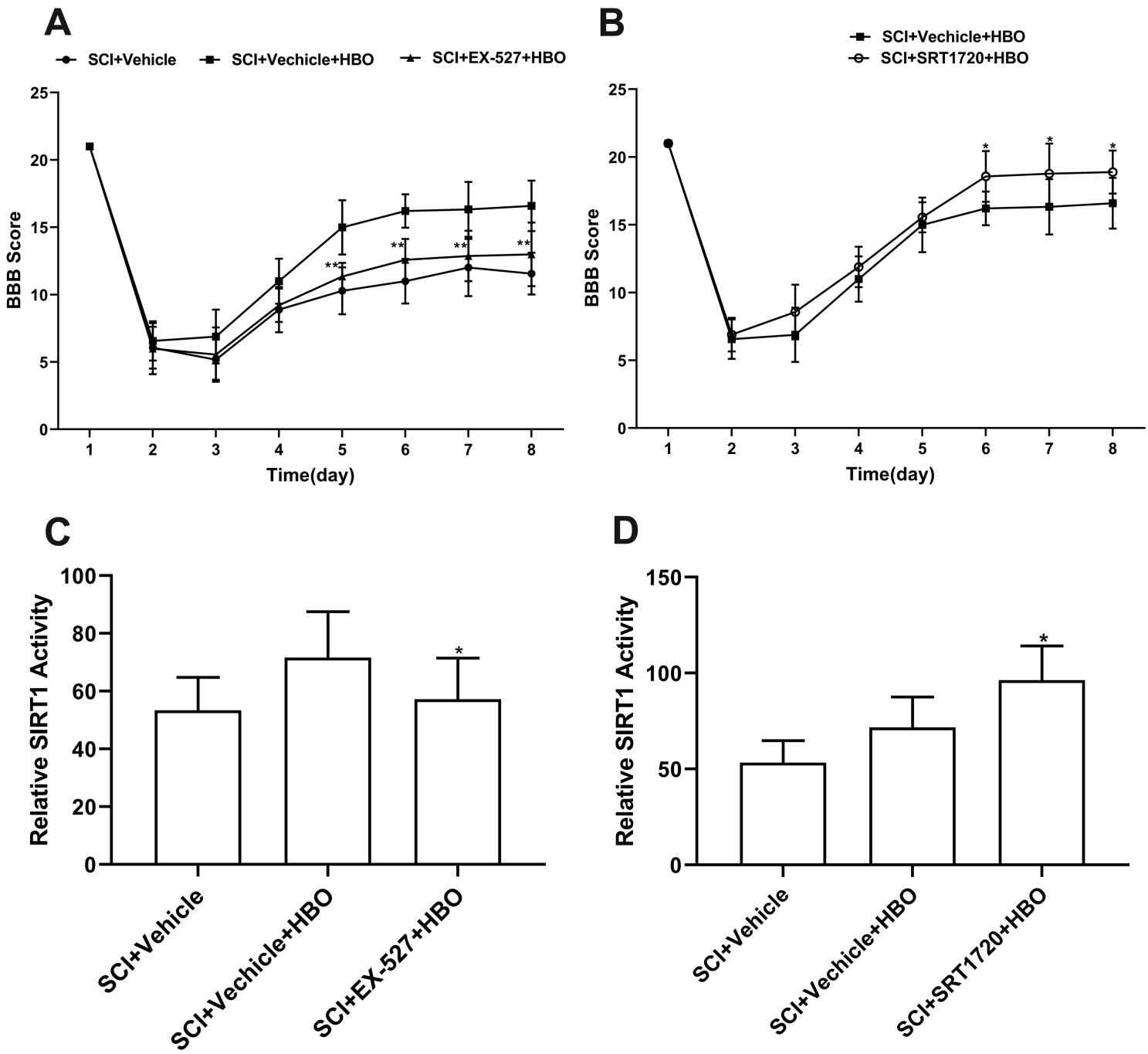

Figure 4

Administration of SIRT1 antagonist EX-527 reversed the beneficial effects of HBO on locomotor recovery, whereas the SIRT1 agonist SRT1720 had synergic effects on the motor function with HBO therapy. (A-B) The SCI injury rats were applicated with both HBO and EX-527 (i.p.) for continuous 7 days (started at 8h postinjury) got the worse BBB scores compared with those with only administration of HBO and vehicle therapy, while the injury rats treated by HBO and intraperitoneal injection SRT1720 had the higher BBB scores versus those with only administration of HBO and vehicle therapy (n=12 per group). (C-D) The EX-

527 inhibited the enhanced activity of SIRT1 induced by HBO treatment, while SRT1720 increased effects of HBO therapy on the activity of SIRT1(n=5 per group). *p<0.05, **p<0.01compared with SCI +vehicle+HBO group.

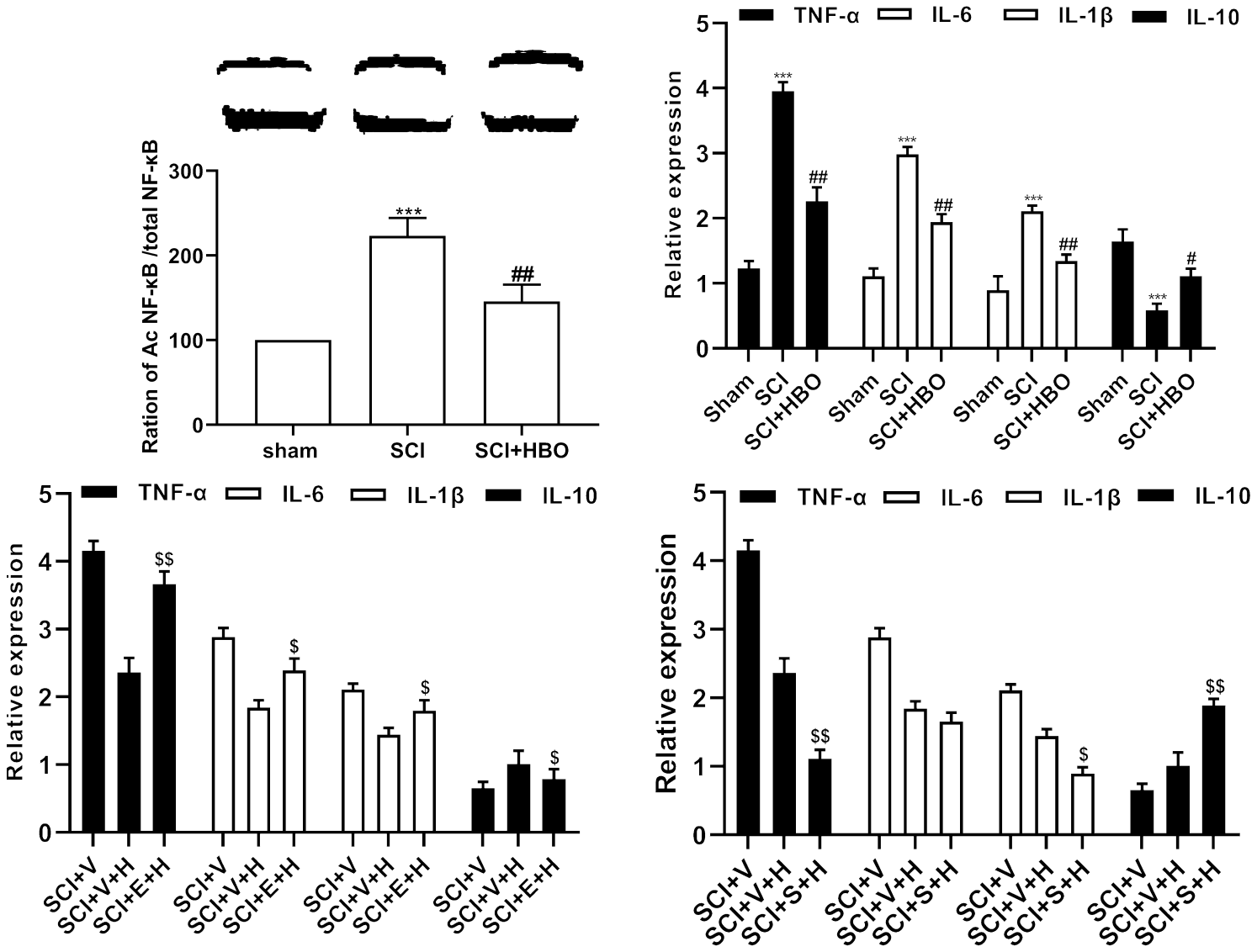

Figure 6

SIRT1 was implicated in the inflammatory cascade induced by traumatic SCI in rats. (A)The enhanced acetylation of NF-κB in SCI rats was remarkably inhibited by HBO treatment (n=4/per group). (B)The increase of pro-inflammatory cytokines TNF-α、IL-6 and IL-1β and decrease of anti-inflammatory cytokine IL-10 in spinal cord of SCI model rats was revered after the SCI rats was administrated with HBO (n=5/per group). (C) The effects of HBO treatment on TNF-α、IL-6, IL-1β and IL-10 was blocked by SIRT1 antagonist EX-527 (n=5/per group). (D) Both application of SIRT1 agonist SRT1720 and HBO promoted the effects of HBO in TNF-α、IL-1β and IL-10 but not IL-6 (n=5/per group). ***p<0.001 compared with sham group, #p<0.05, ##p<0.01 compared with SCI group, $$p<0.01 compared with SCI +vehicle+HBO group.

SCI+V(SCI+Vehicle), SCI+V+H (SCI+Vehicle+HBO), SCI+E+H(SCI+EX-527+HBO), SCI+S+H (SCI+SRT1720+HBO)

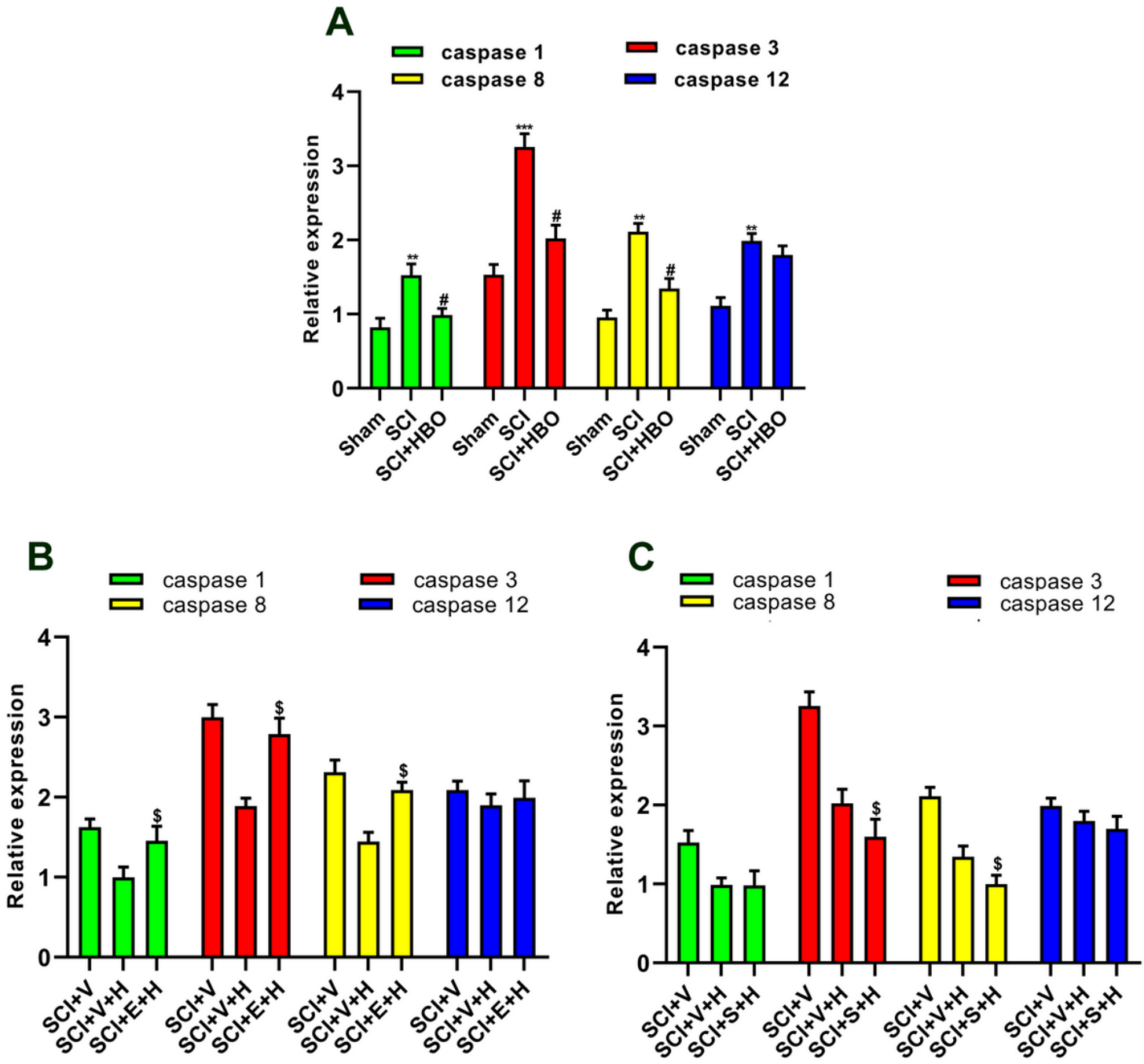

Figure 8

HBO modulated the caspase family in a SIRT1-depentent manner. (A) The increase of expression of caspase 1, caspase 3, caspase 8 but not that of caspase 12 in SCI rats was dramatically alleviated following the HBO treatment (n=4/per group). (B) After application of HBO and SIRT1 antagonist EX-527, the decreased caspase 1, caspase 3 and caspase 8 induced by HBO treatment was reversed (n=5/per

group). (C) The effects of HBO on the increased expression of caspase 3 and caspase 8 was enhanced following intraperitoneal injection of SIRT1 agonist (n=5/per group). **p0.01, ***p0.001 compared with sham group, #p0.05, $p0.05 compared with SCI +vehicle+HBO group. SCI+V(SCI+Vehicle), SCI+V+H (SCI+Vehicle+HBO), SCI+E+H(SCI+EX-527+HBO), SCI+S+H (SCI+SRT1720+HBO)

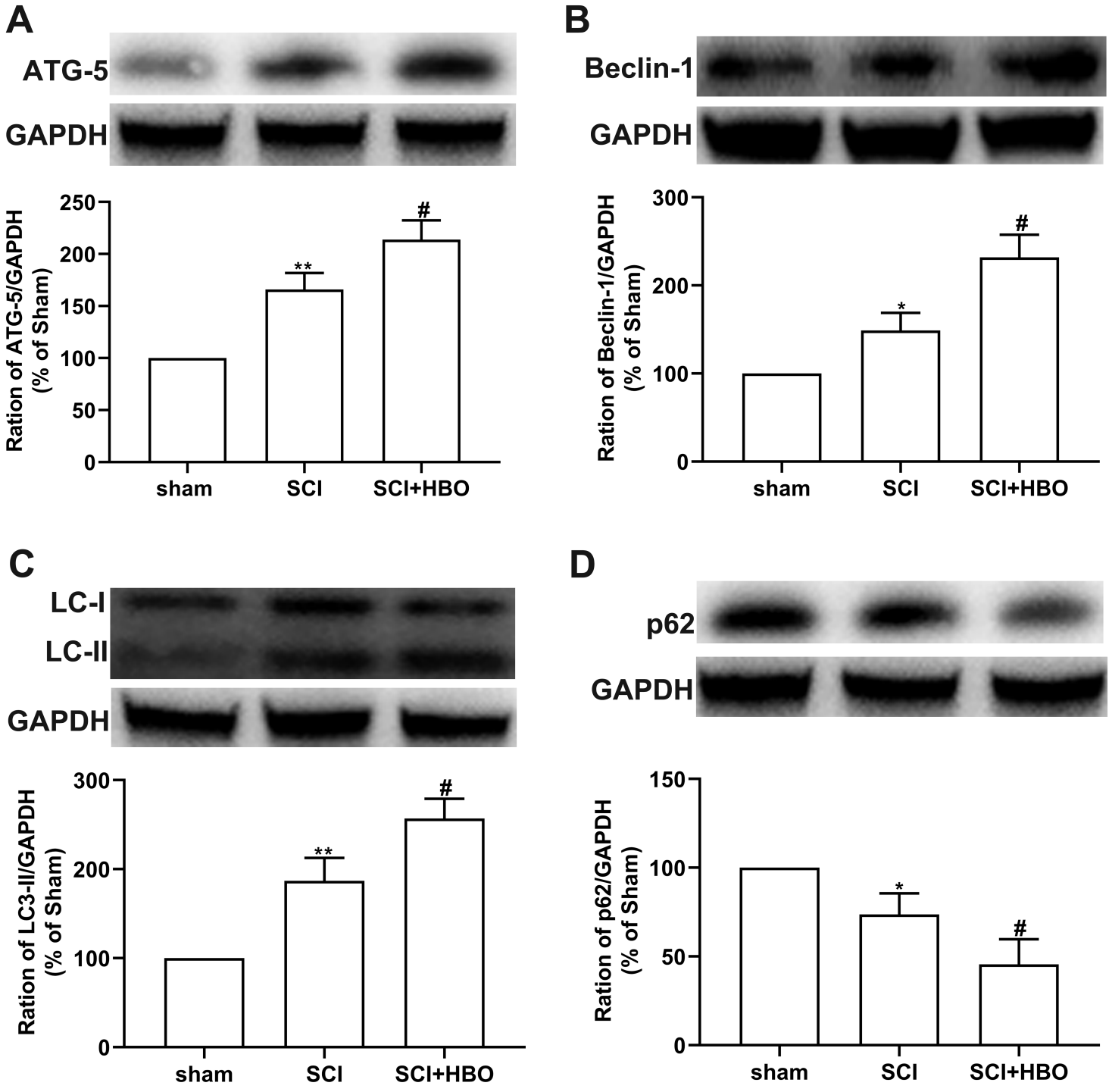

Figure 10

Induction of autophagy in spinal cord of the injury rats was enhanced following HBO treatment. (A-C) The increased expression of autophagy-related protein ATG-5, Beclin-1, LC-II following the injury was

enhanced (n=5 per/group). (D) The decreased expression of p62 was induced by SCI injury and boosted by HBO treatment (n=5 per/group). *p□0.05,**p□0.01 compared with sham group, #p□0.05 compared with SCI group.

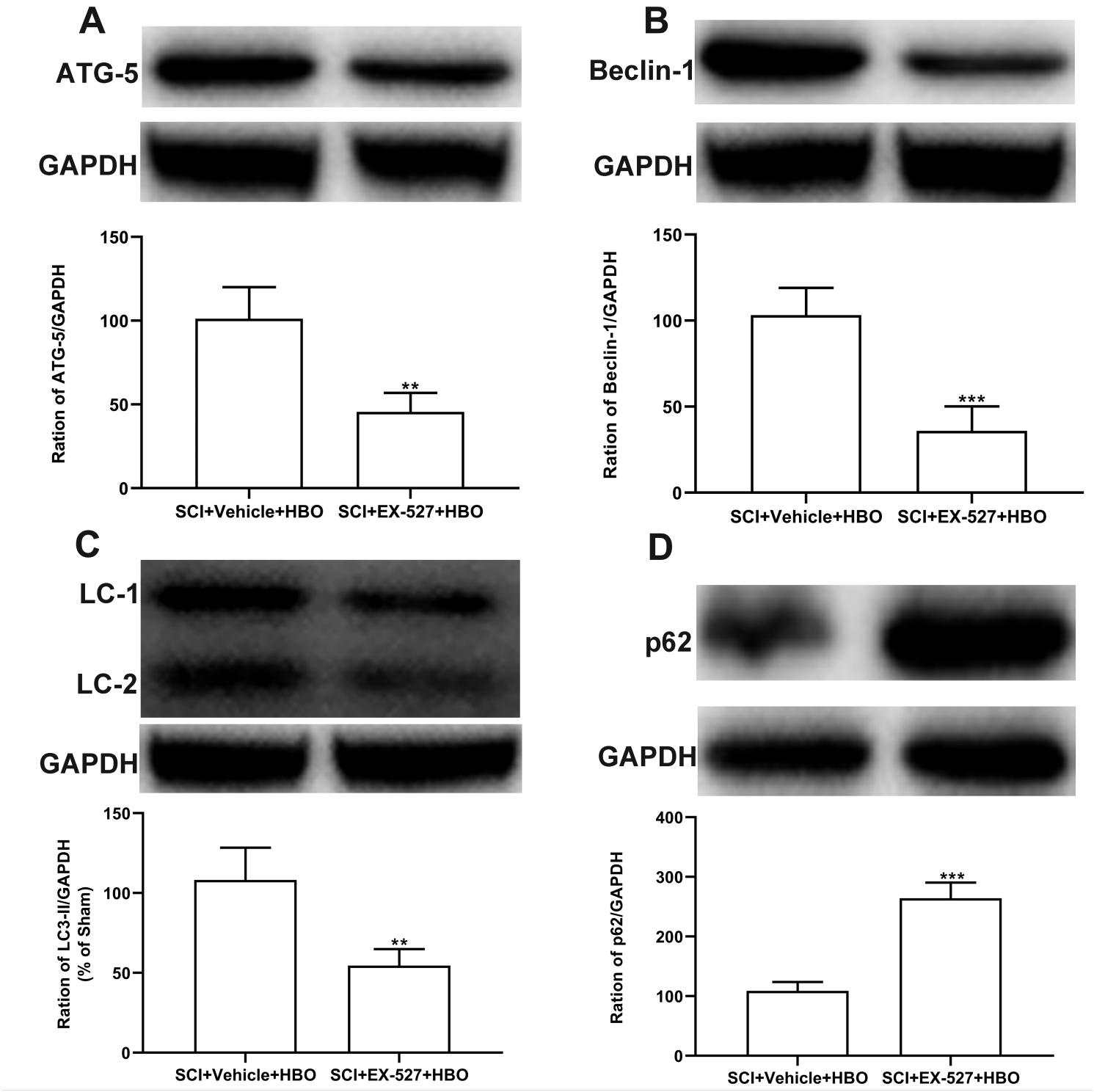

Figure 12

The enhanced autophagy-related protein by HBO treatment reduced after administration of EX-527. (A-C) Intraperitoneal injection of SIRT1 antagonist EX-527 abolished the increased expression of pro-autophagy formation related protein (ATG-5, beclin-1 and LC3-2) in SCI rats following the series HBO therapy,

(D)while the level of autophagy substrate p62 was upregulated by HBO (n=5/per group). **p□0.01, ***p□ 0.001 compared with SCI+Vehicle+HBO group.