Piperine Attenuates the Inflammatory Response, Oxidative Stress and Pyroptosis to Facilitate Recovery After Spinal Cord Injury via Autophagy Enhancement

Haojie Zhang

Wenzhou Medical College Second Affiliated Hospital; Wenzhou Medical University Second Affiliated Hospital

Chenyu Wu

Wenzhou Medical University Second Affiliated Hospital

Jin-Feng Huang

Wenzhou Medical University Second Affiliated Hospital

Yanlin Chen

Wenzhou Medical University Second Affiliated Hospital

Wen-Fei Ni (✉ wenfeini@yeah.net)

Wenzhou Medical University Second Affiliated Hospital

Research

Keywords: spinal cord injury, pyroptosis, inflammation, oxidative stress, piperine

Posted Date: March 5th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-257883/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Spinal cord injury (SCI) is a serious injury that can lead to irreversible motor dysfunction and subsequently result in disability and even death. Due to its complicated pathogenic mechanism, there are no effective drug treatments. Piperine, a natural active alkaloid extracted from black pepper, suppressed inflammation in a previous study. The aim of this study was to investigate the therapeutic effect of piperine in a spinal cord injury model.

Methods: Spinal cord injury was induced in C57BL/6 mice by clamping the spinal cord with a vascular clip (15 g force; Oscar) for 1 min. Eighty mice were divided randomly into the following four groups: The Sham group (n = 20), the SCI+Vehicle group (n = 20), the SCI+ Piperine group (n = 20), and the SCI+ Piperine+3MA group (n = 20). Before SCI and every 2 days post-SCI, evaluations of the Basso mouse scale (BMS) were performed. On day 14 after SCI, inclined plane tests and footprint analyses were performed. On postoperative day 3, the spinal cord was harvested to assess pyroptosis, reactive oxygen species (ROS), inflammation, and autophagy. Qualitative or quantitative analysis of the components of these potential mechanisms was performed by Western blotting (WB), immunofluorescence (IF), quantitative real-time PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA).

Results: Piperine enhanced the functional recovery of spinal cord injury. Additionally, piperine inhibited inflammation, attenuated oxidative stress and pyroptosis, and activated autophagy. However, the effects of piperine on the functional recovery of SCI, ROS-mediated autophagy, inflammation and pyroptosis were reversed by the inhibition of autophagy.

Conclusions: Our experiments demonstrated that piperine facilitated the functional recovery of spinal cord injury by inhibiting the inflammatory response, oxidative stress and pyroptosis, which are mediated by the activation of autophagy.

Introduction

Spinal cord injury (SCI) is one of the most common central nervous system disorders and can result in a devastating loss of motor and sensory function.¹ It can also significantly reduce the quality of life and life expectancy of patients.^{2,3} Approximately 250,000 to 500,000 people suffer from SCI each year worldwide.⁴ However, there is currently no therapy that can achieve an ideal outcome for treating SCI. Therefore, investigation of the pathology and effective treatment of SCI is required.

The pathological process of SCI includes primary injury and secondary injury. Primary injury is caused by direct trauma to the spinal cord. Then, the primary injury can trigger a series of pathologies, including inflammation, oxidative stress and ischemia-reperfusion injury, to cause secondary injury.⁵⁻⁷ Secondary injury is considered an important component of SCI progression and a vital time period for the application of SCI therapy.⁸

Pyroptosis, which is mediated by gasdermin D (GSDMD), is a programmed inflammatory necrotizing cell death process.⁹ The main characteristic of pyroptosis is the activation of the inflammasome, represented by nod-like receptor family pyrin domain-containing 3 (NLRP3). Then, the inflammasome recruits and activates the proteolytic enzyme caspase-1. Finally, the proteolytic enzyme caspase-1 can lead to the cleavage of GSDMD and the maturation of the proinflammatory cytokines interleukin-1 β (pro-IL-1 β) and IL-18.^{10,11} These proinflammatory cytokines can lead to further inflammation. Fleming et al. pointed out that inflammation plays an important role in the expansion of secondary injury and inhibition of recovery of motor function after SCI. Recent studies have indicated that oxidative stress is involved in many neurological disorders (e.g., SCI)¹²⁻¹⁴ In addition, ROS production, which is significantly increased after SCI, is an important factor involved in secondary injury.¹⁵ Previous studies have demonstrated that oxidative stress and the production of ROS play a critical role in NLRP3 activation and the priming step of pyroptosis.^{16,17}

Autophagy, a self-protecting mechanism, facilitates the recycling of damaged organelles and degrades misfolded proteins.^{18,19} Under SCI conditions, moderate activation of autophagy can increase the survival and adaptation of spinal cord neurons.²⁰ Recently, an increasing number of studies have pointed out that activating autophagy can inhibit pyroptosis.²¹⁻²³ Autophagy can inhibit the activation of NLRP3 inflammasomes, which activate pyroptosis, and reduce pro-IL-1 β and IL-18. He et al. showed that activating autophagy can attenuate oxidative stress and reduce ROS production in spinal cord neurons.²⁴ In addition, autophagy can attenuate the inflammatory response.²⁵⁻²⁷ Therefore, autophagy may be a promising target to attenuate the inflammatory response, oxidative stress and pyroptosis of SCI and thus facilitate recovery after SCI.

Piperine, a natural compound, is present in black pepper (*Piper nigrum* Linn) and other related herbs.²⁸ Piperine has been reported to have multiple pharmacological activities, such as anticancer, antimicrobial, anti-asthmatic, antioxidative and anti-inflammatory activities.²⁹⁻³² Recently, one study showed that piperine can suppress systemic inflammation and pyroptosis to protect against bacterial sepsis.³³ Piperine can significantly suppress the activation of the NLRP3 inflammasome and inhibit the release of proinflammatory cytokines to block pyroptosis.³⁴ In addition, piperine can induce autophagy to inhibit the proliferation of prostate cancer cells.³⁵ However, whether piperine has a therapeutic effect on SCI remains unknown. In the present study, the effect of piperine on functional recovery from SCI was investigated in mice. The underlying mechanisms by which piperine-mediated autophagy activation inhibits inflammation, oxidative stress, and pyroptosis were analyzed in the SCI model.

Materials And Methods

Ethical Statement

All animal experiments in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the China National Institutes of Health. The animal experiments were approved by

the Animal Research Committee of Wenzhou Medical University (wydw2017-0022).

Reagents

Piperine was purchased from MedChemExpress (Form: powder, Purity: 98.94%). Pentobarbital sodium was purchased from Solarbio Science & Technology (Beijing, China). Primary antibodies against LC3B, p62, NLRP3, Beclin1, endothelial nitric oxide synthase (eNOS), superoxide dismutase 1 (SOD1), heme oxygenase 1 (HO1), ATG5 and ATP6V1B2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies against iNOS and Cox-2 were obtained from Abcam (Cambridge, UK). Rabbit monoclonal anti-phosphoinositide-3-kinase (VPS34) and anti-cathepsin D (CTSD) antibodies were purchased from the Proteintech Group (Chicago, IL, USA). The primary antibody against GAPDH was obtained from Biogot Technology (AP0063; Shanghai, China). Antibodies against GSDMD and C-CASP1 were obtained from Affinity Biosciences (OH, USA). 3-Methyladenine (3MA) was purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). The fluorescein isothiocyanate (FITC)-conjugated IgG secondary antibody was purchased from Boyun Biotechnology (Nanjing, China), and the horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) secondary antibody was provided by Santa Cruz Biotechnology (Dallas, TX, USA). The 4',6-diamidino-2-phenylindole (DAPI) solution was obtained from Beyotime Biotechnology (Jiangsu, China). The BCA Kit was obtained from Thermo Fisher Scientific (Rockford, IL, USA). The Electrochemiluminescence (ECL) Plus Reagent Kit was acquired from PerkinElmer Life Sciences (Waltham, MA, USA).

SCI model and groups

C57BL/6 mice (male, 20–30 g) were obtained from the Experimental Animal Center of Wenzhou Medical University (License No. SCXK [ZJ] 2005-0019). All experimental procedures followed the Chinese Guidelines of Animal Care and Welfare. This study was approved by the Wenzhou Medical University Animal Care and Use Committee. Eighty mice were divided randomly into the following four groups: The Sham group (n = 20), the SCI+Vehicle group (n = 20), the SCI+ Piperine group (n = 20), and the SCI+ Piperine+3MA group (n = 20). Before surgery, mice were anaesthetized with 1% (w/v) sodium pentobarbital (50 mg/kg). Next, we cut the ligaments and muscles around the T8 spinous process and then performed a laminectomy at the T8 vertebral level to expose the spinal cord. A vascular clip (15 g force; Oscar) was used to clamp the spinal cord for 1 min to induce SCI. The same surgical procedure was performed in the Sham group but without compression of the spinal cord. After surgery, the bladders of the mice were emptied manually every day. In addition, the SCI+ Piperine group was given 10 mg/kg piperine (dissolved in a solution of 2% DMSO in normal saline) by peritoneal injection immediately after SCI and then daily until they were sacrificed. The other groups were injected simultaneously with the same dose of saline. The SCI+ Piperine + 3MA group was treated with 3MA (15 mg/kg) 30 min before piperine injection. In each group, ten mice were for evaluating functional recovery and another ten mice were for follow-up experiments.

Evaluation of locomotor recovery

The locomotor function of mice was assessed using the Basso Mouse Scale (BMS), the inclined plane test and the footprint test. The BMS score ranges from 0 (complete paraplegia) to a score of 9 (normal function). The inclined plane test was used to determine the maximum angle at which the mice could remain on the testing apparatus without falling for least 5 seconds in two positions. All tests above were carried out in an open-field apparatus. We used footprint analysis to assess the rotation angle, base of support and stride length, which measure relative paw placement and regularity. The BMS score were measured before SCI and every 2 days post-SCI. Inclined plane tests and footprint analyses were conducted on day 14 after SCI. Each test was scored by four investigators who were blinded to the experimental groups.

Quantitative real-time PCR (qRT-PCR)

The mRNA levels of inflammatory factors were measured by qRT-PCR. Three days after the surgery, mice were euthanized with 2% (w/v) sodium pentobarbital (200 mg/kg) for euthanizing. Parts of the spinal cord (10 mm) containing the lesion epicenter were harvested at 3 days after the surgery, and total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA by reverse transcription using 1st Strand cDNA Synthesis SuperMix for qPCR (11123ES10, Yeasen). qRT-PCR was performed using SYBR Green reagent (Bio-Rad) and a CFX96® Real-Time PCR System (Bio-Rad). Primers for IL-6, TNF- α , IL-1 β , IFN- γ and GAPDH are listed in the supplementary information. **(Supplementary Table S1)**

Western blot analysis

Total protein was extracted from spinal cord tissues. Next, the extracts were quantified by a BCA kit (Beyotime) and equilibrated to the same protein concentration. We used 12% SDS-polyacrylamide gel electrophoresis to separate the protein samples and then transferred them to polyvinylidene fluoride membranes (Millipore, 0.2 μ m). The membranes were blocked with 5% (w/v) nonfat milk for 2 h at room temperature and subsequently incubated with the primary antibody overnight at 4°C. The membranes were then incubated with the appropriate secondary antibodies for 2 h at room temperature. The bands on the membranes were detected by the ECL Plus Reagent Kit and quantified using Image Lab 3.0 software (Bio-Rad, Hercules, CA, USA).

Immunofluorescence

On postoperative day 3, mice were euthanized, and the spinal cords were collected and fixed in 4% (w/v) paraformaldehyde for 24 h. The samples were embedded in paraffin for transverse paraffin sections. Then, the paraffin sections (approximately 4 mm thick) were mounted on poly-L-lysine-coated slides.

Next, the sections were deparaffinized, rehydrated, washed, and then treated with 10.2 mM sodium citrate buffer for 10 min at 95°C. The sections were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 30 min. After blocking with 10% (v/v) bovine serum albumin in PBS for 30 min, the samples were incubated with antibodies against CASP1 (1:300)/NeuN (1:400), LC3 (1:200)/NeuN (1:400) or p62 (1:200)/NeuN

(1:400) at 4°C overnight. The next day, the samples were incubated with secondary antibodies at room temperature for 1 h. A fluorescence microscope (Olympus, Tokyo, Japan) was used to visualize and assess the spinal cord in six randomly selected fields from each sample.

Detection of MAD and SOD

Mice were sacrificed on postoperative day 3. The spinal cord was harvested and frozen in liquid nitrogen. The level of superoxide dismutase (SOD) in the tissue was measured as an index of tissue antioxidant activity, while malondialdehyde (MDA) was measured as an index of the lipid level in the tissue. First, the spinal cord sample (approximately 100 µg) was dissolved in 1 ml normal saline at 4°C. Then, the supernatant was harvested by centrifugation. The MDA concentration (nmol/mg) and SOD activity (U/m) were assessed. The amount of protein in the sample was calculated by using the BCA Kit. (Jiancheng Bioengineering Institute, Nanjing, China)

Enzyme-linked immunosorbent assay

Parts of the spinal cord (10 mm) containing the lesion epicenter were harvested at 3 days after the surgery. The samples were harvested and washed with PBS and then homogenized in lysis buffer (1× RIPA lysis buffer). The samples were then centrifuged at 15,000 RPM for 15 min at 4°C. The tissue lysate protein concentration was calculated using a BCA kit. The protein levels of IL-1β, IL-18, IL-6, TNF-α and IFN-γ were measured by using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis

All data are shown as the mean ± standard deviation (SD). All statistical analyses were performed by using SPSS software (version 18; IBM Corp., USA). Statistical significance was calculated by one-way analysis of variance (ANOVA) followed by Tukey's analysis or *t*-tests for comparisons between control and treatment groups. In addition, generalized linear mixed models were used to analyze significant differences in the BMS score. We considered **P* < .05 or ***P* < .01 as statistically significant.

Results

Piperine promotes functional recovery

The BMS score were used to assess locomotor function recovery in the Sham, Vehicle, Piperine and Piperine + 3MA groups pre-SCI and every 2 days post-SCI. After surgery, both BMS score were significantly higher in the Sham group than in the Vehicle group at different time points; after piperine treatment, the SCI group had higher BMS score than the SCI group. However, the changes were attenuated by 3-MA (**Figure 1A**). In the inclined plane test, we then tested the inclined plane angle, and in the footprint analysis test, we measured the rotation angle, base of support and stride length in the 4 four groups. The inclined plane angle and stride length results showed that the mice in the Vehicle group had lower scores than those in the Sham group, while piperine restored the scores, and 3-MA reversed the effect of piperine

(Figure 1B, E). Moreover, the rotation angle and base of support in the Vehicle group were higher than those in the Sham group. In contrast, piperine decreased the data above, while 3-MA inhibited the effects of piperine (Figure 1C, D).

Piperine enhances autophagy after SCI

To evaluate the level of autophagy at the spinal cord lesion after SCI, we measured the levels of autophagosomal markers (Beclin1, ATG5, VPS34 and LC3II), autolysosome-related markers (CTSD and ATP6V1B2) and a substrate protein (p62). The immunofluorescence results showed that the percentage of LC3II-positive neurons was higher in the SCI group than in the Sham group. Furthermore, piperine treatment further increased the percentage of LC3II-positive neurons in SCI mice (Figure 2A, B). Similarly, the percentage of p62-positive neurons increased substantially after SCI, while the percentage of p62-positive neurons was lower in the Piperine group than in the SCI group (Figure 2C, D). The Western blotting results showed the expression levels of Beclin1, ATG5, VPS34, CTSD, ATP6V1B2, LC3II and p62 (Figure 2E). The optical density (OD) values for Beclin1, ATG5, VPS34, CTSD, ATP6V1B2, LC3II and p62 were higher in the Vehicle group than in the Sham group (Figure 2F–L). In addition, the changes in the OD values of these markers (except p62) were enhanced in the Piperine group (Figure 2F–L).

Piperine attenuates oxidative stress after SCI

Considering that SCI might result in oxidative stress, we next determined the effects of piperine on SOD, MDA, eNOS and HO-1 levels in the spinal cord. The level of SOD in the tissue was measured as an index of tissue antioxidant activity, and the level of MDA was measured as an index of the lipid level in the tissue. The activity of SOD decreased after SCI, while piperine attenuated the change (Figure 3A). Compared with the Sham group, the SCI group showed a significant decrease in the levels of SOD, eNOS and HO-1. Interestingly, piperine significantly attenuated the abnormally decreased levels of these markers (Figure 3 C, D, E, F). In addition, compared with the Sham group, the SCI group showed an abnormal increase in the level of MDA. However, piperine significantly attenuated the abnormally increased levels of MDA after SCI (Figure 3B).

Piperine attenuates pyroptosis after SCI

To assess the status of pyroptosis, we evaluated the pyroptosis biomarkers NLRP3, GSDMD, C-CASP1, IL-18 and IL-1 β to determine the pyroptosis level in the Sham, SCI + Vehicle and SCI + Piperine groups. As shown in Figure 4A, immunofluorescence staining was performed to show the expression of C-CASP1 in neurons at the lesion (C-CASP1 is labeled in green, the neurons are labeled in red, and the nuclei are labeled in blue). Our results showed that the fluorescence intensity of C-CASP1 was much brighter and the number of C-CASP1-positive cells was much greater in the SCI group than in the Sham group, but piperine significantly decreased the fluorescence intensity of C-CASP1 and diminished the number of C-CASP1 positive cells (Figure 4A, B). As shown in Figure 4B, the Western blotting results revealed the expression levels of NLRP3, GSDMD, and C-CASP1 (Figure 4C). The OD values of NLRP3, GSDMD, and C-CASP1 were higher in the Vehicle group than in the Sham group, while piperine attenuated these changes

(**Figure 4D–F**). The levels of IL-1 β and IL-18 were detected by ELISA, and the results showed that both IL-1 β and IL-18 levels in the Vehicle group were increased compared with those in the Sham group. Meanwhile, piperine significantly reversed the alterations in IL-1 β and IL-18 levels in the mice after SCI (**Figure 4G, H**).

Piperine suppresses inflammation after SCI

Three days after SCI, we used ELISA to evaluate the protein concentrations of proinflammatory cytokines, including IL-1 β , IL-6, TNF- α and IFN- γ , in the spinal cord of the three groups. Owing to the profound impact of the injured spinal cord, the SCI group had significantly increased protein concentrations of proinflammatory cytokines compared to the Sham group, while the administration of piperine significantly reversed the effects of SCI (**Figure 5A–D**). Because incorrect interpretation of inflammation could result when only the protein levels of these proinflammatory cytokines are measured, we used PCR to detect the mRNA levels of these proinflammatory cytokine proteins. Compared with the Sham group, the SCI group exhibited increased IL-1 β , IL-6, TNF- α and IFN- γ mRNA levels, indicating that increased transcription of proinflammatory cytokines may be directly related to SCI. Compared with the SCI group, the Piperine group exhibited significantly lower relative cytokine mRNA levels, suggesting that decreased transcription of these cytokines may be related to treatment with piperine (**Figure 5H–K**). We also detected the expression levels of proinflammatory factors, such as iNos and COX-2. Western blotting revealed that the expression levels of Cox-2 and iNos were increased in the SCI group compared to the Sham group; however, they were inhibited by piperine treatment in the SCI+Piperine group compared with the SCI group (**Figure 5E–G**).

Inhibition of autophagy reverses the effect of piperine on oxidative stress, pyroptosis, inflammation and functional recovery after SCI

After autophagy inhibition, the levels of autophagy, oxidative stress, pyroptosis and inflammation in SCI were evaluated by Western blotting. The expression levels of Beclin1, ATG5, VPS34, CTSD, ATP6V1B2, LC3II, and p62 were detected by Western blotting (**Figure 6A**). The results revealed that the OD values of Beclin1, ATG5, VPS34, CTSD, ATP6V1B2 and LC3II were lower in the SCI+Piperine+3MA group than in the SCI + Piperine group, while a higher OD value of p62 was observed in the SCI + Piperine group (**Figure 6B**). We also detected the expression levels of Cox-2, iNOS, NLRP3, GSDMD, C-CASP1, SOD1, eNOS and HO-1 by Western blotting (**Figure 6 C–E**). The OD values of Cox-2, iNOS, NLRP3, GSDMD, C-CASP1, SOD1, eNOS and HO-1 were higher in the SCI+Piperine+3MA group than in the SCI+ Piperine group (**Figure 6F–H**). We then measured the levels of IL-1 β and IL-18 by ELISA, and the results revealed that both IL-1 β and IL-18 levels in the SCI+Piperine+3MA group were increased compared with those in the SCI+ Piperine group (**Figure 6I, J**).

Discussion

Piperine, an alkaloid isolated from black pepper, has been highly appreciated for decades owing to its pharmacological and medicinal value, which includes anti-inflammatory, anti-arthritis, antidepressive and

anticoagulant activities³⁶. Many studies have proven that piperine plays an important role in the treatment of diverse diseases, including hepatocellular carcinoma³⁷, liver injury³⁸, tuberculosis³⁹, insulin resistance⁴⁰, and colon cancer⁴¹. SCI, which involves serious damage to the spinal cord caused by sustained or sudden trauma, can give rise to a wide range of complications. It remains unknown whether piperine can be used to treat SCI. Our research confirmed that piperine ameliorated SCI, and the potential mechanism included enhancing autophagy to suppress inflammation and ROS-mediated pyroptosis.

Autophagy is a highly conserved lysosomal pathway that involves the bulk degradation of cytoplasmic contents and has been deemed a predominant cause of nonapoptotic cellular death⁴². Autophagy is particularly important in neurons because neurons are terminally differentiated cells and must exist throughout the lifetime of the organism. In neurons, autophagy occurs through both constitutive and stress-induced pathways and catalyzes the degradation of a large number of damaged or aged mitochondria, the endoplasmic reticulum, other cellular organelles and polyribosomes. These two pathways are indispensable in the maintenance of neuronal homeostasis as well as neurodevelopment⁴³. In a previous study, substantial evidence was found that autophagy is directly related to some neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and frontotemporal dementia (FTD)⁴⁴. Boland et al. analyzed both postmortem and experimental models of human tissues, and the results suggested that dysfunction of autophagy might be a predominant contributor to pathogenicity across these diseases⁴⁵. Additionally, autophagy has generally been found to benefit mice after SCI. For example, Yuan et al. found that intermittent fasting exerted a neuroprotective effect after acute SCI via the upregulation of autophagy⁴⁶. Zhu et al. reported that piperine analogs promoted ROS and autophagy and regulated p38 and Akt/mTOR signaling⁴⁷. Thus, we evaluated the relationship between piperine and autophagy by measuring the levels of autophagy-related proteins. Our WB results showed that Beclin1, ATG5, VPS34, CTSD, ATP6V1B2 and LC3II levels were increased after SCI and were further increased in the Piperine group. Immunofluorescence staining for NeuN/LC3II and NeuN/P62 demonstrated that piperine upregulated the level of autophagy in neurons of spinal cord lesions as well. From the above findings, we can draw a conclusion based on our experimental results that piperine enhanced autophagy in SCI.

Pyroptosis, which is characterized by the swelling and rupture of cells, is a specific type of proinflammatory programmed cell death that can lyse cells and cause the release proinflammatory molecules⁴⁸. Piperine reportedly protected macrophages from pyroptosis in a mouse model intraperitoneally infected with *Escherichia coli*³³. In a previous study, it was reported that pyroptosis plays a central role in many diseases, including osteoarthritis⁴⁹, kidney diseases⁵⁰, rheumatoid arthritis⁵¹, and COVID-19⁵². Therefore, we suspected that piperine may suppress pyroptosis in SCI. We evaluated the level of pyroptosis in spinal cord tissue. The main feature of pyroptosis is the activation of the inflammasome, which can recruit and activate the proteolytic enzyme caspase-1, resulting in the cleavage of the pore-forming protein GSDMD protein and the activation of the proinflammatory cytokines interleukin-1b (IL-1b) and IL18⁵³. Consequently, we examined NLRP3, GSDMD, C-CASP1, IL-18 and IL-1β

expression to evaluate the level of pyroptosis in SCI. Western blotting results proved that piperine significantly decreased the levels of NLRP3, GSDMD and C-CASP1, while ELISA showed that piperine obviously decreased the levels of IL-18 and IL-1 β . Additionally, immunofluorescence staining for NeuN/C-CASP1 showed that piperine reduced the number of C-CASP1-positive neurons after SCI. These outcomes suggested that piperine attenuated pyroptosis in SCI.

Reactive oxygen species (ROS), including superoxide and hydrogen peroxide, cause cellular and tissue damage. Consequently, ROS contribute to cell dysfunction and cell death in cellular aging, neurodegeneration and cancer ⁵⁴. The brain has relatively poor enzymatic antioxidant defense and very high aerobic metabolism and blood perfusion. The brain is highly susceptible to oxidative injury resulting from ROS because it produces very high levels of ROS ⁵⁵. Thus, oxidative stress contributes to neuronal injuries such as traumatic brain injury (TBI) and SCI. SOD, an endogenous scavenger of ROS, is one of the main antioxidant enzymes whose function is protecting neurons from oxidative stress ⁵⁶. Malondialdehyde (MDA), which comes from cell membranes, is a product of oxidative lipid degradation and is used as a dependable indicator of oxidative stress ⁵⁷. It was proven in a previous study that piperine acted as a signaling molecule to activate the mitochondrial pathway of apoptosis in hepatocellular carcinoma due to its pro-oxidant-mediated apoptosis-inducing properties ³⁷. Therefore, in the present study, we hypothesized that piperine could attenuate ROS accumulation induced by SCI. In our study, our results revealed increased levels of MDA and decreased activity of SOD in spinal cord tissue from the SCI group compared to that from the Sham group. The abnormal changes in the levels of MDA and activity of SOD were substantially reversed by piperine, which indicated that the neuroprotective effects of piperine might be closely related to its antioxidant benefits. Based on these results, we concluded that piperine inhibited ROS in spinal cord tissue after SCI.

The inflammatory response, which is characterized by a composite of cytokines and chemokines and the infiltration of a large number of leukocytes, has been proven to play an important role in SCI and postinjury recovery ^{58,59}. In secondary SCI (SSCI), the site of injury produces more proinflammatory factors and chemokines, including TNF- α , IL-6, IL-1 β and IFN- γ . Subsequently, a set of complicated immunopathogenic mechanisms finally results in the local demyelination of nerve fibers and the death of neurons and colloid cells ⁶⁰. Yan Li et al. demonstrated that piperine attenuated the inflammatory mediators and nitric oxide synthase activity induced by LPS in NP cells ⁶¹. Therefore, in the present study, we determined whether piperine could reverse the inflammatory response and diminish the inflammatory indicators induced by SCI. In our study, the Western blotting results revealed that the levels of TNF- α , IL-6, IL-1 β and IFN- γ increased after SCI, while piperine attenuated these changes. Then, we examined the content of COX-2 and iNos, and Western blotting showed the same trend. Additionally, we used qRT-PCR to test the mRNA expression levels of TNF- α , IL-6, IL-1 β and IFN- γ . Compared with the Sham group, the SCI group exhibited increased IL-1 β , IL-6, TNF- α and IFN- γ mRNA levels. In contrast, the Piperine group exhibited decreased relative mRNA levels of these cytokines. These results showed that piperine effectively suppressed inflammation.

Inflammasome-mediated pyroptosis and ROS-mediated autophagy are two types of programmed cell death ⁶². In a previous study, we confirmed that the interaction between these cell death programs is very important in the progression of cell death and directly controls the final outcome ^{63,42}. The relationships and interactions among inflammatory, pyroptosis, ROS and autophagy have been widely studied. For example, Cong et al. have reported that electrical stimulation could inhibit. The relationships and interactions among inflammation, pyroptosis, ROS and autophagy have been widely studied. For example, Cong et al. reported that electrical stimulation inhibited pyroptosis in THP-1 macrophages via sirtuin3 activation to promote autophagy and inhibit ROS generation ⁶⁴. Recent evidence revealed that lesions of the injured spinal cord led to mitochondrial damage, which contributed to the accumulation of ROS ⁶⁵. In addition, studies have clarified that elevated levels of ROS can lead to NLRP3 activation and, subsequently, increase the release of IL-1 β and IL-18⁶⁶. Therefore, the ROS-NLRP3 inflammasome pathway has become a promising target for the treatment of human patients with spinal cord injuries. In our study, we hypothesized that piperine facilitates the restoration of spinal cord injury via ROS-induced autophagy and NLRP3-mediated pyroptosis. To confirm the effect of autophagy in SCI, a widely used autophagy inhibitor ⁶⁷, 3-methyladenine (3MA), was used in piperine-treated SCI mice. Our WB results revealed that treatment with 3MA significantly diminished the expression levels of Beclin1, ATG5, VPS34, CTSD, ATP6V1B2, and LC3II and increased the expression levels of P62, COX-2, iNos, NLRP3, GSDMD, and C-CASP1. Additionally, the expression levels of IL-1b and IL-18 increased in the SCI+Piperine+3MA group. The results above demonstrated that 3MA reversed the therapeutic benefits of piperine in SCI mice, confirming that piperine suppresses ROS, attenuates pyroptosis and inhibits inflammation by inducing autophagy (**Figure 7**).

An early study proved that piperine reduced the degree of cerebral edema and attenuated TBI-induced seizures by inhibiting cytokine-activated reactive astrogliosis ⁶⁷. Considering the close relationship between SCI and TBI, we hypothesize that piperine may also play an important role in SCI. However, research has so far proven inconclusive. Thus, our findings proved for the first time that piperine was beneficial for SCI mice due to its neuroprotective effects. In addition, adequate evidence has been found that piperine enhances autophagy in many diseases ^{68,69}. However, pyroptosis is a new cell death type and has not been investigated in SCI. Hence, this is the first study to explore the effect of piperine treatment on pyroptosis in SCI.

Declarations

Acknowledgements

Not applicable.

Funding

This research was supported by the Clinical Scientific Research Project of the Second Affiliated Hospital of Wenzhou Medical University (SAHoWMU-CR2017-08-106), Wenzhou Municipal Science and

Technology Bureau (Y20190078) and Zhejiang Provincial Natural Science Foundation of China (LY17H060009, Y21H060050).

Author contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Availability of data and materials

Data are available under reasonable request.

Ethics approval and consent to participate

The animal experiments were approved by the Animal Research Committee of Wenzhou Medical University (wydw2017-0022).

Consent for publication

Not applicable.

Conflicts of interest

All authors report that there are no conflicts of interest related to the present article.

References

1. Hutson TH, Di Giovanni S. The translational landscape in spinal cord injury: focus on neuroplasticity and regeneration. *Nature reviews Neurology*. 2019;15(12):732-745.
2. Pertici V, Trimaille T, Laurin J, et al. Repair of the injured spinal cord by implantation of a synthetic degradable block copolymer in rat. *Biomaterials*. 2014;35(24):6248-6258.
3. Ahuja CS, Wilson JR, Nori S, et al. Traumatic spinal cord injury. *Nature reviews Disease primers*. 2017;3:17018.
4. Fehlings MG, Tetreault LA, Wilson JR, et al. A Clinical Practice Guideline for the Management of Acute Spinal Cord Injury: Introduction, Rationale, and Scope. *Global spine journal*. 2017;7(3 Suppl):84s-94s.
5. Yang X, Chen S, Shao Z, et al. Apolipoprotein E Deficiency Exacerbates Spinal Cord Injury in Mice: Inflammatory Response and Oxidative Stress Mediated by NF-κB Signaling Pathway. *Frontiers in cellular neuroscience*. 2018;12:142.

6. Greenhalgh AD, Zarruk JG, Healy LM, et al. Peripherally derived macrophages modulate microglial function to reduce inflammation after CNS injury. *PLoS biology*. 2018;16(10):e2005264.
7. Kjell J, Olson L. Rat models of spinal cord injury: from pathology to potential therapies. *Disease models & mechanisms*. 2016;9(10):1125-1137.
8. Tator CH. Biology of neurological recovery and functional restoration after spinal cord injury. *Neurosurgery*. 1998;42(4):696-707; discussion 707-698.
9. Shi J, Gao W, Shao F. Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death. *Trends in biochemical sciences*. 2017;42(4):245-254.
10. Zhang L, Liu H, Jia L, et al. Exosomes Mediate Hippocampal and Cortical Neuronal Injury Induced by Hepatic Ischemia-Reperfusion Injury through Activating Pyroptosis in Rats. *Oxidative medicine and cellular longevity*. 2019;2019:3753485.
11. Hu X, Chen H, Xu H, et al. Role of Pyroptosis in Traumatic Brain and Spinal Cord Injuries. *International journal of biological sciences*. 2020;16(12):2042-2050.
12. Kurian P, Obisesan TO, Craddock TJA. Oxidative species-induced excitonic transport in tubulin aromatic networks: Potential implications for neurodegenerative disease. *Journal of photochemistry and photobiology B, Biology*. 2017;175:109-124.
13. Rana AK, Singh D. Targeting glycogen synthase kinase-3 for oxidative stress and neuroinflammation: Opportunities, challenges and future directions for cerebral stroke management. *Neuropharmacology*. 2018;139:124-136.
14. Özdemir Ü S, Nazıroğlu M, Şenol N, Ghazizadeh V. Hypericum perforatum Attenuates Spinal Cord Injury-Induced Oxidative Stress and Apoptosis in the Dorsal Root Ganglion of Rats: Involvement of TRPM2 and TRPV1 Channels. *Molecular neurobiology*. 2016;53(6):3540-3551.
15. Chen X, Cui J, Zhai X, et al. Inhalation of Hydrogen of Different Concentrations Ameliorates Spinal Cord Injury in Mice by Protecting Spinal Cord Neurons from Apoptosis, Oxidative Injury and Mitochondrial Structure Damages. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. 2018;47(1):176-190.
16. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? *Nature reviews Immunology*. 2010;10(3):210-215.
17. Qiu Z, He Y, Ming H, Lei S, Leng Y, Xia ZY. Lipopolysaccharide (LPS) Aggravates High Glucose- and Hypoxia/Reoxygenation-Induced Injury through Activating ROS-Dependent NLRP3 Inflammasome-Mediated Pyroptosis in H9C2 Cardiomyocytes. *Journal of diabetes research*. 2019;2019:8151836.
18. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature*. 2008;451(7182):1069-1075.
19. Jiang LB, Lee S, Wang Y, Xu QT, Meng DH, Zhang J. Adipose-derived stem cells induce autophagic activation and inhibit catabolic response to pro-inflammatory cytokines in rat chondrocytes. *Osteoarthritis and cartilage*. 2016;24(6):1071-1081.
20. Jiang L, Yuan F, Yin X, Dong J. Responses and adaptations of intervertebral disc cells to microenvironmental stress: a possible central role of autophagy in the adaptive mechanism.

Connective tissue research. 2014;55(5-6):311-321.

21. Jiang C, Jiang L, Li Q, et al. Acrolein induces NLRP3 inflammasome-mediated pyroptosis and suppresses migration via ROS-dependent autophagy in vascular endothelial cells. *Toxicology*. 2018;410:26-40.
22. Li MY, Zhu XL, Zhao BX, et al. Adrenomedullin alleviates the pyroptosis of Leydig cells by promoting autophagy via the ROS-AMPK-mTOR axis. *Cell death & disease*. 2019;10(7):489.
23. Bai Z, Liu W, He D, et al. Protective effects of autophagy and NFE2L2 on reactive oxygen species-induced pyroptosis of human nucleus pulposus cells. *Aging*. 2020;12(8):7534-7548.
24. He JL, Dong XH, Li ZH, Wang XY, Fu ZA, Shen N. Pterostilbene inhibits reactive oxygen species production and apoptosis in primary spinal cord neurons by activating autophagy via the mechanistic target of rapamycin signaling pathway. *Molecular medicine reports*. 2018;17(3):4406-4414.
25. Chen ML, Yi L, Jin X, et al. Resveratrol attenuates vascular endothelial inflammation by inducing autophagy through the cAMP signaling pathway. *Autophagy*. 2013;9(12):2033-2045.
26. Ling H, Chen H, Wei M, Meng X, Yu Y, Xie K. The Effect of Autophagy on Inflammation Cytokines in Renal Ischemia/Reperfusion Injury. *Inflammation*. 2016;39(1):347-356.
27. Matsuzawa-Ishimoto Y, Hwang S, Cadwell K. Autophagy and Inflammation. *Annual review of immunology*. 2018;36:73-101.
28. Wattanathorn J, Chonpathompikunlert P, Muchimapura S, Priprem A, Tankamnerdthai O. Piperine, the potential functional food for mood and cognitive disorders. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 2008;46(9):3106-3110.
29. D'Hooge R, Pei YQ, Raes A, Lebrun P, van Bogaert PP, de Deyn PP. Anticonvulsant activity of piperine on seizures induced by excitatory amino acid receptor agonists. *Arzneimittel-Forschung*. 1996;46(6):557-560.
30. Mittal R, Gupta RL. In vitro antioxidant activity of piperine. *Methods and findings in experimental and clinical pharmacology*. 2000;22(5):271-274.
31. Kim HG, Han EH, Jang WS, et al. Piperine inhibits PMA-induced cyclooxygenase-2 expression through downregulating NF- κ B, C/EBP and AP-1 signaling pathways in murine macrophages. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 2012;50(7):2342-2348.
32. Sunila ES, Kuttan G. Immunomodulatory and antitumor activity of Piper longum Linn. and piperine. *Journal of ethnopharmacology*. 2004;90(2-3):339-346.
33. Liang YD, Bai WJ, Li CG, et al. Piperine Suppresses Pyroptosis and Interleukin-1 β Release upon ATP Triggering and Bacterial Infection. *Frontiers in pharmacology*. 2016;7:390.
34. Peng X, Yang T, Liu G, Liu H, Peng Y, He L. Piperine ameliorated lupus nephritis by targeting AMPK-mediated activation of NLRP3 inflammasome. *International immunopharmacology*. 2018;65:448-457.

35. Ouyang DY, Zeng LH, Pan H, et al. Piperine inhibits the proliferation of human prostate cancer cells via induction of cell cycle arrest and autophagy. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 2013;60:424-430.
36. Haq IU, Imran M, Nadeem M, Tufail T, Gondal TA, Mubarak MS. Piperine: A review of its biological effects. *Phytother Res*. 2020.
37. Gunasekaran V, Elangovan K, Niranjali Devaraj S. Targeting hepatocellular carcinoma with piperine by radical-mediated mitochondrial pathway of apoptosis: An in vitro and in vivo study. *Food Chem Toxicol*. 2017;105:106-118.
38. Sethiya NK, Shah P, Rajpara A, Nagar PA, Mishra SH. Antioxidant and hepatoprotective effects of mixed micellar lipid formulation of phyllanthin and piperine in carbon tetrachloride-induced liver injury in rodents. *Food Funct*. 2015;6(11):3593-3603.
39. Sharma S, Kalia NP, Suden P, et al. Protective efficacy of piperine against Mycobacterium tuberculosis. *Tuberculosis (Edinb)*. 2014;94(4):389-396.
40. Liu C, Yuan Y, Zhou J, Hu R, Ji L, Jiang G. Piperine ameliorates insulin resistance via inhibiting metabolic inflammation in monosodium glutamate-treated obese mice. *BMC Endocr Disord*. 2020;20(1):152.
41. Rehman MU, Rashid S, Arafah A, et al. Piperine Regulates Nrf-2/Keap-1 Signalling and Exhibits Anticancer Effect in Experimental Colon Carcinogenesis in Wistar Rats. *Biology (Basel)*. 2020;9(9).
42. Levine B, Yuan J. Autophagy in cell death: an innocent convict? *J Clin Invest*. 2005;115(10):2679-2688.
43. Stavoe AKH, Holzbaur ELF. Autophagy in Neurons. *Annu Rev Cell Dev Biol*. 2019;35:477-500.
44. Hossain MI, Marcus JM, Lee JH, et al. Restoration of CTSD (cathepsin D) and lysosomal function in stroke is neuroprotective. *Autophagy*. 2020:1-19.
45. Boland B, Yu WH, Corti O, et al. Promoting the clearance of neurotoxic proteins in neurodegenerative disorders of ageing. *Nat Rev Drug Discov*. 2018;17(9):660-688.
46. Yuan W, He X, Morin D, et al. Autophagy induction contributes to the neuroprotective impact of intermittent fasting on the acutely injured spinal cord. *J Neurotrauma*. 2020.
47. Zhu P, Qian J, Xu Z, et al. Piperlonguminine and Piperine Analogues as TrxR Inhibitors that Promote ROS and Autophagy and Regulate p38 and Akt/mTOR Signaling. *J Nat Prod*. 2020;83(10):3041-3049.
48. Xing SS, Yang J, Li WJ, et al. Salidroside Decreases Atherosclerosis Plaque Formation via Inhibiting Endothelial Cell Pyroptosis. *Inflammation*. 2020;43(2):433-440.
49. An S, Hu H, Li Y, Hu Y. Pyroptosis Plays a Role in Osteoarthritis. *Aging Dis*. 2020;11(5):1146-1157.
50. Lin J, Cheng A, Cheng K, et al. New Insights into the Mechanisms of Pyroptosis and Implications for Diabetic Kidney Disease. *Int J Mol Sci*. 2020;21(19).
51. Chadha S, Behl T, Bungau S, et al. Mechanistic insights into the role of pyroptosis in rheumatoid arthritis. *Curr Res Transl Med*. 2020;68(4):151-158.

52. Yap JKY, Moriyama M, Iwasaki A. Inflammasomes and Pyroptosis as Therapeutic Targets for COVID-19. *J Immunol.* 2020;205(2):307-312.
53. Cheng SB, Nakashima A, Huber WJ, et al. Pyroptosis is a critical inflammatory pathway in the placenta from early onset preeclampsia and in human trophoblasts exposed to hypoxia and endoplasmic reticulum stressors. *Cell Death Dis.* 2019;10(12):927.
54. Scott TL, Rangaswamy S, Wicker CA, Izumi T. Repair of oxidative DNA damage and cancer: recent progress in DNA base excision repair. *Antioxid Redox Signal.* 2014;20(4):708-726.
55. Rimessi A, Previati M, Nigro F, Wieckowski MR, Pinton P. Mitochondrial reactive oxygen species and inflammation: Molecular mechanisms, diseases and promising therapies. *Int J Biochem Cell Biol.* 2016;81(Pt B):281-293.
56. Zhao W, Xu Z, Cao J, et al. Elamipretide (SS-31) improves mitochondrial dysfunction, synaptic and memory impairment induced by lipopolysaccharide in mice. *J Neuroinflammation.* 2019;16(1):230.
57. Valdés-Tovar M, Estrada-Reyes R, Solís-Chagoyán H, et al. Circadian modulation of neuroplasticity by melatonin: a target in the treatment of depression. *Br J Pharmacol.* 2018;175(16):3200-3208.
58. Miljkovic D, Momcilovic M, Stojanovic I, Stosic-Grujicic S, Ramic Z, Mostarica-Stojkovic M. Astrocytes stimulate interleukin-17 and interferon-gamma production in vitro. *J Neurosci Res.* 2007;85(16):3598-3606.
59. Chuah MI, Hale DM, West AK. Interaction of olfactory ensheathing cells with other cell types in vitro and after transplantation: glial scars and inflammation. *Exp Neurol.* 2011;229(1):46-53.
60. Olivas AD, Noble-Haeusslein LJ. Phospholipase A2 and spinal cord injury: a novel target for therapeutic intervention. *Ann Neurol.* 2006;59(4):577-579.
61. Li Y, Li K, Hu Y, Xu B, Zhao J. Piperine mediates LPS induced inflammatory and catabolic effects in rat intervertebral disc. *Int J Clin Exp Pathol.* 2015;8(6):6203-6213.
62. Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun.* 2005;73(4):1907-1916.
63. Lockshin RA, Zakeri Z. Apoptosis, autophagy, and more. *Int J Biochem Cell Biol.* 2004;36(12):2405-2419.
64. Cong L, Gao Z, Zheng Y, et al. Electrical stimulation inhibits Val-boroPro-induced pyroptosis in THP-1 macrophages via sirtuin3 activation to promote autophagy and inhibit ROS generation. *Aging (Albany NY).* 2020;12(7):6415-6435.
65. Rao S, Lin Y, Du Y, et al. Designing multifunctionalized selenium nanoparticles to reverse oxidative stress-induced spinal cord injury by attenuating ROS overproduction and mitochondria dysfunction. *J Mater Chem B.* 2019;7(16):2648-2656.
66. Amin FM, Abdelaziz RR, Hamed MF, Nader MA, Shehatou GSG. Dimethyl fumarate ameliorates diabetes-associated vascular complications through ROS-TXNIP-NLRP3 inflammasome pathway. *Life Sci.* 2020;256:117887.

67. Wang X, Zhou G, Liu C, et al. Acanthopanax versus 3-Methyladenine Ameliorates Sodium Taurocholate-Induced Severe Acute Pancreatitis by Inhibiting the Autophagic Pathway in Rats. *Mediators Inflamm.* 2016;2016:8369704.
68. Liu J, Chen M, Wang X, et al. Piperine induces autophagy by enhancing protein phosphatase 2A activity in a rotenone-induced Parkinson's disease model. *Oncotarget.* 2016;7(38):60823-60843.
69. Rather RA, Bhagat M. Cancer Chemoprevention and Piperine: Molecular Mechanisms and Therapeutic Opportunities. *Front Cell Dev Biol.* 2018;6:10.

Figures

Figure 1

Piperine promotes function recovery after SCI. (A) Basso Mouse Scale scores of SCI mice receiving various treatments, and the sham group was used as control. $**p < 0.01$. Data were expressed as mean $\pm$ SD. (B) The results of inclined plane angle in Sham, SCI, Piperine and Piperine +3MA groups. $**p < 0.01$. (C) Respective rotation angle in Sham, SCI, Piperine and Piperine +3MA groups. $**p < 0.01$. (D) Respective base of support in Sham, SCI, Piperine and Piperine +3MA groups. $**p < 0.01$. (E) The outcome of stride length in Sham, SCI, Piperine and Piperine +3MA groups. $**p < 0.01$.

Figure 2

Piperine enhances autophagy after SCI. (A) Immunofluorescence staining for LC3II and NeuN colocalization at the spinal cord lesion after SCI. (B) The quantitative percentage of the LC3II positive neurons at the spinal cord lesion in each group. (C) Immunofluorescence staining for p62 and NeuN colocalization at the spinal cord lesion after SCI. (D) The quantitative percentage of the p62 positive neurons at the spinal cord lesion in each group. (E) Western blotting for Beclin1, ATG5, VPS34, CTSD, ATP6V1B2, LC3II, and p62 expression levels in the Sham, Vehicle and piperine groups. The gels were run under the same experimental conditions, and the cropped blots are shown here. (F-L) Representative quantification data of Beclin1, ATG5, VPS34, CTSD, ATP6V1B2, LC3II, and p62 in each group. **P < 0.01

Figure 3

Piperine attenuated oxidative stress after SCI. (A) Concentration of SOD activity detected by BCA kits. (B) Concentration of MDA content detected by BCA kits. (C) Western blotting for SOD1, eNOS, Ho-1 expression levels in the Sham, Vehicle and Piperine groups. The gels were run under the same experimental conditions, and the cropped blots are shown here. (D-F) Representative quantification data of SOD1, eNOS and Ho-1 in each group. **P<0.01

Figure 4

Piperine attenuates pyroptosis after SCI. (A) Immunofluorescence staining for C-CASP1 and NeuN colocalization at the spinal cord lesion after SCI. (B) The quantitative percentage of the C-CASP-1 positive neurons at the spinal cord lesion in each group. (C) Western blotting for NLRP3, GSDMD and C-CASP1 expression levels in the Sham, Vehicle and Piperine groups. The gels were run under the same experimental conditions, and the cropped blots are shown here. (D-F) Representative quantification data of NLRP3, GSDMD and C-CASP1 in each group. ** $P < 0.01$ versus the sham group; ** $P < 0.01$ (G, H) Enzyme-linked immunosorbent assay (ELISA) results for IL1 β and IL18 expression levels in the Sham, Vehicle, and Piperine groups. ** $p < 0.01$

Figure 5

Piperine suppresses inflammation after SCI. (A-D) Quantification data of TNF-α, IL-6, IL-1β, and IFN-γ measured by ELISA in each group. **P<0.01 (E) Western blotting for COX-2 and iNOS expression levels in the Sham, Vehicle and Piperine groups. The gels were run under the same experimental conditions, and the cropped blots are shown here. (F, G) Representative quantification data of COX-2 and iNOS in each group. **P<0.01 (H-K) Representative quantification data of IL-6, TNF-α, IL-1β and IFN-γ measured by qRT-PCR in each group. **P<0.01

Figure 6

Inhibition of autophagy reverses the effects of piperine on oxidative stress, pyroptosis, inflammation and functional recovery after SCI. (A) Western blotting for Beclin1, ATG5, VPS34, CTSD, ATP6V1B2, LC3II, and p62 expression levels in the Piperine and Piperine+3MA groups. The gels were run under the same experimental conditions, and the cropped blots are shown here. (B) The optical density values of the Beclin1, ATG5, VPS34, CTSD, ATP6V1B2, LC3II and p62 expression levels were quantified and analyzed in each group. $**P < 0.01$ (C-E) Western blotting for the Cox-2, iNos, NLRP3, GSDMD, C-CASP1, SOD1, eNOS and Ho-1 expression levels in the Piperine and Piperine+3MA groups. The gels were run under the same experimental conditions, and the cropped blots are shown here. (F-H) The optical density values of Cox-2, iNos, NLRP3, GSDMD, C-CASP1, SOD1, eNOS and Ho-1 expression levels were quantified and analyzed in the both groups. $**P < 0.01$ (I, J) Enzyme-linked immunosorbent assay (ELISA) results for IL1band IL18 expression levels in the Piperine and Piperine+3MA groups. $**P < 0.01$

Figure 7

Schematic diagram of the effects of piperine on SCI.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementTableS1.docx](#)