

The Influence of Mild Hypothermia Plus Methylprednisolone on Nerve Function and Apoptosis by Inhibiting P38AMPK/Smad2 Pathway

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ABSTRACT

The main aim of this study was to explore the mechanism of mild hypothermia (MH) plus methylprednisolone (MPS) was in spinal cord injury (SCI) therapy. Application of modified Allen's method was for injury of the spinal cord (T10) in rats and treated SCI was with MH or/and MPS immediately. Conduction of Basso-Beattie-Bresnahan (BBB) scores was on all rats at different time points after surgery. Subsequently harvest of the serum and the spinal cord tissues was behind euthanasia of rats. Detection of biochemical indicators, observation of cell apoptosis, and determination of Bax, Bcl-2, p38 MAPK and Smad2 were clarified. We found that MH and MH+MPS could effectively reduce apoptotic cells, dramatically minimize TNF- α , IL-6, IL-8, MDA and Bax contents ($P < 0.01$) and elevate GSH-Px and Bcl-2 level ($P < 0.01$). Additionally, the P38AMPK/Smad2 pathway proteins (phosphorylated p-P38MAPK and Smad2) were notably higher in the model versus the control and the sham, while MH and MH+MPS could patently down-regulate p-P38MAPK and Smad2 protein levels. To conclude, MH+MPS is available to refrain the activation of P38AMPK/Smad2 signal, hence repressing cell apoptosis after SCI, reducing secondary SCI, and benefiting the recovery of nerve function.

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Authors' Contribution

LW designed the research study. QC performed the research. TL and SY provided help and advice on the experiments. XP analyzed the data. LW and CL wrote the manuscript. All authors contributed to editorial changes in the manuscript.

Key words

Methylprednisolone, Mild hypothermia, Spinal cord injury, Apoptosis, P38AMPK/Smad2 pathway

INTRODUCTION

Spinal cord injury (SCI), a serious traumatic illness, affects autonomic functions, and leads to impaired or even absence of motor and sensory functions under the injury site (Prüss *et al.*, 2017), causing an enormous economic pressure for individuals and the medical system (Alizadeh *et al.*, 2019). The characteristics of tissue injury related to the injury site after SCI are myelin degradation, cell death, axon loss and destruction of the blood spinal cord barrier (Gensel *et al.*, 2012; Oyibo, 2011). Secondary injuries begin to occur quickly after the main damage, and this process keeps for a period of time, resulting in gradual damage to the spinal cord tissue around the lesion (Schwab *et al.*, 2006). The acute phase of secondary injury

after spinal damage begins to lead to SCI, causing vascular harm, neurotransmitter accumulation (excitatory toxicity), formation of free radicals, inflammation and cell death (Hou and Rabchevsky, 2014; von Leden *et al.*, 2017). Among them, cell death mainly appears in the secondary injury mechanism, with responding to all kinds of injury-induced regulators through a variety of mechanisms, and it will affect neurons and glial cells after SCI (Almad *et al.*, 2011; Beattie *et al.*, 2002; Dyck *et al.*, 2019). Thus, exploring the cellular and molecular mechanisms of SCI and developing novel efficient therapies for this destructive disease are vital.

Methylprednisolone is a corticosteroid that has been identified to mitigate spinal inflammatory cascade reaction after SCI. For instance, it was found that methylprednisolone represses the proliferation of endogenous neural stem cells in non-human primates SCI (Ye *et al.*, 2018). In the meantime, a study manifests that methylprednisolone combined with amniotic mesenchymal stem cells improve inflammation and neuronal apoptosis after SCI in rats (Gao *et al.*, 2014). However, frequent high-dose methylprednisolone injection can influence patients' quality of life and cause a variety of side effects, such as pneumonia, gastric ulcer, leukemia, infection and

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neuropathy (Chikuda *et al.*, 2014; Karabey-Akyurek *et al.*, 2017). Therefore, it is necessary to further understand the mechanism of methylprednisolone's action on SCI, which will be beneficial for reducing its side effects in humans.

Glucose is the main energy source of the central nervous system (Liu *et al.*, 2015). Ischemia and hypoxia after SCI results in insufficient glucose supply, which is not conducive to neuronal repair (Okon *et al.*, 2013). AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that participates in various biological functions and is a key factor in glucose metabolism, which is closely linked with glucose transport and mitochondrial function (Carling, 2017). Zinc modulates spinal cord and neuronal glucose metabolism and promotes functional recovery after spinal cord injury through AMPK signaling (Hu *et al.*, 2021). Similarly, betulin controls AMPK-mTOR-TFEB signaling pathway to enhance autophagy to repress pyroptosis in SCI (Wu *et al.*, 2021).

Hence, the study was to uncover the character of mild hypothermia (MH) plus methylprednisolone (MPS) on apoptosis after SCI, and its mechanism of activation of related pathways, offering potential therapy and theoretical foundation for mitigating secondary SCI and restoring nerve function.

MATERIALS AND METHODS

Experimental materials

Sixty healthy adult female SD rats, weighing 220-250g (Hunan SJA Laboratory Animal Co., Ltd), MPS (MACKLIN), Trizon Reagent (CW0580S, CWBIO), Ultrapure RNA Ultra-pure RNA extraction kit (CW0581M, CWBIO), HiFiScript cDNA first-strand synthesis kit (CW2569M, CWBIO), UltraSYBR Mixture (CW0957M, CWBIO), fluorescence PCR instrument (CFX Connect™ real-time, Bio-Rad Laboratories), RIPA cell lysate (C1053, APPLYPGEN), BCA Protein Assay Kit (CW0014S, CWBIO), PVDF Membrane (IPVH00010, Millipore), skimmed milk powder special for sealing (P1622, APPLYPGEN), bovine serum albumin (BSA) (A8020, Solarbio), ultra-sensitive luminescent solution (RJ239676, Thermo Fisher Scientific), internal reference primary antibody: Mouse Monoclonal Anti-GAPDH (TA-08, ZSGB-Bio, 1/2000), secondary antibody: HRP-labeled goat anti-mouse IgG (H+L) (ZB-2305, ZSGB-Bio, 1/2000), the target primary antibody: Rabbit Anti-TGF-β1 (bs-0068R, Bioss, 1/500), Rabbit Anti-Smad2 (AF6449, Affinity, 1/1000), Rabbit Anti-Smad3 (ab40854, Abcam, 1/1000), secondary antibody: HRP-labeled goat anti-rabbit IgG (H+L) (ZB-2301, ZSGB-Bio, 1/2000), ultrahigh sensitivity chemiluminescence imaging system (Chemi Doc™ XRS+, Bio-Rad Laboratories).

SCI model construction and treatment

Firstly, the rats were anesthetized via intraperitoneal injection of 10% chloral hydrate. The back hairs of the anesthetized rats were shaved, and the skin was disinfected with iodophor. The skin was longitudinally with the T11 thoracic spine as the midpoint, about 3 to 4 cm. After incising the skin, muscles were removed on the T10 and T11 thoracic vertebrae, and T10 and T11 lamina were exposed. Using small scissors and hemostatic forceps, T10 and T11 lamina were carefully removed to expose the spinal cord. A self-made pad was gently stuck on the exposed epidural surface. Subsequently, 12g percussion stick and a self-made percussion device was used to hit the gasket vertically from a 7 cm height free fall from the graduated sleeve. After stressing the gasket, spinal cord was damaged. Manifestation of ruptured middle spinal cord, intramedullary hemorrhage and reperfusion affirmed acute SCI. Ultimately, suture of the muscles, fascia and skin in sequence, and repetitive disinfection of the wound were done.

Grouping

Animal groups were comprising control, sham, model, MH, MH + MPS groups. Among them, normal reared rats were in the control, the rats with simple operation but not hitting spinal cord were in the sham, SCI model rats were in the model, MH group comprised after modelling. The rats were given cooling down and then rewarmed. After modelling in the MH+MPS group, the rats were given cooling down, intraperitoneal injection of MPS (100 mg/kg, once daily, for 3 d), and warmth. Conduction of Basso-Beattie-Bresnahan (BBB) scores in all rats was different time points after surgery. Euthanasia of the rats in groups was conducted with harvest of spinal cord tissues for subsequent researches.

ELISA

The rat blood sera were used to detect the levels of TNF-α, IL-1β, IL-8 and MPO according to the ELISA kit manufacturer's instructions.

Detection of oxidative stress indicators

The oxidative stress indicators were detected in each group at 24 h, 48 h, 72 h and 7 d. After anesthesia, we opened the rats' thoracic cavity, exposed their heart and aortic roots, intubated the left ventricle to the aortic root, cut the right atrial appendage, and perfused 200 ml heparin containing saline until the outflow fluid was clear, and then exposed the spinal cord again, taking of 1 cm of spinal cord tissue was with the injured segment as the center. After tissue homogenization, detection of MDA content (nmol/mg) and GSH-Px content (U/g) as per kit instructions.

TUNEL staining

Tissue sections were dewaxed in the xylene, hydration with gradient ethanol. Proteinase K working solution (50 µg/mL) was added dropwise to each sample, and incubated at 37°C for 30 min. Later, tissues were thoroughly washed with PBS, and sufficient amount of TUNEL detection solution was added to each section. Finally, sections were sealed with anti-fluorescence quencher and observed under a fluorescence microscope.

Flow cytometry

The rat spinal cord tissues were excised from each group, and detached into single-cell suspensions by trypsin. Meanwhile, the cell density was adjusted to 5×10^5 cells/mL. Cells were then incubated with Annexin V and PI, and 10^6 cells/mL were used for the detection using flow cytometry (C6). Data was analyzed using CFlow Plus software.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Spinal cord tissues were grinded into powder using the liquid nitrogen in the presence of TRIzol lysis solution for the extraction of total RNA. UV-Vis spectrophotometer was used to measurement the concentration and purity of RNA (OD260/OD280). HiFiScript cDNA first-strand synthesis kit was used to synthesize the cDNA via reverse transcription. Fluorescent PCR instrument was used for RT-qPCR. Primer sequences are listed in Table I (synthesized by General Biosystems (Anhui) Co., Ltd.), with β -actin as a loading control, and the relative expression of Bax, Bcl-2, p38 MAPK and Smad2 was calculated as per $2^{-\Delta\Delta Ct}$ method (Table I).

Table I. Primer sequences of Bax, Bcl-2, p38 MAPK, Smad2 and β -actin.

Primer	Sequence (5' → 3')
Bax F	GCGATGAAGTGGACAACAAC
Bax R	GCAAAGTAGAAAAGGGCAACC
Bcl-2 F	GCGTCAACAGGGAGATGTCA
Bcl-2 R	TTCCACAAAGGCATCCCAGC
p38 MAPK F	CGAGAATGTGATTGGTCTGTTGG
p38 MAPK R	TCTTCATTACAGCGAGGTTG
Smad2 F	TGAAGTGTCTCCTACCACTCTCT
Smad2 R	CACTCCCCTTCCTATATGCCTTCTG
β -actin F	GCCATGTACGTAGCCATCCA
β -actin R	GAACCGCTCATTGCCGATAG

Western blot

Sample (50 mg) was added in RIPA buffer and homogenized using the homogenizer (65HZ, 60s). It was then centrifuged to isolate total protein. BCA kit was used to measure total protein. Subsequently, protein was denatured in presence of loading buffer and subjected to SDS-PAGE, and then transfer to the membrane. The membranes were then incubated with primary antibodies (anti-TLR4, anti-MyD88, anti-NALP3, anti-Collagen I, anti-survivin), followed by incubation with HRP-labeled secondary antibodies. ECL luminescent liquid was used to the membrane and exposed in a gel imaging system. Ultimately, "Quantityone" software was used for analyzing the protein expression.

Statistical analysis

Statistical analysis of all data was via Graphpad Prism7 with exhibition as mean $\pm$ standard deviation (SD). Analysis of the inter-group notable differences was via T-test, one-way and two-way analysis of variance (ANOVA), and $*P < 0.05$.

RESULTS

Motor function score and rat spinal cord morphology

Table II shows the motor function scores of the 5 groups at different times after surgery were clarified. BBB scores of the model at different time points were strikingly reduced versus the control ($P < 0.01$). Besides, versus the model, the BBB score of the MH and MH+MPS was elevated ($P < 0.05$). Manifestation of the spinal cord of each group 7 d after surgery was in Figure 1. The spinal cords of the control and the sham were morphologically homogeneous, with smooth surface, while that of the model, the MH and the MH+MPS displayed some degree of variation (Table II).

Table II. Motor function scores at different times after surgery (BBB score)

Group	24 h	48 h	72 h	7 d
Control	21	21	21	21
Sham	19.67 $\pm$ 0.58	21	21	21
Model	0**	0**	1**	5.33 $\pm$ 0.58**
MH	0	0.67 $\pm$ 0.58	1.33 $\pm$ 0.58	6.67 $\pm$ 1.53
MH+ MPS	0	0.33 $\pm$ 0.58	1.33 $\pm$ 0.58	8 $\pm$ 1#

Note ** $P < 0.01$ vs. control group; # $P < 0.05$ vs. model group

Effect of MH+MPS on inflammatory response after SCI

To look into the changes of inflammatory response in the therapy of SCI with MH+MPS, detection of IL-8, IL-6 and TNF- α contents was clarified. Consequently,

TNF- α was plainly elevated in the sham and the model versus the control, whereas MH and MH+MPS tellingly reduced TNF- α ($P < 0.01$). IL-6 and IL-8 contents were brilliantly elevated in the model versus the control, while MH and MH+MPS saliently reduced IL-6 and IL-8 ($P < 0.01$) (Fig. 2).

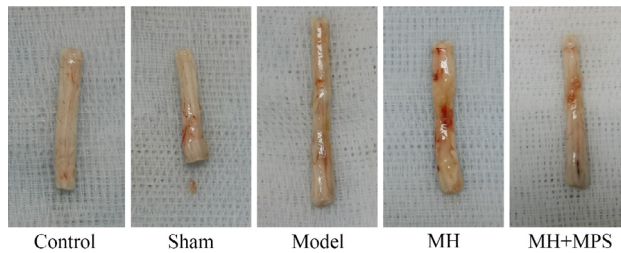


Fig. 1. Spinal cord of each group 7 d after surgery. MH, mild hypothermia; MH+MPS, mild hypothermia plus methylprednisolone.

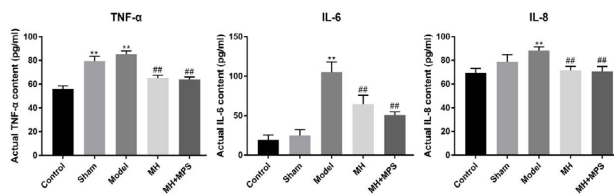


Fig. 2. Effect of hypothermia and methylprednisolone on TNF- α , IL-6 and IL-8 of spinal cord during treatment of spinal injury. For Abbreviations, see Fig. 1. ** $P < 0.01$ vs. the control; ## $P < 0.01$ vs. the model.

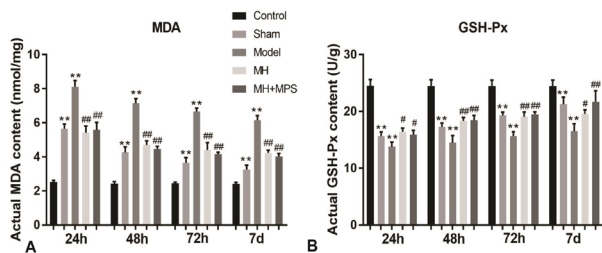


Fig. 3. Effect of hypothermia and methylprednisolone on MDA (A) and GSH-Px (B) content in spinal cord during treatment of spinal injury. For Abbreviations, see Fig. 1. ** $P < 0.01$ vs. the control; ## $P < 0.01$ vs. the model.

Effect of MH+MPS on oxidative stress after SCI

To dig into the alterations of oxidative stress in SCI therapy with MH+MPS, detection of MDA and GSH-Px was in the injured spinal cord. As a result, MDA in the sham and the model in each time period was memorably elevated versus the control ($P < 0.01$), whereas MH and MH+MPS were significantly diminished MDA content

after SCI ($P < 0.01$) (Fig. 3A). Further, GSH-Px content in each time period was manifestly lower in the sham and the model versus the control ($P < 0.01$), while MH and MH+MPS observably elevated the GSH-Px content after SCI ($P < 0.05$) (Fig. 3B).

Effect of MH+MPS on cell apoptosis after SCI

To delve into whether MH+MPS will affect cell apoptosis after SCI, determination of apoptosis of spinal cord cells was crucial. Consequently, in contrast to control group and sham group, the apoptotic cells in the model augmented. Besides, versus the model, MH and MH+MPS reduced cell apoptosis (Fig. 4). Flow cytometry revealed that the spinal cord cell apoptosis rate of control group and sham group were $0.6\% \pm 0.4\%$ and $0.7\% \pm 0.3\%$, respectively; apoptosis rate in the model was $6.2\% \pm 1.0\%$; apoptosis rate in the MH and the MH+MPS were $5.1\% \pm 0.7\%$ and $4.6\% \pm 0.8\%$ separately, which were eminently reduce versus the model ($P < 0.05$) (Fig. 5). Above results evidenced that MH+MPS is available to curb cell apoptosis after SCI.

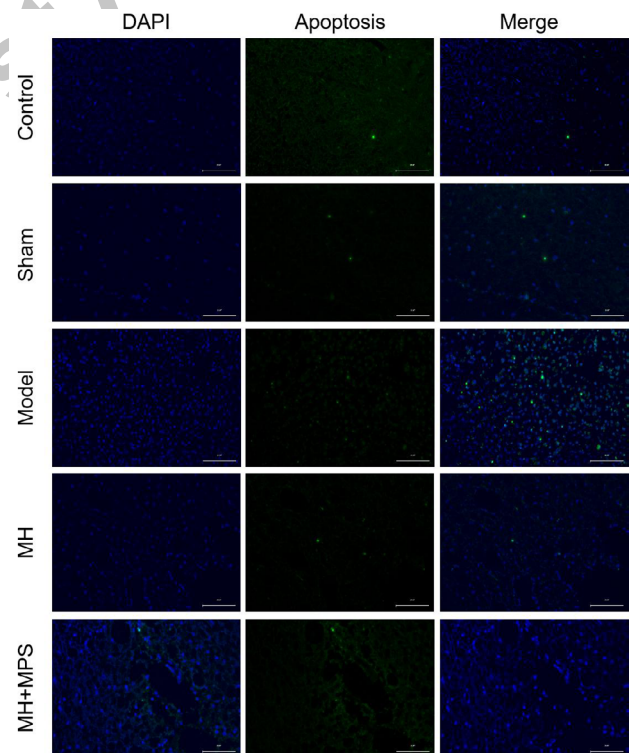


Fig. 4. Effect of hypothermia and methylprednisolone on apoptosis of cells (Tunel staining) in spinal cord during treatment of spinal injury. For Abbreviations, see Fig. 1.

The mechanism of action of MH+MPS on SCI

To further probe into the mechanism of MH+MPS

to inhibit apoptosis after SCI, examination of apoptosis-related protein (Bax and Bcl-2) and P38AMPK/Smad2 pathway protein level was clarified. RT-qPCR results showed that versus the model, MH and MH+MPS significantly down-regulated Bax mRNA level ($P < 0.05$); versus the control, Bcl-2 in the model was significantly diminished ($P < 0.01$), whereas MH+MPS further dwindled Bcl-2 expression level ($P < 0.01$); for P38AMPK/Smad2 pathway related protein expression, P38AMPK and Smad2 levels in model group were dramatically declined ($P < 0.01$), while MH+MPS escalated P38AMPK expression ($P < 0.01$), and further decreased Smad2 expression level ($P < 0.01$) (Fig. 6A).

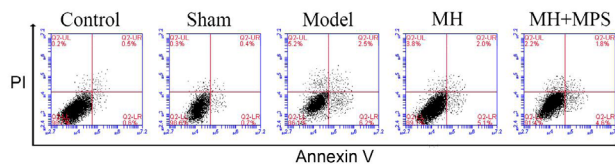


Fig. 5. Effect of hypothermia and methylprednisolone on apoptosis of cells (Flow cytometry) in spinal cord during treatment of spinal injury. For Abbreviations, see Fig. 1.

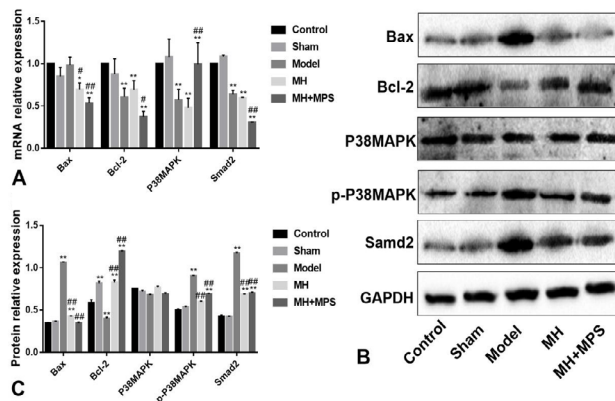


Fig. 6. Expression levels of P38AMPK/Smad2 pathway proteins in spinal cord during treatment of spinal injury. A: RT-qPCR to assay apoptosis-related gene and P38AMPK/Smad2 pathway gene. B-C: Western blot to assay apoptosis-related and P38AMPK/Smad2 pathway proteins. For Abbreviations, see Fig. 1. * $P < 0.05$, ** $P < 0.01$ vs. the control, # $P < 0.05$, ## $P < 0.01$ vs. the model.

It was unveiled that versus the control, Bax in model group was tellingly waxed, while Bcl-2 was compelling waned ($P < 0.01$); MH and MH+MPS could dramatically diminish Bax protein level, and up-regulate Bcl-2 protein level ($P < 0.01$) (Fig. 6B, C). Moreover, P38AMPK protein level did not alter notably in each group. Phosphorylated p-P38MAPK and Samd2 were markedly elevated in the

model versus the control and the sham ($P < 0.01$), while MH and MH+MPS could considerably down-regulate p-P38AMPK and Samd2 protein level ($P < 0.01$) (Fig. 6B, C). Above-mentioned results expounded that MH+MPS can restrain the activation of P38AMPK/Smad2 signal, thereby reining cell apoptosis after SCI.

DISCUSSION

The clinical outcome of SCI depends on the severity and site of the lesion, and SCI is possible to lead to absence of sensory or motor function under the injury site. Studies have demystified that in the first few hours after spinal injury, apoptotic features appear in oligodendrocytes, and the cell density exhibits a downward trend (Xu *et al.*, 2008). We established rat SCI model to found that Bax expression level waxed significantly, whereas Bcl-2 expression level waned significantly, indicating that the prepared SCI model activated cell apoptosis.

MH therapy is extensively employed in therapy of various acute injuries of central nervous system. Cell death shows up in the secondary SCI, and necrosis and apoptosis are considered to be the two cranial cell death mechanisms after SCI (Crowe *et al.*, 1997; Wang *et al.*, 2013). Studies have unveiled that the factors that cause cell necrosis include large accumulation of toxic blood components (Juliet *et al.*, 2009), glutamate excitotoxicity (Gudz *et al.*, 2006), ATP depletion (Matute *et al.*, 2007), the release of pro-inflammatory cytokine (Jana and Pahan, 2007), and the formation of free radicals (Takahashi *et al.*, 2003), all of which stimulate cells to swell and dissolve rapidly. MH treatment can reduce the metabolic rate (Maxwell *et al.*, 1999), calpain-mediated proteolysis and mitochondrial dysfunction (Clifton *et al.*, 1993; Marion *et al.*, 1993), nerve excitability, immune and inflammatory response (Corbett *et al.*, 1997), free radical production (Jia *et al.*, 2008), etc. MH treatment can prevent apoptosis induced viacell damage. Cell apoptosis is the cell death mechanism after the occurrence of SCI. Apoptosis represents programmed cell death, which is an energy-dependent mode that starts primary injury. In the process of apoptosis, cells shrink and are eventually engulfed, in which inflammatory response is not induced. Studies have uncloaked that in rat SCI, cell apoptosis first occurs 4 hours after injury, and reaches a peak on day 7 (Beattie *et al.*, 2000). Fas-deficient mice showed notably meliorated functional recovery after SCI, manifested by decreased macrophage infiltration and decreased expression of inflammatory cytokines, thus causing a pronounced decrease in apoptosis and inflammatory reaction (Yu and Fehlings, 2011). In this study, MH and MH+MPS reduced inflammation and oxidative stress while decreasing the

production of apoptotic cells in the spinal tissue, and significantly repressed Bax expression and up-regulated Bcl-2, indicating that MH+MPS was available to repress apoptosis after SCI, and combination therapy has a synergistic effect and can perfect the therapeutic effect.

It has been corroborated that ghrelin has anti-inflammatory and neuroprotective properties in the SCI model. The administration of ghrelin represses the activation of the p38 AMPK/NF- κ B pathway, which in turn releases nerve growth factor (proNGF), revealing that refraining the activation of AMPK and JUN pathways has anti-inflammatory and neuroprotective effects (Lee and Yune, 2014). In the current study, repressing P38AMPK and Smad2 protein expression was MH+MPS, thereby refraining apoptosis after SCI. Among them, MH can curb P38AMPK and Smad2 expression at the gene and protein level, whereas MPS up-regulates P38AMPK and down-regulates Smad2 at the gene level. It shows that MH and MPS have different targets, acting on the P38AMPK/Smad2 pathway. Therefore, combination therapy can improve the repair of SCI in rats.

MH+MPS can suppress the activation of P38AMPK/Smad2 signal, hence repressing cell apoptosis after SCI, reducing secondary SCI, and benefiting the recovery of nerve function.

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Availability of data and materials

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

Ethics statement

The animal experiment research protocol was approved by the Ethics Committee of Guizhou Orthopedic Hospital and performed in accordance with the guidelines for the care and use of experimental animals. The experiment was approved by the Ethics Committee of the Guizhou Orthopedic Hospital (NO.t-a309).

Statement of conflict of interests

The authors have declared no conflict of interest.

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