Article

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Metformin Protects against Spinal Cord Injury through Inhibiting Neuronal Apoptosis and Enhancing Autophagy Mediated by Endoplasmic Reticulum Stress

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Background: Spinal cord injury (SCI) is a spinal nerve dysfunction caused by trauma, resulting in irreversible and destructive spinal cord impairments. The present research was formulated to identify the biological functions of metformin in SCI and to probe into the intrinsic mechanisms.
Methods: SCI rat models were established using Allen's method and SCI rats were intraperitoneally injected with 20 or 100 mg/kg metformin. Besides, endoplasmic reticulum (ER) stress was induced in PC12 cells by administration of 1 μM thapsigargin. Then, 100 μmol/mL metformin was given to PC12 cells. Afterwards, Basso-Beattie-Bresnahan (BBB) locomotor scoring for detecting hindlimb locomotor function, hematoxylin and eosin (H&E) staining for detecting pathological changes, cell counting kit-8 (CCK-8) assay for detecting viability, TdT-mediated dUTP nick-end labeling (TUNEL) staining for detecting apoptosis, immunofluorescence and western blot for detecting autophagy and ER stress response were performed.
Results: Results of *in vivo* experiments revealed that metformin promoted functional recovery, reduced lesion size, attenuated apoptosis in spinal cord tissues, strengthened autophagy and repressed ER stress after SCI. Additionally, *in vitro* experimental results evidenced that elevation of ER stress partly abolished the suppressing effects of metformin on neuronal apoptosis and reversed the promoting effects of metformin on autophagy.
Conclusions: To sum up, metformin can protect against SCI by repressing neuronal apoptosis and enhancing autophagy, depending on inhibition of X-box binding protein 1 (XBP-1)-mediated ER stress.

Keywords: metformin; spinal cord injury; apoptosis; autophagy; endoplasmic reticulum stress

Introduction

Spinal cord injury (SCI), a kind of traumatic disease, can lead to severe lower limb dysfunction [1,2]. Nowadays, accompanied by the rapid development of transportation and industry, the incidence of SCI is increasing steadily [3]. Till now, there is a lack of ideal treatment drugs for SCI [4]. Hence, exploration of effective, secure and dependable drug for SCI is still one of the main hotspots in basic and clinical research.

Metformin, an oral hypoglycemic drug, has been broadly adopted in type 2 diabetes mellitus [5]. With the development of in-depth research, metformin has attracted more extensive attention. Metformin treatment could promote functional recovery after SCI by suppressing neuron injury and apoptosis [6,7]. However, the underlying molecular mechanism of metformin in improving functional recovery has not been fully understood till now.

Endoplasmic reticulum (ER) is responsible for correctly folding newly synthesized proteins [8]. The abnormal accumulation of misfolded or unfolded proteins will induce ER stress. Long term high-intensity ER stress will lead to over stress and cell death [9,10]. As a molecular chaperone in ER, glucose regulated protein 78 (GRP78) can be activated in response to ER stress [11]. X-box binding protein 1 (XBP-1) is the main regulatory factor in ER stress [12]. Wu *et al*. [13] report that downregulation of GRP78 could activate unfolded protein response (UPR) signaling and autophagy. Importantly, it has been proved that apoptosis elicited by ER stress is implicated in SCI [14]. Additionally, literature has verified that metformin could attenuate cerebral damage by inhibiting the expressions of GRP78 and XBP-1 [15].

Autophagy is a highly conserved self-degradation event in which lysosomes are responsible for the clearance of damaged organelles and macromolecules [16]. Cell autophagy is a double-edged sword. Moderate autophagy can protect cells while excessive autophagy will bring about autophagic cell death [17]. Abnormal autophagy occurs in the pathology process of SCI [18]. Recent research reported that transcription factor E3 (TFE3) can facilitate SCI recovery through repressing ER stress and augmenting autophagy

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flux [19]. Although metformin's role in improving functional recovery after SCI from the two aspects: autophagy flux stimulation [20] and the relief of ER stress [21] has been noticed, the roles of autophagy and ER stress have not been thoroughly elucidated. In the present work, whether metformin protects against SCI through suppressing XBP-1-mediated ER stress was deeply investigated.

Herein, hindlimb locomotor function, pathological changes, neuronal viability, apoptosis of spinal cord neurons, autophagy and ER stress response were evaluated to identify the specific role of metformin in SCI and to probe into the molecular mechanism.

Materials and Methods

Establishment of SCI Rat Model

Adult male Sprague Dawley (SD) rats (220–250 g) obtained from the Animal Center of the Chinese Academy of Science (Shanghai, China) were housed in a controlled environmental condition at 22 ± 2.0 °C. Based on Allen's method, the SCI rat model was established [22]. Rats were subjected to anesthesia using 1% sodium pentobarbital (40 mg/kg, i.p.). Laminectomy was implemented to expose the T9-T11 lamina and weight impact contusions were executed on the exposed posterior spinal cord surface. SCI model was successfully established when the body trembled, the tail swayed, and a fluttering retraction occurred in the hind limbs and body. SD rats were divided into four groups: (I) Sham group (N = 10), including healthy controls that were subjected to sham operation; (II) SCI model group (N = 10), SCI rat model receiving no treatment; (III) 20 mg/kg MET group (N = 10), SCI rat model receiving 20 mg/kg metformin treatment; (IV) 100 mg/kg MET group (N = 10), SCI rat model receiving 100 mg/kg metformin treatment. In order to avoid large difference in injury levels, Basso-Beattie-Bresnahan (BBB) score for evaluating hindlimb locomotor function was conducted to assess the severity of injury after SCI induction and SCI rats with little difference in injury levels were screened for the experiments. SCI rats were intraperitoneally injected with 20 or 100 mg/kg metformin (317240, Sigma-Aldrich, St. Louis, MO, USA), respectively, immediately after surgery [23]. After 28 days, a lethal dose of pentobarbital was administered to SD rats and the spinal cord samples were subjected to histopathological examination. Experiments were performed under a project license (No. IACUC FJMU 2022-0566) granted by the Institutional Ethics Committee of Fujian Medical University Union Hospital and Fujian Medical University.

Basso-Beattie-Bresnahan (BBB) Score

BBB score was performed to evaluate the restoration of hindlimb locomotor function [24] in rats on 14, 28 d after SCI establishment.

Hematoxylin and Eosin (H&E) Staining

The injured spinal cord was fixed in 4% formaldehyde after cardiac perfusion, then embedded with paraffin and sliced into 4 μm sections after dehydration. Subsequently, the sections were stained with a commercial H&E kit (G1120, Solarbio, Beijing, China) and photographed under an optical microscope (BX53, Olympus, Tokyo, Japan).

Cell Culture

PC12 cells (CRL-1721, ATCC, Manassas, VA, USA) were cultivated in DMEM (11966025, Gibco, Carlsbad, CA, USA) containing 10% FBS (A5669701, Gibco, Carlsbad, CA, USA) and 1% antibiotics (15070063, Gibco, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere with 5% CO_2. By querying ExPASy Cellosaurus databases (https://www.cellosaurus.org/), the cell line (PC12 cells) used in the present research is not misidentified or cross-contaminated. The cell line (PC12 cells) was free of mycoplasma contamination.

Cell Treatment

ER stress was induced in PC12 cells by administration of 1 μM thapsigargin (586005, Sigma-Aldrich, St. Louis, MO, USA) for 20 h [25]. Then, 100 μmol/mL metformin was given to PC12 cells for 24 h [26].

Cell Transfection

The XBP-1 lentivirus (Ov-XBP-1) were synthesized and purified from Genechem (Shanghai, China). Polymerase chain reaction (PCR) was used to amplify XBP-1 cDNA, followed by insertion of the viral vector. PC12 cells were transduced with lentivirus using co-transfection reagent polybrene (REVG0001, Genechem, Shanghai, China). Then, 3 μg/mL puromycin was given to PC12 cells for 7 days to select the stable-expressed cells. The *XBP-1* primers: forward, 5′-GCTCTAGAGCCACCATGGTGGT GGTGGCAGCGGCGC-3′ and reverse, 5′-CGGGATCCC TAGAGGCTTGGTGTATACATGGTC-3′.

Cell Viability

96-well plates were seeded with PC12 cells (5 × 10^3/well). After treatment described above, 10 μL of cell counting kit-8 (CCK-8) solution was given to PC12 cells for 2 h of cultivation at 37 °C. The microplate reader (Model 680 XR, Bio-Rad, Hercules, CA, USA) recorded $OD_{450\ nm}$ of each well. Cell viability (%) = (OD value of experimental group – OD value of blank group)/(OD value of control group – OD value of blank group) × 100%.

Cell Apoptosis

Apoptosis of spinal cord tissues and PC12 cells was measured using TdT-mediated dUTP nick-end labeling (TUNEL) Apoptosis Detection kit (Roche, Basel, Switzerland). Briefly, following fixation in 4% formaldehyde

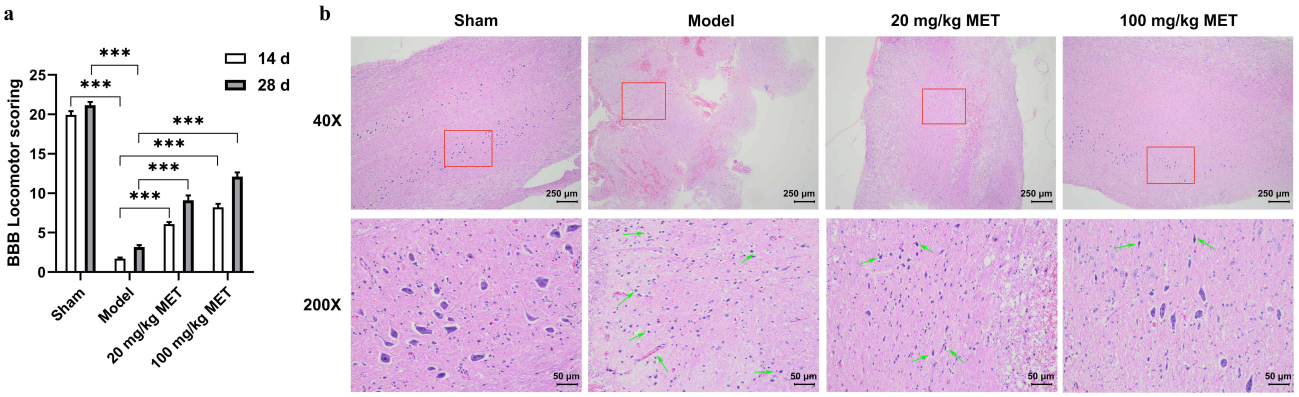

Fig. 1. Metformin promotes functional recovery and reduces lesion size after SCI. Spinal cord injury (SCI) rats were intraperitoneally injected with 20 or 100 mg/kg metformin. N = 10. (a) Motor function at 14 d and 28 d after SCI was assessed using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale. (b) Hematoxylin and eosin (H&E) staining of spinal cord tissues, arrows indicate Nissl dissolution; magnification, 40×, scale bar = 250 μm; magnification, 200×, scale bar = 50 μm. *** $p < 0.001$.

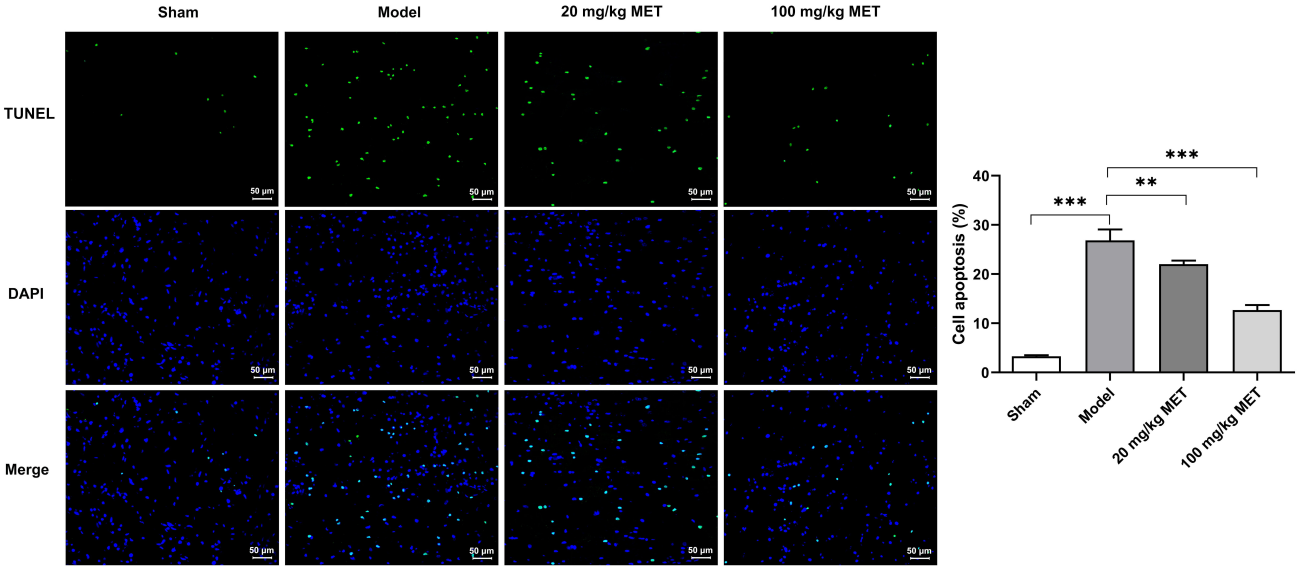

Fig. 2. Metformin attenuates apoptosis in spinal cord tissues of SCI rats. SCI rats were intraperitoneally injected with 20 or 100 mg/kg metformin. TdT-mediated dUTP nick-end labeling (TUNEL) staining of apoptosis in spinal cord tissues, scale bar = 50 μm. ** $p < 0.01$, *** $p < 0.001$.

and permeabilization with 0.2% Triton X-100, samples were then labeled by TUNEL. Next, 4', 6-diamidino-2-phenylindole (DAPI, C1002, Beyotime, Shanghai, China) was applied to counterstain sections. Representative images under fluorescence microscope were captured and Image J (version 1.37, National Institute of Health, Bethesda, MD, USA) was adopted to calculate the percentage of TUNEL-positive cells.

Reverse Transcription-Quantitative PCR

RNA extracted from PC12 cells by employing TRIzol reagent was converted into cDNA using cDNA Synthesis Kit (K1622, Invitrogen, Carlsbad, CA, USA). Next, SYBR Premix Ex Taq reagents (RR820B, Takara, Tokyo, Japan) were employed to perform qPCR reaction on ABI 7500 quantitative PCR instrument (ABI 7500, Applied Biosystems, Foster City, CA, USA). The expression of gene was determined by $2^{-\Delta\Delta Ct}$ method. The sequences of primers: *XBP-1*: forward 5′-ACACGCTTGGGGATGGATGC-3′, reverse 5′-CCATGGGAAGATGTTCTGGG-3′; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): forward 5′-CTGCACCACCAACTGCTTAG-3′, reverse 5′-AGGTCCACCACTGACACGTT-3′.

Western Blot Analysis

The concentration of protein extracted from spinal cord tissues and PC12 cells by employing RIPA lysis buffer was determined via BCA method. 8% or 12% SDS-PAGE-separated protein was transferred to PVDF membranes (IPFL20200, Millipore, Billerica, MA,

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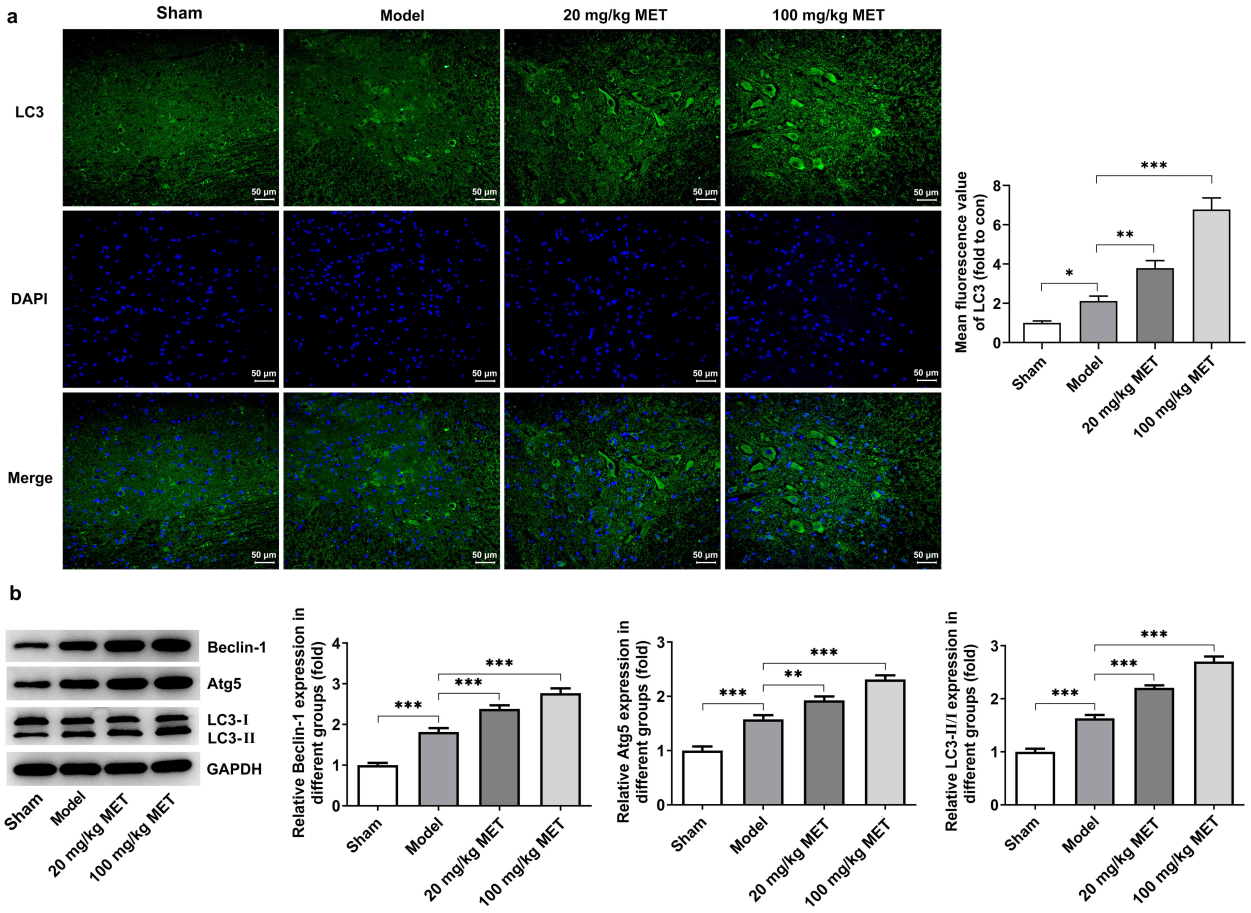

Fig. 3. Metformin strengthens autophagy in SCI rats. SCI rats were intraperitoneally injected with 20 or 100 mg/kg metformin. (a) Immunofluorescence staining of light chain 3 (LC3) expression, scale bar = 50 μm. (b) Western blot analysis of Beclin-1, autophagy-related gene 5 (Atg5), LC3-I as well as LC3-II expressions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

USA). Membranes were then incubated with primary antibodies (Abcam, Cambridge, MA, USA) including anti-Beclin-1 (ab207612, 1:2000), anti-autophagy-related gene 5 (Atg5) (ab228668, 1:3000), anti-LC3 (ab221794, 1:1000), anti-GRP78 (ab21685, 1:1000), anti-DNA damage inducible transcript 3 (CHOP) (ab11419, 1:200), anti-Caspase-12 (ab62484, 1:2000), anti-Pro-caspase-12 (ab8117, 1:1000), anti-inositol-requiring enzyme-1 (IRE-1) (ab37073, 1:1000), anti-XBP-1 (ab37152, 1:1000) as well as anti-GAPDH (ab9485, 1:2500) overnight at 4 °C. On the second day, membranes were incubated with secondary antibody (ab205718, 1:50,000; ab205719, 1:20,000, Abcam, Cambridge, MA, USA) for 2 h at room temperature. With the application of an electrochemiluminescence (ECL) kit (WBULS0100, Millipore, Billerica, MA, USA), protein signals were developed. Image J (version 1.37, National Institute of Health, Bethesda, MD, USA) was adopted to analyze band intensities.

Immunofluorescence

After fixation in 4% formaldehyde, permeabilization with 0.1% Triton X-100 and blocking in 10% goat serum, samples were then incubated with primary antibodies anti-LC3 (ab48394, 1:1000, Abcam, Cambridge, MA, USA) or anti-GRP78 (ab227865, 1:1000, Abcam, Cambridge, MA, USA) overnight at 4 °C. On the next day, samples were subjected to 1 h of cultivation with FITC conjugated secondary antibody (ab150077, 1:1000, Abcam, Cambridge, MA, USA) at room temperature. Subsequently, DAPI was employed to counterstain for 5 min. Images were observed and photographed with the help of fluorescence microscope.

Statistical Analysis

Data of 3 independent repeats were expressed as mean ± SD. Comparisons among multiple groups were conducted by employing one-way analysis of variance followed by Tukey's post hoc test. Two-way analysis of variance followed by Tukey's post hoc test was employed for analyses of BBB score and CCK-8 assay. Differences were considered as statistically significant when $p < 0.05$.

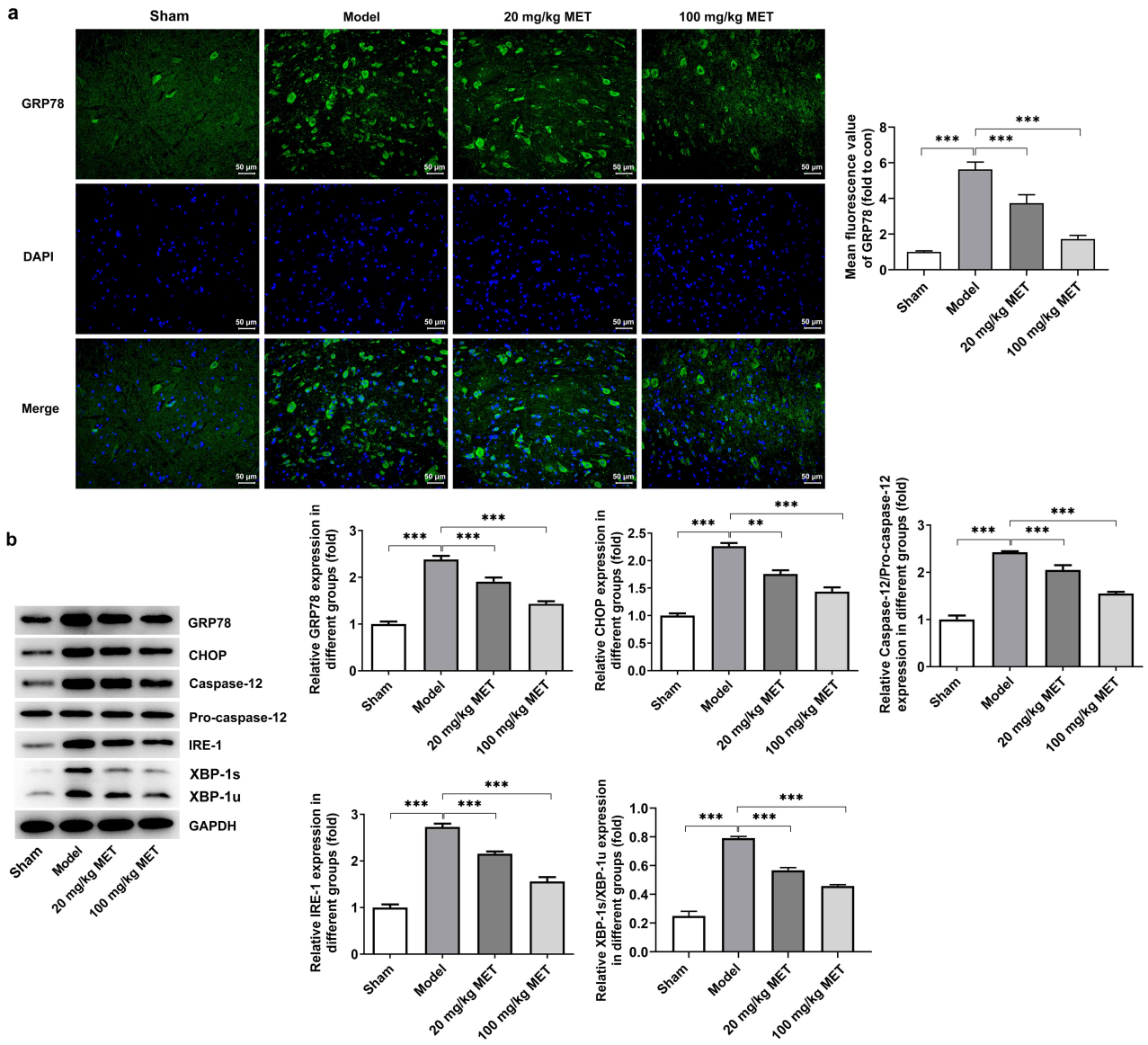

Fig. 4. Metformin suppresses endoplasmic reticulum (ER) stress caused by SCI. SCI rats were intraperitoneally injected with 20 or 100 mg/kg metformin. (a) Immunofluorescence staining of glucose regulated protein 78 (GRP78) expression, scale bar = 50 μm. (b) Western blot analysis of GRP78, DNA damage inducible transcript 3 (CHOP), Caspase-12, Pro-caspase-12, inositol-requiring enzyme-1 (IRE-1), XBP-1s as well as XBP-1u expressions. ** $p < 0.01$, *** $p < 0.001$. XBP-1, X-box binding protein 1.

Results

Metformin Promotes Functional Recovery and Reduces Lesion Size after SCI

SCI rat models were established to evaluate the influences of metformin on SCI *in vivo*. Motor function on 14 d and 28 d after SCI was assessed using BBB locomotor rating scale. BBB scores of the sham group were about 20 normally, whereas SCI rats displayed extremely low BBB scores. BBB scores of SCI rats receiving metformin treatment significantly increased. Metformin recovered the motor function of SCI rats to a certain extent (Fig. 1a). The structure of rat spinal cord in the sham group was normal. The contours of neurons were clear and the nucleolus were clearly visible. In SCI rats, multifocal hemorrhage in spinal cord, a large number of neuron necrosis and dissolution of Nissl body were observed. As expected, metformin increased motor neurons and reduced lesion size in spinal cord. Besides, pathological observation showed that structure damage and cell swelling were relieved upon metformin treatment, and high dosage of metformin achieved better curative effects (Fig. 1b).

Metformin Attenuates Apoptosis in Spinal Cord Tissues of SCI Rats

For the measurement of apoptosis in the spinal cord, TUNEL staining was executed. In comparison with rats in the sham group, distinctly increased TUNEL-positive cells

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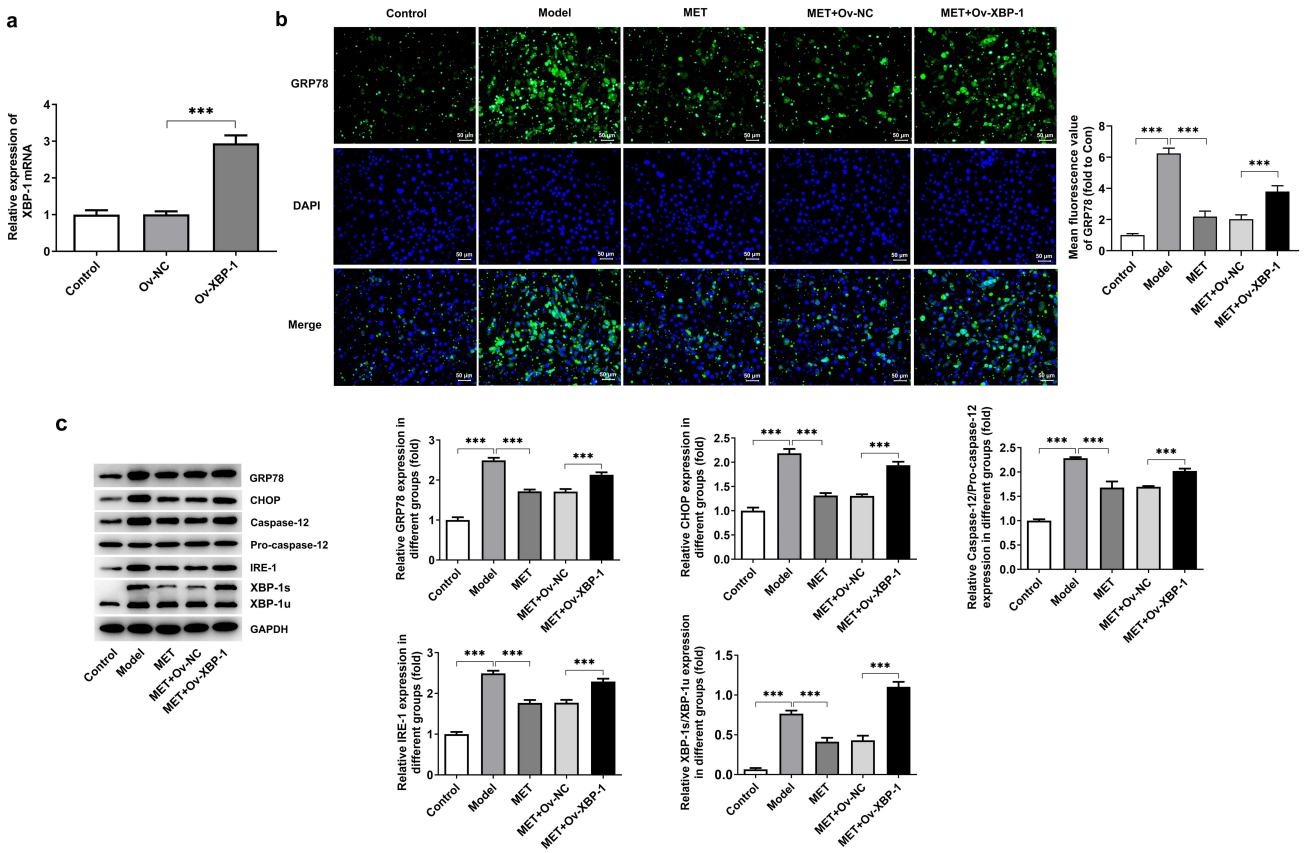

Fig. 5. Metformin relieves ER stress in thapsigargin-treated PC12 cells by repressing XBP-1 expression. ER stress was induced in PC12 cells by administration of 1 μM thapsigargin for 20 h. Then, 100 μmol/mL metformin was given to PC12 cells for 24 h. Treated-PC12 cells were transfected with Ov-XBP-1 or Ov-NC. (a) RT-qPCR analysis of XBP-1 mRNA level. (b) Immunofluorescence staining of GRP78 expression, scale bar = 50 μm. (c) Western blot analysis of GRP78, CHOP, Caspase-12, Pro-caspase-12, IRE-1, XBP-1s as well as XBP-1u expressions. *** $p < 0.001$.

were observed in SCI rats, suggesting that SCI caused obvious apoptosis in spinal cord tissues. In contrast, metformin dose-dependently decreased the number of TUNEL-positive cells, reducing apoptosis in spinal cord tissues of SCI rats (Fig. 2).

Metformin Strengthens Autophagy in SCI Rats

Immunofluorescence staining revealed that SCI resulted in the accumulation of autophagy factor LC3 and metformin treatment further enhanced LC3 accumulation in SCI rats (Fig. 3a). Moreover, SCI led to the elevation of Beclin-1, Atg5 as well as LC3 II/I and metformin treatment reinforced the enhancement on the levels of proteins above (Fig. 3b). To conclude, metformin further activated autophagy in rats after SCI.

Metformin Suppresses ER Stress Caused by SCI

In view of the vital role of ER stress during SCI, ER stress response in SCI rats and the influence of metformin were also investigated. Immunofluorescence staining displayed increased GRP78 fluorescence in rats after SCI and metformin reduced GRP78 fluorescence in SCI rats (Fig. 4a). Additionally, elevated GRP78, DNA damage inducible transcript 3 (CHOP), Caspase-12, inositol-requiring enzyme-1 (IRE-1) as well as XBP-1s/1u levels caused by SCI were partially reversed upon metformin treatment (Fig. 4b). In a word, metformin could suppress ER stress caused by SCI.

Metformin Relieves ER Stress in Thapsigargin-Treated PC12 Cells by Repressing XBP-1 Expression

Furthermore, PC12 cells were employed to probe into the intrinsic mechanisms in the protective effects of metformin against SCI *in vitro*. ER stress was induced in PC12 cells by administration of 1 μM thapsigargin for 20 h. Then, 100 μmol/mL metformin was given to PC12 cells for 24 h. Ov-XBP-1 was introduced into PC12 cells to upregulate XBP-1 expression (Fig. 5a). Increased GRP78 fluorescence was observed in thapsigargin-treated PC12 cells and metformin reduced GRP78 fluorescence. Then, the reduction of GRP78 fluorescence caused by metformin treatment in thapsigargin-treated PC12 cells was reversed upon up-regulation of XBP-1 (Fig. 5b). Besides, thapsigargin treat-

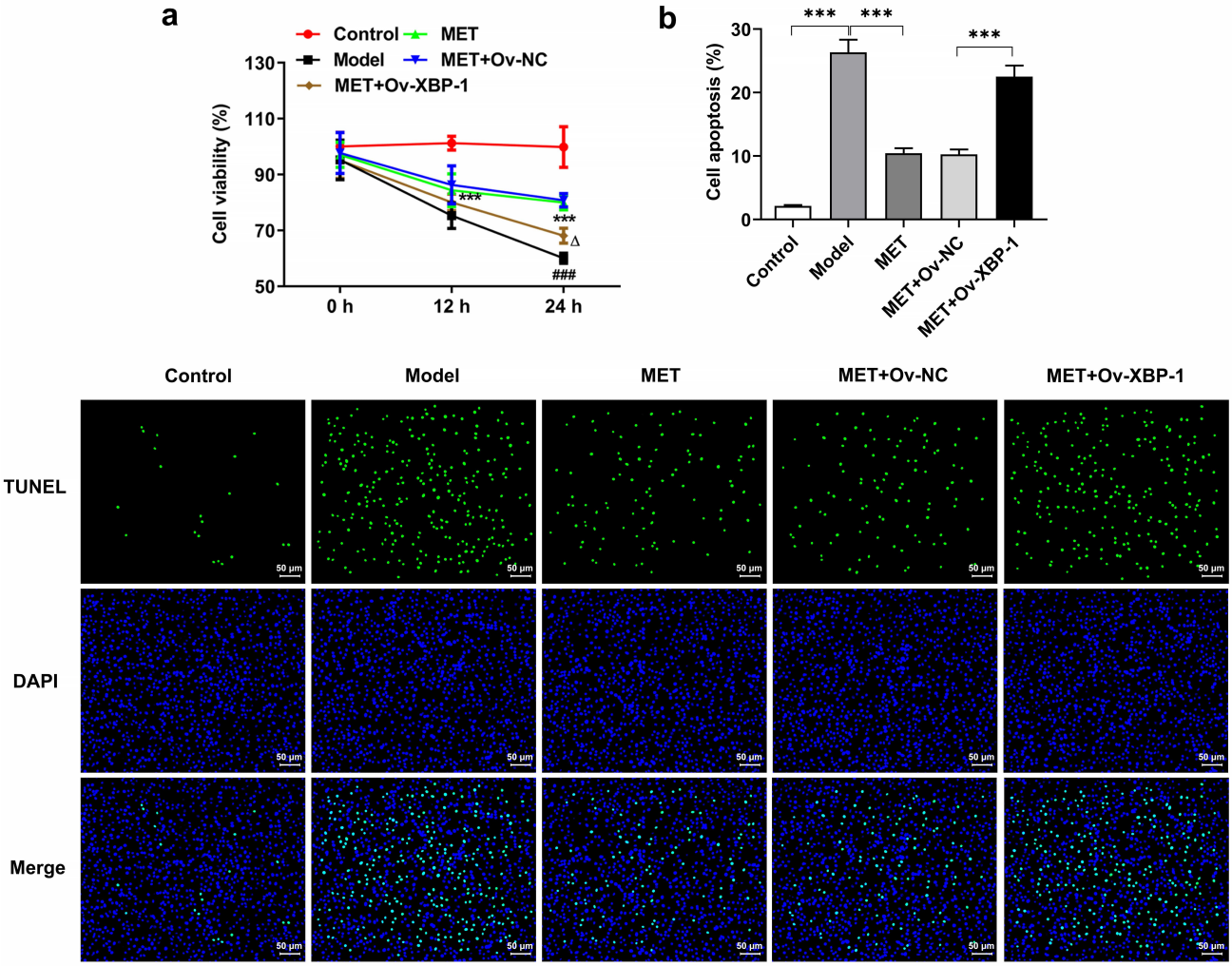

Fig. 6. Metformin attenuates neuronal apoptosis by suppressing XBP-1-mediated ER stress. ER stress was induced in PC12 cells by administration of 1 μM thapsigargin for 20 h. Then, 100 μmol/mL metformin was given to PC12 cells for 24 h. Treated-PC12 cells were transfected with Ov-XBP-1 or Ov-NC. (a) Cell counting kit-8 (CCK-8) assay of neuronal viability; Model group vs. Control group, *** $p < 0.001$; MET group vs. Model group, $^{###}$ $p < 0.001$; MET+Ov-XBP-1 vs. MET+Ov-NC, $^{\Delta}$ $p < 0.05$. (b) TUNEL staining of neuronal apoptosis, scale bar = 50 μm, *** $p < 0.001$.

ment elevated GRP78, CHOP, Caspase-12, IRE-1 as well as XBP-1s/1u levels in PC12 cells, which were partly abrogated by metformin. The suppressing effects of metformin on the levels of proteins above were also reversed upon up-regulation of XBP-1 (Fig. 5c). To sum up, metformin could relieve thapsigargin-induced ER stress by repressing XBP-1 expression in PC12 cells.

Metformin Attenuates Neuronal Apoptosis by Suppressing XBP-1-Mediated ER Stress

Obvious cell injury was observed in thapsigargin-treated PC12 cells. The enhancement of the viability of PC12 cells caused by metformin was abolished upon activation of XBP-1-mediated ER stress (Fig. 6a). In addition, TUNEL staining revealed that elevation of ER stress abolished the suppressing effects of metformin on neuronal apoptosis (Fig. 6b).

Metformin Strengthens Autophagy by Suppressing XBP-1-Mediated ER Stress

Increased LC3 accumulation and Beclin-1, Atg5 and LC3 II/I levels were observed in thapsigargin-treated PC12 cells. Thapsigargin treatment caused a weak elevation of autophagy in PC12 cells. Metformin enhanced LC3 accumulation as well as Beclin-1, Atg5 and LC3 II/I levels, further promoting autophagy in thapsigargin-treated PC12 cells. Then, XBP-1 overexpression inhibited the promoting effects of metformin on LC3 accumulation and Beclin-1, Atg5 and LC3 II/I levels, partly abrogating the further promotion of metformin on autophagy. In general, metformin strengthened autophagy by suppressing XBP-1-mediated ER stress in thapsigargin-treated PC12 cells (Fig. 7a,b).

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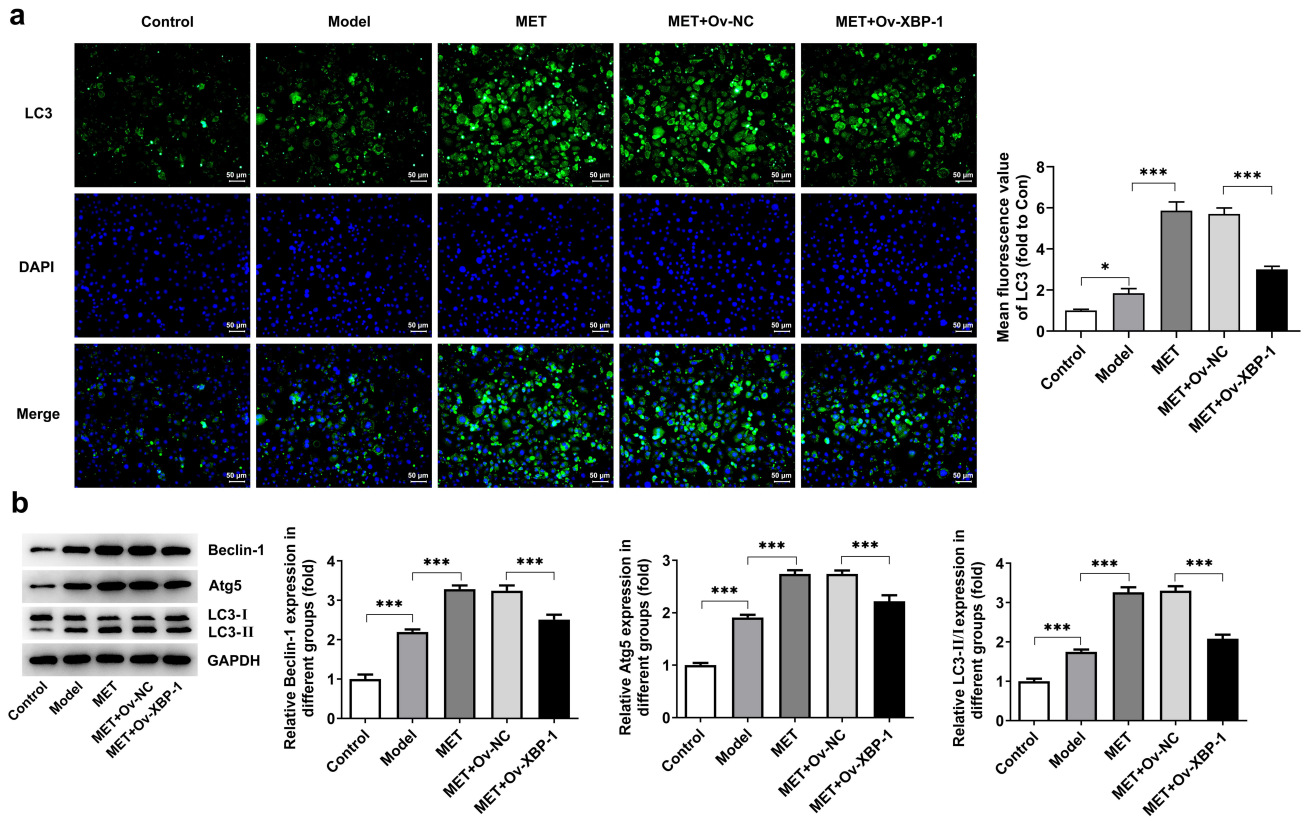

Fig. 7. Metformin strengthens autophagy by suppressing XBP-1-mediated ER stress. ER stress was induced in PC12 cells by administration of 1 μM thapsigargin for 20 h. Then, 100 μmol/mL metformin was given to PC12 cells for 24 h. Treated-PC12 cells were transfected with Ov-XBP-1 or Ov-NC. (a) Immunofluorescence staining of LC3 expression, scale bars = 50 μm. (b) Western blot analysis of Beclin-1, Atg5, LC3-I as well as LC3-II expressions. * $p < 0.05$, *** $p < 0.001$.

Discussion

As a highly disabling disease, SCI brings a heavy burden to patients and society. Recent studies of ER stress on various nervous system diseases have aroused wide concern [27,28]. ER stress is usually triggered by aberrant aggregation of unfolded proteins and misfolded proteins in ER [8,9]. Unfolded proteins could induce the activation of PERK/ATF6 signaling, and then upregulate expressions of chaperones including GRP78, followed by activation of CHOP as well as other ER stress-related proteins, to induce cell apoptosis [29]. Previous studies have proved that CHOP, GRP78 and XBP-1 are elevated after SCI and ER stress-induced apoptosis participates in the response to SCI [9,14]. In our current work, activation of ER stress and obvious neuronal apoptosis was observed in the spinal cord of rats after SCI.

Metformin, a synthetic derivative of guanidine, has been used as a hypoglycemic drug for several decades [5]. Accompanied by the extensive application of metformin, the biological functions of metformin on numerous other diseases were investigated. For instance, Zhang *et al.* [6] report that metformin could promote motor functional recovery and inhibit neuroinflammatory response and neuronal apoptosis in SCI rats. Wang *et al.* [7] reveal that metformin could suppress neuronal apoptosis and repair neurites after SCI. Researches above verify that metformin exhibits excellent neuro-protective effects against SCI. In the present research, it was validated that metformin promoted functional recovery, reduced lesion size and attenuated apoptosis in spinal cord tissues after SCI. In addition, metformin suppressed ER stress caused by SCI. Zhu *et al.* [14] discover that FGF22 administration could promote the recovery of spinal cord lesions by decreasing ER stress-associated proteins. Importantly, studies have confirmed that metformin could adjust ER stress/URP-mediated cell death by regulating UPR and protein synthesis [26,30]. Besides, a recent study by researcher Kokott-Vuong [31] also stated that metformin appears to downregulate the pro-apoptotic PERK arm of UPR. Herein, this work proved that metformin could alleviate neuronal apoptosis to boost the recovery of SCI by suppressing XBP-1-mediated ER stress.

Recent studies illustrate that autophagy is a 'self-eating' pathway involved in ER stress after SCI [27,32]. Literature authenticates that SCI could inhibit autophagy to induce neuronal apoptosis and autophagy activators could prevent neuron death to impede the deterioration of SCI [33]. Besides, increasing evidence suggests that metformin

plays a significant role in the regulation of autophagy. Chuan *et al*. [34] indicate that pretreatment with metformin could promote the activation of AMPK-induced autophagy and inhibit ER stress response to mitigate brain damage following cardiac arrest/cardiopulmonary resuscitation. The present work confirmed that metformin could enhance autophagy and reduce neuronal apoptosis to protect against SCI by suppressing XBP-1-mediated ER stress.

Conclusions

To sum up, metformin promoted functional recovery, reduced lesion size, attenuated neuronal apoptosis, strengthened autophagy and repressed ER stress after SCI. Moreover, the suppressing effects of metformin on neuronal apoptosis and the promoting effects of metformin on autophagy were reversed upon upregulation of XBP-1. Mechanically, metformin could alleviate neuronal apoptosis and enhance autophagy to protect against SCI by suppressing XBP-1-mediated ER stress. Findings above highlighted the protective property of metformin, developing novel strategies for SCI therapies.

Availability of Data and Materials

Data sets during the present study are available from the corresponding authors on reasonable request.

Author Contributions

JDL, ZTSG, WGL and GC contributed to the concept and designed the research study. JDL, ZTSG and ZYW performed the research and contributed to the analysis and interpretation of the data. JDL, ZTSG, WGL and GC wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Experiments were performed under a project license (No. IACUC FJMU 2022-0566) granted by the Institutional Ethics Committee of Fujian Medical University Union Hospital and Fujian Medical University in compliance with its guidelines for the care and use of animals.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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