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ORIGINAL PAPER

Melatonin Enhances Autophagy and Reduces Apoptosis to Promote Locomotor Recovery in Spinal Cord Injury via the PI3K/AKT/mTOR Signaling Pathway

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Abstract
Spinal cord injury (SCI) leads to neuronal death resulting in central nervous system (CNS) dysfunction; however, the pathogenesis is still poorly understood. Melatonin (MT), a hormone secreted mainly by the pineal gland, is associated with neuroprotective effects against SCI. Enhanced autophagy can promote the recovery of locomotor function and reduce apoptosis after SCI. Interestingly, MT increases autophagy in SCI in vivo. Nevertheless, the ability of MT to increase autophagy and decrease apoptosis, and the potential effects on the recovery of motor neurons in the anterior horn after SCI remain to be clarified. In this study, we discovered that MT treatment improved motor function recovery in a rat SCI model. Indeed, MT upregulated the expression of the phosphatidylinositol 3-kinase (PI3K), while expression of protein kinase B (AKT) and mammalian target of rapamycin (mTOR) was downregulated after SCI. Additionally, MT increased the expression of autophagy-activating proteins, while the expression of apoptosis-activating proteins in neurons was decreased following SCI. Furthermore, autophagy was inhibited, while apoptosis was induced in SCI model rats and lipopolysaccharide (LPS)-stimulated primary neurons by treatment with MT, the PI3K inhibitor 3-methyladenine (3-MA) and mTOR inhibitor Rapamycin (Rapa). Collectively, our results suggest that MT can improve the recovery of locomotor function by enhancing autophagy as well as reducing apoptosis after SCI in rats, probably via the PI3K/AKT/mTOR signaling pathway.

Keywords Spinal cord injury · Melatonin · Autophagy · PI3K · AKT · mTOR signaling pathway

Introduction

Spinal cord injury (SCI) is a severe disorder of the central nervous system (CNS) caused by major trauma such as falling from a significant height and road traffic accidents. The morbidity associated with SCI in China is 23.7–60.6/million of the population [1, 2]. There are two phases of acute SCI, primary and secondary (main composition) SCI. The latter leads to pathophysiological processes, such as apoptosis, edema, scarring and inflammation in the injured tissue, which seriously affect the conductive function of the nerves in the corresponding spinal cord segments [3, 4]. Neuronal apoptosis caused by secondary SCI can lead to neuronal dysfunction in patients [5]. Recovery of spinal cord function after acute SCI is difficult.

Autophagy is self-destructive process in which cellular proteins or organelles are encapsulated into vesicles that fuse with lysosomes to form autophagosomes, the contents of which are finally degraded to modify cellular metabolism [6, 7]. In our previous studies, autophagy was shown to improve the recovery of neurological function after acute SCI in a short period of time [8, 9]. Interestingly, apoptosis was shown to increase after inhibiting autophagy of the spinal cord in SCI model rats, a phenomenon that has also been reported [10–12]. Moreover, several studies showed increased levels of autophagy in the damaged area after acute SCI, while also indicating that inhibiting autophagy significantly enhances apoptosis [13–15]. We suggested that enhancing autophagy can lead to a reduction

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in apoptosis and promotion of neurological function recovery after SCI. Therefore, effective strategies for enhancing autophagy to protect against SCI are urgently required.

In recent years, pharmacotherapeutic protection of neurons following SCI has received extensive attention [16, 17]. Melatonin (MT) is a type of neuro-hormone that is produced and secreted by the pineal gland as well as the surrounding tissues during the hours of darkness [18, 19]. Several studies have demonstrated that MT can promote the recovery of motor function after SCI by protecting neurons against oxidation, which indicates the therapeutic potential of MT for SCI [20, 21]. Furthermore, a meta-analysis revealed that MT inhibited neural cell apoptosis to promote locomotor recovery in a rat model of SCI [22] with a similar high level of efficacy that was observed in our previous study [8]. Moreover, Park reported that MT can significantly enhance autophagy to promote neuroprotection [23]. Liu and our previous studies showed that enhancing autophagy can inhibit apoptosis in the spinal cord and induce neurological repair after SCI in vivo [24, 25]. Similarly, our studies indicated that MT induced autophagy in the spinal cord and promote functional recovery of motor neurons in a rat model of SCI. Hence, we hypothesized the existence of a link between MT and autophagy after SCI. In addition, few studies have focused on the relationship between MT, autophagy and SCI. Thus, we further explored the ability of MT to induce autophagy and promote nerve function recovery after SCI both in vivo and in vitro.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway is associated with autophagy, nerve regeneration, inflammation and apoptosis in the CNS [26, 27]. In our previous studies, we showed that enhancing autophagy reduced apoptosis and induced locomotor recovery after acute SCI in vivo via the PI3K/AKT/mTOR signaling pathway [25]. Similarly, our findings indicated that MT regulates the expression of the key components of the PI3K/AKT/mTOR signaling pathway SCI at day 7 in a rat model of SCI.

However, in our previous and other people's studies, the ability of MT to ameliorate SCI and the relationship between MT and the PI3K/AKT/mTOR signaling pathway after SCI are still poorly understood. Therefore, this study, we investigated the potential of MT to reduce neuronal damage and inhibit apoptosis, while enhancing autophagy against acute SCI via the PI3K/AKT/mTOR signaling pathway.

Methods

Animals and Experimental Protocols

Male Sprague–Dawley rats (aged 8–10 weeks, 240–260 g) were provided by the Animal Experimental Center of Jinzhou Medical University (SCXK [Liao] 2014-0004). All animals were housed in cages under specific pathogen-free (SPF) conditions. Animal maintenance and experimental protocols were conducted in accordance with the NIH Guidelines for the care and use of laboratory animals. The rats were randomly divided into three groups (n = 5/group) to determine the optimal concentration of MT (Sigma, USA). In the sham group, an incision was made in the skin and the tissue was blunt-dissected to destroy the lamina at the T9–10 segment without injuring the spinal cord tissue. In the vehicle group, the spinal cord was injured at the T10 segment using a spinal cord impactor according to the method described by Allen [14, 28]. In the MT group, the spinal cord was injured using the same method that was used in the vehicle group. Rats were then treated with MT (12.5 mg/kg/day) by intraperitoneal injection (i.p.) for 7 days; this protocol was based on our previous study [8]. All groups were then housed under SPF conditions prior to experiments.

Cell Viability Assays and Treatment

Primary neurons from the spinal cord of fetal rats were cultivated in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) without fetal bovine serum (FBS, Gibco, USA) for 8 h, then the cells were cultivated in Neurobasal media (Gibco, USA) composed of B27 (Gibco, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used to evaluate cell viability. In brief, primary neurons were treated with MT (0, 5, 10, 20, 40, 80, and 100 μM) and lipopolysaccharide (LPS, 100 ng/ml, Sigma, USA) for 24 h at 37 °C. MTT (20 μM, Sigma-Aldrich) was then added to each well and plates were incubated for 4 h at 37 °C. Dimethyl sulfoxide (150 μl, Sigma-Aldrich) was then added to each well and the absorbance at 490 nm was recorded. Neurons were divided into seven groups: control, LPS, LPS + 3-methyladenine (3-MA, 60 μM, Selleck, USA), LPS + MT, LPS + 3-MA + MT, LPS + Rapa and LPS + rapamycin (Rapa, 10 nM, Selleck, USA) + MT.

BBB Scores

Basso, Beattie and Bresnahan scores (BBB scores) were used to evaluate the motor function of rats on days 1, 3, 7, 14 and 28 after SCI. Briefly, the BBB scores ranged from 0 (complete paralysis) to 21 (without injury) based on observation of the movement and coordination of the ankles, hips, knees and trunk after SCI. BBB scoring was performed by four experienced researchers using a double-blind protocol.

Nissl Staining

On day 7, rats were anesthetized with 10% chloral hydrate, followed by perfusion with saline and 4% paraformaldehyde. The spinal cord section T8–L2 was removed and fixed in 4% paraformaldehyde. After 2 days, 40 tissue sections (thickness, 20 µm; measured using a single-blind method) were prepared with a cryostat (Leica CM3050S; Heidelberg, Germany) and stored at −20 °C. For staining, sections were dried at room temperature (30 min) and then immersed in the chloroform/ethanol (1: 1) overnight. Sections were then washed three times with PBS and immersed in cresyl violet (40 s, 37°C) before washing once with distilled water. Sections were then treated sequentially with 95% ethanol, absolute ethanol, and xylene and mounted with neutral gum for photography under light microscopy.

Western Blot Analysis

On day 7 after SCI, the spinal cord tissue was extracted and total proteins were measured using bicinchoninic acid (BCA) assays according to the manufacturer's instructions. Proteins (20 µg) were separated by SDS-PAGE and transferred to PVDF membranes. After blocking with 5% skimmed milk in TBST (0.1% Tween20, 100 mM NaCl, and 10 mM Tris–HCl, pH 7.4) for 1 h, the membranes were incubated overnight at 4 °C with the following primary detection antibodies: p-PI3K (Abcam, Cambridge, UK, 1: 1,000), p-mTOR (Abcam, 1: 1,000), p-AKT (Abcam, 1: 1000), LC3-B (Abcam, 1: 1000), p62 (Abcam, 1: 1000), Beclin-1 (Abcam, 1: 1000), NeuN (Abcam, 1: 800), cleaved Caspase 3 (Abcam, 1: 500), cleaved Caspase 9 (Abcam, 1: 500), active Caspase 3 (Abcam, 1: 800), active Caspase 9 (Abcam, 1: 1,000), Bax (Abcam, 1: 1000), Bcl-2 (Abcam, 1: 1000), β-actin (Abcam, 1: 1000), and GAPDH (Abcam, 1: 1000). Membranes were then incubated with the corresponding secondary detection antibody (Abcam, goat anti-rabbit IgG or goat anti-mouse IgG, 1: 10,000). Signals were detected using an electrochemiluminescence (ECL) reagent (Sigma, USA) and analyzed using absorbance (A) values with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

RNA Extraction and Quantitative Polymerase Chain Reaction (Q-PCR)

Total RNA was extracted from neurons with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (1 µg) was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The cDNA obtained was subjected to real time polymerase chain reactions (Q-PCR) with the SYBR Green reagent (Roche, Indianapolis, IN, USA) using the following primers: GAPDH, Forward 5′-CCCACGGCAAGTTCAACGG-3′, Reverse 5′-CTTTCCAGAGGGGCCATCCA-3′; lc3b, Forward 5′-CCATGCCGTCCGAGAAGACCTTC-3′, Reverse 5′-CGACCAGCTTCCGCTGGTAACGTC-3′; Casp3, Forward 5′-TACCCTGAAATGGGCTTGTGT-3′, Reverse 5′-GTTAACACGAGTGAGGATGTG-3′; and Bax, Forward 5′-GGATGCGTCCACCAAGAAGC-3′, Reverse 5′-CCTACGCAGGTGGTTCTTCG-3′. The relative mRNA abundance was calculated using the ΔΔCt method, and gene expression levels were normalized against GAPDH.

Immunofluorescence and TUNEL Staining

Frozen sections were prepared from tissue obtained on day 7 after SCI using the same protocol as that used for Nissl staining. Sections were dried at room temperature for 30 min, washed in phosphate-buffered saline (PBS) for 15 min and permeabilized with Triton X-100 (0.3%, diluted in PBS) at room temperature for 10 min. After blocking with sheep serum (Sigma, USA) at room temperature for 2 h, the sections were incubated with a primary antibody for the detection of NeuN (Abcam, 1: 1000), then immersed in equilibration buffer containing TUNEL stain (enzyne solution and labeling solution, TUNEL Apo-Green Detection Kit, Biotool, USA). After NeuN staining, the other sections were incubated with Alexa Fluor®568 (1:500, Life technologies, USA) for immunofluorescence staining. Finally, all the sections were stained with DAPI solution (Abcam, 1: 1000) for 10 min at room temperature. Subsequently, the sections were observed under a fluorescence microscope (Olympus, Japan), and the optical density of the immunofluorescence detected in the sections was analyzed by ImageJ2x software.

Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM) and groups were compared by one-way ANOVA, and Dunnett's post-hoc tests. $P < 0.05$ was considered to indicate statistical significance. Statistical analysis was performed using SPSS 17.0 software.

Result

MT Improved Locomotor Function Recovery and Increased the Number of Spinal Cord Neurons at Day 7 After SCI

In our previous study, MT was shown to promote the recovery of motor capabilities [8], which was consistent with similar reports by Lee and Li [29, 30]. In addition, our previous studies showed that BBB scores can be used to evaluate behavioral changes in rats following SCI [31].

BBB scores are based on the activity of the joints such as the ankle, knee, hip, and tail. In this study, we rigorously adopted BBB scores to evaluate motor function of rats on days 1, 3, 7, 14, and 28 after SCI. The sham group exhibited consistent BBB scores of 21 throughout the study. Compared with vehicle group, the scores in the MT group were significantly increased at days 7 ($P < 0.05$), 14 ($P < 0.05$) and 28 ($P < 0.01$) after SCI (Fig. 1). These observations suggested that MT treatment influenced locomotor recovery from day 7 after SCI; therefore, we evaluated the neuroprotective effects of MT at day 7 after SCI.

Fogerson reported that increasing the number of neurons in the white matter can promote functional recovery of motor neurons after CNS injury [32]. Therefore, we studied the survival of neurons to evaluate the neuroprotective effects of MT on motor neurons by Nissl staining and Western blot analysis of the average number of neurons in the spinal cord at day 7 after SCI [31]. MT significantly increased the number of neurons in the spinal cord ventral horn at day 7 after SCI ($P < 0.05$, Fig. 2a, b). Furthermore, Western blot analysis showed that expression of the neuronal marker NeuN was significantly increased in the in spinal cord at day 7 after SCI in the MT group ($P < 0.05$ vs. the vehicle group). These data unexpectedly suggested that MT improves neuronal recovery following acute SCI in rats.

MT Enhanced Neuronal Autophagy at Day 7 After Acute SCI, Probably via the PI3K/AKT/mTOR Signaling Pathway

According to Hong, MT increases the level of autophagy after SCI over a relatively brief time period [23]. This is in accordance with our previous study showing that enhanced autophagy promotes locomotor function recovery after SCI [14]. Thus, we next investigated the ability of MT to induce autophagy and the potential effects on the expression of autophagy-activating proteins, such as LC3B and Beclin-1.

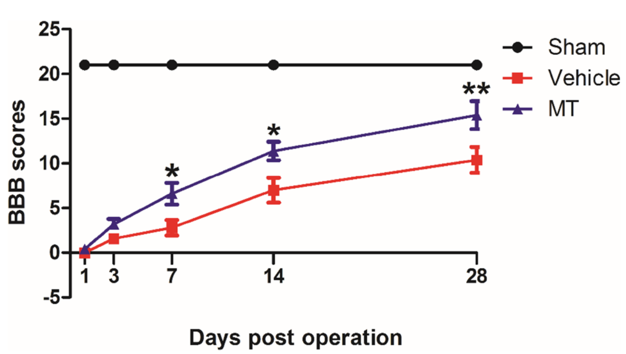


Fig. 1 Basso, Beattie and Bresnahan (BBB) scores were used to show the effect of MT on locomotor function in SCI model rats. Data are presented as the mean ± SD. (*$P < 0.05$, **$P < 0.01$ vs. the vehicle group, n = 5)

In preliminary experiments, autophagy was evaluated by Western blot analysis of LC3-II/LC3-I (LC3B degradation products) and Beclin-1. MT significantly increased the expression of LC3-II/LC3-I ($P < 0.01$), Beclin-1 ($P < 0.05$) and p62 ($P < 0.05$) at day 7 after SCI (Fig. 3a, e–g). To further evaluate the ability of MT to increase autophagy in spinal cord neurons at day 7 after SCI, we used immunofluorescence to show the proportion of LC3B (red)-positive neurons (NeuN, green). Compared with vehicle ($P < 0.05$) and sham ($P < 0.01$) groups, MT increased the proportion of LC3B-positive neurons at day 7 after SCI (Fig. 4).

We have previously demonstrated that activation of the PI3K/AKT/mTOR signaling pathway leads to increased spinal cord neuron autophagy after SCI [13]. In this study, we showed that p-PI3K, p-mTOR and p-AKT expression was reduced in the MT group compared with that in the vehicle group ($P < 0.05$, $P < 0.01$) (Fig. 3a–d). Thus, these data suggested that MT enhances autophagy in spinal cord neurons at day 7 after SCI via the PI3K/AKT/mTOR signaling pathway.

MT Inhibited Neuronal Apoptosis in the Spinal Cord Neurons After SCI

Previous reports have indicated that apoptosis is the cause of damage to neurons as well as white matter after SCI [33]. Therefore, we investigated the ability of MT to decrease apoptosis and reduce damage after SCI. As shown in Fig. 5, MT significantly decreased the expression of apoptosis-activating proteins (cleaved Caspase 3, cleaved Caspase 9, active Caspase 3, active Caspase 9 and Bax; $P < 0.01$, $P < 0.05$) at day 7 after SCI, while expression of apoptosis-inhibiting proteins was increased (Bcl-2; $P < 0.05$). Similarly, MT reduced the number of TUNEL (green)-positive neurons (NeuN, red) in the spinal cord at day 7 after SCI ($P < 0.01$, Fig. 6). These data suggested that MT inhibits apoptosis in neurons after SCI in rats.

MA Inhibits Neurons, Autophagy and Induces Apoptosis with the Treatment of Melatonin in SCI Model Rats

We investigated the effects of 3-MA to decrease the number of neurons, autophagy and increase apoptosis with the treatment of MT after SCI in rats. Figure 7a, b show that 3-MA can decrease the number of neurons after MT treatment in SCI model. As shown in Fig. 7c, compared with SCI alone, the LC3B expression was significantly decreased by 3-MA treatment (Fig. 7c, $P < 0.01$). In addition, compared with the SCI + MT group, LC3B expression was significantly decreased in the SCI + 3-MA + MT group (Fig. 7c, $P < 0.01$), while Caspase 3 expression was increased (Fig. 7c, $P < 0.01$).

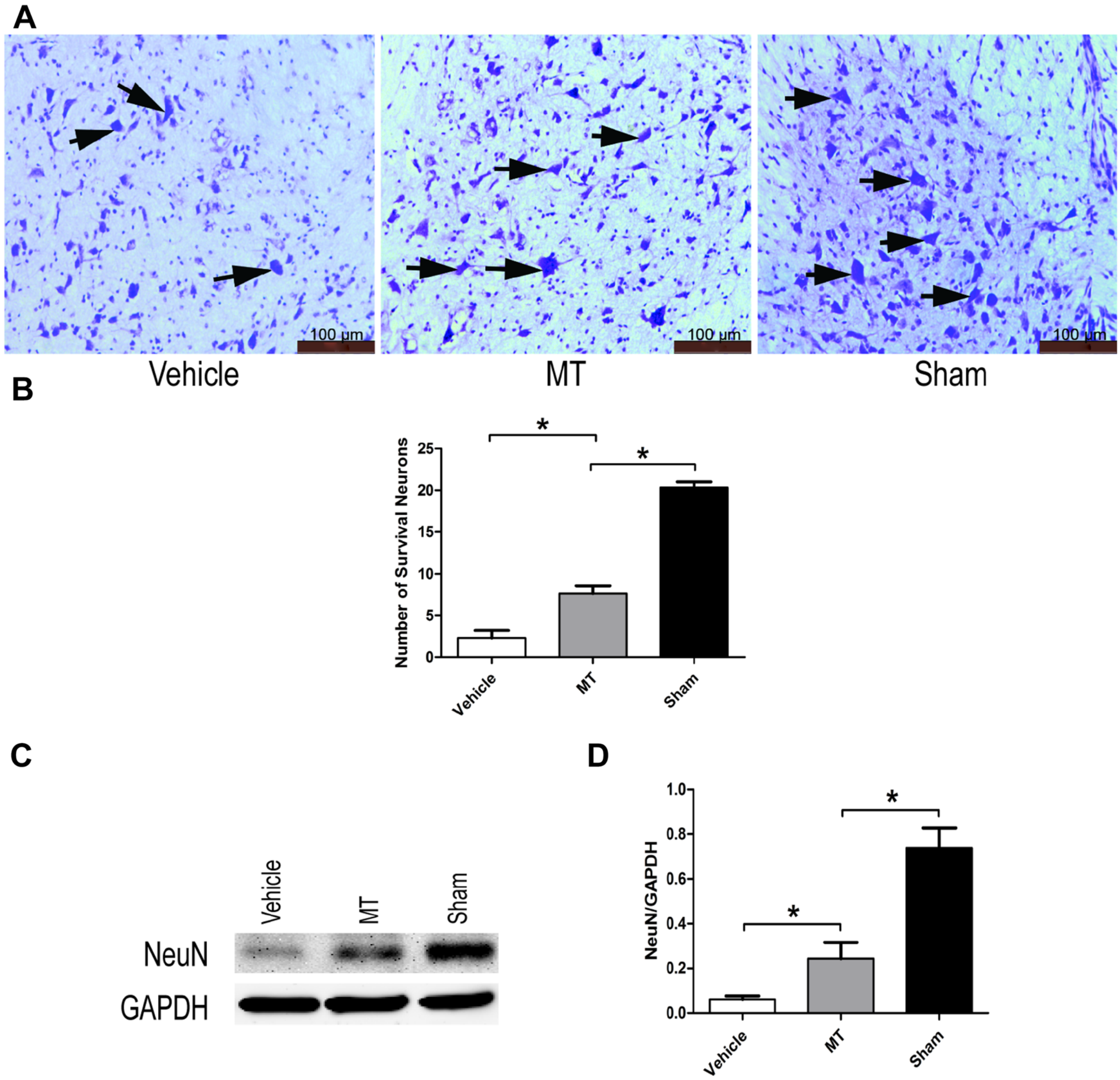


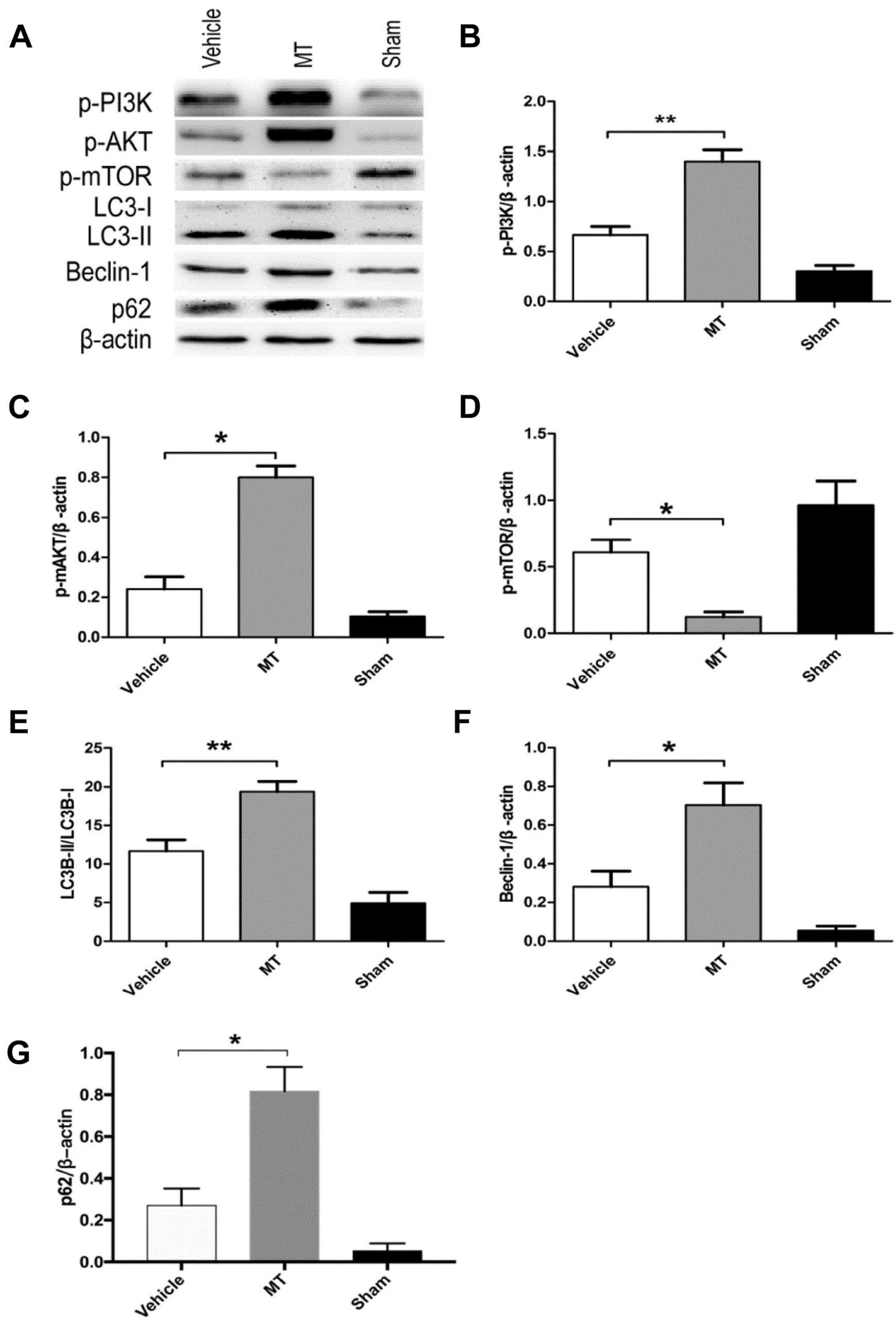
Fig. 2 Nissl staining and Western blot analysis were used to show the effect of MT on the survival rate of neurons in SCI model rats at day 7. **a**, **b** Nissl staining and number of surviving neurons. **c**, **d** Western blot analysis of NeuN expression. Data are presented as the mean ± SD. (* $P < 0.05$ vs. the MT group, n = 5, scale bar = 100 μm)

Selection of the Optimum Concentration of MT in LPS-Induced Primary Neurons

Primary spinal cord neurons were treated with MT (0, 5, 10, 20, 40, 80, and 100 μM) for 24 h before cell viability was analyzed by MTT assay. Compared with the 0 μM control group, there were no significant differences in cell viability following MT treatment at 40 μM (Fig. 8a); therefore, this was selected as the optimum concentration for use in investigations of the effects of MT on LPS-stimulated primary neurons.

Anti-autophagic and Anti-apoptotic Effects of MT were Reduced by Inhibition of the PI3K/AKT/mTOR Signaling Pathway in Primary Spinal Cord Neurons

To evaluate the ability of MT to enhance autophagy and inhibit apoptosis via activation of the PI3K/AKT/mTOR signaling pathway, we treated primary neurons with LPS to simulate an injury model prior to treatment then with MT, the PI3K inhibitor (3-MA) and the mTOR inhibitor (Rapa). Expression of the autophagy-activating LC3B gene and the apoptosis-activating genes Caspase 3 and Bax in


A
Vehicle
MT
Sham
p-PI3K
p-AKT
p-mTOR
LC3-I
LC3-II
Beclin-1
p62
β-actin
B
**
p-PI3K/β -actin
C
*
p-mAKT/β -actin
D
*
p-mTOR/β -actin
E
**
LC3B-II/LC3B-I
F
*
Beclin-1/β -actin
G
*
p62/β–actin

◂**Fig. 3** Western blot analysis was used to show the effect of MT on autophagy and PI3K/AKT/mTOR signaling in SCI model rats at day 7. **a** Western blot of PI3K/AKT/mTOR signaling pathway proteins (p-PI3K, p-AKT, p-mTOR) and autophagy-activating proteins (LC3B and Beclin-1). **b**–**g** Statistical analysis of Western blot. Data are presented as the mean ± SD. (*$P < 0.05$, **$P < 0.01$ vs. the vehicle group, n = 5)

primary neurons was then analyzed by Q-PCR (Fig. 8b–d). Compared with LPS alone, the LC3B expression was significantly decreased by 3-MA treatment (Fig. 8b, $P < 0.01$). In addition, compared with the LPS + MT group, LC3B expression was significantly decreased in the LPS + 3-MA + MT group (Fig. 8b, $P < 0.01$), while Caspase 3 and Bax expression was increased (Fig. 8c and d, $P < 0.01$). However, compared with 3-MA treatment, the expression of LC3B and Caspase 3 were opposite after Rapa treatment (Fig. 8e, f). These results indicated that MT inhibited autophagy and induced apoptosis in PI3K inhibitor (3-MA)-treated neurons after SCI.

Discussion

In this study, we investigated the pathological role of apoptosis and autophagy in locomotor dysfunction after SCI. Our findings confirmed that MT induced a marked recovery of nerve function by promoting autophagy and inhibiting apoptosis. Thus, we further explored the mechanisms underlying the neuroprotective effects MT in SCI in vivo. We showed that MT enhanced autophagy and inhibited apoptosis in neurons, and ameliorated locomotor dysfunction at day 7 after SCI via activation of the PI3K/AKT/mTOR signaling pathway. We aim to provide the evidence that the neuroprotective effects of MT are mediated by enhancing autophagy and reducing apoptosis in neurons to improve locomotor recovery after SCI both in vitro and in vivo.

Several studies showed that high levels of apoptosis in neurons results in effects such as neuronal death, scar formation, histological damage, and locomotor dysfunction [34–36]. Many studies have indicated that Caspase 3 (an interleukin-converting enzyme-like protease) and Caspase 9 (a member of the Caspase family activated by the release of cytochrome c) are related to the activation of apoptosis in rat neurons after acute SCI [37, 38]. Similarly, Bax and Bcl-2, which act as pro-apoptotic and anti-apoptotic factors, respectively, form a heterodimer during acute SCI [39, 40]. Our previous studies demonstrated that increased expression of pro-apoptotic proteins (Caspase 3, Caspase 9 and Bax) after SCI led to locomotor dysfunction and cell death [31]. These findings suggest that the high levels apoptosis caused by SCI can exacerbate neuronal damage. In spite of many studies indicated autophagy not always

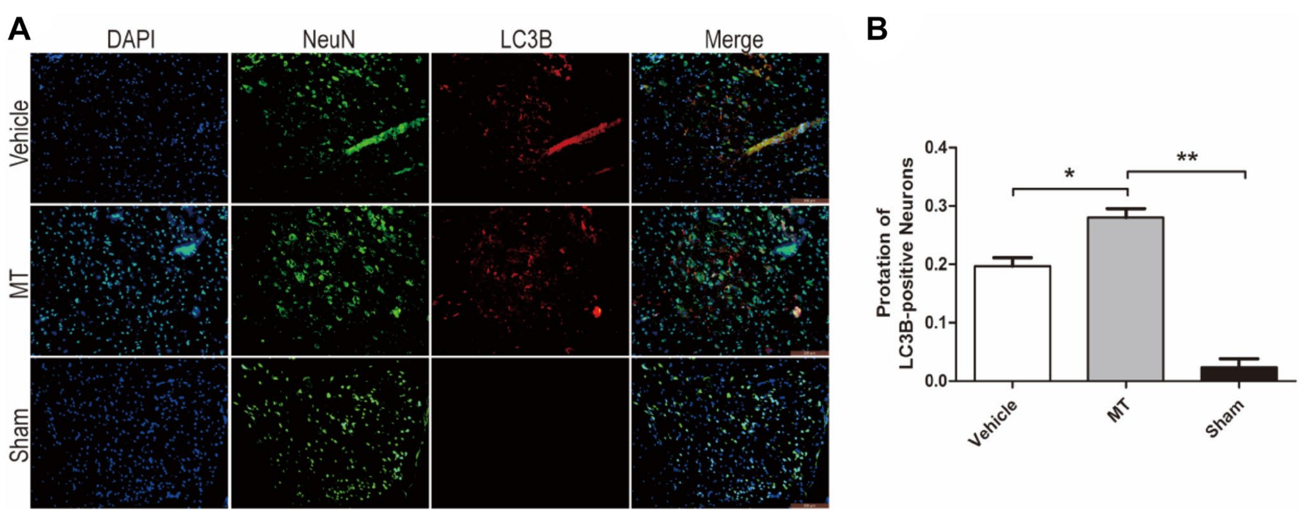


Fig. 4 Immunofluorescence analysis was used to show the effect of MT on autophagy-activatingprotein (LC3B) expression in the spinal cord neurons in SCI model rats at day 7. **a** Immunofluorescence of LC3B (red), NeuN (green) and DAPI (blue). **b** Statistical analysis of immunofluorescence as a proportion of the LC3B-positive neurons. Data are presented as the mean ± SD. (*$P < 0.05$, **$P < 0.01$ vs. the MT group, n = 5, scale bar = 50 μm)

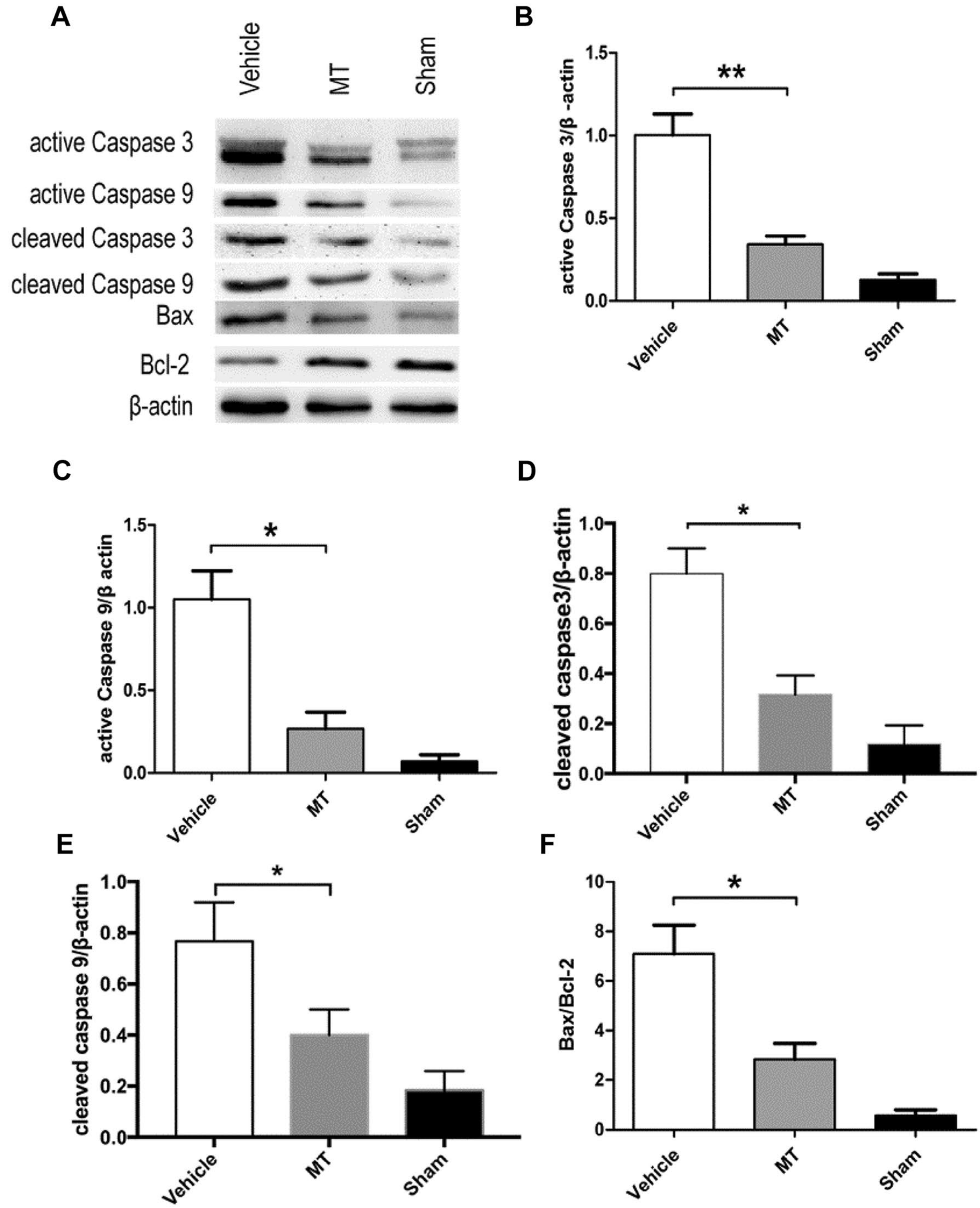


Fig. 5 Western blot analysis was used to show the effect of MT on apoptosis-related protein expression in SCI model rats at day 7. **a** Western blot of apoptosis-related protein (active Caspase 3, active Caspase 9, cleaved Caspase 3, cleaved Caspase 9, Bax and Bcl-2). **b–d** Statistical analysis of Western blot. Data are presented as the mean ± SD. (*$P<0.05$, ** $P<0.01$ vs. the MT group, n = 5)

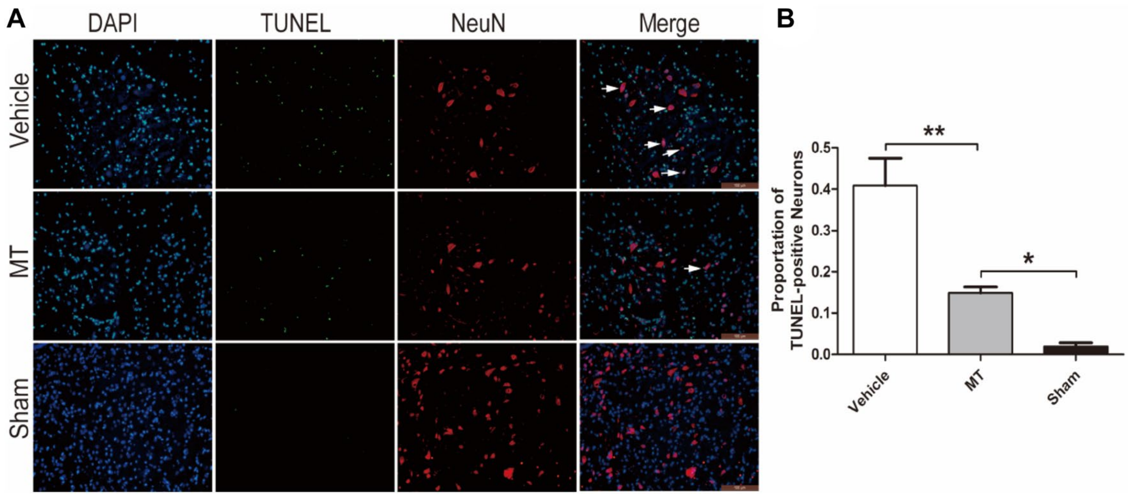


Fig. 6 Immunofluorescence analysis was used to show the effect of MT on TUNEL-positive neurons in spinal cord tissue in SCI model rats at day 7. **a** Immunofluorescence of TUNEL (green), NeuN (red) and DAPI (blue). **b** Statistical analysis of TUNEL staining as a proportion of the TUNEL-positive neurons. Data are presented as the mean ± SD. (* $P < 0.05$, ** $P < 0.01$ vs. the MT group, n = 5, scale bar = 100 μm)

related to the reduction of apoptosis, while our previous studies showed that enhanced autophagy significantly inhibited apoptosis in the spinal cord and improved locomotor function after acute SCI in vivo [25, 41]. Therefore, to determine the mechanism underlying the anti-apoptotic effects of MT in SCI model rats, we focused on autophagy.

Autophagy is an important phase in CNS disorders, such as in Alzheimer's disease (AD), Parkinson's disease (PD), and SCI [42, 43]. Autophagy exerts neuroprotective effects against acute SCI via a mechanism that involves processes such as the inhibition of apoptosis and axon regeneration [44, 45]. However, autophagy is called as "double-edged sword" and some studies reported that, autophagy inhibition seems to be neuroprotective after injury, the phenomenon may related to the different stimulation and tissues [46, 47]. Interestingly, Recent study indicated that MT markedly enhanced neuroprotection against neural damage in the spinal cord in vivo via anti-oxidative stress [20]. Moreover, MT significantly increases autophagy in the CNS in vivo [48]. Similarly, our results showed that MT reduced the expression of apoptosis-activating proteins (active Caspase 3, active Caspase 9 and Bax) and the number of TUNEL-positive neurons at day 7 after acute SCI, while expression of autophagy-activating proteins (LC3B and Beclin-1) and the number of LC3B-positive neurons were increased. Therefore, we suggest that MT enhances autophagy and reduces apoptosis after acute SCI in rats. However, few studies have defined the molecular mechanism by which MT activates autophagy, while reducing apoptosis to improve locomotor function after acute SCI.

Recent studies showed that an optimal dose of MT provided neuroprotective effects in vivo, while lower or higher doses had almost no effect. This phenomenon may relate to the activation of important mitogen activated protein kinases. Similarly, the results of MTT assays of cell viability showed the 40 μM MT was the optimum dose for survival of primary spinal cord neurons. Furthermore, Liu showed that 3-MA inhibited autophagy and accelerated locomotor dysfunction after SCI in rats via the PI3K/AKT/mTOR signaling pathway [24]. Our results showed that p-PI3K, p-AKT and p-mTOR (the key components of the PI3K/AKT/mTOR signaling

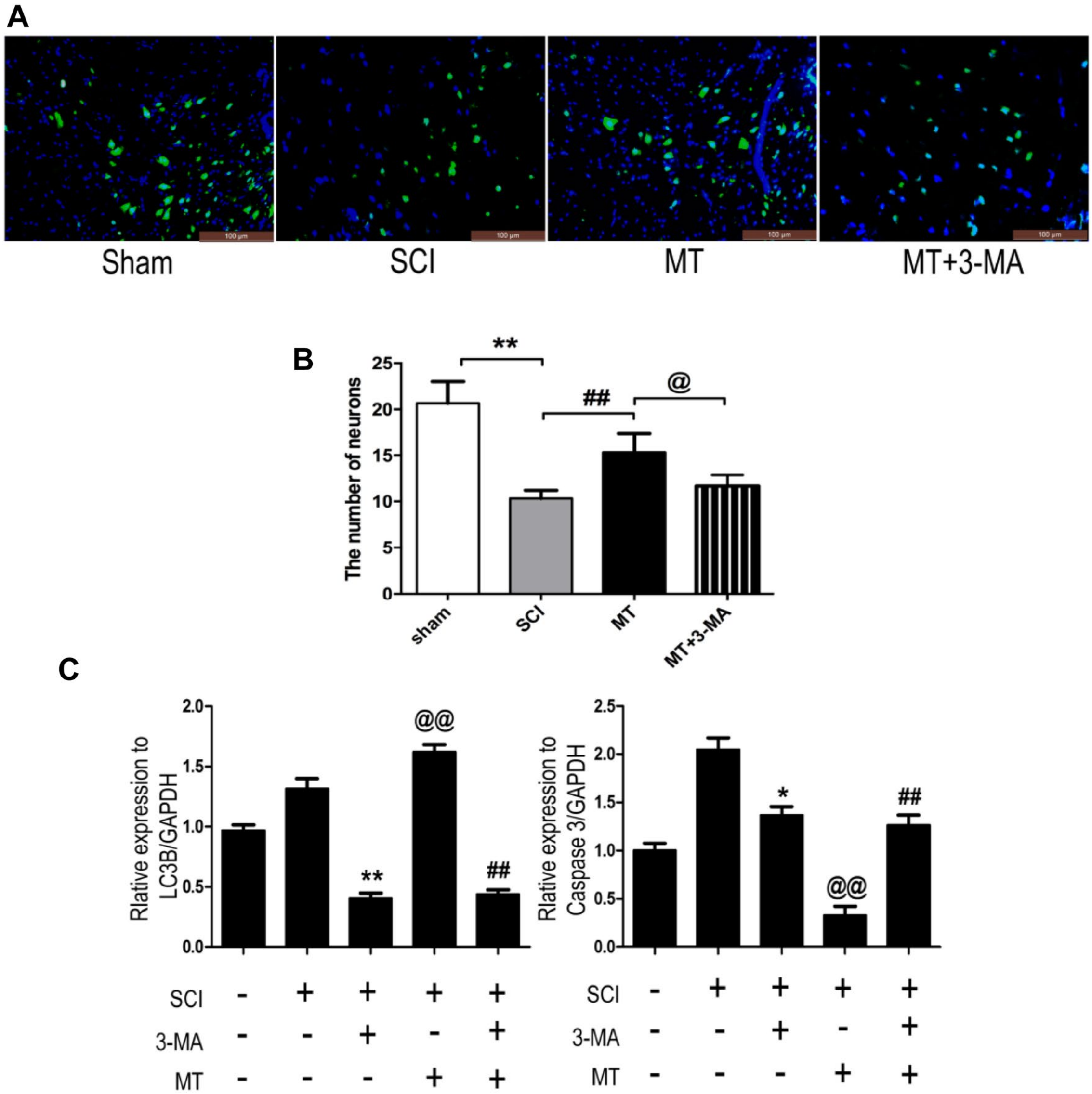


Fig. 7 Immunofluorescence (NeuN green) was used to show the number of neurons, and Q-PCR analysis was used to show the effects of MT on the expression of genes encoding autophagy-activating proteins (LC3B) and apoptosis-activating proteins (Caspase 3) after PI3K inhibitor (3-MA) treatment in SCI model rats. Data are presented as the mean ± SD (*$P < 0.05$, **$P < 0.01$ vs. the SCI group, @@$P < 0.01$ vs. the SCI + 3-MA group, ##$P < 0.01$ vs. the SCI + MT group)

pathway) are activated by the induction of autophagy in spinal cord neurons after acute SCI [25]. This is consistent with other studies demonstrating that MT enhances autophagy following liver injury via the PI3K/AKT/mTOR signaling pathway [49]. We confirmed that that the neuroprotective effect of MT is mediated through induction of autophagy and inhibition of apoptosis via the PI3K/AKT/mTOR signaling pathway using the PI3K inhibitor, 3-MA.

Conclusion

In summary, this study has revealed that MT can improve locomotor function of rats after acute SCI. Additionally, MT was shown to enhance neuronal survival and increase autophagy while reducing apoptosis in spinal cord neurons in SCI model rats probably via the PI3K/AKT/mTOR signaling pathway. Therefore, MT is implicated as a potentially effective therapeutic agent in acute SCI.

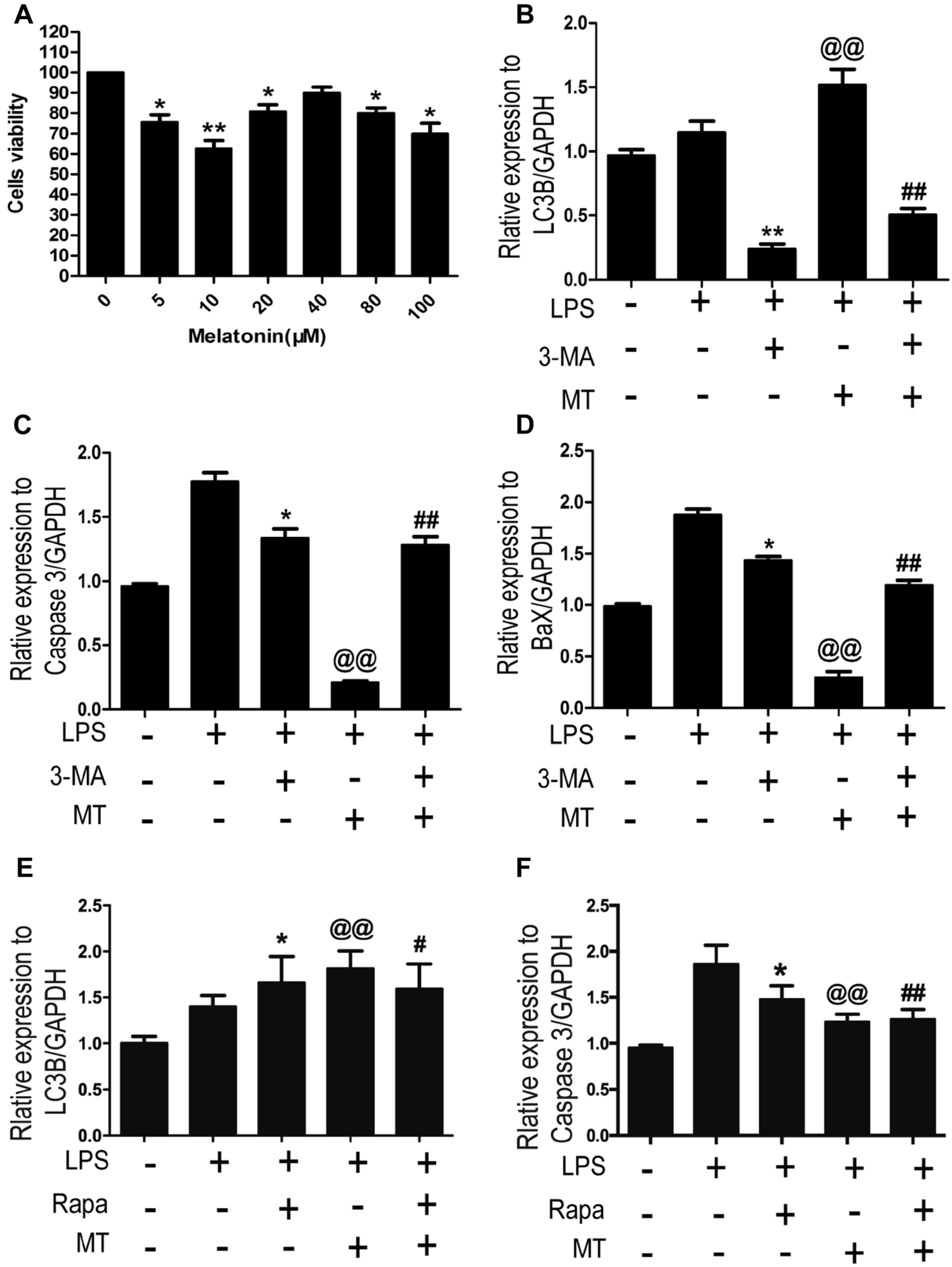


Fig. 8 Q-PCR analysis was used to show the effect of MT on the expression of genes encoding autophagy-activating proteins and apoptosis-activating proteins after PI3K and mTOR inhibitor treatment in primary neurons after LPS injury. **a** Cell viability after MT (0, 5, 10, 20, 40, 80, and 100 μM) treatment (*$P<0.05$, ** $P<0.01$ vs. the 0 μM group). **b**–**f** Statistical analysis of Q-PCR of Caspase 3, Bax and LC3B. Data are presented as the mean ± SD (**a** *$P<0.05$; B-D: **$P<0.01$ vs. the LPS group, @@$P<0.01$ vs. the LPS + 3-MA group, ##$P<0.01$ vs. the LPS + MT group; E–F: *$P<0.05$ vs. the LPS group, @@$P<0.01$ vs. the LPS + Rapa group, ##$P<0.01$ vs. the LPS + Rapa group)

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Compliance with Ethical Standards

Conflict of interest All authors declare no conflict of interest.

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