



Tanshinone IIA alleviates oxidative damage after spinal cord injury *in vitro* and *in vivo* through up-regulating miR-124



Gu Gong^a, Yiqi Gu^b, Yunfeng Zhang^a, Wanguo Liu^a, Li Li^c, Juan Li^{d,*}

^a Department of Orthopedics, China-Japan Union Hospital of Jilin University, Changchun 130033, Jilin, China

^b Department of Breast Surgery, China-Japan Union Hospital of Jilin University, Changchun 130033, Jilin, China

^c Department of Physiology, School of Basic Medical Sciences, Shenzhen University Health Sciences Center, Shenzhen 518060, China

^d School of Public Health Jilin University, Changchun 130021, Jilin, China

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ABSTRACT

Aims: Spinal cord injury (SCI) is a damage of spinal cord caused by trauma or diseases. Here, we explored the effects of tanshinone IIA (Tan IIA) on SCI oxidative damage *in vitro* and *in vivo*.

Materials and methods: *In vitro*, PC-12 cells were treated by H₂O₂ to stimulate oxidative injury. Then, the effects of Tan IIA on cell viability, apoptosis and autophagy were assessed by CCK-8 assay, flow cytometry assay and western blotting, respectively. The expression of miR-124 was measured by qRT-PCR, and whether Tan IIA exerted effects on H₂O₂-treated PC-12 cells through modulating miR-124 was verified. *In vivo*, Sprague-Dawley rats were induced SCI using a weight drop device. Then, the effects of Tan IIA on motor function recovery of rats, myeloperoxidase (MPO) activity in damaged tissue and tissue cell proliferation, apoptosis and autophagy were investigated, respectively.

Key findings: *In vitro*, H₂O₂ stimulation reduced cell viability, and induced cell apoptosis and autophagy. Those alterations were mitigated by Tan IIA treatment. Tan IIA treatment reversed the H₂O₂-induced down-regulation of miR-124. Silence of miR-124 reversed the effects of Tan IIA on H₂O₂-treated PC-12 cells, as well as activation of JNK and p38MAPK pathways. *In vivo*, Tan IIA treatment alleviated the SCI-induced enhancement of MPO activity in damaged tissue, apoptosis and autophagy of damaged tissue cells, and promoted the motor function recovery of rats.

Significance: Tan IIA attenuated oxidative damage after SCI *in vitro* and *in vivo* might be through up-regulating miR-124 and then inactivating JNK and p38 MAPK pathways.

1. Introduction

Spinal cord injury (SCI) is a traumatic event that leads to permanent disability and significantly lowers life quality [1]. It also incurs substantial financial burden to both individual's family and the community [2]. Reported by the World Health Organization and The International Spinal Cord Society, 40–80 new cases per million people (250,000 to 500,000 new cases) are estimated to develop SCI annually worldwide [3]. Despite of the developments of modern medicine, there is no effective strategy to restore the neurological function after SCI, which makes the curative strategies of SCI become a hotspot recently.

The progression of SCI comprises of two steps including primary injury and secondary injury. Primary mechanical insults, such as traumas (accidents, sports injuries and falls), spinal stenosis, osteoarthritis, abscess and tumors, lead to primary damage at the injury epicenter [4]. Then, a cascade of events such as oxidative stress,

demyelination, apoptosis and inflammation are triggered, and if those events are poorly controlled, more extensive damages may be induced [5]. Since the primary injury is abrupt and irreversible, accumulating evidence aims to treat SCI through preventing or delaying secondary injury. For instance, chlorogenic acid ameliorates SCI through inhibiting inflammatory response in rat SCI models [6]. Interleukin-33 has also been reported to improve functional recovery after SCI via decreasing secondary injury [7].

Tanshinone IIA (Tan IIA) is a natural phenanthrene-quinone that is isolated from the dried roots of *Salvia miltiorrhiza* [8]. Despite of the traditional application in coronary heart diseases and cerebrovascular diseases, recent literatures revealed its potential function in tumor therapy [9,10]. Moreover, the anti-oxidant, anti-inflammatory and anti-apoptotic properties of Tan IIA have been well documented in more and more reports. Tan IIA has been proved to repress inflammation and oxidative stress in a rat model of cirrhosis [11]. Another study also

* Corresponding author at: School of Public Health Jilin University, No.1163, Xinmin Street, Changchun 130021, Jilin, China.

E-mail address: juanli0112@sohu.com (J. Li).

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reported the protective role of Tan IIA in renal damage through repressing oxidative stress and inflammation [12]. However, literatures focused on the functional roles of Tan IIA in SCI are limited. A previous literature once demonstrated the anti-inflammatory and anti-apoptotic roles of Tan IIA in rats with SCI [13]. Whether there is an inhibitory effect of Tan IIA on oxidative stress after SCI is not clear.

MicroRNAs (miRNAs) are a group of small and endogenous RNA transcripts in cells without protein-coding activity [14]. It is demonstrated that miRNAs play critical roles in the regulation of multiple cellular biological processes and participate in the pathogenesis of many human diseases, including SCI [14,15]. miRNA-124 (miR-124) is abundantly expressed in neurons in the mammalian central nervous system and involved in the regulation of gene expression during embryonic neurogenesis and postnatal neural differentiation [16]. Xu et al. indicated that miR-124 in neurons was reduced after SCI, while over-expression of miR-124 could promote the repair of SCI [17].

In our study, we explored the effects of Tan IIA on SCI *in vitro* and *in vivo*. *In vitro*, the rat pheochromocytoma (PC-12) cells were treated by H₂O₂ to stimulate oxidative injury. The possible regulatory mechanism about the anti-injury role of miR-124 was also investigated. *In vivo*, Sprague-Dawley rats were induced SCI using a weight drop device. In our opinion, the findings of our research will be helpful for further understanding the therapeutic mechanism of Tan IIA for SCI prevention and treatment.

2. Materials and methods

2.1. Cell culture and treatments

Rat PC-12 cells (Cell Bank of Type Culture Collection, Chinese Academy of Science, Shanghai, China) were cultivated in flasks with a density of 1×10^4 cells/mL. The basal medium was Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (both Sigma, St. Louis, MO, USA). Cells were maintained in a humidified incubator that was filled with 5% CO₂ and 95% air at 37 °C. Culture medium was replaced by fresh ones every other day. After attachments, cells were incubated in DMEM containing increasing concentrations of H₂O₂ (0, 12.5, 50, 100 and 200 µM) for 6 h to explore the adequate inductive dosage of H₂O₂. For treatment with Tan IIA, cells were pre-treated with increasing concentrations of Tan IIA (0, 1, 2, 3 and 4 µM; Sigma) for 9, 18, 27 or 36 h prior to H₂O₂ treatments.

2.2. Cell transfection

miR-124 inhibitor and its negative control (NC) were synthesized by GenePharma (Shanghai, China). At 70–80% confluence, miRNAs were transfected into cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

2.3. Cell viability assay

Viability of PC-12 cells was determined by Cell Counting Kit-8 (CCK-8) assay. Briefly, 5×10^3 cells were plated into the each well of 96-well plates, followed by incubation at 37 °C overnight. After desired treatments, the culture medium was replaced by fresh DEME containing 10% CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD, USA). After that, cells were incubated at 37 °C for 1 h. A Microplate Reader (Bio-Rad, Hercules, CA, USA) was utilized for measurements of the absorbance at 450 nm.

2.4. Apoptosis assay

Apoptosis of PC-12 cells was assessed by double staining with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated

Annexin V. Briefly, after desired treatments, cells were collected in phosphate buffered saline (PBS). Then, after rinsing, cells were suspended in binding buffer from the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen). According to the recommended protocols, cells were stained with FITC-Annexin V and PI at room temperature for 15 min. Cells were washed with binding buffer, and then were subjected to flow cytometry analysis using a FACS can (Beckman Coulter, Fullerton, CA, USA). Percentage of apoptotic cells was analyzed by using FlowJo software (Tree Star, San Carlos, CA, USA).

2.5. Quantitative reverse transcription PCR (qRT-PCR)

Total RNAs of PC-12 cells were extracted by using a miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Then, RNAs (500 ng) were reversely transcribed into cDNAs using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) as recommended by the supplier. The thermal-cycling conditions were 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C. The following real-time PCR was performed using the TaqMan Universal Master Mix II (Applied Biosystems), and the PCR conditions were as follows: an initial incubation at 95 °C for 10 min, then 40 cycles consisting of 95 °C for 15 s and 60 °C for 1 min. The relative expression of miR-124 was analyzed according to the 2^{-ΔΔCt} method [18]. U6 was used as the internal control.

2.6. Western blotting

After desired treatments, cells or tissues were lysed in RIPA buffer (Beyotime Biotechnology, Shanghai, China). Supernatants of cell lysates were collected, and the contents of proteins in the supernatants were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Proteins were then separated by SDS-PAGE, followed by blotting to the polyvinylidene difluoride (PVDF) membranes. Then, these PVDF membranes were blocked by 5% skimmed milk powder in TBST (25 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) for 1 h, followed by incubation with primary antibodies against B cell lymphoma-2 (Bcl-2; ab196495), Bcl-2-associated X protein (Bax; ab182733), cleaved caspase-3 (ab49822), pro caspase-3 (ab90437), microtubule-associated protein 1 light chain-3B (LC3B; ab48394), Beclin-1 (ab62557), p62/sequestosome 1 (p62; ab155686), β-actin (ab8229, all Abcam, Cambridge, UK), caspase-9 (9508), c-Jun N-terminal kinase (JNK; 9252), phospho (p)-JNK (9251), c-Jun (9165), p-c-Jun (2993), p38MAPK (9212) or p-p38MAPK (9211, all Cell Signaling Technology, Beverly, MA, USA) at 4 °C overnight. After rinsing in TBST, membranes were incubated with secondary antibodies (goat anti-rabbit, ab205718; goat anti-mouse, ab205719, both Abcam) marked by horseradish peroxidase for 1 h at room temperature. The proteins in the PVDF membranes were visualized by using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).

2.7. SCI model in adult rats

Thirty Sprague-Dawley rats (7–8 weeks, 218–257 g) were obtained from the Experimental Animal Center of Jilin University (Chuangchun, China). After being fed in our facility for 1 week, except 10 rats were used as control (Sham group), all others were induced SCI using a weight drop device as previously described [19] and randomly divided into SCI group or SCI + Tan IIA group with 10 rats in each group. The rats were kept in a temperature-controlled house under a 12 h light/dark cycle with free access to food and water. In the SCI group, the rats were subjected to SCI using an impactor. In the SCI + Tan IIA group, the rats were subjected to SCI and treated with an intraperitoneal injection of Tan IIA. Tan IIA was administered 1 h before operation (50 mg/kg). From day 1 to 7 post-SCI, Tan IIA was administered (20 mg/kg) once a day at the same time. At day 1 (24 h after SCI), three rats in each group were sacrificed using cervical dislocation and the

damaged tissues (T9-T10) was cut to detect the myeloperoxidase (MPO) activity. At day 3, four rats in each group were sacrificed and the damaged tissues were also cut. Western blotting was conducted to detect the protein levels of main factors involved in cell apoptosis and cell autophagy. TUNEL assay was conducted to assess the rate of apoptotic cells. A motor function recovery test ($n = 3$) was performed every two days from day 0 to day 10. After experiment, all rats were sacrificed.

Experiments using rats were performed in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animal and were approved by the Ethics committee of China-Japan Union Hospital of Jilin University (Changchun, China).

2.8. MPO activity

MPO activity was measured in the spinal cord tissue as previously described [20]. Briefly, each tissue sample was weighted, homogenized in homogenate medium [0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (PH 7)] and centrifuged at 20,000g for 30 min at 4 °C. Then, an aliquot of the supernatant was incubated with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM peroxide (H_2O_2). The rate of change in absorbance was measured spectrophotometrically at 460 nm. The MPO activity was defined as the quantity of enzyme required to degrade 1 mmol of H_2O_2 per min at 37 °C, expressed as units of MPO/g wet tissue.

2.9. TUNEL assay

The paraffin-embedded tissue slices were dewaxed, washed with PBS, and digested with proteinase K in a wet box for 30 min at 37 °C. After washing with PBS, the slides were dipped in TUNEL reaction mixture, and incubated for 1 h at 37 °C. Then, followed by washing, the sections were incubated with converter-AP for 30 min at 37 °C and washed with PBS. Subsequently, the sections were stained with NBT/BCIP substrate solution for 1 h, and the signals were observed under a microscope (Nikon, Japan). The cells with purple nuclei were considered dead. The number of TUNEL positive cells was counted.

2.10. Behavioral examination

A motor function recovery test was scored in accordance with the rules of Basso, Beattie and Bresnahan (BBB) [21], which comprise 21 criteria for the movement of lower limbs, from complete paralysis to complete mobility. Two additional investigators blinded to treatment and grade observed the mechanically contused animals.

2.11. Statistical analysis

Each experiment was repeated three times. The results were presented as the mean \pm standard error of the mean (SEM). Statistical analysis was performed using Graphpad Prism 5 software (GraphPad, San Diego, CA, USA). The P -values between two groups were calculated using t -test and P -values between more than three groups were calculated using the one-way analysis of variance (ANOVA). A $P < 0.05$ was considered as a significant difference.

3. Results

3.1. Tan IIA mitigates H_2O_2 -induced decreases of cell viability and increase of apoptosis in PC-12 cells

First of all, the adequate dosage of H_2O_2 to induce oxidative stress was explored. PC-12 cells were stimulated with diverse concentrations of H_2O_2 , followed by measurements of cell viability. Compared with non-treated cells, cell viability was significantly reduced by 100 μM H_2O_2 ($F = 13.72$, $P < 0.05$) and 200 μM H_2O_2 ($P < 0.01$, Fig. 1A).

However, 12.5–50 μM H_2O_2 had no significant effects on PC-12 cell viability. Accordingly, 200 μM H_2O_2 was used to induce oxidative stress in subsequent experiments. Then, the effects of H_2O_2 on cell apoptosis were examined. Results by flow cytometry presented that percentage of apoptotic cells was markedly enhanced by H_2O_2 treatments as relative to the control ($P < 0.01$, Fig. 1B). Western blotting results showed that H_2O_2 down-regulated the expression of Bcl-2 while up-regulated the expressions of Bax, c/p-caspase-9 and c/p-caspase-3 ($F = 313$, $P < 0.01$ or $P < 0.001$, Fig. 1C), which was consistent with the results by flow cytometry assay. Those above results indicated that H_2O_2 reduced cell viability and enhanced apoptosis in PC-12 cells. Afterwards, PC-12 cells were stimulated with diverse concentrations of Tan IIA, and the results in Fig. 1D showed that 4 μM Tan IIA could significantly reduce viability of PC-12 cells as relative to the control ($F = 43.69$, $P < 0.05$). Therefore, 1 and 2 μM Tan IIA were used in the following experiments. As evidence from Fig. 1E, the effect of H_2O_2 on cell viability was remarkably alleviated by 1 or 2 μM Tan IIA pre-treatment for same times (18 h, $F = 60.75$, $P < 0.05$ or $P < 0.01$). Fig. 1F illustrated that 2 μM Tan IIA pre-treatment also notably alleviated the H_2O_2 -induced PC-12 cell viability inhibition in a time-dependent manner ($F = 34.05$, $P < 0.05$ or $P < 0.01$). Fig. 1G and H displayed that the percentage of apoptotic cells and expression of apoptosis-related proteins were dramatically attenuated by 1 or 2 μM Tan IIA pre-treatment for 18 h compared with the H_2O_2 group ($F = 32.35$ and 90.13, respectively, $P < 0.05$ or $P < 0.01$). Collectively, we concluded that Tan IIA could ameliorate the oxidative stress-induced alteration of cell viability and apoptosis in PC-12 cells.

3.2. Tan IIA attenuates H_2O_2 -induced autophagy in PC-12 cells

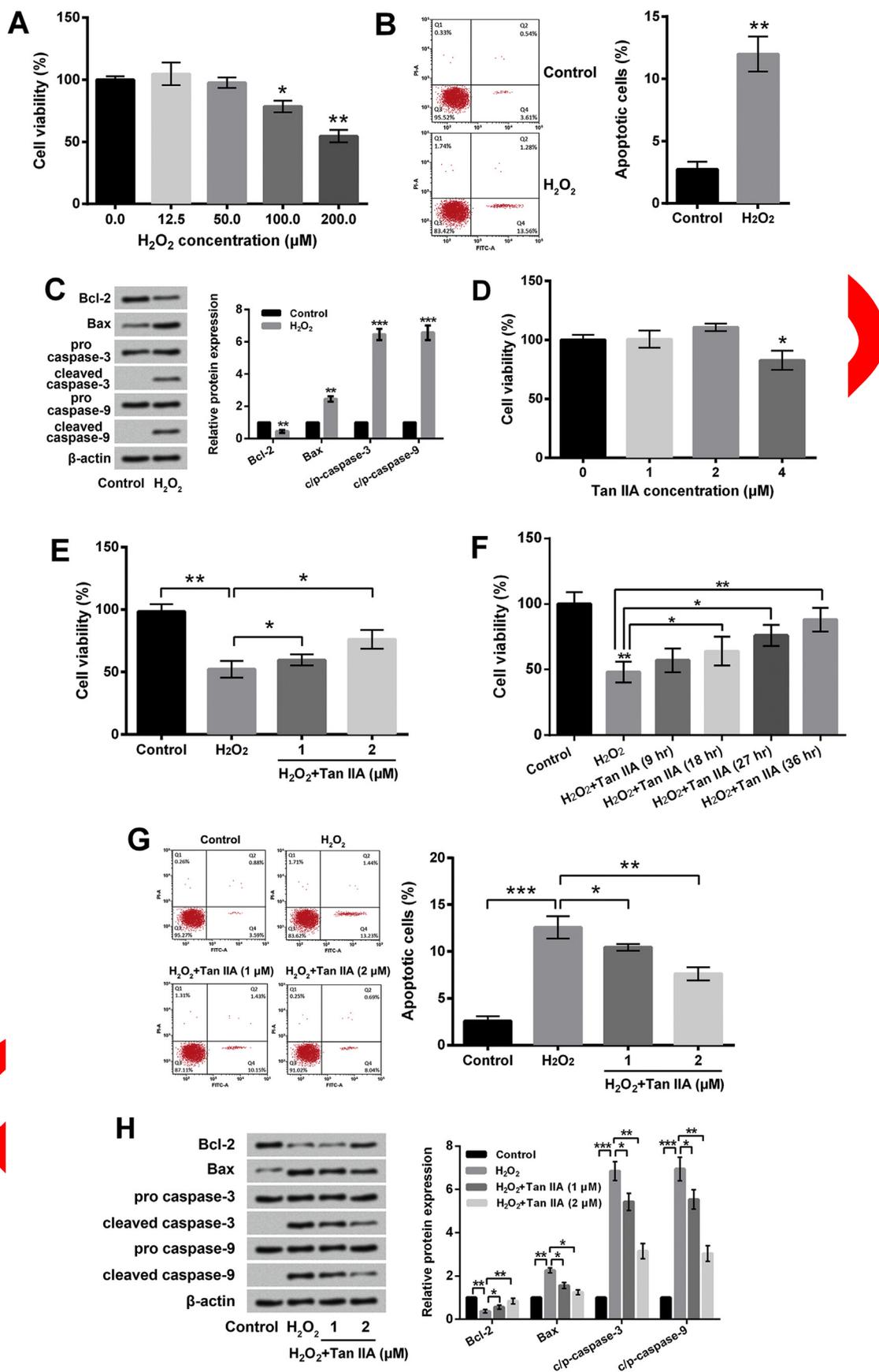
Next, the expression of autophagy markers after treatments with H_2O_2 and/or Tan IIA was investigated. Results in Fig. 2 presented that expressions of LC3B-II and Beclin-1 were observably up-regulated, whereas expression of p62 was down-regulated by H_2O_2 treatments ($F = 105.9$, $P < 0.01$ or $P < 0.001$). Alteration of those proteins induced by H_2O_2 was found to be mitigated by co-stimulation with Tan IIA (1–2 μM , $P < 0.05$, $P < 0.01$ or $P < 0.001$). Thus, we concluded that Tan IIA could ameliorate the oxidative stress-induced autophagy in PC-12 cells.

3.3. Tan IIA up-regulates miR-124 expression

miR-124 is abundantly expressed in neurons and plays critical roles in SCI (Zhao, Zhang, 2015b). So, we detected the expression of miR-124 in PC-12 cells after H_2O_2 and/or Tan IIA treatment. In Fig. 3A, miR-124 level in the H_2O_2 group was notably lower than that in the control group ($P < 0.05$). In Fig. 3B, miR-124 level in the H_2O_2 + Tan IIA group was dramatically higher than that in the H_2O_2 group ($F = 53.11$, $P < 0.01$). Those results indicated that Tan IIA could reverse the H_2O_2 -induced down-regulation of miR-124 and implied that miR-124 might participate in the effects of Tan IIA on H_2O_2 -treated PC-12 cells.

3.4. Tan IIA affects PC-12 cells through up-regulating miR-124 expression

Following experiments were performed to verify whether Tan IIA affect PC-12 cells through regulating miR-124 expression. miR-124 inhibitor was transfected into PC-12 cells to down-regulated the expression of miR-124. qRT-PCR results in Fig. 4A showed miR-124 level in cells transfected with miR-124 inhibitor was significantly lower than that in cells transfected with NC ($F = 79.05$, $P < 0.01$), indicating that miR-124 inhibitor could successfully silenced miR-124. Then, cells (transfected or non-transfected) were treated with H_2O_2 and/or Tan IIA, followed by assessments of cell viability, apoptosis and autophagy. We found that Tan IIA-induced increase of cell viability ($F = 135.96$, $P < 0.05$, Fig. 4B), decrease of apoptotic cells ($F = 40.22$, $P < 0.05$, Fig. 4C), alterations of proteins related to apoptosis ($F = 51.36$,



(caption on next page)

Fig. 1. Effects of H_2O_2 on PC-12 cell viability and apoptosis were attenuated by Tanshinone IIA (Tan IIA) treatments. (A) Cell viability was tested by CCK-8 assay after 12.5–200 μM H_2O_2 treatment for 6 h. (B) Cell apoptosis was detected by flow cytometry assay after 200 μM H_2O_2 treatment for 6 h. (C) The protein levels of Bcl-2, Bax, Caspase 3 and Caspase 9 in cells were measured by western blot analysis after 200 μM H_2O_2 treatment for 6 h. (D) After Tan IIA (1–4 μM) treatment for 18 h, cell viability was tested by CCK-8 assay. (E) After Tan IIA (1–2 μM) pre-treatment for 18 h and/or 200 μM H_2O_2 treatment for 6 h, cell viability was detected by CCK-8 assay. (F) After 2 μM Tan IIA pre-treatment for 9–36 h and/or 200 μM H_2O_2 treatment for 6 h, cell viability was detected by CCK-8 assay. (G–H) After Tan IIA (1–2 μM) pre-treatment for 18 h and/or 200 μM H_2O_2 treatment for 6 h, cell apoptosis were assessed by flow cytometry assay; the protein levels of Bcl-2, Bax, Caspase 3 and Caspase 9 in cells were tested by western blot analysis. Non-treated cells were served as control. Data were presented as the mean \pm SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

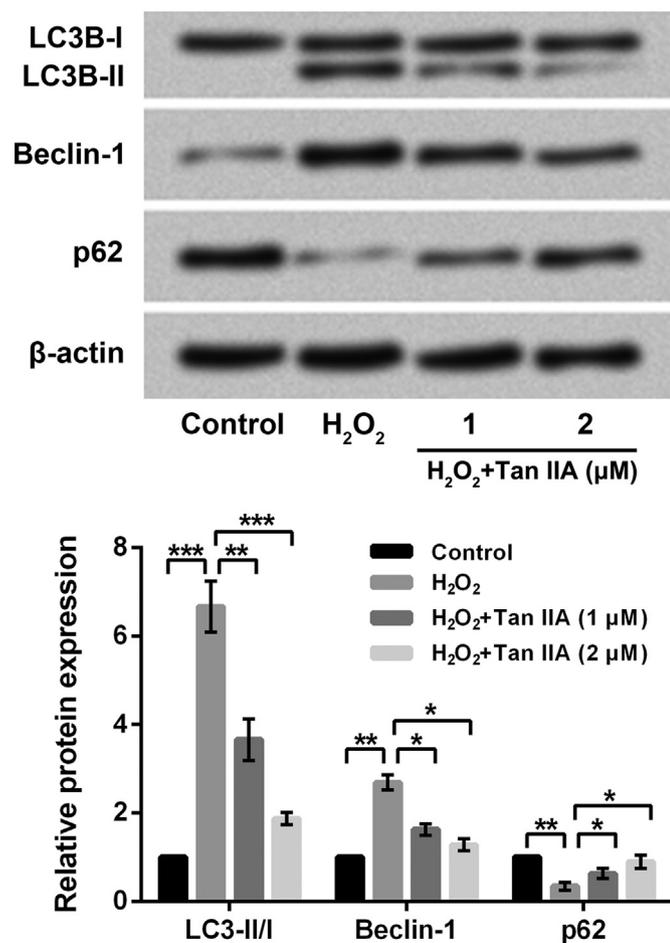


Fig. 2. Effects of H_2O_2 on PC-12 cell autophagy were mitigated by Tanshinone IIA (Tan IIA) treatments. After Tan IIA (1–2 μM) pre-treatment for 18 h and/or 200 μM H_2O_2 treatment for 6 h, the protein levels of LC3, Beclin-1 and p62 in cells were tested by western blot analysis. Non-treated cells were served as control. Data were presented as the mean \pm SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

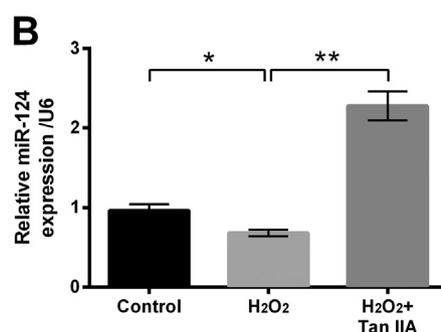
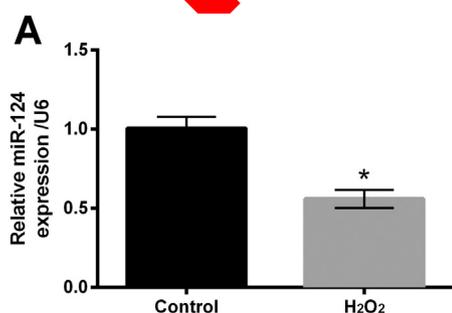


Fig. 3. Effects of H_2O_2 on miR-124 level in PC-12 cells were reversed by Tanshinone IIA (Tan IIA) treatments. (A) Expression of miR-124 in cells was measured by qRT-PCR after 200 μM H_2O_2 treatment for 6 h. (B) After Tan IIA (2 μM) pre-treatment for 18 h and/or 200 μM H_2O_2 treatment for 6 h, the miR-124 level in cells were measured by qRT-PCR. Non-treated cells were served as control. Data were presented as the mean \pm SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

$P < 0.05$ or $P < 0.01$, Fig. 4D) and autophagy ($F = 75.45$, $P < 0.05$ or $P < 0.01$, Fig. 4E) were all notably reversed by miR-124 inhibition, as compared to the H_2O_2 + Tan IIA + NC group. Those results implied that Tan IIA affected PC-12 cells via up-regulating miR-124 expression.

3.5. Tan IIA inhibits the JNK and p38MAPK pathways via up-regulating miR-124 expression

JNK and p38MAPK pathways have been demonstrated to be involved in the regulation of cellular oxidative stress after SCI [22,23]. Therefore, we explored the activations of the JNK and p38MAPK pathways in PC-12 cells after H_2O_2 and/or Tan IIA treatment or miR-124 inhibitor transfection. Western blotting results showed that the expressions of p-JNK, p-c-Jun and p-p38MAPK were all up-regulated in cells treated with H_2O_2 , and these up-regulations were all observably decreased by Tan IIA. Moreover, the effects of Tan IIA on expressions of key kinases in the JNK and p38MAPK pathways were reversed by miR-124 inhibition ($F = 48.40$ and 75.6 , respectively, $P < 0.05$, $P < 0.01$ or $P < 0.001$, Fig. 5A–B). Therefore, we concluded that Tan IIA could inhibit the JNK and p38MAPK pathways through up-regulating miR-124 expression in PC-12 cells under oxidative stress.

3.6. Tan IIA alleviated oxidative stress, apoptosis and autophagy after SCI in adult rats

Finally, to explore the effects of Tan IIA on SCI *in vivo*, Sprague-Dawley rats were subjected to SCI using a weight drop device. Fig. 6A showed that compared to SCI group, the rats in SCI + Tan IIA group have higher BBB scores ($F = 34.22$, $P < 0.01$). Fig. 6B displayed that the MPO activity was significant increased after SCI ($F = 115.4$, $P < 0.001$), while Tan IIA treatment notably alleviated the SCI-induced increase of MPO activity ($P < 0.01$). The rate of TUNEL positive (+) cells in SCI + Tan IIA group was lower than in SCI group ($F = 143.6$, $P < 0.05$, Fig. 6C). Moreover, the results of western blotting in Fig. 6D presented that Tan IIA treatment remarkably alleviated the SCI-induced up-regulations of Bax, c/p-caspase 3 and c/p-caspase 9, as well as down-regulation of Bcl-2 ($F = 105.8$, $P < 0.01$). Fig. 6E illustrated that Tan IIA treatment also obviously attenuated the SCI-induced up-regulations of LC3-II/LC3-I and Beclin-1, as well as down-regulation of p62 ($F = 153.3$, $P < 0.05$ or $P < 0.01$). Taken together, these above findings suggested that Tan IIA also could relieve SCI *in vivo*.

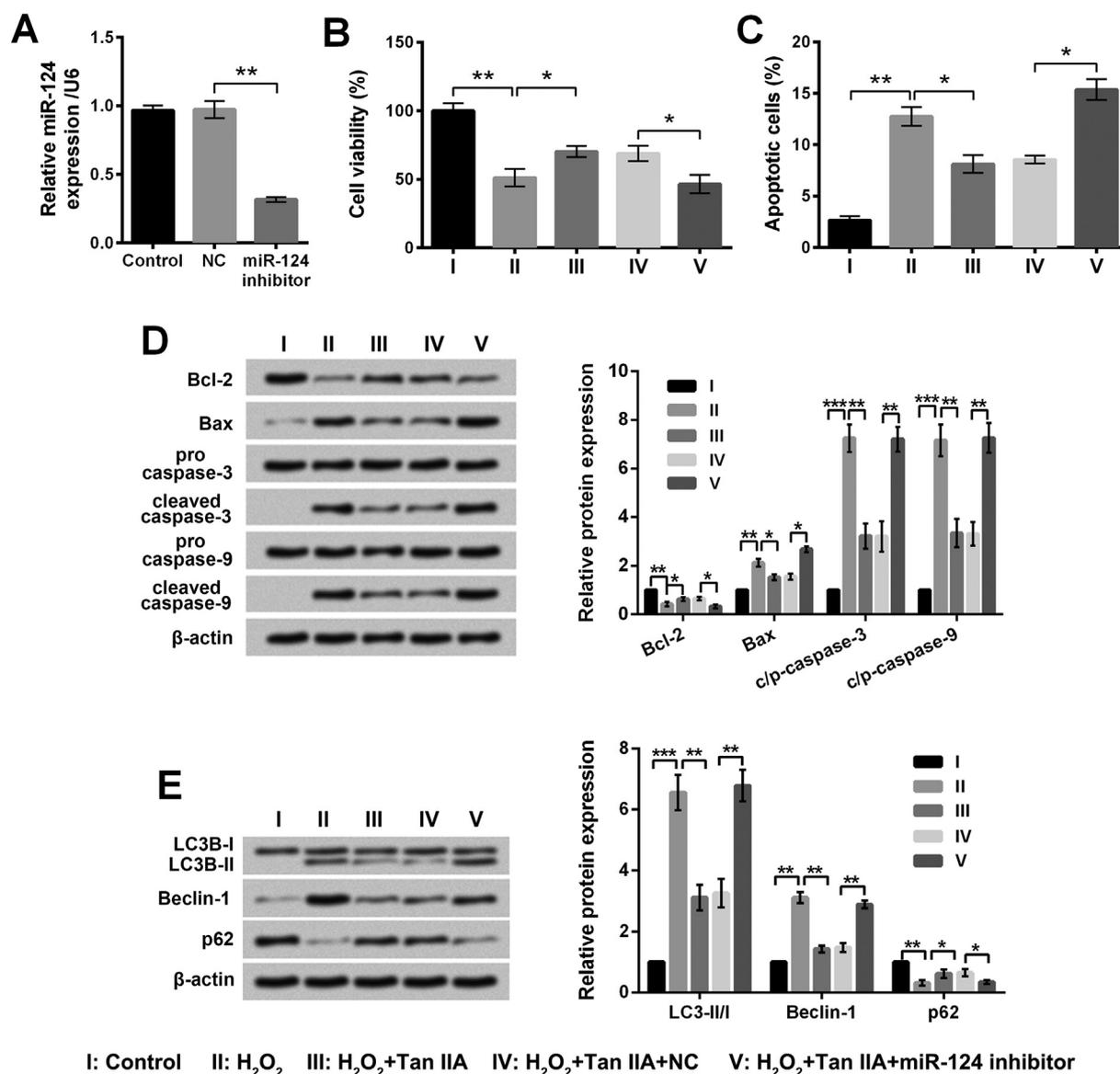


Fig. 4. Effects of Tanshinone IIA (Tan IIA) on cell viability, apoptosis and autophagy were reversed by miR-124 inhibition in H₂O₂-treated PC-12 cells. (A) After miR-124 inhibitor transcription, the expression of miR-124 in cells was measured by qRT-PCR. After Tan IIA (2 μM) pre-treatment for 18 h and/or 200 μM H₂O₂ treatment for 6 h or miR-124 inhibitor transcription, (B–C) cell viability and cell apoptosis were assessed by CCK-8 assay and flow cytometry assay, respectively. (D–E) Western blot analysis was conducted to measure the protein levels of Bcl-2, Bax, Caspase 3, Caspase 9, LC3, Beclin-1 and p62. Non-treated cells were served as control. Data were presented as the mean ± SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

4. Discussion

Great efforts have been made to improve the prognosis of SCI, but the outcome of therapy for this disease is unsatisfied. In the current research, we analyzed the effect of Tan IIA on SCI *in vitro* and *in vivo*. *In vitro*, PC-12 cells were exposed to H₂O₂ to simulate SCI secondary damage due to oxidative stress. We found that Tan IIA could notably alleviate the H₂O₂-induced PC-12 cell viability inhibition, cell apoptosis and cell autophagy. Meanwhile, we found that Tan IIA could up-regulate the expression of miR-124 in H₂O₂-treated PC-12 cells. Suppression of miR-124 could alleviate the effects of Tan IIA on H₂O₂-induced PC-12 cell viability inhibition, cell apoptosis and cell autophagy, as well as JNK and p38MAPK pathways activation. *In vivo*, we revealed that Tan IIA also could mitigate SCI by reducing MPO activity, inhibiting cell apoptosis and autophagy and promoting motor function recovery.

As a major contributor to secondary damage in SCI, oxidative stress is a promising therapeutic target [24,25]. After the primary injury,

elevated intracellular calcium causes increase of reactive oxygen species (ROS) production, resulting in oxidative stress [26]. H₂O₂, which is membrane-permeable, is an important ROS, and high level of H₂O₂ was observed in mitochondria following SCI [27]. Therefore, we constructed *in vitro* cell model to mimic secondary injury of SCI using H₂O₂ stimulation.

Due to SCI, not only the spinal cord neurons but also the white matter axons which transfer signals between body and brain are impaired, and the axons in adult central nervous system (CNS) are failed to regenerate spontaneously since the supportive milieu is lost [28]. Therefore, the neuronal cell growth after SCI is of great importance. Accordingly, we studied the effects of Tan IIA on PC-12 cell viability and apoptosis. Results showed that H₂O₂-induced decrease of cell viability and increase of apoptosis were both attenuated by Tan IIA treatments. Mitochondrion is susceptible to oxidative stress, and the mitochondrial dysfunction is interrelated with oxidative damage, leading to catalysis of other secondary injury in SCI [27]. In mitochondria-dependent apoptosis pathway, Bcl-2 family members (Bax

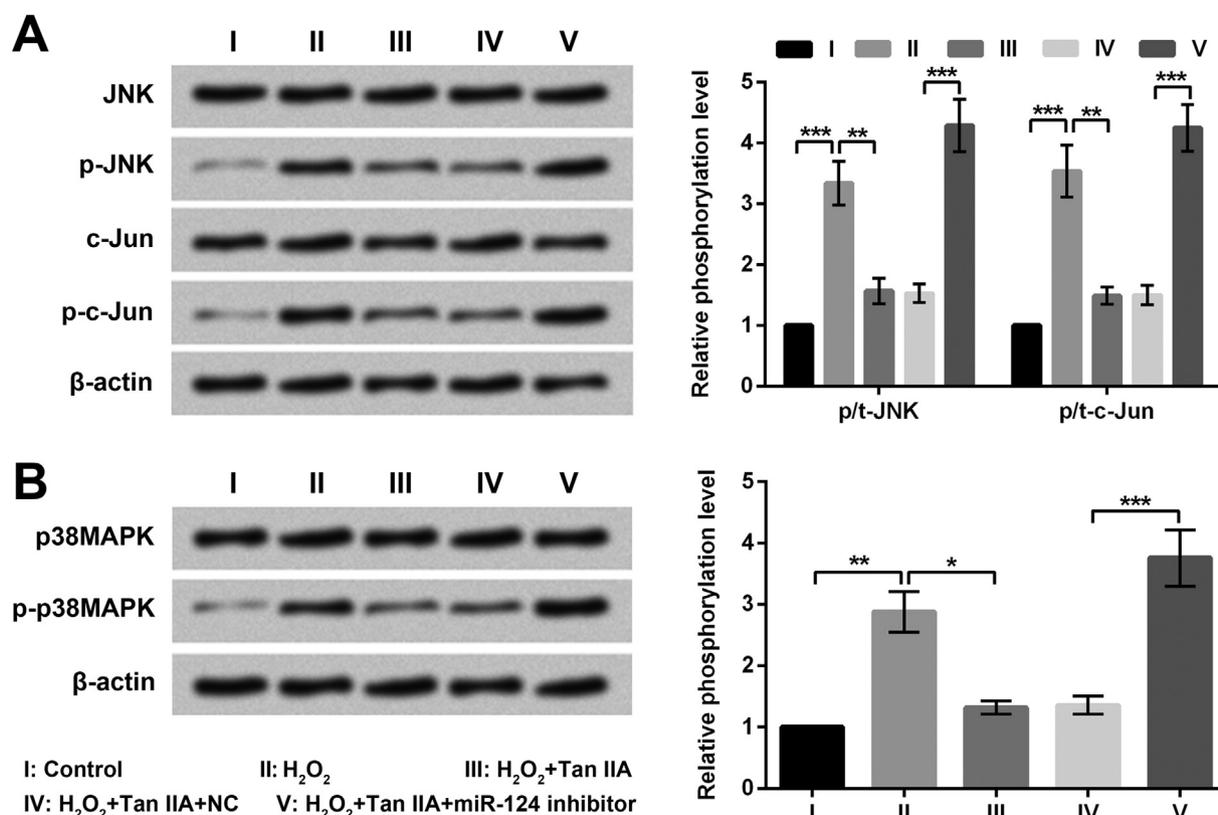


Fig. 5. Tanshinone IIA (Tan IIA) inhibited the JNK and p38MAPK pathways through up-regulating miR-124 expression in H₂O₂-treated PC-12 cells. After Tan IIA (2 μM) pre-treatment for 18 h and/or 200 μM H₂O₂ treatment for 6 h or miR-124 inhibitor transcription, the protein levels of phospho (p)-JNK, JNK, p-c-Jun and c-Jun (A), as well as p-p38MAPK and p38MAPK (B) were examined by western blot analysis. Non-treated cells were served as control. Data were presented as the mean ± SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and Bcl-2) are implicated in ROS-induced apoptosis [29]. Thus, the expressions of Bax and Bcl-2 as well as caspases that lie downstream of mitochondrial pathway were examined by Western blot analysis. The up-regulation of anti-apoptotic Bcl-2, and down-regulations of pro-apoptotic Bax, cleaved caspase-9 and cleaved caspase-3, induced by Tan IIA, combinatorially illustrated that Tan IIA might repress apoptosis via inhibiting the intrinsic apoptosis pathway.

Autophagy is a catabolic mechanism, by which degradation of cytoplasmic proteins and organelles are facilitated in lysosome to maintain cellular homeostasis [30]. ROS is reported as the upstream modulator of autophagy, and ROS production can positively regulate autophagy by diverse mechanism [31]. Kanno et al. have suggested autophagy and cell death induced by autophagy are both increased in damaged neural tissues after SCI [32]. Therefore, we next explored the alteration of autophagy when cells were treated with H₂O₂ and/or Tan IIA. Accordingly, autophagy was analyzed through testing the expression of three popular autophagy markers including LC3B, Beclin-1 and p62 [33]. Up-regulation of LC3B-II and Beclin-1 as well as down-regulation of p62 indicated that oxidative stress induced autophagy in PC-12 cells. Those alterations were attenuated by Tan IIA, presenting that Tan IIA could decrease oxidative stress-induced autophagic damage in PC-12 cells.

miRNAs are small regulatory RNAs that cannot code protein while can interact with mRNA transcripts of target-genes. Recently, an extensive body of evidence supports that miRNAs are involved in functional recovery after SCI [34,35]. miR-124 is an essential regulator abundantly expressed in the nervous system [36]. Zhao et al. have reported that miR-124 expression in neurons is down-regulated within 7 days after SCI [37]. Gong et al. have proved that silence of miR-124 can promote cell apoptosis and induce expression of autophagy-associated proteins [38]. Therefore, we next explored the relationship

between Tan IIA and miR-124 expression. Herein, we interestingly found miR-124 expression was down-regulated by oxidative stress, and the down-regulation was reversed by Tan IIA. Moreover, we also found miR-124 inhibition could reverse the effects of Tan IIA on PC-12 cells. Those results indicated that Tan IIA might up-regulate miR-124 expression thereby affect PC-12 cells.

The JNK pathway can transduce extra-cellular signals, and it can be activated in response to a range of stresses, including oxidative stress [39]. When the JNK is phosphorylated and activated, the downstream c-Jun was phosphorylated in the amino-terminal domain [40]. Together, the p38MAPK pathway is also consolidated to be another oxidative stress-sensitive pathway in rat nucleus pulposus cells [41]. Therefore, we finally explored the involvements of these two pathways. Results in our study demonstrated that the JNK and p38MAPK pathways were both inhibited by Tan IIA through up-regulating miR-124 expression in PC-12 cells.

Finally, we also assessed the effects of Tan IIA on SCI *in vivo*. We demonstrated that Tan IIA exhibited a protective effect against SCI in rats. The administration of Tan IIA promoted motor function recovery of the hind limbs, inhibited MPO activity, cell apoptosis and cell autophagy in damaged tissues.

In conclusion, we found Tan IIA could attenuate oxidative stress after SCI *in vitro* and *in vivo*. Tan IIA alleviated H₂O₂-induced decrease of cell viability, and increases of apoptosis and autophagy in PC-12 cells at least in part via up-regulating miR-124 and then inactivating the JNK and p38MAPK pathways. Our study revealed an innovative regulatory mechanism of Tan IIA in protection against oxidative stress, which might provide novel therapeutic strategies for SCI treatments.

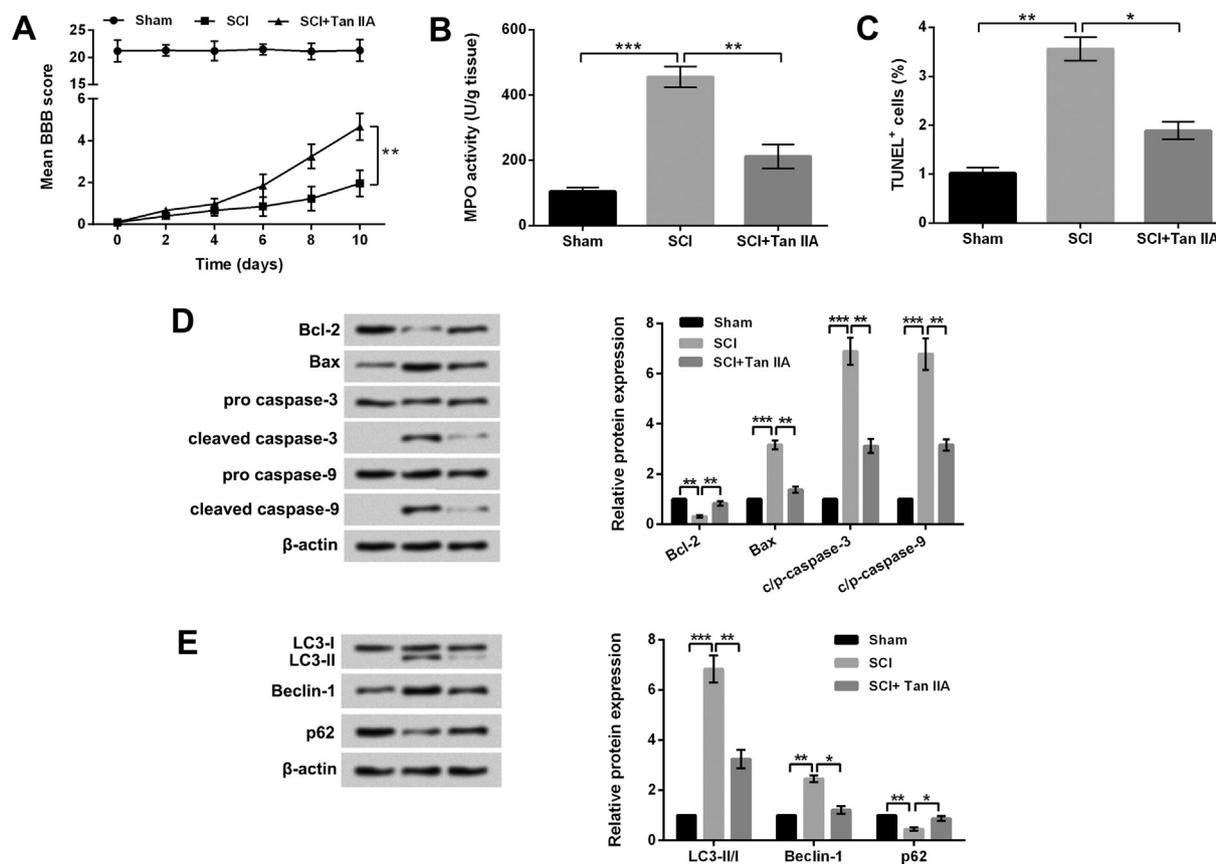


Fig. 6. Effects of Tan IIA on traumatic-induced SCI in adult rats. (A) A motor function recovery test was accessed from day 0 to day 10 after SCI and the BBB scores were recorded. (B) Myeloperoxidase (MPO) activity was measured after SCI for 24 h. (C) TUNEL assay was conducted to test cell apoptotic in the damaged tissues after SCI for 3 days. (D–E) Western blot analysis was performed to detect the protein levels of Bcl-2, Bax, Caspase 3, Caspase 9, LC3, Beclin-1 and p62 in the damaged tissues after SCI for 3 days. Non-treated rats were served as control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Authors' contributions

Juan Li contributed to the conception and design of the study and manuscript drafting. Other authors were involved in the acquisition, analysis, and interpretation of the data. Gu Gong contributed more in this work and as the first author.

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Competing interests

The authors declare that they have no competing interests.

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