



Polydatin alleviates traumatic spinal cord injury by reducing microglial inflammation via regulation of iNOS and NLRP3 inflammasome pathway



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ABSTRACT

Polydatin is a glucoside of resveratrol with lots of functional properties in the central nervous system, such as anti-edema, anti-oxidation and anti-inflammation. The purpose of this study was to evaluate the effects of polydatin on traumatic spinal cord injury (SCI) and explore the relative mechanisms. SCI models were established using the weight-drop method in rats, additionally, single polydatin administration (20, 40 mg/kg body weight) remarkably improved motor function of SCI rat, along with decreased nitric oxide (NO) generation and inflammatory factor (IL-1 β , IL-6 and TNF- α) production in spinal cord tissues. Similar to the results of in vivo experiments, the inflammatory response was aggravated with the intervention of lipopolysaccharide (LPS) in BV2 microglia. However, polydatin treatment (1, 2 and 4 μ M) inhibited iNOS expression, decreased NLRP3 inflammasome activation, which subsequently relieved microglial inflammation. Above all, our data indicated that polydatin possessed neuroprotective effects in SCI rats, possibly by suppressing iNOS expression and NLRP3 inflammasome activation in microglia.

1. Introduction

Traumatic spinal cord injury (SCI), a terrible incident, is chiefly caused by motor vehicle accidents, violence and falls [1]. Not surprisingly, the epidemiological studies found that SCI mainly occurred among young men and caused permanent neurological deficits that dramatically degraded their life quality [2]. Since there is no standardized cross-regional assessment method, it is difficult to calculate the accurate prevalence of SCI in the worldwide at present. However, it is roughly estimated that the incidence of reported SCI events varies in the range of 12 to 65 cases/million depending on area [3]. The representative features of SCI are relatively extensive, including limb paralysis, sensation loss in lower extremity and uracratia or uroschisis [4,5]. The occurrence of SCI triggers a series of complex events at the cellular and molecular levels and the neurological impairments are composed of two related injury phases, namely primary and secondary damage [6]. The primary impairments to spinal cord result in severe neuron necrosis, which is unable to be recovered by all applicable means so far [7]. In the secondary involves spinal cord edema, ischaemia, axon demyelination and neuron loss [8,9]. For the

secondary injury, abnormal mitochondrial activity, hyperactive microglia as well as inflammatory response also lead to permanent neurological impairments [10,11].

Increasing evidences suggest that the accumulation of inflammatory cytokines around the damaged spinal cord is one of the important harmful factors for SCI pathological manifestations [12,13]. Recent experiments have shown that a variety of pro-inflammatory cytokine, such as macrophage migration inhibitory factor (MIF), interleukin-1 β (IL-1 β), IL-6 and tumor necrosis factor- α , were consistently increased after compression-induced spinal cord injury [14,15]. Administration of anti-inflammatory medications modulated microglia polarization with M1/M2 phenotype shifts, reduced neuroinflammatory response, and subsequently inhibiting the generation and expansion of SCI-induced secondary tissue damages [16–18]. Currently, cellular transplantation has emerged as an available treatment option for SCI. However, the principle of cell transplantation is still focused on attenuating microglia activation, inhibiting the associated cytokine production and reducing inflammation and spinal cord damages [19,20]. Hence, we believe that modulating the inflammatory disturbance that is mainly induced by hyperactive microglia has been recognized as a promising therapeutic

Abbreviations: ASC, apoptosis-associated speck-like protein containing; CNS, central nervous system; IL-1 β , interleukin-1 β ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NLRP3, NOD-like receptor protein 3; NO, nitric oxide; SCI, spinal cord injury

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strategy for prevention of SCI.

Nitric oxide (NO), the gaseous chemical messenger, is the smallest molecule with known biological activity. It is well understood that NO at low concentrations is closely related to the physiological process of the central nervous system (CNS), such as synaptic plasticity, receptor function and neurotransmitter release [21]. Under pathological conditions, including SCI, NO is largely synthesized by inducible nitric oxide synthase (iNOS) that is abundantly expressed in microglia, astrocytes and vascular smooth muscle cells [22]. Whereas, large amounts of NO contribute to oxidative stress and inflammation, forming neurological disorders [23]. For another, NOD-like receptor protein 3 (NLRP3) inflammasomes, consisted of NLRP3, apoptosis-associated speck-like protein containing (ASC) and caspase-1, promote the cleavage of caspase-1, and thereby improving the immature inflammatory factors to become mature inflammatory factors [24,25]. Moreover, treatment with the pharmacologic inhibitor of NLRP3 inflammasome, BAY 11–7082 or A438079, improved neurological recovery after SCI surgery in the mouse SCI models [26]. These findings indicate that NO molecules and NLRP3 inflammasomes are the possible targets for SCI treatment.

Nowadays, high-dose methylprednisolone, possessing strong anti-oxidant and anti-inflammatory properties, is commonly used in the treatment of acute SCI. However, considering the adverse effects of methylprednisolone, the therapeutic effect is often unsatisfactory. Therefore, it is necessary to develop effective medications for SCI. In the present study, we detected the possible effects of polydatin on inflammatory response after traumatic SCI in rats and investigated the potential molecular mechanism by using lipopolysaccharide (LPS)-stimulated BV2 microglia.

2. Materials and methods

2.1. Drugs and reagents

Polydatin (purity \geq 95%, No: P109978) was purchased from Shanghai Aladdin biochemical technology Co., Ltd. (Shanghai, China). Pyrrolidine dithiocarbamate (PDTC) was obtained from MedChemExpress (USA). NO and BCA protein assay kits were respectively obtained from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China) and Beyotime Institute of Biotechnology Co., Ltd. (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits for IL-1 β , IL-6 and TNF- α (for mouse and rat) were all from Hangzhou Lianke Biotechnology Co., Ltd. (Hangzhou, China).

2.2. Animals and surgical treatment

Adult male Sprague-Dawley (SD) rats (220–250 g, License number: SCXK (Liaoning, China) 2015-0001) were used to establish SCI model. The rats were maintained in suitable cages under standard laboratory condition before any animal experiments. All the experimental procedures were strictly adhered to the Guide for Care and Use of Laboratory Animals and approved by Shengjing Hospital of China Medical University. A total of 176 rats were randomly assigned into the following four groups of 44 rats each: I. Control (Rats only received T8 laminectomy); II. SCI (Rats received SCI surgery); III. SCI + 20 mg/kg polydatin (Rats received SCI surgery and 20 mg/kg polydatin treatment); IV. SCI + 40 mg/kg polydatin (Rats received SCI surgery and 40 mg/kg polydatin treatment). After the rats were anesthetized with 30 mg/kg pentobarbital sodium solution, T8 laminectomy was conducted and the collision injury on the spinal cord was formed by a 10 g impactor falling from a height of 5 cm. The control rats only underwent T8 laminectomy. The rats received a single intraperitoneal injection of polydatin (20, 40 mg/kg) or equal volume of saline 30 min after the SCI surgery. In each group, eight rats were randomly selected to evaluate the motor function on day 0, 1, 4, 7, 10 and 14 post-SCI surgery and the remaining rats were sacrificed 24 h after the SCI surgery (Fig. 1). In the

remaining, eight rats for measuring wet and dry weight of the spinal cord, eight rats for analyzing pathological changes, eight rats for detecting biochemical indicators, six rats for evaluating mRNA and total protein expression and six rats for testing nucleoprotein expression.

2.3. Behavioral assessment

The open-field test was carried out to assess motor recovery of SCI rats following the SCI surgery. According to the Basso-Beattie-Bresnahan (BBB) locomotion scale, the score graded from 0 to 21 represented the motor capacity of SCI rats [27]. Score 21 indicated normal athletic ability and score 0 represented severe nerve damage. Behavioral evaluations were performed by trained personnel who were blinded to grouping.

2.4. Edema evaluation

Spinal cord edema as a consequence of SCI was detected utilizing the wet to dry weight mode [28]. The separated spinal cord tissues near the lesion site were instantly weighed on an electronic balance, and then the wet weight was recorded. Afterwards, the samples were dried at 80 °C for 48 h to the constant weight (dry weight). Spinal cord edema degree in each sample was calculated as the wet weight to dry weight ratio.

2.5. Histological and immunohistochemical analysis

After perfusion with ice-cold saline and 4% paraformaldehyde, the obtained spinal cord tissues around the lesion epicenter were fixed in 4% paraformaldehyde, embedded in paraffin blocks and cut (thickness: 5 μ m) along the cross section. Simply, the spinal cord slices were stained with hematoxylin solution and counterstained with eosin as previously described [29], and then the morphological alterations were examined with a light microscopy at 200 \times magnification. For the immunohistochemical analysis, all the sections were subjected to the similar immunohistochemical staining procedures [30]. Generally, the spinal cord sections blocked by goat serum were incubated with rabbit anti-iNOS primary antibody (Bioss, China; dilution 1:100) overnight at 4 °C and biotin-labeled Goat Anti-Rabbit IgG (H + L) (Beyotime Institute of Biotechnology, China; dilution 1:200) for another 30 min at 37 °C in sequence. Finally, diaminobenzidine (DAB, Solarbio, China) as the chromogen was used to visualize the immunocomplex that were observed under a light microscopy at 400 \times magnification.

2.6. Cell culture and treatment

BV2 mouse microglia were grown in DMEM medium (Gibco, USA) with 10% fetal bovine serum (FBS; BI, USA) in a humidified 5% CO₂ incubator at 37 °C. When the BV2 microglia reached 70% confluency, the cells were randomly divided into the following five groups: I. Blank (BV2 microglia without LPS stimulation); II. LPS (BV2 microglia only treated with 100 ng/ml LPS for 24 h); III. 1 μ M polydatin (BV2 microglia co-treated with 100 ng/ml LPS and 1 μ M polydatin for 24 h); IV. 2 μ M polydatin (BV2 microglia co-treated with 100 ng/ml LPS and 2 μ M polydatin for 24 h); V. 4 μ M polydatin (BV2 microglia co-treated with 100 ng/ml LPS and 4 μ M polydatin for 24 h). VI. PDTC (BV2 microglia pre-incubated with 10 μ M PDTC for 1 h and treated with 100 ng/ml LPS for 24 h). After the incubation, the cells and culture medium were respectively collected for the subsequent study.

2.7. Inflammatory cytokine and NO detection

The obtained spinal cord tissues were homogenized in PBS on ice and the supernatants were separated by centrifugation at 2500 rpm for 10 min. In addition, the cell medium was harvested after completing the above intervention and centrifuged at 1000 rpm for 10 min to obtain

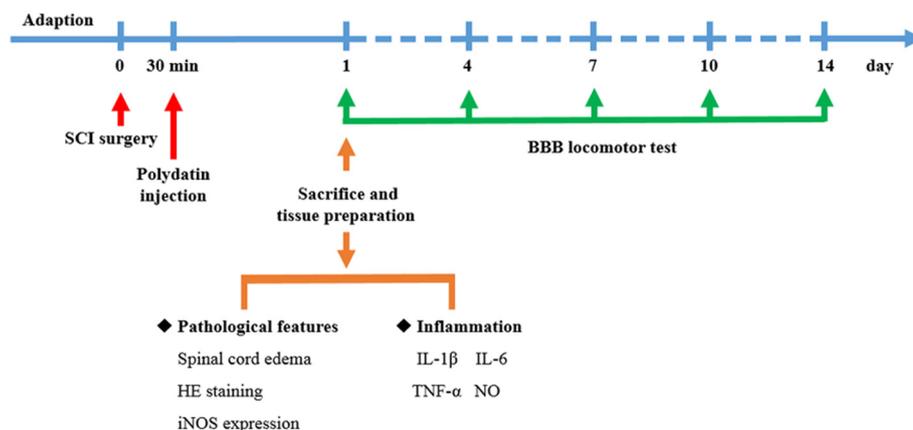


Fig. 1. Timeline for the experimental procedure.

the supernatants. According to the required protocols, the concentrations of IL-1 β , IL-6, and TNF- α were quantified by the corresponding ELISA kits. The levels of NO in the spinal cord tissues and the cell supernatant were measured by commercial NO assay kit according to manufacturer's instructions.

2.8. NF- κ B immunofluorescence

Paraformaldehyde-fixed BV2 cells were permeabilized with 0.1% TritonX-100 and blocked by goat serum. In turn, the cells were incubated overnight at 4 °C with NF- κ B primary antibody (Proteintech, China; dilution 1:50) and Cy3-labeled goat anti-rabbit IgG (H + L) (Beyotime Institute of Biotechnology, China; dilution 1:400) for 1 h at room temperature. Afterwards, the BV2 microglia were incubated with 4', 6-diamidino-2-phenylindole (DAPI) to stain cell nuclei. Finally, immune-positive cells were visualized under fluorescence microscope at 400 \times magnification.

2.9. Real-time polymerase chain reaction (RT-PCR)

Total RNA of the spinal cord tissues and BV2 cells was isolated by Trizol reagent to synthesize cDNA with super M-MLV reverse transcriptase (BioTeke, China) and the primers. Quantitative Fluorescence Analysis was performed in Exicycler 96 System (Bioneer, Korea) and $2^{-\Delta\Delta CT}$ method was used to calculate the data. The primers sequences were showed in Table 1.

2.10. Western blotting

Western blotting was performed as previously described [31], simply, the total proteins of rat spinal cord and intervened BV2 microglia were extracted, separated by SDS-PAGE gels, and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes that were blocked with 5% skimmed milk for 2 h at the room temperature. Subsequently, the PVDF membranes were treated with the following primary antibodies overnight at 4 °C and HRP-conjugated secondary antibody (Beyotime Institute of Biotechnology, China; dilution 1:5000) for 45 min at 37 °C. Ultimately, enhanced chemiluminescent (ECL) reaction was used to visualize the target protein bands and

their density were digitized by Gel-Pro-Analyzer.

The primary antibodies were listed as followed: iNOS (BOSTER, China; dilution 1:500), p-NF- κ B (KeyGen, China; dilution 1:500), p-I κ B (Bioss, China; dilution 1:500), NLRP3 (Abcam, USA; dilution 1:300), ASC (Proteintech, China; dilution 1:300), caspase-1 (Abcam, USA; dilution 1:1000), cleaved caspase-1 (Cell Signaling Technology, USA; dilution 1:1000), IL-1 β (BOSTER, China; dilution 1:300) and β -actin (KeyGen, China; dilution 1:500).

2.11. Statistical analysis

Data were presented as the mean \pm S.D. Values were compared using One-way analysis of variance (ANOVA) followed by Newman-Keuls test and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of polydatin on results of SCI

As shown in Fig. 2A, SCI elicited the impairments of motor function in rats within 14 days ($p < 0.05$ versus Sham) and polydatin treatment (20, 40 mg/kg, $p < 0.05$ versus SCI) improved locomotor activity that was represented by BBB scores. Besides, the administration of polydatin (20, 40 mg/kg, $p < 0.05$ versus SCI) significantly alleviated the edema in spinal cord and inhibited the morphological changes (Fig. 2B and C). The data indicated that polydatin possessed the ability of alleviating SCI-related symptoms.

3.2. Effects of polydatin on NO generation in SCI rats

As shown in Fig. 3A–C, the procedure of SCI increased iNOS expressions at protein and mRNA levels ($p < 0.05$ versus Sham), which could be observably down-regulated by polydatin treatment (20, 40 mg/kg, $p < 0.05$ versus SCI). Consistent with the trend of iNOS expression, the elevated NO contents in the spinal cord tissues of SCI rats were distinctly decreased by polydatin (20, 40 mg/kg, $p < 0.05$ versus SCI; Fig. 3D). The data indicated that polydatin could diminished iNOS expressions, and thereby reducing NO generation in the spinal cord tissues of SCI rats.

Table 1

The primers used in the present study.

		Forward (5'-3')	Reverse (5'-3')
Rat	iNOS	CTTGGAGCGAGTTGTGGATTG	ACCTCTGCCTGTGCGTCTCTT
	β -Actin	GGAGATTACTGCCTGGCTCCTAGC	GGCCGGACTCATCGTACTCCTGCTT
Mouse	iNOS	GCAGGGAATCTTGGAGCGAGTTG	GTAGGTGAGGGCTTGCTGAGTG
	β -Actin	CTGTGCCATCTACGAGGGCTAT	TTTGATGTACAGCACGATTTC

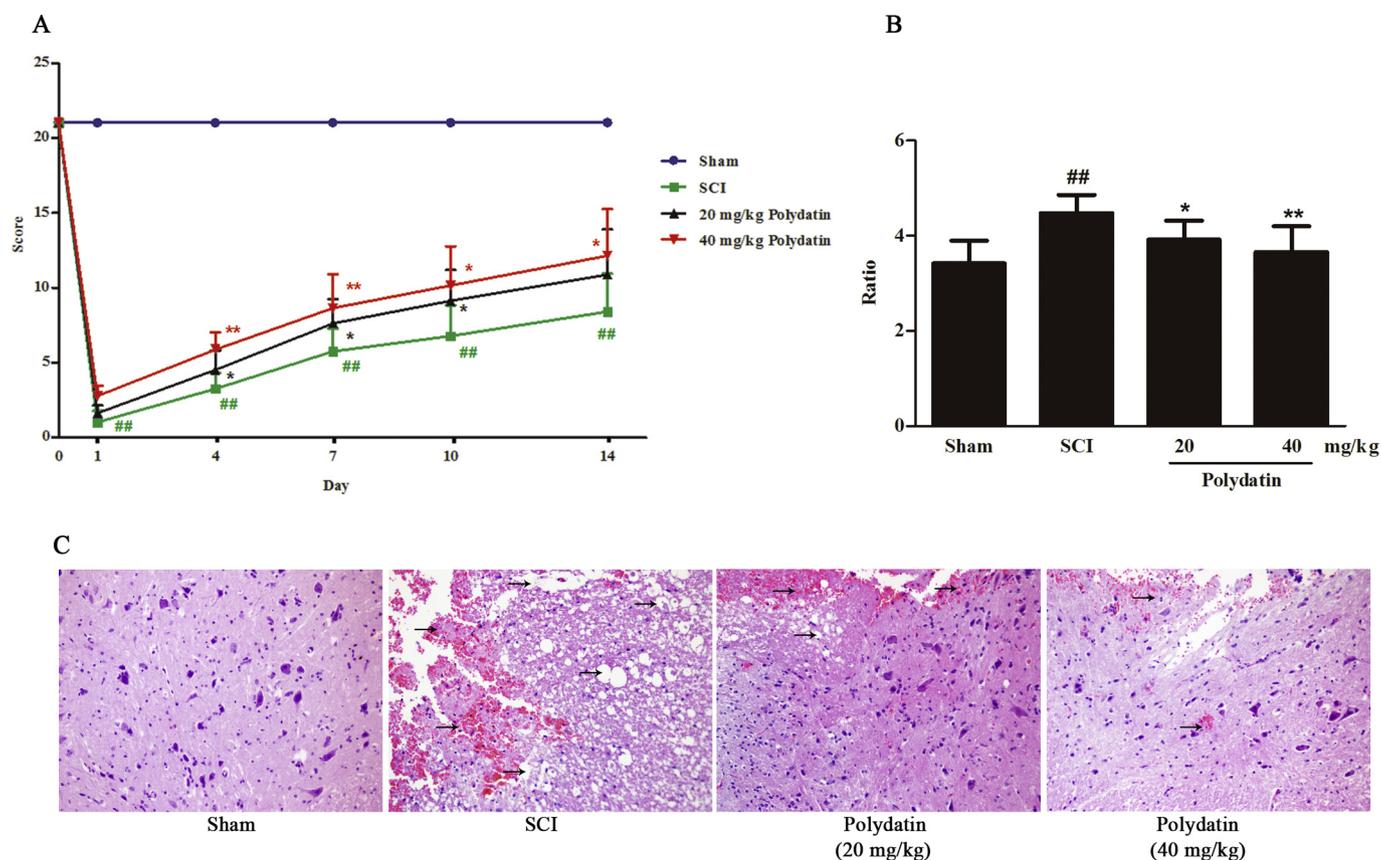


Fig. 2. Effects of polydatin on results of SCI. (A) The average Basso-Beattie-Bresnahan (BBB) scores were shown on 0, 1, 4, 7, 10 and 14 day post-SCI. (B) Ratio of wet to dry spinal cord weight 24 h post-SCI. (C) H&E staining results in spinal cord 24 h post-SCI (Scale bar = 100 μ m). SCI: spinal cord injury group. Data are reported as means \pm S.D. (n = 8). # $p < 0.05$ and ## $p < 0.01$ versus Sham; * $p < 0.05$ and ** $p < 0.01$ versus SCI.

3.3. Effects of polydatin on IL-1 β , IL-6 and TNF- α production in SCI rats

As shown in Fig. 4A–C, the levels of pro-inflammatory factors, such as IL-1 β , IL-6 and TNF- α , were visibly elevated in the spinal cord of model SCI rats compared to the control ones ($p < 0.05$). The single injection of polydatin (20, 40 mg/kg, $p < 0.05$ versus SCI) significantly decreased IL-1 β , IL-6 and TNF- α concentrations. The data indicated that polydatin suppressed inflammatory cytokine generation in the spinal cord tissues post-SCI.

3.4. Effects of polydatin on IL-1 β , IL-6 and TNF- α generation in LPS-stimulated BV2 microglia

As shown in Fig. 5A–C, the stimulation of LPS (100 ng/ml) accelerated the release of IL-1 β , IL-6 and TNF- α in BV2 microglia ($p < 0.05$ versus Blank). Whereas, after the incubation with polydatin (1, 2 and 4 μ M, $p < 0.05$ versus LPS), the productions of IL-1 β , IL-6 and TNF- α were clearly restrained. The data indicated that polydatin mitigated inflammation induced in activated microglia.

3.5. Effects of polydatin on NF- κ B activation in LPS-stimulated BV2 microglia

As shown in Fig. 6A, 100 ng/ml LPS stimulation significantly increased NF- κ B p65 fluorescence strength ($p < 0.05$ versus Blank), which could be reversed by polydatin treatment (1, 2 and 4 μ M, $p < 0.05$ versus LPS). Additionally, the phosphorylated levels of NF- κ B p65 were also declined, along with down-regulated phosphorylated I κ B after polydatin incubation (1, 2 and 4 μ M, $p < 0.05$ versus LPS, Fig. 6B and C). Importantly, the effects of polydatin were the same as the administration of PDTTC (10 μ M). The data indicated that polydatin

inhibited LPS-induced NF- κ B activation in BV2 microglia.

3.6. Effects of polydatin on NO production in LPS-stimulated BV2 microglia

As shown in Fig. 7A and B, the administration of 100 ng/ml LPS promoted the expressions of iNOS at both protein and mRNA levels ($p < 0.05$ versus Blank) in BV2 cells and polydatin treatment (1, 2 and 4 μ M, $p < 0.05$ versus LPS) could reverse these changes. Similar to the results of in vivo experiments, LPS-induced increase of NO release was also reduced by polydatin (1, 2 and 4 μ M, $p < 0.05$; Fig. 7C). The data indicated that polydatin adjusted NO productions that were triggered by microglia activation.

3.7. Effects of polydatin on NLRP3 inflammasome pathway in LPS-stimulated BV2 microglia

As shown in Fig. 8A, the expressions of NLRP3 inflammasome-related proteins were detected in LPS-induced BV2 microglia in present or absent of polydatin. The stimulation of 100 ng/ml LPS accelerated the expression of NLRP3 and its associated proteins, including ASC, cleaved caspase-1 and IL-1 β ($p < 0.05$ versus Blank) in BV2 microglia. In addition, polydatin treatment (1, 2 and 4 μ M, $p < 0.05$ versus LPS) prominently inhibited these protein expressions. The data indicated that polydatin suppressed the activation of NLRP3 inflammasomes in LPS-treated BV2 microglia.

4. Discussion

Polydatin, 3,4',5-trihydroxystilbene-3- β -D-glucoside, is also known as piceid and mainly existed in the root of *Polygonum cuspidatum* that is widely used as one of the folk medicines in Asia [32]. Polydatin has

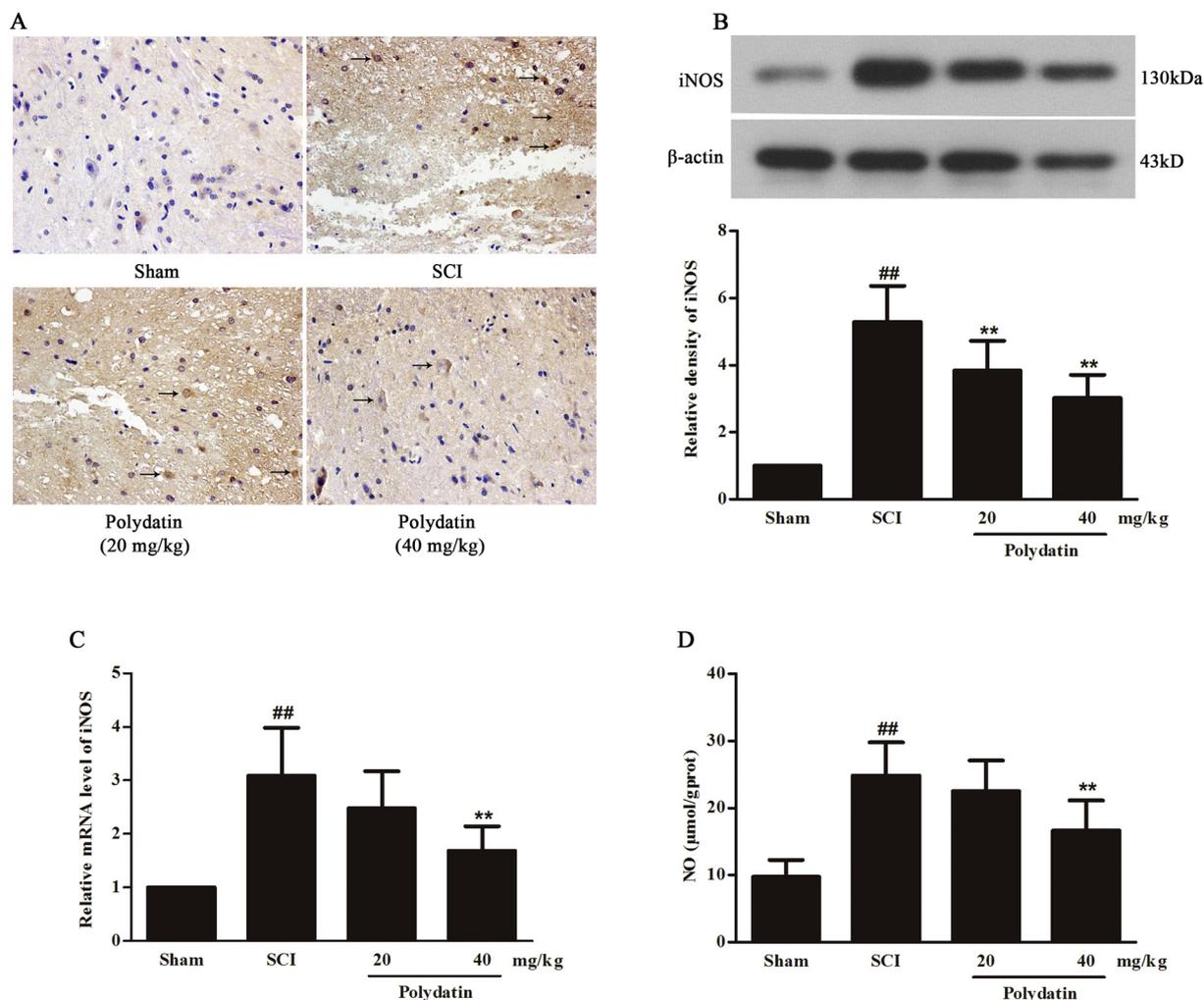


Fig. 3. Effects of polydatin on NO generation in SCI rats. (A) Immunohistochemistry of iNOS in the spinal cord tissues 24 h post-SCI (Scale bar = 50 μm). (B) Western blotting and relative protein levels for iNOS in spinal cord 24 h post-SCI. (C) RT-PCR and relative mRNA levels for iNOS in spinal cord 24 h post-SCI. (D) NO contents in spinal cord 24 h post-SCI. SCI: spinal cord injury group. Data are reported as means ± S.D. (n = 8). # *p* < 0.05 and ## *p* < 0.01 versus Sham; * *p* < 0.05 and ** *p* < 0.01 versus SCI.

numerous activities, such as promising anti-oxidant and anti-inflammatory capacity [33]. In the study, we have investigated the effects of polydatin on neuroinflammation in trauma-induced SCI rats and explored the underlying mechanisms in LPS-treated BV2 microglia. Here, the data showed that the motor dysfunction in SCI model rats, which was accompanied by increased NO generations and elevated inflammatory cytokines (IL-1β, IL-6 and TNF-α). Polydatin treatment

was able to alleviate SCI-induced locomotor loss and normalize neuroinflammation by inhibiting iNOS and NLRP3 inflammasome signaling in microglia.

SCI was induced by a modified weight-drop method in rats, which were thought to be the appropriate models employed in our study. In our trials, the open field test based on Basso-Beattie-Bresnahan (BBB) scoring system was used to assess the recovery of limb motor capacity

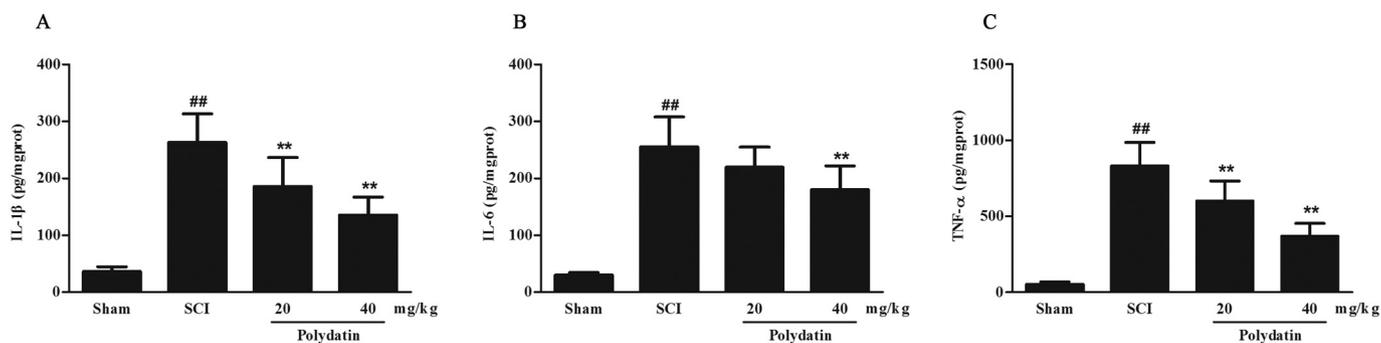


Fig. 4. Effects of polydatin on IL-1β, IL-6 and TNF-α production in SCI rats. (A) IL-1β levels in spinal cord 24 h post-SCI. (B) IL-6 levels in spinal cord 24 h post-SCI. (C) TNF-α levels in spinal cord 24 h post-SCI. SCI: spinal cord injury group. Data are reported as means ± S.D. (n = 8). # *p* < 0.05 and ## *p* < 0.01 versus Sham; * *p* < 0.05 and ** *p* < 0.01 versus SCI.

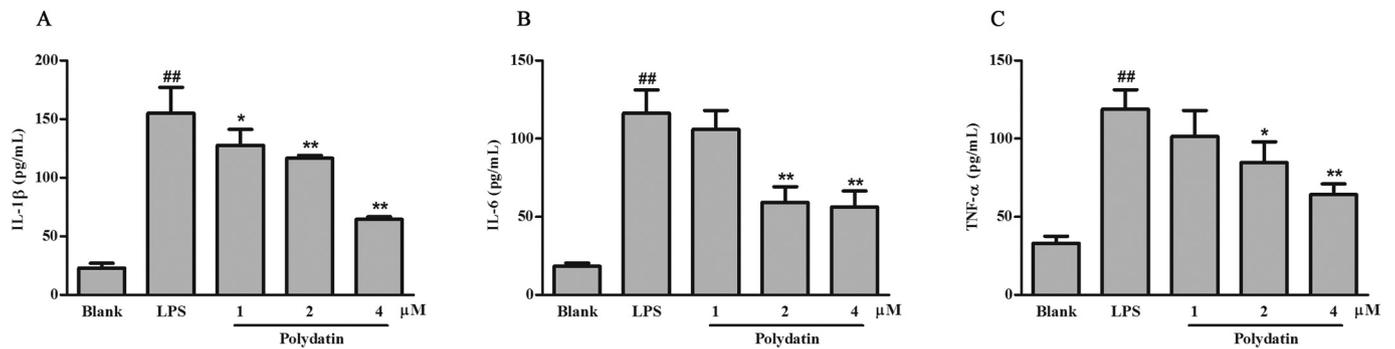


Fig. 5. Effects of polydatin on IL-1 β , IL-6 and TNF- α generation in LPS-stimulated BV2 microglia. (A) IL-1 β production in LPS-induced BV2 microglia. (B) IL-6 production in LPS-induced BV2 microglia. (C) TNF- α production in LPS-induced BV2 microglia. Data are reported as means \pm S.D. (n = 3). # p < 0.05 and ## p < 0.01 versus Blank; * p < 0.05 and ** p < 0.01 versus LPS.

on day 0, 1, 4, 7, 10 and 14 post-SCI surgery. The behavioral performance was gradually improved during the process and poor motor ability was also observed in model SCI rats, which was consistent with the previous experiments [34]. In addition, our results supported that the BBB scores of polydatin-treated SCI rats were significantly up-regulated since the fourth day post-SCI. With the improvement of motor ability in lower limbs, the degrees of edema and the morphological

changes in the traumatized spinal cord tissues were also reversed after polydatin administration in SCI rats, indicating that polydatin could ameliorate SCI-related neurological dysfunction.

It has been noted that increased inflammatory response, the distinct hallmark of the secondary spinal injury, is mediated by resident microglia and the infiltrated immune cells, such as blood-borne monocytes [35,36]. It has been shown that trauma-induced inflammatory response

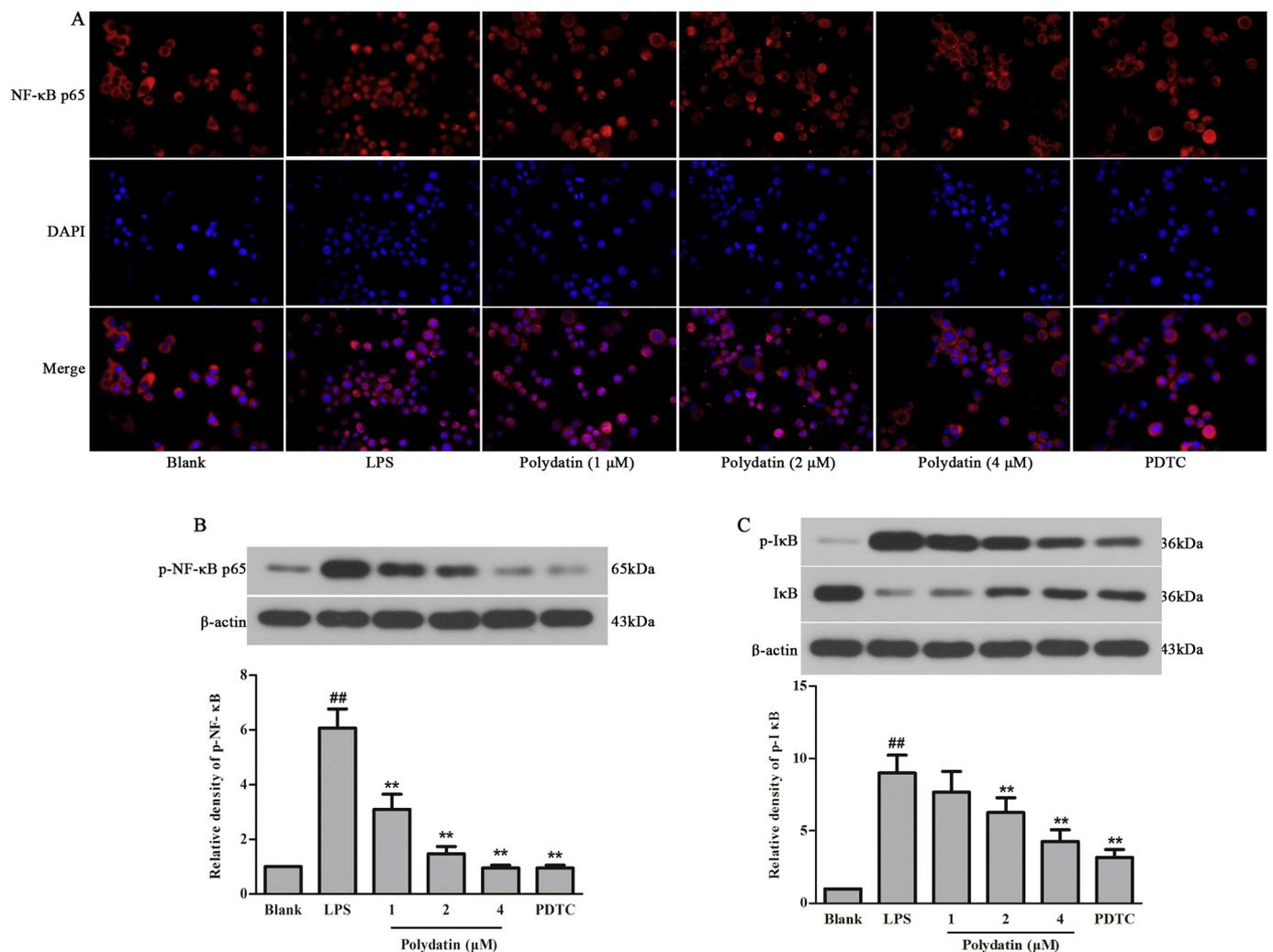


Fig. 6. Effects of polydatin on NF- κ B activation in LPS-stimulated BV2 microglia. (A) Immunofluorescence of NF- κ B p65 in LPS-induced BV2 microglia (Scale bar = 50 μ m). (B) Western blotting and relative protein levels for p-NF- κ B p65 in LPS-induced BV2 microglia. (C) Western blotting and relative protein levels for p-I κ B in LPS-induced BV2 microglia. Data are reported as means \pm S.D. (n = 3). # p < 0.05 and ## p < 0.01 versus Blank; * p < 0.05 and ** p < 0.01 versus LPS.

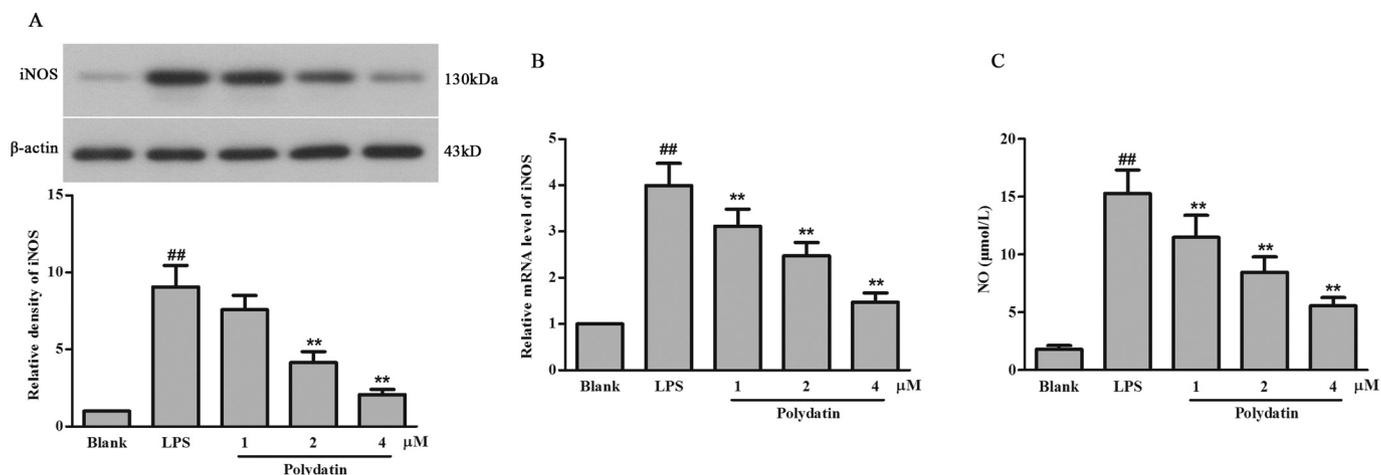


Fig. 7. Effects of polydatin on NO production in LPS-stimulated BV2 microglia. (A) Western blotting and relative protein levels for iNOS in LPS-induced BV2 microglia. (B) RT-PCR and relative mRNA levels for iNOS in LPS-induced microglia. (C) NO production in LPS-induced microglia. Data are reported as means ± S.D. (n = 3). # *p* < 0.05 and ## *p* < 0.01 versus Blank; * *p* < 0.05 and ** *p* < 0.01 versus LPS.

was closely related to the pathogenesis of the secondary injury process [37,38]. NO at low concentration plays an important role in physiological processes of neurons, whereas, high levels of NO that were produced by increased iNOS in lesion sites possess neurotoxicity [22]. NO at low concentration plays an important role in physiological processes of neurons, whereas, high levels of NO that were produced by increased iNOS in lesion sites possess neurotoxicity [39]. We showed that the concentrations of NO were down-regulated with decreased iNOS expressions after polydatin treatment. Since multiple inflammatory factors were involved in the injury process of SCI, we next examined spinal IL-1β, IL-6 and TNF-α levels after SCI surgery, which could be

normalized by polydatin administration. Prominently enhanced anti-inflammatory capacity of spinal cord tissues might contribute to the neuroprotection of polydatin.

Following SCI, the primary injury triggers the secondary cascade through biochemical and inflammatory event, in addition, the spinal cord tissues around the mechanical trauma were extremely vulnerable to the secondary injury on account of microglial products [40]. Microglia, the primary resident macrophages in the central nervous system, are the key elements in maintaining the normal function of neurons [41]. Given that microglia exert their physiological effects via the modulation of synaptic pruning. Their functions alters with the

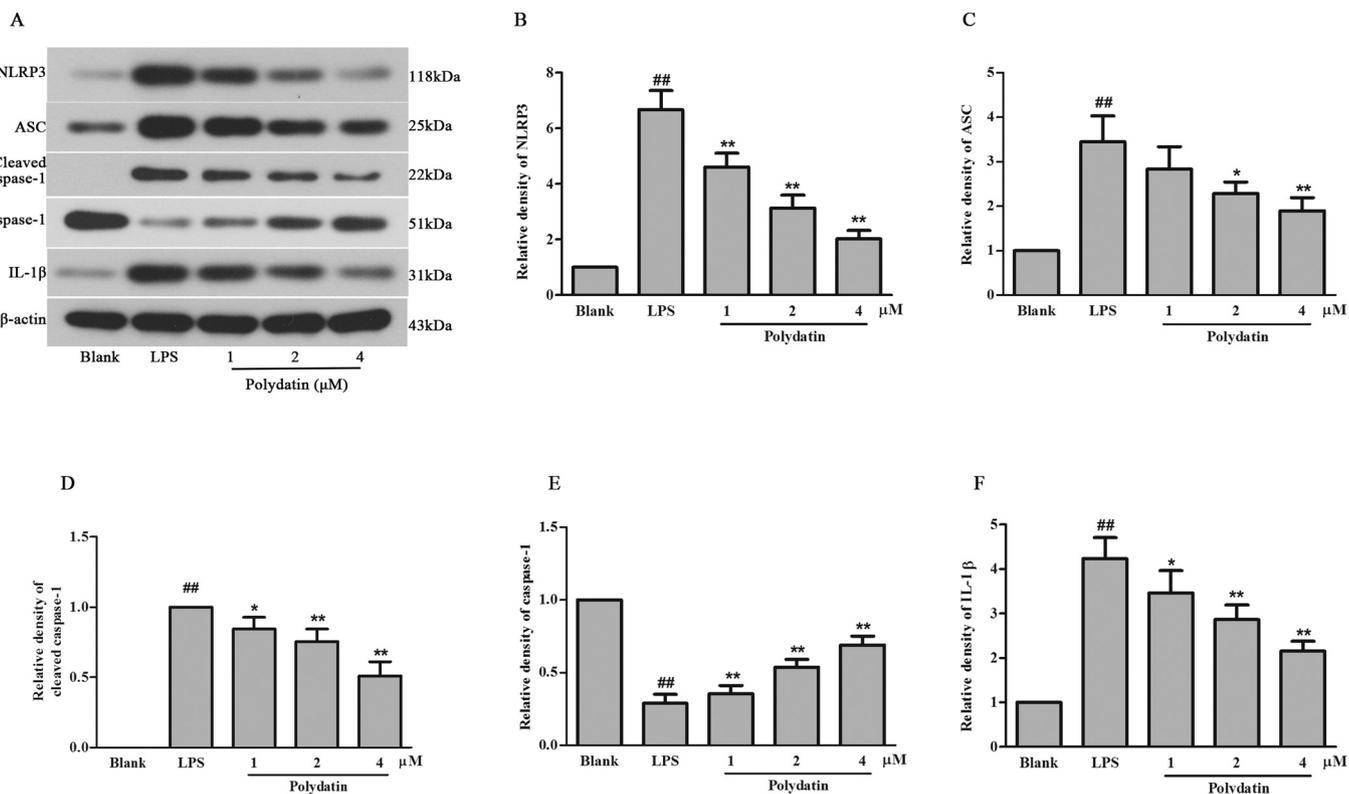


Fig. 8. Effects of polydatin on NLRP3 inflammasome pathway in LPS-stimulated BV2 microglia. (A) Western blotting for NLRP3-related proteins in LPS-induced BV2 microglia. (B) Relative protein levels for NLRP3. (C) Relative protein levels for ASC. (D) Relative protein levels for cleaved caspase-1. (E) Relative protein levels for caspase-1. (F) Relative protein levels for IL-1β. Data are reported as means ± S.D. (n = 3). # *p* < 0.05 and ## *p* < 0.01 versus Blank; * *p* < 0.05 and ** *p* < 0.01 versus LPS.

changes of microenvironment, microglia are hyper-activated and release multiple inflammatory factors in response to injury. The over-produced inflammatory cytokines not only directly impair neuronal function, but also recruit more inflammatory cells to infiltrate the lesion site, thereby deteriorating the secondary injury and disturbing behavioral development after SCI [42]. Based on the above, we attempted to sort out the associated mechanisms of polydatin in alleviating the nerve damage caused by SCI. It is generally accepted that exposing neurons to inflammatory factors contributed to their abnormality, apoptosis and death during activation of microglia [43]. Additionally, it is also well understood that the production of inflammatory cytokine is regulated by nuclear factor-kappa B (NF- κ B) and its downstream molecules [44]. Therefore, we used a NF- κ B specific inhibitor PDTC to examine the influence of polydatin on NF- κ B activation in LPS-treated BV2 microglia. In our study, polydatin treatment inhibited inflammatory factor IL-1 β , IL-6 and TNF- α generation in LPS-treated BV2 microglia. Besides, there was a decrease in NF- κ B activation after polydatin administration, which was consistent with the effects of PDTC. Next, iNOS expression and NO production were both measured in LPS-treated BV2 microglia and the results suggested that polydatin could restore these up-regulations. Recently, it was found that activation of NLRP3 inflammasomes in activated microglia cells in vitro or in vivo was an important signaling leading to progression of neurodegeneration [45]. NLRP3 inflammasome has emerged as a critical element of inflammatory process and is regarded as a potential target in SCI [46]. The present data demonstrated that the expressions of NLRP3 inflammasome-related protein, including NLRP3, ASC, cleaved caspase-1 and IL-1 β , were observably decreased with polydatin treatment in LPS-stimulated BV2 microglia. Our study elucidated that polydatin treatment could inhibit the activation of iNOS and NLRP3 inflammasome in microglia.

In conclusion, our results demonstrate that polydatin improved SCI-induced motor dysfunction in rats. Due to the effective anti-inflammatory property, polydatin reduced inflammation of the spinal cord tissues, blocked NF- κ B regulating iNOS and NLRP3 inflammasome expression in microglia, and eventually improved behavior performance post-SCI. Although more studies are required to confirm the direct regulation of polydatin on inflammation via NF- κ B regulating iNOS and NLRP3 inflammasome pathway, this study indicates that polydatin possesses therapeutic potential for SCI-related motor dysfunction.

Disclosure

The authors declared no conflict of interest.

References

- [1] A. Prochazka, Targeted stimulation of the spinal cord to restore locomotor activity, *Nat. Med.* 22 (2016) 125–126.
- [2] S. Okada, The pathophysiological role of acute inflammation after spinal cord injury, *Inflamm. Regen.* 36 (2016) 20.
- [3] R. Hamid, M.A. Averbeck, H. Chiang, A. Garcia, R.T. Al Mousa, S.J. Oh, A. Patel, M. Plata, G. Del Popolo, Epidemiology and pathophysiology of neurogenic bladder after spinal cord injury, *World J. Urol.* (May 11 2018) (Epub ahead of print).
- [4] S. Samantary, A. Das, D.C. Matzelle, S.P. Yu, L. Wei, A. Varma, S.K. Ray, N.L. Banik, Administration of low dose estrogen attenuates persistent inflammation, promotes angiogenesis, and improves locomotor function following chronic spinal cord injury in rats, *J. Neurochem.* 137 (2016) 604–617.
- [5] V. Dietz, A. Curt, Neurological aspects of spinal-cord repair: promises and challenges, *Lancet Neurol.* 5 (2006) 688–694.
- [6] P. Freund, N. Weiskopf, N.S. Ward, C. Hutton, A. Gall, O. Ciccarelli, M. Craggs, K. Friston, A.J. Thompson, Disability, atrophy and cortical reorganization following spinal cord injury, *Brain* 134 (2011) 1610–1622.
- [7] T. Genovese, E. Mazzon, E. Esposito, C. Muià, R. Di Paola, C. Crisafulli, P. Bramanti, S. Cuzzocrea, Inhibition of tyrosine kinase-mediated cellular signalling by Tyrphostins AG126 and AG556 modulates secondary damage in experimental spinal cord trauma, *Neuropharmacology* 52 (2007) 1454–1471.
- [8] N.A. Silva, N. Sousa, R.L. Reis, A.J. Salgado, From basics to clinical: a comprehensive review on spinal cord injury, *Prog. Neurobiol.* 114 (2014) 25–57.
- [9] K. Hamann, R. Shi, Acrolein scavenging: a potential novel mechanism of attenuating oxidative stress following spinal cord injury, *J. Neurochem.* 111 (2009) 1348–1356.
- [10] A.M. Louw, M.K. Kolar, L.N. Novikova, P.J. Kingham, M. Wiberg, J. Kjems, L.N. Novikov, Chitosan polyplex mediated delivery of miRNA-124 reduces activation of microglial cells in vitro and in rat models of spinal cord injury, *Nanomedicine* 12 (2016) 643–653.
- [11] X.Z. Liu, X.M. Xu, R. Hu, C. Du, S.X. Zhang, J.W. McDonald, H.X. Dong, Y.J. Wu, G.S. Fan, M.F. Jacquin, C.Y. Hsu, D.W. Choi, Neuronal and glial apoptosis after traumatic spinal cord injury, *J. Neurosci.* 17 (1997) 5395–5406.
- [12] P. Popovich, D. McTigue, Damage control in the nervous system: beware the immune system in spinal cord injury, *Nat. Med.* 15 (2009) 736–737.
- [13] X.F. Wang, L.D. Huang, P.P. Yu, J.G. Yu, F. Liu, L. Wang, X.M. Xu, P.H. Lu, Upregulation of type I interleukin-1 receptor after traumatic spinal cord injury in adult rats, *Acta Neuropathol.* 111 (2006) 220–228.
- [14] M. Koda, Y. Nishio, M. Hashimoto, T. Kamada, S. Koshizuka, K. Yoshinaga, S. Onodera, J. Nishihira, H. Moriya, M. Yamazaki, Up-regulation of macrophage migration-inhibitory factor expression after compression-induced spinal cord injury in rats, *Acta Neuropathol.* 108 (2004) 31–36.
- [15] M.E. Schwab, D. Bartholdi, Degeneration and regeneration of axons in the lesioned spinal cord, *Physiol. Rev.* 76 (1996) 319–370.
- [16] Q. Zhang, G. Bian, P. Chen, L. Liu, C. Yu, F. Liu, Q. Xue, S.K. Chung, B. Song, G. Ju, J. Wang, Aldose reductase regulates microglia/macrophages polarization through the cAMP response element-binding protein after spinal cord injury in mice, *Mol. Neurobiol.* (2014) 662–676.
- [17] N. Uezono, Y. Zhu, Y. Fujimoto, T. Yasui, T. Matsuda, M. Nakajo, M. Abematsu, T. Setoguchi, S. Mori, H.K. Takahashi, S. Komiyama, M. Nishibori, K. Nakashima, Prior treatment with anti-high mobility group Box-1 antibody boosts human neural stem cell transplantation-mediated functional recovery after spinal cord injury, *Stem Cells* 36 (2018) 737–750.
- [18] S.A. Myers, K.R. Andres, T. Hagg, S.R. Whittemore, CD36 deletion improves recovery from spinal cord injury, *Exp. Neurol.* 256 (2014) 25–38.
- [19] K.K. Veeravalli, V.R. Dasari, A.J. Tsung, D.H. Dinh, M. Gujrati, D. Fassett, J.S. Rao, Human umbilical cord blood stem cells upregulate matrix metalloproteinase-2 in rats after spinal cord injury, *Neurobiol. Dis.* 36 (2009) 200–212.
- [20] P.H. Lerou, G.Q. Daley, Therapeutic potential of embryonic stem cells, *Blood Rev.* 19 (2005) 321–331.
- [21] S. Moncada, R.M.J. Palmer, E.A. Higgs, Nitric oxide: physiology, pathophysiology, and pharmacology, *Pharmacol. Rev.* 43 (1991) 109–142.
- [22] A. Conti, M. Miscusi, S. Cardali, A. Germanò, H. Suzuki, S. Cuzzocrea, F. Tomasello, Nitric oxide in the injured spinal cord: synthases cross-talk, oxidative stress and inflammation, *Brain Res. Rev.* 54 (2007) 205–218.
- [23] S.S. Gross, M.S. Wolin, Nitric oxide: pathophysiological mechanisms, *Annu. Rev. Physiol.* 57 (1995) 737–769.
- [24] K. Mortezaee, N. Khanlarkhani, C. Beyer, A. Zendedel, Inflammation: its role in traumatic brain and spinal cord injury, *J. Cell. Physiol.* 233 (2018) 5160–5169.
- [25] A. Zendedel, S. Johann, S. Mehrabi, M. Taghi Joghataei, G. Hassanzadeh, M. Kipp, C. Beyer, Activation and regulation of NLRP3 inflammasome by intrathecal application of SDF-1 α in a spinal cord injury model, *Mol. Neurobiol.* 53 (2016) 3063–3075.
- [26] W. Jiang, M. Li, F. He, S. Zhou, L. Zhu, Targeting the NLRP3 inflammasome to attenuate spinal cord injury in mice, *J. Neuroinflammation* 14 (2017) 207.
- [27] D.M. Basso, M.S. Beattie, J.C. Bresnahan, A sensitive and reliable locomotor rating scale for open field testing in rats, *J. Neurotrauma* 12 (1995) 1–21.
- [28] A.V. Leonard, E. Thornton, R. Vink, NK1 receptor blockade is ineffective in improving outcome following a balloon compression model of spinal cord injury, *PLoS One* 9 (2014) e98364.
- [29] J. He, J. Zhao, X. Peng, X. Shi, S. Zong, G. Zeng, Molecular mechanism of MiR-136-5p targeting NF- κ B/A20 in the IL-17-mediated inflammatory response after spinal cord injury, *Cell. Physiol. Biochem.* (2017) 1224–1241.
- [30] T. Qin, F. Fang, M. Song, R. Li, Z. Ma, S. Ma, Umbelliferone reverses depression-like behavior in chronic unpredictable mild stress-induced rats by attenuating neuronal apoptosis via regulating ROCK/Akt pathway, *Behav. Brain Res.* 317 (2017) 147–156.
- [31] S. Li, X. Sun, L. Xu, R. Sun, Z. Ma, X. Deng, B. Liu, Q. Fu, R. Qu, S. Ma, Baicalin attenuates in vivo and in vitro hyperglycemia-exacerbated ischemia/reperfusion injury by regulating mitochondrial function in a manner dependent on AMPK, *Eur. J. Pharmacol.* 815 (2017) 118–126.
- [32] Q. Hong Meng, H. Bao Liu, J. Bo Wang, Polydatin ameliorates renal ischemia/reperfusion injury by decreasing apoptosis and oxidative stress through activating sonic hedgehog signaling pathway, *Food Chem. Toxicol.* 96 (2016) 215–225.
- [33] D. Arslan-Acaroz, F. Zemheri, H.H. Demirel, I. Kucukkurt, S. Ince, A. Eryavuz, In vivo assessment of polydatin, a natural polyphenol compound, on arsenic-induced free radical overproduction, gene expression, and genotoxicity, *Environ. Sci. Pollut. Res.* 25 (2018) 2614–2622.
- [34] G. Casili, D. Impellizzeri, M. Cordaro, E. Esposito, S. Cuzzocrea, B-cell depletion with CD20 antibodies as new approach in the treatment of inflammatory and immunological events associated with spinal cord injury, *Neurotherapeutics* 13 (2016) 880–894.
- [35] Y. Taoka, K. Okajima, M. Uchiba, K. Murakami, S. Kushimoto, M. Johno, M. Naruo, H. Okabe, K. Takatsuki, Role of neutrophils in spinal cord injury in the rat, *Neuroscience* 79 (1997) 1177–1182.
- [36] J.C. Fleming, M.D. Norenberg, D.A. Ramsay, G.A. Dekaban, A.E. Marcillo, A.D. Saenz, M. Pasquale-Styles, W.D. Dietrich, L.C. Weaver, The cellular inflammatory response in human spinal cords after injury, *Brain* 129 (2006) 3249–3269.
- [37] W. Peng, M.L. Cotrina, X. Han, H. Yu, L. Bekar, L. Blum, T. Takano, G.-F. Tian, S.A. Goldman, M. Nedergaard, Systemic administration of an antagonist of the ATP-

- sensitive receptor P2X7 improves recovery after spinal cord injury, *Proc. Natl. Acad. Sci.* 106 (2009) 12489–12493.
- [38] J. Kjell, L. Olson, Rat models of spinal cord injury: from pathology to potential therapies, *Dis. Model. Mech.* 9 (2016) 1125–1137.
- [39] R. Pannu, I. Singh, Pharmacological strategies for the regulation of inducible nitric oxide synthase: neurodegenerative versus neuroprotective mechanisms, *Neurochem. Int.* 49 (2006) 170–182.
- [40] K.D. Beck, H.X. Nguyen, M.D. Galvan, D.L. Salazar, T.M. Woodruff, A.J. Anderson, Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment, *Brain* 133 (2010) 433–447.
- [41] M. Colonna, O. Butovsky, Microglia function in the central nervous system during health and neurodegeneration, *Annu. Rev. Immunol.* 35 (2017) 441–468.
- [42] K.M. Lenz, L.H. Nelson, Microglia and beyond: innate immune cells as regulators of brain development and behavioral function, *Front. Immunol.* 9 (2018) 698.
- [43] M.T. Heneka, M.P. Kummer, E. Latz, Innate immune activation in neurodegenerative disease, *Nat. Rev. Immunol.* 14 (2014) 463–477.
- [44] L.M. Kingeter, B.C. Schaefer, Malt1 and cIAP2-Malt1 as effectors of NF- κ B activation: kissing cousins or distant relatives? *Cell. Signal.* 22 (2010) 9–22.
- [45] M. Gold, J. El Khoury, β -Amyloid, microglia, and the inflammasome in Alzheimer's disease, *Semin. Immunopathol.* 37 (2015) 607–611.
- [46] J.P. de Rivero Vaccari, W.D. Dietrich, R.W. Keane, Activation and regulation of cellular inflammasomes: gaps in our knowledge for central nervous system injury, *J. Cereb. Blood Flow Metab.* 34 (2014) 369–375.