Inflammatory Response and Cell Apoptosis are Inhibited by Indirubin-3'-Oxime via PI3K/Akt Pathway in Rat Model of Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) main cause of motor dysfunction results in loss of feelings partially or completely. The current study investigated indirubin-3'-oxime (IR3O) for treatment of SCI in rat model and evaluated the related mechanism. Rats in model SCI and ID30 groups were subjected to laminectomy at 8^{th} thoracic vertebra level. Vertebral column was secured by clamping T6 and T10 and SCI model was established by dripping a hammer. Treatment groups received 0.25, 0.5, 0.75 and 1.0 mg/kg doses of ID30 daily for 2-weeks post-surgery. Treatment with ID30 effectively improved BBB score in rats with SCI in dose-based manner. Accumulation of water in spinal cord tissues was alleviated significantly on treatment of SCI rats with ID30. ID30 treatment significantly alleviated SCI mediated higher serum levels of TNF-α and cytokines (IL-1β and IL-6) in SCI rats. In ID30 treated SCI rats SOD, CAT and GSH activities were significantly alleviated. The SCI mediated increased cleaved caspase-3 and -9 levels were alleviated by ID30 treatment significantly. Moreover, ID30 treatment suppressed SCI mediated elevation of PGE2, COX-2 levels and significantly ($P<0.05$) elevated PPAR-γ expression. The ID30 treatment of SCI rats significantly ($P<0.05$) elevated PI3K and Akt phosphorylation. Thus, ID30 inhibited edema and improved BBB score in rats with SCI by targeting pro-inflammatory cytokines and oxidative response. Moreover, in SCI rats ID30 treatment down-regulated apoptotic proteins, promoted PPAR-γ activation and elevated PI3K/Akt phosphorylation. Thus, ID30 has potential to be studied further for development of therapeutic strategy for SCI.

Introduction

Spinal cord injury (SCI) leads to motor dysfunction and loss of feelings partially or completely and is one of the most serious injuries of the nervous system. The main causes of SCI include road accidents, fall from tall trees or buildings and sports injuries (Arora et al. 2015). Reports reveal that annually around 40 people in every 10-lakh population suffer from SCI throughout the world (Arora et al. 2015). Most of these cases are of working age group which adversely impacts the economic status of families and countries (Dudley-Javoroski and Shields 2013). Till date treatment of SCI is a challenge for clinicians and people dealing with drug development programs. Thus, SCI treatment comprising of nerve damage inhibition or recovery using chemotherapies has great significance globally.

Mechanical injury caused to spinal cord by the external impact is irreversible and can't be treated effectively (Harper et al. 2014; López-Larraz et al. 2012). The subsequent damage following primary SCI including inflammation, excessive activation of glutamate receptors, water accumulation, overload of calcium and peroxidation of lipids comprise of secondary SCI (Harper et al. 2014). These processes together trigger the death of neurons within a time period ranging from few days to weeks (Jia et al. 2013). Thus, neuronal and glial cells are eliminated excessively following primary SCI and severity increases during secondary SCI (Jia et al. 2013). The SCI mediated damage is not only confined to residual neurons but also extends to surroundings of the injured region (Laubacher et al. 2015). Inflammation plays vital role in aggravation of the complications following secondary SCI (Rosety-Rodriguez et al. 2014). The most adverse factor associated with secondary SCI is apoptosis and

therefore its inhibition alleviates nerve damage (Rosety-Rodriguez et al. 2014). Numerous pathways activated due to apoptosis play active role in elimination of cells during different processes (Amin et al. 2014).

The superfamily of nuclear transcription factors which are ligand activated consist of peroxisome proliferator-activated receptor (PPAR) as a member (Yi et al. 2008). Reports demonstrate that agonists for PPAR inhibit inflammation and suppress apoptosis of neurons in the brain (Yi et al. 2008; McTigue 2008). Promotion of nerve growth factors and phosphorylation of phosphoinositide 3-kinase (PI3K)/Akt pathway is involved in inhibition of neuronal apoptosis (Chae and Kim 2009). Therefore, neural function may be improved by inducing NGF proliferation and inducing PI3K/Akt activation in order to inhibit apoptosis (Kim et al. 2004). Indirubin skeleton has been investigated initially for kinase inhibitor potential against CDK and GSK3b (Polychronopoulos et al. 2004; Marko et al. 2001). The current study investigated indirubin-3'-oxime (IR3O) for treatment of SCI in rat model and evaluated the related mechanism.

Materials And Methods

Animals

Sixty Male Sprague Dawley rats (195-205 g weight; 7-week old) were provided by the Vital River Laboratories, Animal Technology Co., Ltd (Beijing, China). All rats obtained were housed individually at 24 ± 1˚C temperature, under 60% humidity and exposed to 12 h light followed by 12 h dark cycles. Free access was given to laboratory chow and water to all the rats *ad libitum*. Guidelines released by the National Institute of Health China were followed for all protocols on rats (Jiang et al., 2015). Study approval was given by the Animal Care and Use Committee, Second Military Medical University, Shanghai, China.

Surgical protocol

Rats were assigned randomly to six groups of 10 each, model SCI, sham and four ID30 treatment (at 0.25, 0.5, 0.75 and 1.0 mg/kg doses) groups. Rats in model SCI and ID30 treatment groups were give sodium pentobarbital (30 mg/kg) intraperitoneally prior to laminectomy at 8^{th} thoracic vertebra level in order to carefully expose spinal cord. Then, vertebral column was secured by clamping T6 and T10 and SCI model was established by dripping a hammer. Wound was sutured and rats were housed under sterile conditions. SCI model and sham groups were given normal saline (100 µl) whereas treatment groups received 0.25, 0.5, 0.75 and 1.0 mg/kg doses of ID30 daily for 2-weeks post-surgery.

Rat behaviour analysis and water content measurement

Motor function of rats following ID30 treatment was evaluated at week 1 and 2 using the 0-21-point Basso, Beattie and Bresnahan (BBB) scale. For this purpose, rats were freely allowed to walk in an open field for 4 min to observe the hindlimbs for assigning the score (Mukhamedshina et al., 2018). On day

31st of the surgery, rats were sacrificed, spinal cord tissues were extracted and subsequently weighed to record wet weight. Then these tissues were dried for 48 h at 80˚C and weighed again to record the dry weight. Accumulation of water in spinal cord tissues was calculated using the formula: (dry weight/wet weight) x100%.

Determination of inflammatory and oxidative factors

Blood samples were taken from the rats on day 31st, centrifuged at 4˚C for 15 min at 3,000 x g to isolate the serum. The serum samples were stored at -80˚C for determination of various factors by ELISA assay. The commercially available kits (Nanjing Jiancheng Biology Engineering Institute, Nanjing, China) were used to determine TNF-α (cat. no. H052), IL-6 (cat. no. H007), IL-1β (cat. no. H002), MDA (cat. no. A003-1), SOD (cat. no. A001-1-1), GSH (cat. no. A006-2) and GSH-PX (cat. no. A005) activities. Spectrophotometry was used for measurement of absorbance at 450 nm wavelength using Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Western blotting

Tissues of spinal cord were kept in ice-cold saline and treated with RIPA buffer for 40 min to get homogenized tissues. Centrifugation was performed at 4˚C for 15 min at 13,000 x g to collect the supernatant and protein content was determined by BSA assay (Bio-Rad). Electrophoresis was carried out by loading protein samples (40 µg/lane) on 10% SDS-PAGE gel followed by transfer to PVD membranes. Membranes were treated for 1 h with 5% non-fat milk powder and Tween-20 (TBST) at 37˚C followed by incubation for overnight with primary antibodies at 4˚C. The antibodies used were against COX-2 (sc-7951), PPAR-γ (sc-9000), PI3K (sc-7175), Akt (sc-8312), p-Akt (sc-7985-R) and GAPDH (sc-25778). Membranes after TBST washing were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies for 1 h. The BeyoECL Moon (Beyotime, China) was used for observing protein bands and Image Lab™ software for analysis.

Statistical analysis

The data are presented as the mean ± standard deviations of three measurements. For analysis of the data version 17.0; SPSS software (SPSS, Inc., Chicago, IL, USA) was used. Analysis of the data was made using one-way analysis of variance and Dunnett's post-hoc test. At $P<0.05$ values were considered to indicate a statistically significant difference.

Results

ID3O elevated BBB score

Spinal cord injury led to a prominent reduction in BBB score relative to the rats in sham group (Figure 1). However, ID30 administration to SCI rats effectively improved BBB score in dose-based manner relative to untreated group. The BBB score was significantly increased on treatment of SCI rats with 0.25, 0.5, 0.75

and 1.0 mg/kg doses of ID30. Treatment with ID30 at 1.0 mg/kg doses increased BBB score in SCI rats to the level of sham group.

Figure 1. Effect of ID30 on BBB score in SCI rats. Treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg ID30 was followed by determination of BBB score. *P<0.05, **P<0.02 vs. SCI group.

ID30 alleviated water accumulation in SCI rat spinal cord tissues

Water collection in tissues of spinal cord was significantly higher in rats with SCI (Figure 2). Accumulation of water in spinal cord tissues was alleviated significantly on treatment of SCI rats with ID30. Although ID30 significantly prevented SCI induced accumulation collection in spinal cord tissues at 0.25, 0.5, and 0.75 mg/kg concentrations but the effect was maximum at 1.0 mg/kg doses.

Figure 2. Effect of ID30 on spinal tissue water accumulation in SCI rats. ID30 treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg doses was followed by measurement of water content in spinal cord tissues. *P<0.05, **P<0.02 vs. SCI group.

ID30 decreased pro-inflammatory factors

In SCI rats the levels of TNF-α as well as other cytokines such as IL-1β and IL-6 were significantly higher in serum (Figure 3). However, ID30 administration significantly down-regulated SCI mediated higher serum levels of TNF-α and cytokines (IL-1β and IL-6). Decrease in serum TNF-α and cytokine (IL-1β and IL-6) levels by ID30 were significant at 0.25, 0.5, and 0.75 mg/kg doses and maximum at 1.0 mg/kg concentration in SCI rats.

Figure 3. Effect of ID30 on inflammatory cytokines in SCI rats. ID30 treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg doses was followed by determination of serum TNF-α, IL-1β and IL-6 levels. *P<0.05, **P<0.02 vs. SCI group.

Oxidative stress is inhibited by ID30

In rats with SCI the activities of SOD, CAT and GSH were significantly (P<0.05) higher in serum relative sham group (Figure 4). The MDA level in SCI rat serum was significantly lower than sham group. ID30 treatment alleviated SOD, CAT and GSH activities significantly in SCI rats at 0.25, 0.5, 0.75 and 1.0 mg/kg doses. Moreover, the MDA level was significantly promoted in SCI rats on treatment with 0.25, 0.5, 0.75 and 1.0 mg/kg doses of ID30 in dose-based manner.

Figure 4. Effect of ID30 on oxidative stress factors. The ID30 treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg doses was followed by determination of (A) SOD, (B) CAT and (B) GSH activities. (D) The level of MDA was also determined. *P<0.05, **P<0.02 vs. SCI group.

ID30 decreased caspase activities

The levels of cleaved caspase-3 and -9 in tissues of spinal cord were increased significantly by SCI in rats relative to sham group (Figure 5). ID30 treatment at 0.25, 0.5, 0.75 and 1.0 mg/kg doses alleviated SCI mediated increased cleaved caspase-3 and -9 levels in dose-based manner. Cleaved caspase-3 and -9 expression was significantly alleviated by ID30 treatment at 0.25, 0.5 and 0.75 mg/kg doses in SCI rats. Treatment with ID30 at 1.0 mg/kg concentration reduced cleaved caspase-3 and -9 levels close to that of sham group.

Figure 5. Effect of ID30 on caspase cleavage. ID30 treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg doses was followed by ELISA assay for determination of (A) caspase-3 and (B) caspase-9 activities. *P<0.05, **P<0.02 vs. SCI group.

Inhibition of PGE2 expression by ID30

The expression of PGE2 was significantly (P<0.05) promoted by SCI in rats relative to sham group (Figure 6). ID30 treatment at 0.25, 0.5, 0.75 and 1.0 mg/kg doses suppressed SCI mediated elevation of PGE2 expression in dose-based manner. In SCI rats treatment with 1.0 mg/kg doses of ID30 reduced PGE2 expression close to that of the sham group.

Figure 6. Effect of ID30 on PGE2 expression. Treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg ID30 was followed by evaluation of PGE2 expression. *P<0.05, **P<0.02 vs. SCI group.

Elevated PPAR-γ and suppressed COX-2 expression by ID30

The SCI caused a significant suppression in PPAR-γ and elevated COX-2 expression in rats (Figure 7). ID30 administration to SCI rats at 0.25, 0.5, 0.75 and 1.0 mg/kg significantly (P<0.05) elevated PPAR-γ expression and suppressed COX-2 level in dose-based manner. In SCI rats, promotion of PPAR-γ and reduction of COX-2 level was maximum by ID30 treatment at 1.0 mg/kg.

Figure 7. Effect of ID30 on PPAR-γ/COX-2 expression. Treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg ID30 was followed by evaluation of PPAR-γ/COX-2 expression by western blotting. *P<0.05, **P<0.02 vs. SCI group.

ID30 promoted Akt and PI3K activation

In rats subjected to SCI the phosphorylation of PI3K and Akt was significantly lower in tissues of spinal cord (Figure 8). However, ID30 administration of SCI rats at 0.25, 0.5, 0.75 and 1.0 mg/kg doses significantly (P<0.05) elevated PI3K and Akt phosphorylation in dose-based manner. The PI3K and Akt phosphorylation in SCI rats was promoted to maximum level on treatment with 1.0 mg/kg doses of ID30.

Figure 8. Effect of ID30 on PI3K/Akt phosphorylation. The ID30 treatment of SCI rats with 0.25, 0.5, 0.75 or 1.0 mg/kg was followed by western blot assay or determination of phosphorylated PI3K and Akt levels. *P<0.05, **P<0.02 vs. SCI group.

Discussion

This study demonstrated that ID30 prevents oxidative, inflammatory and edema mediated tissue damage in spinal cord of rats arising due to spinal injury. Effective improvement in BBB score by ID30 treatment in SCI rats was found to be associated with the up-regulation of phosphorylated PI3K/Akt expression.

Severe disability because of motor dysfunction in SCI patients has adverse impact on economy of families and nations in general (Tian et al. 2009). Therefore, therapeutic strategies to recover SCI patients or alleviate SCI mediated nerve damage are of utmost significance. Among primary and secondary classes of SCI studies have found that latter is reversible and can be stopped (Paterniti et al. 2009; Jiang et al. 2007). Therefore, recovery of SCI patient is possible at secondary stage and once this stage is crossed severe disability arises (Jiang et al. 2007). Primary response of secondary injury in patients with SCI is the development of inflammation and water accumulation (Tian et al. 2009). In the current study ID30 treatment increased BBB score which was significantly reduced in rats following SCI. This indicated that ID30 has potential to prevent nerve damage caused by SCI in the rat model. The ID30 treatment also resulted in inhibition of water accumulation and edema development in spinal tissues of SCI rats.

Inflammation, excessive cytokine secretion and toxins leading to excitation trigger neuronal apoptosis via activation of the concerned genes (Uchida et al. 2012). Generally, genes regulating Bax, caspase-3 and Bcl-2 are influenced by various factors to tiger cellular apoptosis (Zhang et al. 2016; Mizuno et al. 2015). Injuries to nervous system have been reported to trigger apoptosis excessively in various study models (Mizuno et al. 2015). In the present study ID30 effectively inhibited excessive release of TNF-α and interleukins in the spinal tissues of rats with SCI. These factors were secreted at higher levels in rat spinal tissues following spinal cord injury. The ID30 treatment also significantly alleviated SOD, CAT and GSH activities in SCI rat spinal cord tissues. Treatment with ID30 led to a significant promotion in MDA level in rats with SCI compared to model group. Caspase-3 and − 9 activities in rats with SCI were alleviated effectively on treatment with ID30 in dose-based manner.

Involvement of PPAR-γ has been identified in several pathological as well as physiological processes (Griggs et al. 2015). Moreover, PPAR-γ agonist injection in cerebral cortex leads to a marked reduction in damage to neurons and suppresses apoptosis thereby demonstrating neuroprotective role (Paterniti et al. 2013; Park et al. 2007). Activation of PPAR-γ has been found to target radical generation in patients suffering from ischemia mediated inflammation and multiple sclerosis (Yi et al. 2008). Several signalling pathways activated by inflammatory responses to mediate inflammation are inhibited by PPAR-γ activation (Paterniti et al. 2013). The current study demonstrated that ID30 treatment suppressed SCI mediated elevation of PGE2 expression in SCI rat spinal cord tissues. Moreover, ID30 treatment of SCI rats significantly elevated PPAR-γ expression and suppressed COX-2 level. The PI3K/Akt pathway is triggered by multiple extracellular signals and is associated with proliferation and survival of cells (Isele et al. 2007). The involvement of this pathway has also been revealed in pathological processes and physiological functioning of the neurons (Isele et al. 2007). Moreover, neuronal proliferation, formation of myelin and plasticity of synapses is also regulated by PI3K/Akt pathway (Zhang et al. 2015; Felix et al.

2014). The present study showed that ID30 treatment of SCI rats significantly promoted PI3K and Akt phosphorylation in spinal cord tissues. In summary, ID30 inhibited edema and improved BBB score in rats with SCI by targeting pro-inflammatory cytokines and oxidative response. Moreover, in SCI rats ID30 treatment down-regulated apoptotic proteins, promoted PPAR-γ activation and elevated PI3K/Akt phosphorylation. Thus, ID30 has potential to be studied further for development of therapeutic strategy for SCI.

Declaration

Declarations

Acknowledgement

None

Availability of data and material

All the related data and material can be obtained from the authors.

Authors' contributions

Lv Wang designed and conceived the study. Yan Zhu, Lixue Wu, Qiuxiang Zhou, Yueyue Yan and Jinlong Qu, performed all the experimental work. Yan Zhu and Lixue Wu were also involved in data handling and processing. All the authors contributed equally in preparing the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

The experiments received approval from the Beijing Jishuitan Hospital, Beijin, China. The animal study protocols were priorly approved by animal ethical review board of Beijing Jishuitan Hospital, Beijin, China.

Consent to publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

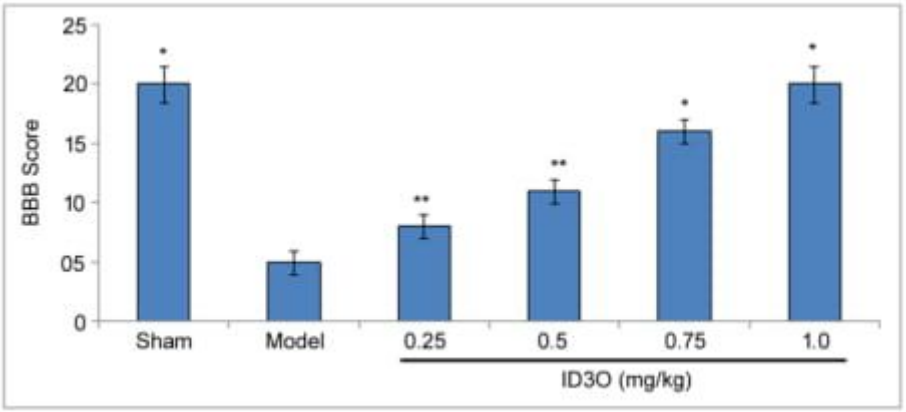

Figure 1

Effect of ID30 on BBB score in SCI rats. Treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg ID30 was followed by determination of BBB score. *P<0.05, **P<0.02 vs. SCI group.

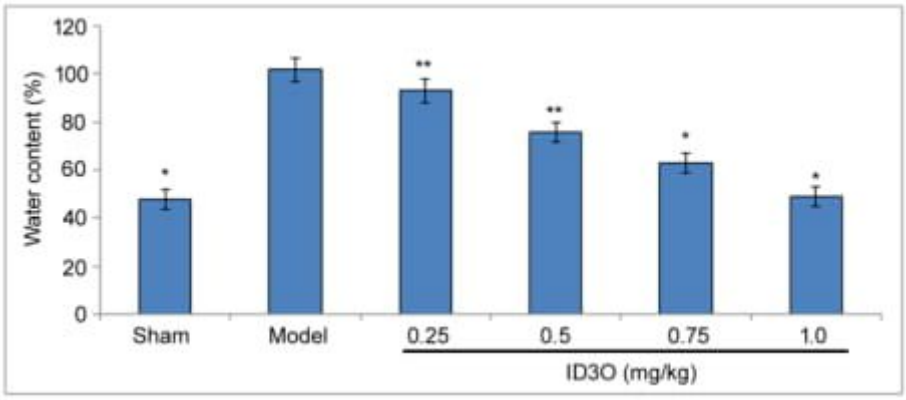

Figure 2

Effect of ID30 on spinal tissue water accumulation in SCI rats. ID30 treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg doses was followed by measurement of water content in spinal cord tissues. *P<0.05, **P<0.02 vs. SCI group.

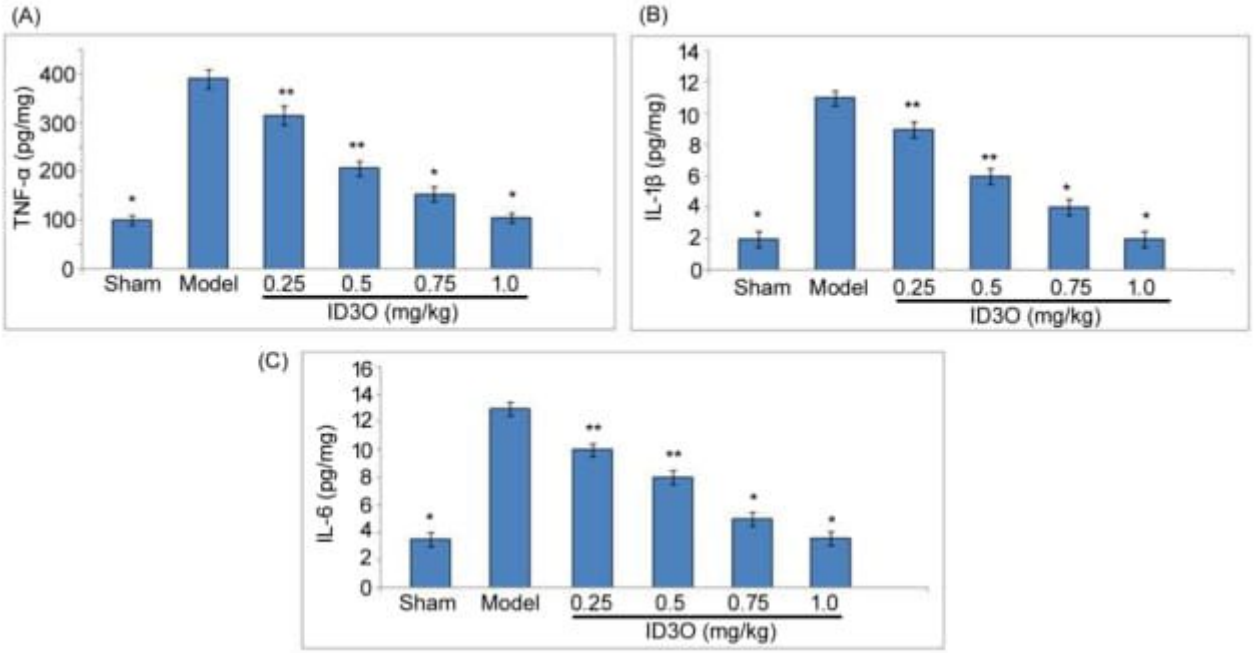

Figure 3

Effect of ID30 on inflammatory cytokines in SCI rats. ID30 treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg doses was followed by determination of serum TNF-α, IL-1β and IL-6 levels. *P<0.05, **P<0.02 vs. SCI group.

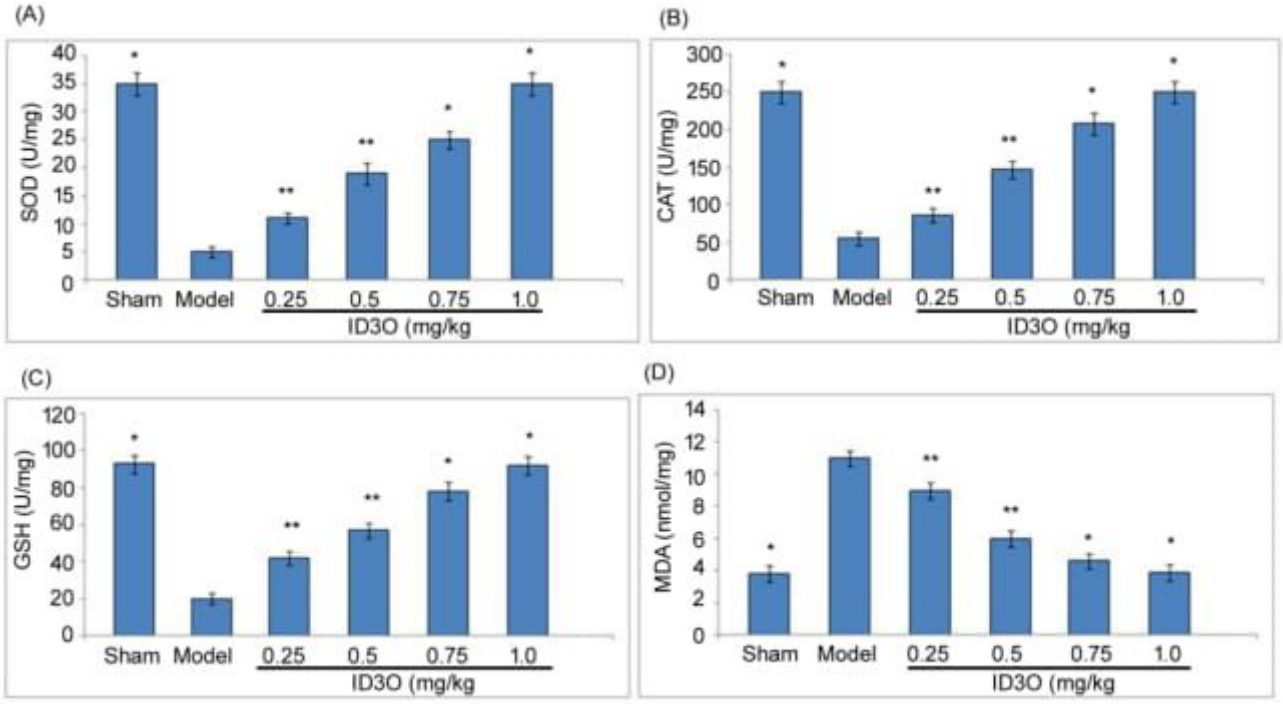

Figure 4

Effect of ID30 on oxidative stress factors. The ID30 treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg doses was followed by determination of (A) SOD, (B) CAT and (B) GSH activities. (D) The level of

MDA was also determined. *P<0.05, **P<0.02 vs. SCI group.

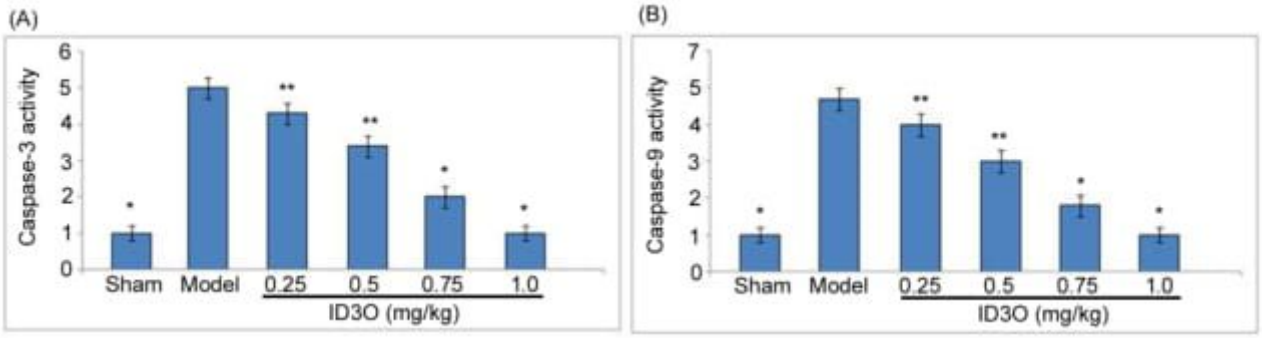

Figure 5

Effect of ID30 on caspase cleavage. ID30 treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg doses was followed by ELISA assay for determination of (A) caspase-3 and (B) caspase-9 activities. *P<0.05, **P<0.02 vs. SCI group.

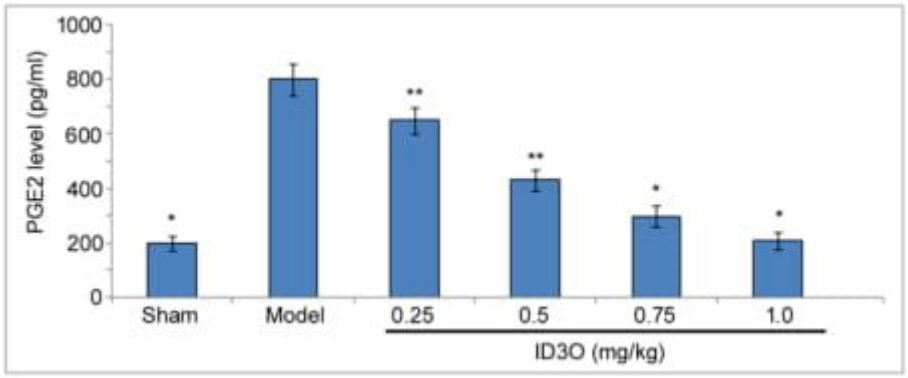

Figure 6

Effect of ID30 on PGE2 expression. Treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg ID30 was followed by evaluation of PGE2 expression. *P<0.05, **P<0.02 vs. SCI group.

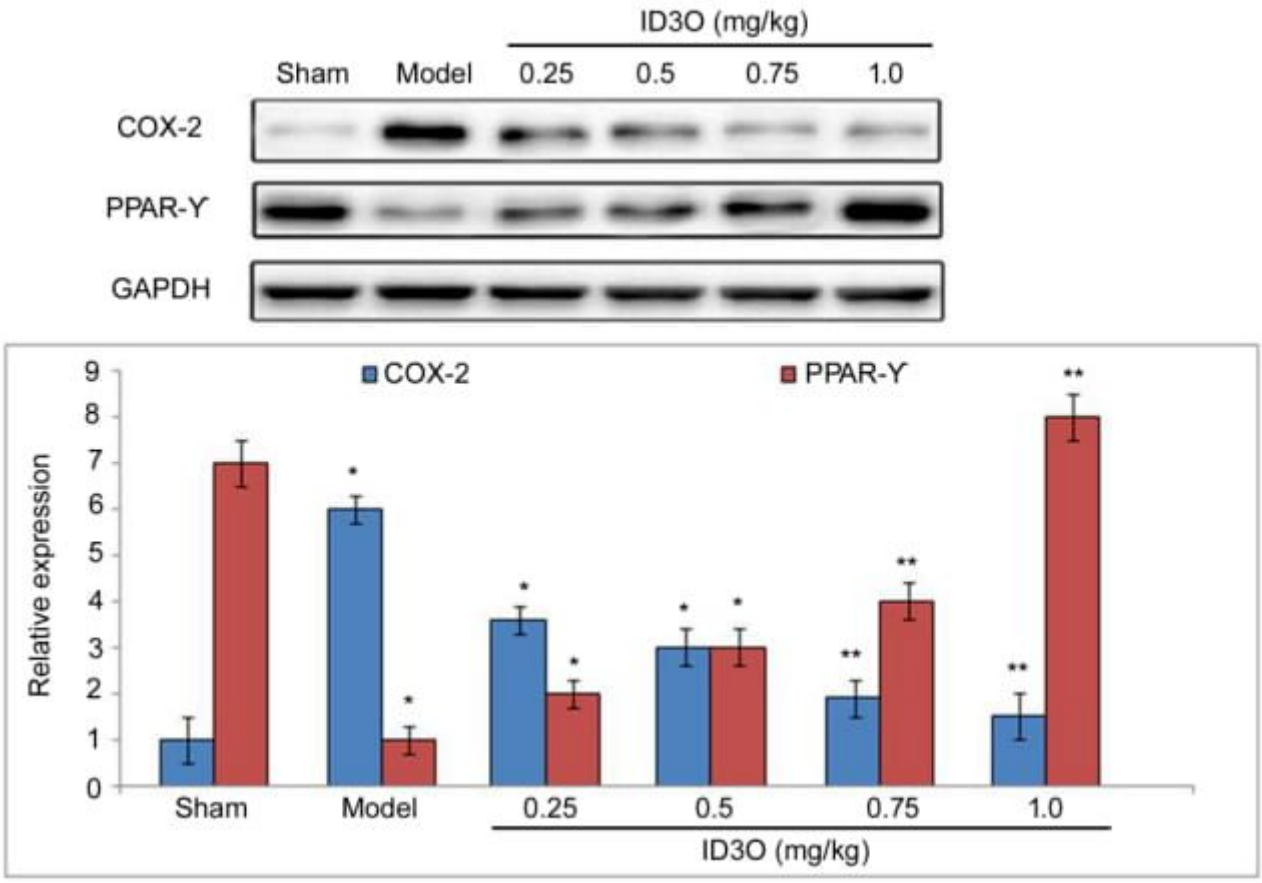

Figure 7

Effect of ID30 on PPAR-γ/COX-2 expression. Treatment of SCI rats with 0.25, 0.5, 0.75 and 1.0 mg/kg ID30 was followed by evaluation of PPAR-γ/COX-2 expression by western blotting. *P<0.05, **P<0.02 vs. SCI group.

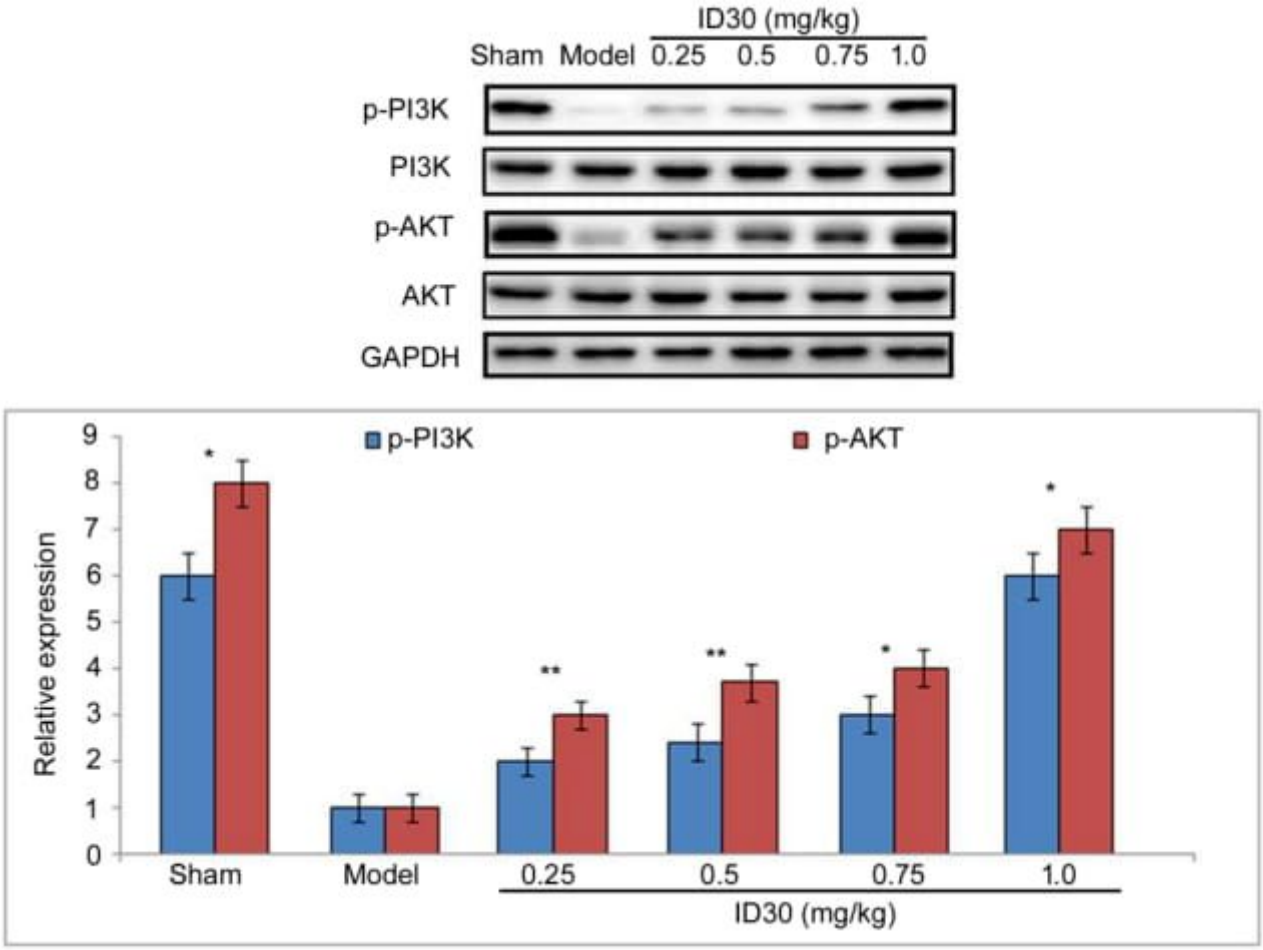

Figure 8

Effect of ID30 on PI3K/Akt phosphorylation. The ID30 treatment of SCI rats with 0.25, 0.5, 0.75 or 1.0 mg/kg was followed by western blot assay or determination of phosphorylated PI3K and Akt levels. *P<0.05, **P<0.02 vs. SCI group.