

Original Article

Angelica Sinensis restores motor function and reduces inflammation as well as apoptosis in CD1 mice with spinal cord injury

Xiao-Zhang Ying^{1*}, Bo Zhu^{1*}, Qi Zheng¹, Shi-Yuan Shi¹, Yi-Fan Wang¹, Jian Shen¹, Ming-Feng Zheng¹, Ke-Feng Zhang²

¹Department of Orthopedics, Integrated Chinese and Western Medicine Hospital of Zhejiang Province, Hangzhou 310003, Zhejiang, China; ²Department of Spine Surgery, Jining No.1 People's Hospital, Jining 272011, Shandong, China. *Equal contributors.

Received August 23, 2015; Accepted March 3, 2016; Epub March 15, 2016; Published March 30, 2016

Abstract: In this study the possible effects of Angelica Sinensis extract on traumatic SCI mice were explored. Four experimental groups of mice were used (n=15 each), which included Sham + Vehicle group where only laminectomy was performed, Sham + Angelica Sinensis (AS) group where only laminectomy was performed, SCI + Vehicle group where SCI was induced through clip compression technique and SCI/AS group where after inducing SCI, AS was used intraperitoneally to observe the possible therapeutic effects compared to controls. Locomotor function in experimental mice improved after 20 days of treatment with AS evident by BMS scaled score performed by blind observers and TUNEL assay also showed antiapoptotic effect. It was observed that NF- κ B p65 levels and phosphorylated NF- κ B p65 levels were significantly reduced after treatment with AS. Proinflammatory cytokines, TNF- α and IL-1 β levels were also reduced alongwith MPO activity in treated mice. Statistical analysis was performed by one way ANOVA followed by Bonferroni *post hoc* analysis for multiple comparisons and a *p* value of less than 0.01 was considered significant. It was observed that Angelica Sinensis (20 mg/kg, body weight dose) restored locomotor function alongwith antiapoptotic effects, reduced infiltration of neutrophils and attenuation of other inflammatory markers that helps in the reduction of inflammation which is an important part of secondary phase of SCI pathology.

Keywords: SCI, MPO activity, proinflammatory cytokines, Angelica Sinensis, Bcl-2 and Bax levels, NF- κ B p65, phosphorylated NF- κ B p65

Introduction

Spinal cord injury (SCI) has been a cause of concern due to its complex pathophysiology [1]. Although advances in medical care and pharmacology have improved patients outcome however, the problems related to neuronal injury are still persistent. It has been observed that traumatic injury to the spinal cord causes death to a number of neurons which not only pose a problem regarding their regeneration but the death of neurons continue for hours after traumatic SCI [2]. This phase of spinal cord injury after mechanical injury is termed as "secondary phase" where different biochemical, molecular and cellular events continue damaging the injured site. As a result inflammatory responses then participate in amplification of secondary

damage [3]. After SCI microglia are activated and macrophages cross the blood brain barrier (BBB) where they act as intrinsic spinal phagocytes. As a result proinflammatory cytokines, reactive oxygen species (ROS) and nitrogen species are released [4, 5].

Injury to spinal cord is important in central nervous system (CNS) as it causes sensory and motor dysfunction in patients. Morphological changes also occur in tissues after mechanical injury to spinal cord which includes hemorrhage, ischemia and edema [6, 7]. It has been demonstrated that activation of MAPK signaling pathways is a crucial step in inflammatory responses [8]. Studies have shown that NF- κ B is one of the major transcription factors that help in the regulation of production of proin-

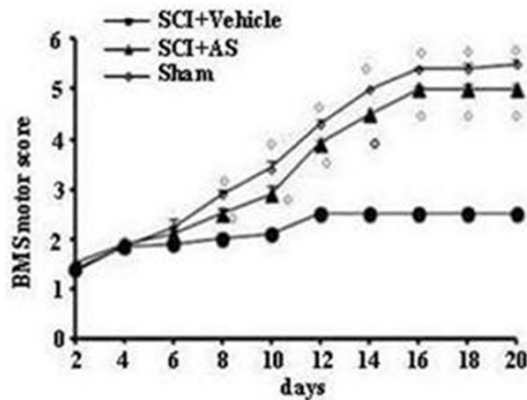


Figure 1. Motor function recovery can be seen at enhanced levels in mice treated with Angelica Sinensis as apparent from the graphical representation of BMS scaled motor score, where * $P < 0.01$ and $^{**}P < 0.01$.

flammatory cytokines in the CNS [9, 10]. In secondary injury after SCI it has been demonstrated that apoptosis is a major event which is regulated by caspase and Bcl-2 families [11-13]. As apoptosis is involved in secondary damage and degeneration therefore it can lead to functional disability in the spinal cord. For treating SCI such a therapy can be effective that can be aimed at multiple targets.

Angelica Sinensis also known as Danggui is a traditional Chinese medicine that has been used in variety of gynecological disorders with demonstrated clinical efficacy [14]. Different other uses of compounds extracted from the roots of *A. Sinensis* include increase of myocardial blood flow and reduction in tissue damage caused by radiation [15-17]. For the treatment of cancer patients it has been demonstrated to possess a number of advantages and little toxicity [18].

Therefore, in this study we decided to explore the possible therapeutic effects of *A. Sinensis* roots extract on locomotor function recovery after SCI in mice as well as the possible effects on apoptosis and inflammation that cause a major problem in secondary damage after mechanical injury in SCI.

Materials and methods

In this study adult male CD1 mice (25-30 g) were used. Mice were kept in controlled environment and complete care was taken according to the ethical guideline of Integrated

Chinese and Western Medicine Hospital of Zhejiang Province, Hangzhou, China. For inducing SCI a clip compression model was used [19]. After anaesthetizing mice using chloral hydrate (400 mg/kg body weight) an incision was made on midline of the back longitudinally and paravertebral muscles were exposed which were dissected for exposing T5-T8 vertebrae. After exposing spinal cord by four levels T5-T8 laminectomy, SCI was induced at T6-T7 level using aneurysm clip with a 24 g force. Spinal cord was compressed for 1 minute in injured groups while only laminectomy was performed in sham animals. 1.0cc saline was used subcutaneously for making up the blood volume lost during surgery in all animals. These animals were housed in controlled environment for 20 days and food, water was provided to them. During this time period bladder was emptied manually until normal bladder function of the mice was restored.

Mice were divided into four groups randomly that included Group 1: sham + vehicle group where only laminectomy was performed and mice were treated with vehicle, $n=15$, Group 2 sham + AS (Angelica Sinensis), here AS was administered after 1 hour and 6 hour of performing laminectomy, $n=15$, Group 3 was SCI + vehicle group where mice were administered vehicle at 1 hour and 6 hour after performing SCI, $n=15$, Group 4 where mice were treated with AS (20 mg/kg, body weight, intraperitoneally) at 1 hour and 6 hour after performing SCI. For studying locomotor function recovery a separate set of mice ($n=15$) for each group was used where AS was administered at 1 hour and 6 hour after SCI as well as daily once for a period of 19 days, where sham groups served as control for comparisons.

After 24 h animals were deeply anesthetized with sodium pentobarbital and then perfused transcardially using phosphate buffered saline (PBS, 0.1 M) and 4% paraformaldehyde afterwards in 0.1 M PBS at pH 7.4. Tissue segments were removed, 1 cm on each side of the lesion and embedded in paraffin, then these were cut longitudinally for exposing posterior area of spinal cord and performing other immunohistochemical procedures.

Motor function recovery of mice ($n=15$) for each group was assessed for 20 days using Basso Mouse Scale (BMS) open field score [20] which

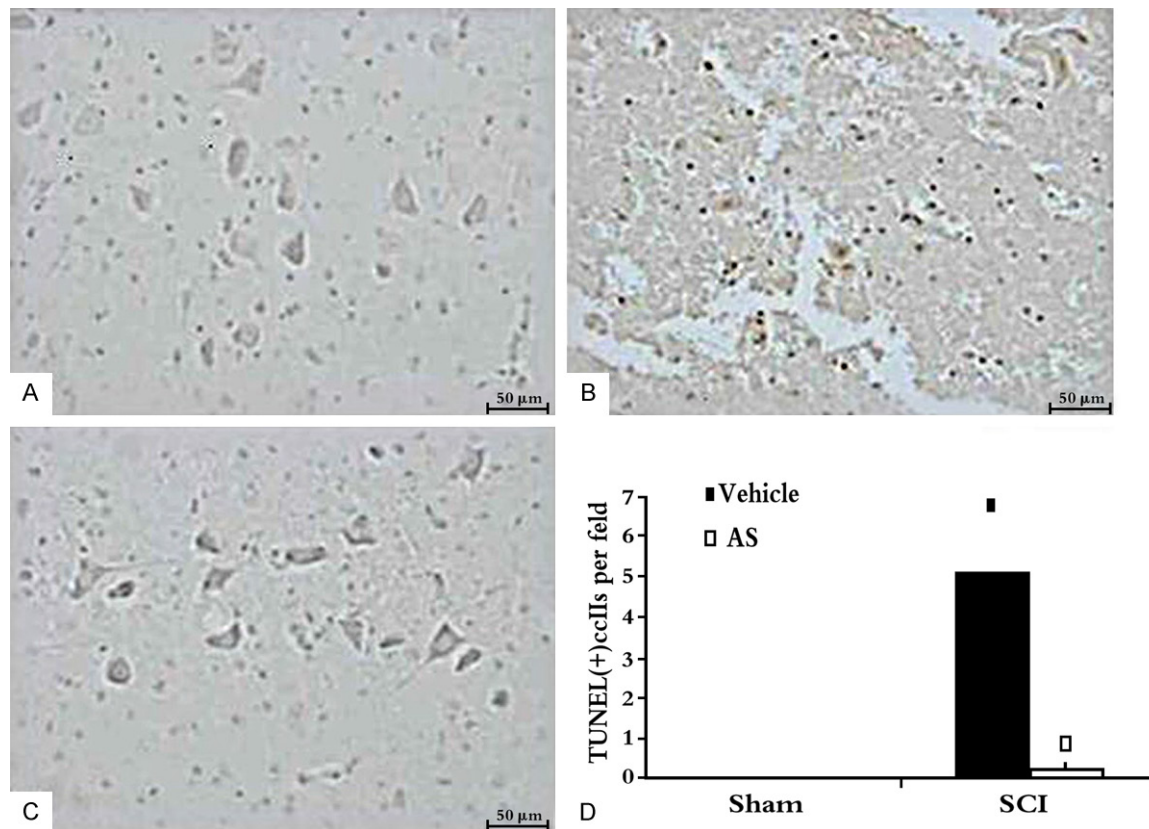


Figure 2. Figure showing result of TUNEL-like staining. A. Shows sham control where almost no apoptotic cells are seen. B. Shows the presence of apoptotic cells in untreated SCI mice spinal tissues and intracellular apoptotic fragments which are dark brown in color. C. Shows significant reduction in the presence of apoptotic cells in SCI mice treated with AS. D. Representative figure showing results for positive presence of apoptotic cells counted in 10 fields per slide by blind observer, where significant presence in untreated mice can be seen as compared to untreated mice, here $^*P<0.01$ and $^{\circ}P<0.01$.

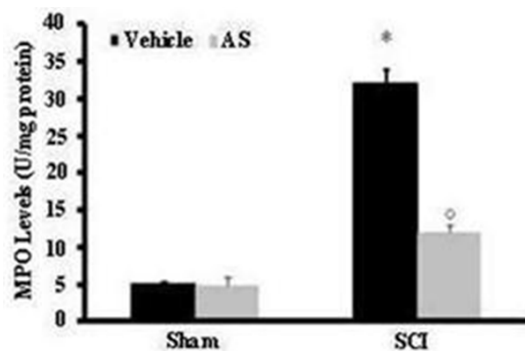


Figure 3. Increased MPO activity can be observed in mice after 24 h of injury however treatment did attenuate MPO activity in spinal cord tissues studied, where, $^*P<0.01$ and $^{\circ}P<0.01$.

is a 0 to 9 points scale ranging from complete paralysis at 0 to effective hind limb function at 9. Evaluations were made by two blind observers for each group.

For analysis of phospho NF- κ B p65 (serine 536), NF- κ B p65, Bax and Bcl-2 through western blotting, spinal cord extracts were prepared according to previous reports with slight modifications [21]. At first filters were blocked with 5% non-fat dried milk (AppliChem GmbH Germany) in PBS at room temperature for 40 minutes and then specific antibodies were used, phospho-NF- κ B p65 (serine 536) (Cell Signaling, 1:1,000), or anti-Bax (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), or anti-Bcl-2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), or anti-NF- κ B p65 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) in PM with 0.1% Tween-20 (Sigma-Aldrich, Milan, Italy) (PMT) at 4°C, overnight. For incubation of membranes peroxidase conjugated bovine anti mouse IgG secondary antibody or peroxidase conjugated goat anti rabbit IgG antibody (1:2000, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room tem-

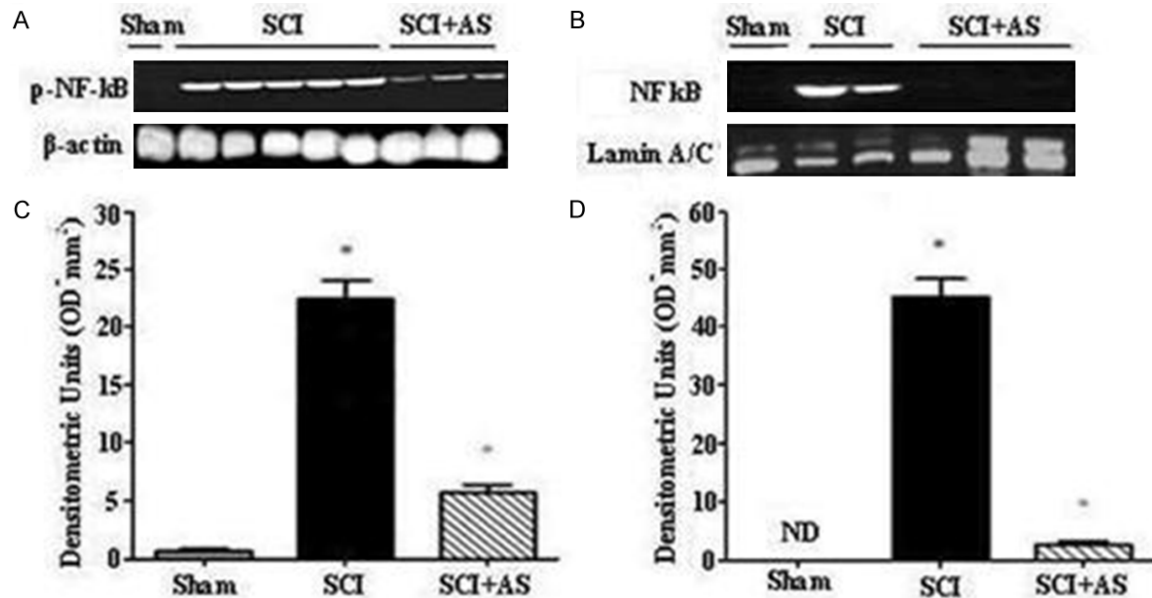


Figure 4. Western blot figures showing the effect of Angelica Sinensis treatment on SCI. A. Shows Western blots where significant increase on phosphorylation of Ser536 of NF-κB p65 can be seen in SCI mice compared to other groups. B. Western blots show significant decrease in levels of NFκB in AS treated mice with traumatic SCI. C. Shows the levels of phosphorylation of Ser 536 of NF-κB p65 compared to other groups where * $P < 0.01$ and * $P < 0.01$. D. Shows the levels of nuclear protein NFκB where significant decrease can be observed in spinal cord tissues where AS treatment was observed as compared to SCI untreated group, * $P < 0.01$ and * $P < 0.01$.

perature. Antibodies against β-actin and lamin A/C (1:10,000 Sigma Aldrich, Milan, Italy) were used. Then relative bands of phospho NF-κB, phospho NF-κB p65, Bax and Bcl-2 were quantified using densitometry.

Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Assay was conducted using TUNEL Assay Kit (Apotag, HRP kit DBA, Milan, Italy according to manufacturer's instructions and previously described reports [12] and signals were visualized using diaminobenzidine.

Polymorphonuclear leukocytes accumulation was determined through MPO activity according to previously described reports [22] and it was reported in units/mg protein. For measuring levels of TNF-α and IL-1β tissues colorimetric commercial kit DuoSet ELISA development Systems (R&D systems, Milan, Italy) was used. Tissue extracts were used after homogenizing in PBS containing 2 mmol/L of phenyl-methylsulfonyl fluoride.

All the experiments were performed in triplicate and values expressed in results section as mean ± SEM. Analysis of all the results was carried out by one way ANOVA followed by Bon-

ferroni *post-hoc* analysis for performing multiple comparisons where p value less than 0.01 was considered significant, while BMS scale data was analyzed using Mann-Whitney test and p value less than 0.01 was considered significant.

Results

Motor function recovery yielded positive results that indicated a better recovery of SCI mice after treatment with Angelica Sinensis (**Figure 1**). TUNEL staining showed that apoptotic cells were absent in sham operated animals and significant appearance was observed in SCI mice that were left untreated however, treated mice showed significant reduction in the presence of apoptotic cells (**Figure 2**).

Influx of leukocytes was studied using myeloperoxidase (MPO) activity and elevated levels were observed in tissues with spinal cord injury as compared to sham operated animals while a much attenuated level was observed in treated experimental models (**Figure 3**).

Western blot analysis performed for the evaluation of phosphorylation of Ser536 on NF-κB

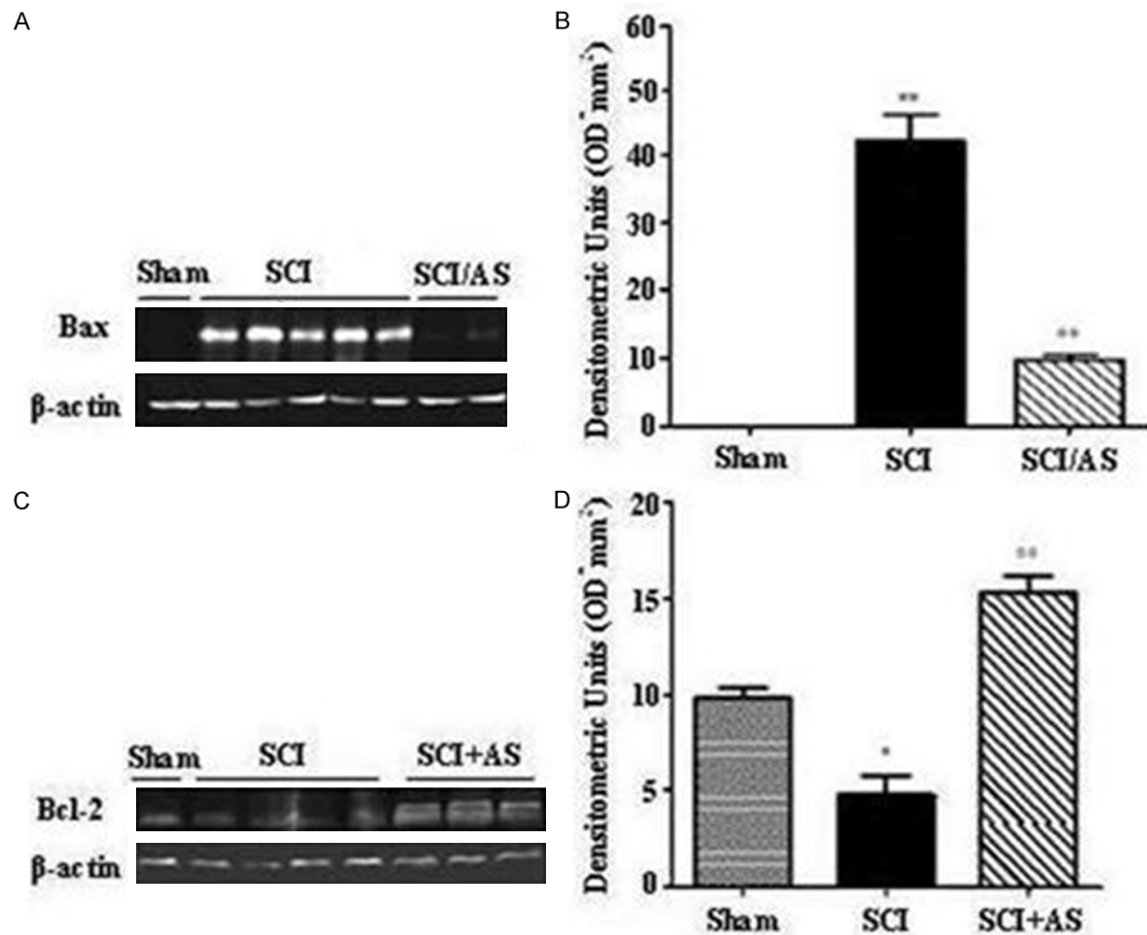


Figure 5. Representative Western blot showing relative Bax and Bcl-2 levels standardized against β -actin. A. An increase in Bax levels of SCI in untreated mice can be seen while a significant attenuation of Bax levels can be observed in mice treated with Angelica Sinensis (AS). B. Densitometric analysis of Bax levels shows a significant difference in Bax levels in SCI untreated mice, SCI treated mice and sham control where, ** $P < 0.01$ and * $P < 0.01$. C. Western blots show an increased expression of Bcl-2 expression in treated mice while a significantly attenuated expression was found in untreated mice and sham control. D. A significantly attenuated level of Bcl-2 expression can be observed in untreated SCI mice and a basal level expression in sham control while a decreased level was observed in SCI mice, where, * $P < 0.01$ and ** $P < 0.01$.

subunit p65 and NF- κ B p65 showed that after SCI a significant increase occurred in phosphorylation of Ser 536 on NF- κ B p65 at 24 h after injury while treatment with Angelica Sinensis showed significant decrease in levels of NF- κ B p65, the levels in SCI group were also elevated in comparison with sham operated animals (**Figure 4**).

Western blotting was performed for Bax and Bcl-2 on spinal cord tissues that showed a strong positive result for blots on tissues taken from SCI group for Bax while sham operated animals showed negative results for Bax, however, tissues from AS treated group with SCI showed positive result for Bax blotting but the

degree of positivity was low in this group. A different result was observed for Bcl-2 where sham operated mice gave strong positive bands for Bcl-2 and in SCI mice bands were significantly low however, in treated animals it was observed that AS treatment restored the levels of Bcl-2 in spinal tissues (**Figure 5**).

In order to understand the effect of AS treatment on proinflammatory cytokines levels the level of TNF- α and IL-1 β were established in all the groups. Here it was observed that levels of both these proinflammatory cytokines increased in tissues from SCI group which was untreated as compared to significantly reduced levels in AS treated group with SCI and almost

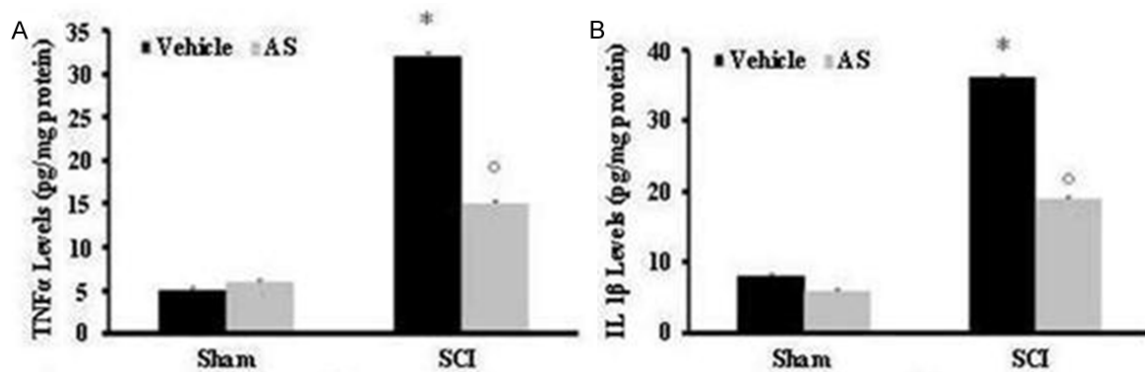


Figure 6. Effect of Angelica Sinensis treatment on levels of TNF- α and IL-1 β in traumatic mice. A. A significant increase in the levels of TNF- α can be seen in rats with SCI after 24 h while treatment with AS decreased the levels as compared with untreated mice and sham controls, * $P < 0.01$ and * $P < 0.01$. B. Similarly attenuated levels for IL-1 β can be seen in treated animals as compared to sham control and untreated mice, * $P < 0.01$ and * $P < 0.01$.

negligible amounts in sham operated animals (Figure 6).

Discussion

Spinal Cord Injury has a complex pathophysiology and it is divided into two phases distinctively. A primary phase that is caused by traumatic injury and then a secondary phase is characterized by inflammatory responses. These responses then cause severe devastation and hinder the outcome for functional recovery in such patients. Different problems accompanying these responses include apoptosis, necrosis of neurons and development of scar tissue [23].

Different events accompanying inflammatory responses and then leading to damage after spinal cord injury include infiltration of neutrophils which are the first cells to arrive at the site of injury and this results in the release of reactive mediators which cause damage to endothelial cells and as a result vascular permeability is increased which in turn causes increased movement of immune cells to the spinal cord and increasing damage [8]. MPO activity was evaluated to determine the effect of Angelica Sinensis treatment on traumatic SCI mice that showed a marked decrease in treated mice as compared to mice where only SCI was induced.

It has been observed that macrophages and microglia are dominant in the immune responses after SCI and play major role in secondary damage caused in patients [3]. Proinflammatory cytokines that are released such as TNF- α and IL-1 β play a major role towards demyelination of axons as well as cell death [24, 25].

Another important factor in secondary injury of SCI is apoptosis that can be triggered by a variety of factors contributing towards demyelination and also results in loss of cells [26, 27]. TUNEL assay showed an increase in apoptosis after spinal cord injury, while Angelica Sinensis treatment attenuated apoptotic response. It was also observed that proapoptotic Bax levels decreased significantly in treated mice while antiapoptotic protein levels of Bcl-2 protein increased. These protein levels are important for the development of apoptosis and it was established by the BMS score scales that locomotor functional recovery in mice was also restored in treated mice.

Different studies have shown therapeutic effects of Angelica Sinensis in different experimental models, however, to the best of our knowledge this is the first report of the use Angelica Sinensis on functional locomotor recovery in mice after traumatic SCI and it has been concluded that the factors leading towards secondary pathology of SCI are attenuated to a significant extent by the use of 20 mg/kg AS dose intraperitoneally. This study can further help to understand and develop targeted therapies for pathophysiological mechanisms of spinal cord injuries.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ke-Feng Zhang, Department of Spine Surgery, Jining No.1 People's Hospital, 6 Jiankang Road, Jining 272011, Shandong, China. Tel: 0086-537-2253104; Fax: 0086-537-2253104; E-mail: zkf1979@126.com

References

- [1] Maegele M, Riess P, Sauerland S, Bouillon B, Hess S, McIntosh TK, Mautes A, Brockmann M, Koebke J, Knifka J and Neugebauer EA. Characterization of a new rat model of experimental combined neurotrauma. *Shock* 2005; 23: 476-481.
- [2] Profyris C, Cheema SS, Zang D, Azari MF, Boyle K and Petratos S. Degenerative and regenerative mechanisms governing spinal cord injury. *Neurobiol Dis* 2004; 15: 415-436.
- [3] Chan CC. Inflammation: beneficial or detrimental after spinal cord injury? *Recent Pat CNS Drug Discov* 2008; 3: 189-199.
- [4] Genovese T, Mazzon E, Crisafulli C, Di-Paola R, Muia C, Bramanti P and Cuzzocrea S. Immunomodulatory effects of etanercept in an experimental model of spinal cord injury. *J Pharmacol Exp Ther* 2006; 316: 1006-1016.
- [5] Park E, Velumian AA and Fehlings MG. The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. *J Neurotrauma* 2004; 21: 754-774.
- [6] Young W. Secondary injury mechanisms in acute spinal cord injury. *J Emerg Med* 1993; 11: 13-22.
- [7] Taoka Y and Okajima K. Role of leukocytes in spinal cord injury in rats. *J Neurotrauma* 2000; 17: 219-229.
- [8] Carlson SL, Parrish ME, Springer JE, Doty K and Dossett L. Acute inflammatory response in spinal cord following impact injury. *Exp Neurol* 1998; 151: 77-88.
- [9] Barnes PJ and Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; 336: 1066-1071.
- [10] Li Q and Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2002; 2: 725-734.
- [11] Cohen GM. Caspases: the executioners of apoptosis. *Biochem J* 1997; 326: 1-16.
- [12] Esposito E, Mazzon E, Paterniti I, Impellizzeri D, Bramanti P and Cuzzocrea S. Olprinone attenuates the acute inflammatory response and apoptosis after spinal cord trauma in mice. *PLoS One* 2010; 5: 9 e12170.
- [13] Liu XZ, Xu XM, Hu R, Du C, Zhang SX, McDonald JW, Dong HX, Wu YJ, Fan GS, Jacquin MF, Hsu CY and Choi DW. Neuronal and glial apoptosis after traumatic spinal cord injury. *J Neurosci* 1997; 17: 5395-5406.
- [14] Yamada H, Kiyohara H, Cyong JC, Kojima Y, Kumazawa Y and Otsuka Y. Studies on polysaccharides from *Angelica acutiloba*-part 1. Fractionation and biological properties of polysaccharides. *Planta Medica* 1984; 50: 163-167.
- [15] Kim SH, Lee SE, Oh H, Kim SR, Yee ST, Yu YB, Byun MW and Jo SK. The radioprotective effects of Bu-Zhong-Yi-Qi-Tang: a prescription of traditional Chinese medicine. *Am J Chin Med* 2002; 30: 127-137.
- [16] Wang X, Wei L, Ouyang JP, Muller S, Gentils M, Cauchois G and Stoltz JF. Effects of an angelica extract on human erythrocyte aggregation, deformation and osmotic fragility. *Clin Hemorheol Microcirc* 2001; 24: 201-205.
- [17] Xie F, Li X, Sun K, Chu Y, Cao H, Chen N, Wang W, Liu M, Liu W and Mao D. An experimental study on drugs for improving blood circulation and removing blood stasis in treating mild chronic hepatic damage. *J Trad Chin Med* 2001; 21: 225-231.
- [18] Cai HB and Luo RC. Prevention and therapy of radiation induced pulmonary injury with traditional Chinese medicine. *Di Yi Jun Yi Da Xue Xue Bao* 2003; 23: 958-960.
- [19] Rivlin AS and Tator CH. Effect of duration of acute spinal cord compression in a new acute cord injury model in the rat. *Surg Neurol* 1978; 10: 38-43.
- [20] Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM and Popovich PG. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. *J Neurotrauma* 2006; 23: 635-659.
- [21] Bethea JR, Castro M, Keane RW, Lee TT, Dietrich WD and Yezierski RP. Traumatic spinal cord injury induces nuclear factor-kappaB activation. *J Neurosci* 1998; 18: 3251-3260.
- [22] Mullane KM, Kraemer R and Smith B. Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J Pharmacol Methods* 1985; 14: 157-167.
- [23] Hausmann ON. Post-traumatic inflammation following spinal cord injury. *Spinal Cord* 2003; 41: 369-378.
- [24] Blight AR. Macrophages and inflammatory damage in spinal cord injury. *J Neurotrauma* 1992; 9: S83-91.
- [25] Gomes-Leal W, Corkill DJ and Picanco-Diniz CW. Systematic analysis of axonal damage and inflammatory response in different white matter tracts of acutely injured rat spinal cord. *Brain Res* 2005; 1066: 57-70.
- [26] Li GL, Farooque M, Holtz A and Olsson Y. Apoptosis of oligodendrocytes occurs for long distances away from the primary injury after compression trauma to rat spinal cord. *Acta Neuropathol* 1999; 98: 473-480.
- [27] Li M, Ona VO, Chen M, Kaul M, Tenneti L, Zhang X, Stieg PE, Lipton SA and Friedlander RM. Functional role and therapeutic implications of neuronal caspase-1 and -3 in a mouse model of traumatic spinal cord injury. *Neuroscience* 2000; 99: 333-342.