Int J Clin Exp Pathol 2015;8(6):6779-6785
www.ijcep.com /ISSN:1936-2625/IJCEP0007195

Original Article

Angelica Sinensis attenuates inflammatory reaction in experimental rat models having spinal cord injury

Jun Xu[1], Xiao-Qiang E[2], Hui-Yong Liu[3], Jun Tian[1], Jing-Long Yan[1]

[1]*Department of Orthopedics, The Second Affiliated Hospital of Harbin Medical University, Harbin 150001, China;*
[2]*Department of Orthopedics, The First Affiliated Hospital of Harbin Medical University, Harbin 150081, China;*
[3]*Department of Orthopedics, Hulan District Hospital of Traditional Chinese Medicine, Hulan 150500, China*

Received February 18, 2015; Accepted April 13, 2015; Epub June 1, 2015; Published June 15, 2015

Abstract: This study was aimed to evaluate the effect of Angelica Sinensis on experimental rat models in which spinal cord injury was induced by studying different factors. Different factors causing inflammation play a key role in pathophysiology of SCI. Here three groups of rats (n=15, each was used). These included a sham control group where only laminectomy was performed, SCI group where SCI was induced and AS/SCI group where although SCI was induced but Angelica Sinensis was also administered to study its effect and draw a comparison with control. The expression of I-kBα and NF-kB p65 was also studied using western blotting and after recording optical density (OD) values of western blots. MPO activity was used to measure the effect of 20 mg/kg Angelica Sinensis. The levels of proinflammatory cytokines TNF-α, IL-1β and IL-6 were also studied. As compared with SCI group and sham control it was observed that Angelica Sinensis significantly reduced the expression of I-kBα and NF-kB p65, ($P<0.05$), while MPO activity was also significantly reduced. Proinflammatory cytokine level was also reduced in treated group as compared to both other groups. On the basis of this study we concluded that the use of 20 mg/kg Angelica Sinensis in rat models can attenuate the secondary damage caused by SCI and thus help in controlling the pathology of SCI in rats.

Keywords: Spinal cord injury, angelica sinensis, proinflammatory cytokines, inflammation in SCI, MPO activity

Introduction

A major cause of disability, spinal cord injury (SCI) is attributed to different factors that include mechanical factors as well as other mechanisms caused by the trauma [1]. Such trauma causes disruption of tissues as well as irreversible axonal injuries that cause death of neurons [2]. Other factors like excitotoxicity and vascular abnormalities also contribute to the pathology of SCI where neuronal death continues even after many hours of the induction of SCI [3-5]. Secondary damage plays its part in increasing the extent of pathological implications of SCI where inflammation as well as apoptosis play there vital role [6].

One of the major processes that complicate pathophysiology of SCI is inflammatory reactions which are triggered causing secondary damage [7, 8]. Inflammatory cells produce proinflammatory cytokines like TNF-α, IL-1β and IL-6 which can be seen in a large amount at the site of injury [9, 10]. In central nervous system (CNS) rate of apoptosis is increased that involves both neurons and glia where cytokines related to the TNF superfamily are considered as a specific cause [11, 12]. Different studies have shown several pathways that play an important role in such cases, for example, activation of MAPK signaling pathway has been regarded as an important step in inflammatory responses [13]. The production of proinflammatory cytokines in CNS is also modulated by a major transcription factor NF-kB [14, 15].

In traditional Chinese medicine the root of Angelica Sinensis is known as Danggui and it is used in various gynecological disorders and its clinical efficacy has been demonstrated [16]. Several other uses of compounds extracted and purified from the roots of A. Sinensis include increasing the myocardial blood flow and reducing tissue damages induced by radia-

tion [17-19]. It has been widely used in treating cancer patients with a number of advantages and little toxicity while its clinical efficacy has also been demonstrated [20].

In this study we decided to explore the effect of Angelica Sinensis on several factors leading to inflammation as well as apoptosis in pathological mechanisms underlying spinal cord injury. SCI was induced in rats and purified extract of A. Sinensis was tested to study the effect on proinflammatory cytokines, myeloperoxidase activity. The expression of NF-kB p65 and I-kB-α has been studied. In this way an effort was made to study the possible use of this extract in treating SCI pathology.

Materials & methods

In this study male Sprague-Dawley rats weighing 220-250 g each were used. All the experimental conditions were approved by the Ethics Committee of Harbin Medical University, Harbin, China and Hulan District Hospital of Traditional Chinese Medicine, Hulan, China. Animals were kept under standard and controlled conditions with 12 h light/dark cycles. Spinal Cord injury was induced using a NYU Impactor as previously described [21]. 4% sodium pentobarbital (40 mg/kg, i.p) was used for anesthesia in all animals. Paraverteberal muscles were exposed by making an incision along middle of the back and laminectomy was performed at T9-T11 level. Cord was exposed and dura was left undamaged where weight drop impact was made using 10 g rod at the exposed dorsal surface of the cord after which skin and lesioned muscles were sutured in layers before using 1.0 ml subcutaneous injection of saline for making up for volume blood lost during surgery.

Experimental design included three groups of rats (n=15 each) named as SCI group (where spinal cord injury was induced), SCI/AS group (where Angelica Sinensis was used for treating SCI) and Sham control group (where only laminectomy was performed and no SCI was induced). In SCI/AS group all the animals were given intraperitoneal injection of AS at a concentration of 20 mg/kg after the operation. At 24 h after injury rats were sacrificed using cervical dislocation where spinal cord was exposed from T1-T12 and at the site of injury the damaged tissue from T9 to T10 was cut for further analysis.

According to the previous reports [22] myeloperoxidase (MPO) activity was determined in spinal cord tissue to observe the accumulation of polymorphonuclear leukocytes. At 24 h after SCI injured tissues extracted from all the groups (n=15) were weighed for estimating myeloperoxidase activity. Tissue samples were homogenized in 0.5% (w/v) hexadecyltrimethyl-ammonium bromide that was dissolved in 10 mM potassium phosphate buffer at pH 7.0 and then centrifuged at 20,000× g for 30 min at 4°C. Supernatant was taken and an aliquot was incubated with a solution of 1.6 mM tetramethyle benzidine and 0.1 mM peroxide (H_2O_2). Optical density (OD) was measured using spectrophotometer at 460 nm. MPO activity is the quantity of enzyme required to degrade 1 mmol of H_2O_2 per minute at 37°C and is expressed as units of MPO/g wet tissue.

The levels of proinflammatory cytokines for each group (n=15) was determined after dissecting the lesion site and homogenizing it in PBS at 24 h after SCI. It was then centrifuged at 4°C for 15 min at 900 g. Supernatant was collected and concentration of TNF-α, IL-1β and IL-6 was measured using respective ELISA kits (R&D systems, Minneapolis, MN).

According to the previously described reports [23] NF-kB p65 and phosphorylated I-kBα levels were tested, however slight modifications were made. Spinal cord segments with injury epicenter of 10 mm size were used for total protein extraction by Total Protein Extraction Kit (Applygen Technologies Inc, Beijing, China). Concentration of proteins was determined using BCA protein assay kit (Applygen Technologies Inc, Beijing, China) according to the manufacturer's protocol. After boiling samples for 5 minutes those were diluted using sample buffer after which 50 μg protein obtained from each sample was loaded on 4-20% polyacrylamide gel for performing electrophoresis for separation and then those were transferred to polyvinylidene diflouride membrane. The membrane was then incubated with specific primary antibodies mouse anti rat NF-kB p65 monoclonal antibody (1:1000; Cruz Biotechnology Santa Cruz, CA, USA); monoclonal rabbit anti-rat phosphorylated I-kBα (Ser32) antibody (1: 500; Cell Signaling Technology, Danvers, MA, USA). For visualizing reactive bands horseradish peroxidase conjugated anti rabbit or anti mouse IgG antibodies (1:2000;

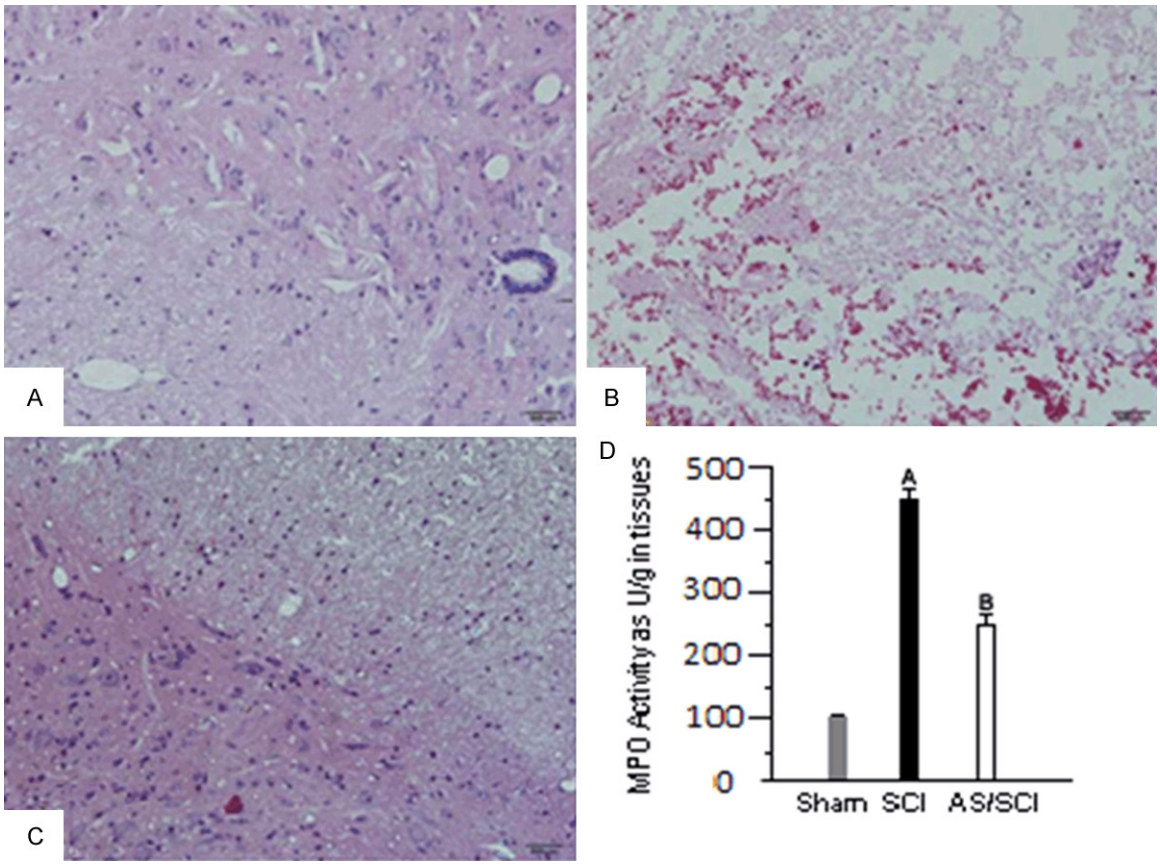

Figure 1. Effects of the treatment of Angelica Sinensis at 24 h after SCI. A. Shows histological changes in Sham group where normal histology can be observed. B. Significant changes in the histology of spinal cord can be seen having loose structured tissues, hemorrhage and edema at 24 h after SCI. C. A significant difference can be observed in AS/SCI group showing protective effect of Angelica Sinensis in AS/SCI group. D. Graphic illustration shows increase in MPO activity in SCI compared with sham control ($P^A<0.05$), however, significant decrease in MPO activity is observed in AS/SCI group ($P^B<0.01$).

Jackson, West Grove, PA, USA) and ECL Western blotting kit (Applygen Technologies Inc, Beijing, China) were used according to the manufacturer's protocol. X-ray films were exposed for 10 s to 1 min and membranes were visualized using polyclonal rabbit anti-actin antibody (1:500; Santa Cruz Co., Santa Cruz, CA, USA) for detecting actin in the samples which was used as a loading control. With the help of Gel-Pro analyzer 4.0 software optical density (OD) of the samples was determined.

Statistical analysis of the data was carried out using SPSS 13.0 software (SPSS, Chicago, IL, USA) where Mean ± SD value expressed the experimental data. One way analysis of variance was used to analyze the results followed by Bonferroni *post hoc* test for making multiple comparisons. *P* value of less than 0.05 was considered significant.

Results

Several studies have shown that histological changes are associated with the influx of leukocytes into the spinal cord, therefore, we used MPO activity assay at 24 h after SCI in order to study the effect of Angelica Sinensis on infiltration of neutrophiles. It was observed that there was a marked increase in MPO activity in SCI group as compared to sham group where MPO activity was found negligible, however, treatment of SCI with a dose of Angelica Sinensis resulted in a significant decrease in MPO activity in AS/SCI group compared to the control. The results are shown in **Figure 1**.

A significant increase in the expression of phosphorylated I-kBα was observed 24 hours after the SCI in traumatic rats, however treatment with AS reduced the expression of phosphorylated I-kBα, where $P<0.05$ (**Figure 2**).

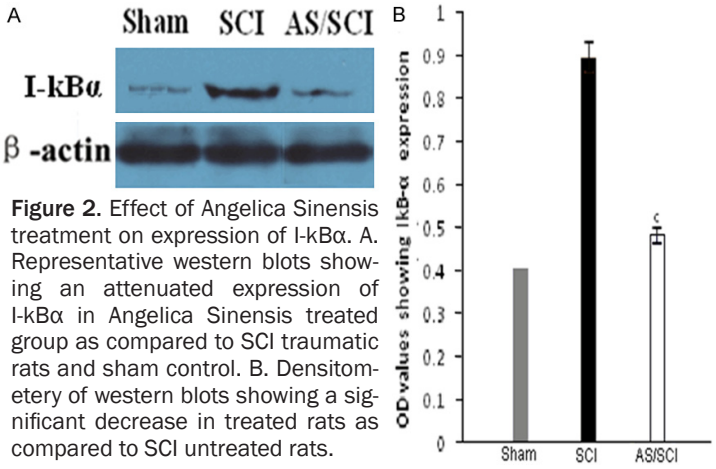

Figure 2. Effect of Angelica Sinensis treatment on expression of I-kBα. A. Representative western blots showing an attenuated expression of I-kBα in Angelica Sinensis treated group as compared to SCI traumatic rats and sham control. B. Densitometery of western blots showing a significant decrease in treated rats as compared to SCI untreated rats.

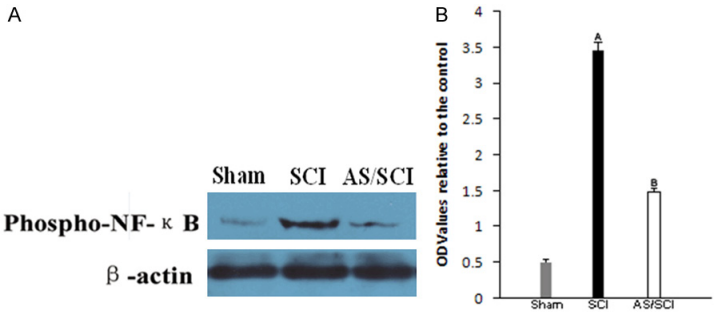

Figure 3. Effect of Angelica Sinensis treatment on NF-kB pathway. A. Western blots showing basal expression of phosphorylated NF-kB in sham group and increased level of expression in SCI group while a decreased expression can be seen in AS/SCI group. B. Graph shows OD values relative to the control showing densitometery of western blots where a significant increase in pNF-kB can be seen in SCI group compared to the control (P^A<0.05) while decrease is observed in AS/SCI group (P^B<0.01).

It was observed that expression of phosphorylated NF-kB remained at a basal level in sham group, while a significant increase was observed in SCI group. However, in AS/SCI group there was a significant decrease in the expression of NF-kB that helped us to conclude that treatment of SCI using Angelica Sinensis can result in inhibition of NF-kB pathway (**Figure 3**).

The effect of Angelica Sinensis on expression of proinflammatory cytokines like TNF-α, IL-1β and IL-6 was also observed. We performed study for evaluation of proinflammatory cytokines and mRNA level expression at 6 h, 12 h and 24 h after SCI and it was observed that proinflammatory cytokines level peaked at 6h after SCI and decreased subsequently (data not shown here). Here we therefore decided to evaluate the protein expression of TNF-α, IL-1β and IL-6 at 6h after SCI. At this point an increased level of these cytokines can be observed in SCI group compared to control while a decreased level is observed in AS/SCI group as shown in **Figure 4**.

Discussion

In this study the potential therapeutic effect of Angelica Sinensis on SCI rat models was studied based on protective effect for secondary damages caused by inflammation related factors in SCI. Different experimental models have been developed to cause major events in SCI in rats [24] and one of the most widely used models for this purpose is contusion injury. Here NYU impactor is used by dropping weight and causing rapid insult that leads to primary damage. It is done by exposing the dorsal surface of spinal cords and results in immediate or transient contusions.

It has been reported in several studies that A. Sinensis exhibits anti-inflammatory, antifibrotic and antiproliferative properties due to which it is used in the treatment of cancers, ulcers and other diseases. There have been different pharmacological evidences showing that extracts of A. Sinensis can improve local as well as systemic blood flow that benefits such patients [25-27]. Therefore, in this study we decided to explore the possible anti-inflammatory effects of A. Sinensis so that it can be explored as a potential therapeutic agent for treating the complicated spinal cord injury pathologies.

In SCI inflammatory responses are triggered where inflammatory cells release proinflammatory mediators along with neurotoxins that can generate reactive oxygen species and nitrogen species and it can result in damage to the cells [28, 29]. Several invaders occupy the injury site and neutrophiles are the first of them among leukocytes that invade the injured site of spinal cord [30-32]. As MPO activity is an indicator of polymorphonuclear leucocytes accumulation

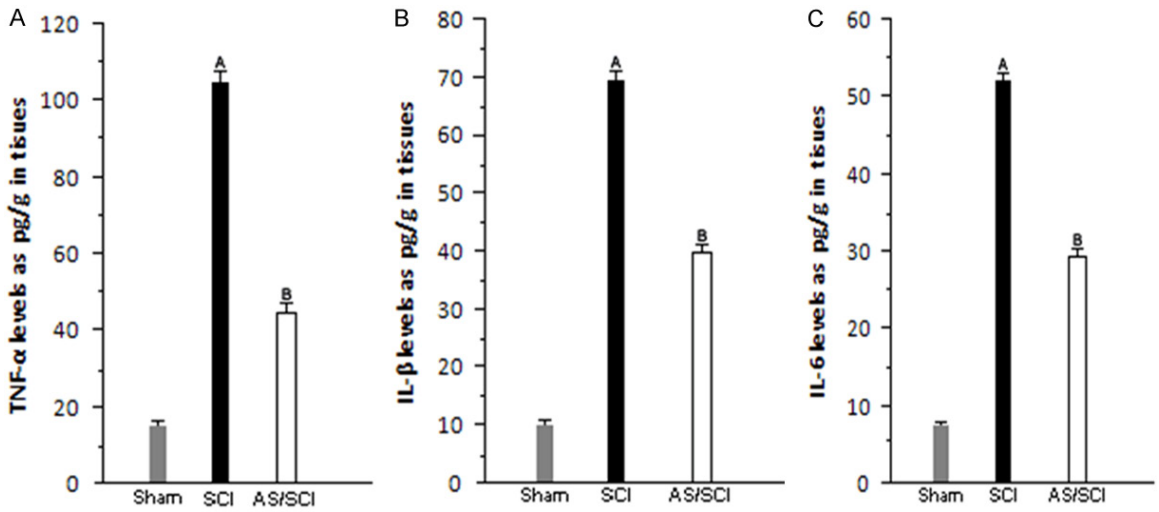

Figure 4. Effect of treatment of Angelica Sinensis on proinflammatory cytokines levels. A. Decrease in the level of TNF-α can be seen in AS/SCI group (P^B<0.01) as compared to control and significant increase can be seen in SCI group (P^A<0.05). B. Significant decrease can be observed in IL-1β level in AS/SCI treated group as compared to significant increase in SCI group when compared to control. C. Again a decrease in level of IL-6 can be seen in AS/SCI treated group compared to SCI group and sham control.

because myeloperoxidase, a lysosomal protein is stored in azurophilic granules of neutrophiles we studied MPO activity and downregulation of neutrophiles in A. Sinensis treated SCI rats showed potential protective effects of A. Sinensis in rat models, because in rats with SCI where A. Sinensis was not administered an increased MPO activity was observed.

Studies have demonstrated that proinflammatory cytokines like TNF-α, IL-1β and IL-6 play a major role in development of SCI [22, 33]. There are several pathways that modulate these factors like MAPK and NF-kB pathways [34-36]. Many studies have demonstrated previously that mRNA levels and protein levels of proinflammatory cytokines are increased in SCI damaged tissues along with the activation of inflammation related pathways [37-40].

In this study the decrease in levels of proinflammatory cytokines also helped us in concluding that Angelica Sinensis plays an important role in the downregulation of the production of proinflammatory cytokines that results in a comparatively lesser damage as opposed to the upregulation in untreated spinal cord injury rat models. Therefore, we can conclude that A. Sinensis possesses the potential of possible therapeutic use for treating spinal cord injuries however, this is a preliminary study in this regard because to the best of our knowledge this is the first report of the use of extract of A. Sinensis in rat models with SCI and more studies will be helpful in future in exploring the potential protective effects of this compound in treating SCI.

Acknowledgements

This work was supported by The National Science Foundation of China (NO. 81301530), the scientific research project of Education Department of Heilongjiang Province (NO. 12541531) and the scientific research project of Health Department of Heilongjiang Province (NO. 2012-602).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jun Tian, Department of Orthopedics, The Second Affiliated Hospital of Harbin Medical University, 246 Xuefu Street, Nangang District, Harbin 150001, Heilongjiang, China. E-mail: tianjun722@126.com

References

[1] Profyris C, Cheema SS, Zang D, Azari MF, Boyle K, Petratos S. Degenerative and regenerative mechanisms governing spinal cord injury. Neurobiol Dis 2004; 15: 415-36.

[2] Tator CH. Update on the pathophysiology and pathology of acute spinal cord injury. Brain Pathol 1995; 5: 407-13.
[3] Liu D, Xu GY, Pan E, McAdoo DJ. Neurotoxicity of glutamate at the concentration released upon spinal cord injury. Neuroscience 1999; 93: 1383-9.
[4] Tator CH, Koyanagi I. Vascular mechanisms in the pathophysiology of human spinal cord injury. J Neurosurg 1997; 86: 483-92.
[5] Hausmann ON. Post-traumatic inflammation following spinal cord injury. Spinal Cord 2003; 41: 369-78.
[6] Lu J, Ashwell KW, Waite P. Advances in secondary spinal cord injury: role of apoptosis. Spine 2000; 25: 1859-66.
[7] Blight AR. Macrophages and inflammatory damage in spinal cord injury. J Neurotrauma 1992; 9 Suppl 1: S83-S91.
[8] Nakamura M, Houghtling RA, MacArthur L, Bayer BM, Bregman BS. Differences in cytokine gene expression profile between acute and secondary injury in adult rat spinal cord. Exp Neurol 2003; 184: 313-25.
[9] Young W. Secondary injury mechanisms in acute spinal cord injury. J Emerg Med 1993; 11 Suppl 1: 13-22.
[10] Akira S, Hirano T, Taga T, Kishimoto T. Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). FASEB J 1990; 4: 2860-67.
[11] Robertson J, Beaulieu JM, Doroudchi MM, Durham HD, Julien JP, Mushynski WE. Apoptotic death of neurons exhibiting peripherin aggregates is mediated by the proinflammatory cytokine tumor necrosis factor-alpha. J Cell Biol 2001; 155: 217-26.
[12] Satoh JI, Kuroda Y. Alpha-synuclein expression is upregulated in NTera2 cells during neuronal differentiation but unaffected by exposure to cytokines and neurotrophic factors. Parkinsonism Relat Disord 2001; 8: 7-17.
[13] Carlson SL, Parrish ME, Springer JE, Doty K, Dossett L. Acute inflammatory response in spinal cord following impact injury. Exp Neurol 1998; 151: 77-88.
[14] Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997; 336: 1066-71.
[15] Li Q, Verma IM. NF-kappaB regulation in the immune system. Nat Rev Immunol 2002; 2: 725-34.
[16] Yamada H, Kiyohara H, Cyong JC, Kojima Y, Kumazawa Y, Otsuka Y. Studies on polysaccharides from *Angelica acutiloba*-part 1. Fractionation and biological properties of polysaccharides. Planta Medica 1984; 50: 163-7.
[17] Kim SH, Lee SE, Oh H, Kim SR, Yee ST, Yu YB, Byun MW, Jo SK. The radioprotective effects of Bu-Zhong-Yi-Qi-Tang: a prescription of traditional Chinese medicine. Am J Chin Med 2002; 30: 127-37.
[18] Wang X, Wei L, Ouyang JP, Muller S, Gentils M, Cauchois G, Stoltz JF. Effects of an angelica extract on human erythrocyte aggregation, deformation and osmotic fragility. Clin Hemorheol Microcirc 2001; 24: 201-5.
[19] Xie F, Li X, Sun K, Chu Y, Cao H, Chen N, Wang W, Liu M, Liu W, 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: 225-31.
[20] Cai HB, Luo RC. Prevention and therapy of radiation induced pulmonary injury with traditional Chinese medicine. Di Yi Jun Yi Da Xue Xue Bao 2003; 23: 958-60.
[21] Gruner JA. A monitored contusion model of spinal cord injury in the rat. J Neurotrauma 1992; 9: 123-8.
[22] Yang L, Jones NR, Blumbergs PC, Van Den Heuvel C, Moore EJ, Manavis J, Sarvestani GT, Ghabriel MN. Severity-dependent expression of pro-inflammatory cytokines in traumatic spinal cord injury in the rat. J Clin Neurosci 2005; 12: 276-84.
[23] Han X, Wang SY, Zhang Z, Lü DC, Liu HR. BMS-345541 inhibited nuclear factor kappa B expression and improved locomotor function recovery in rats after acute spinal cord injury. Neural Regen Res 2011; 6:1775-79.
[24] Beattie MS, Hermann GE, Rogers RC, Bresnahan JC. Cell death in models of spinal cord injury. Prog Brain Res 2002; 137: 37-47.
[25] M. Gao, Zhang JH, Zhou FX, Xie CH, Han G, Fang SQ, Zhou YF. Angelica sinensis suppresses human lung adenocarcinoma A549 cell metastasis by regulating MMPs/TIMPs and TGF-β1. Oncol Rep 2012; 27: 585-93.
[26] Yang C, Niu S, Yu L, Zhu S, Zhu J, Zhu Q. The aqueous extract of Angelica sinensis, a popular Chinese herb, inhibits wear debris-induced inflammatory osteolysis in mice. J Surg Res 2011; 176: 476-83.
[27] Chao WW, Hong YH, Chen ML, Lin BF. Inhibitory effects of Angelica sinensis ethyl acetate extract and major compounds on NF-κB transactivation activity and LPS-induced inflammation. J Ethnopharmacol 2010; 129: 244-49.
[28] Bao F, Liu D. Peroxynitrite generated in the rat spinal cord induces apoptotic cell death and activates caspase-3. Neuroscience 2003; 116: 59-70.
[29] Bao F, Liu D. Hydroxyl radicals generated in the rat spinal cord at the level produced by impact injury induce cell death by necrosis and apoptosis: protection by a metalloporphyrin. Neuroscience 2004; 126: 285-95.

[30] Bethea JR. Spinal cord injury-induced inflammation: a dual-edged sword. Prog Brain Res 2000; 128: 33-42.
[31] Chatzipanteli K, Yanagawa Y, Marcillo AE, Kraydieh S, Yezierski RP, Dietrich WD. Posttraumatic hypothermia reduces polymorphonuclear leukocyte accumulation following spinal cord injury in rats. J Neurotrauma 2000; 17: 321-32.
[32] Trivedi A, Olivas AD, Noble-Haeusslein LJ. Inflammation and spinal cord injury: Infiltrating leukocytes as determinants of injury and repair processes. Clin Neurosci Res 2006; 6: 283-92.
[33] Merrill JE, Benveniste EN. Cytokines in inflammatory brain lesions: helpful and harmful. Trends Neurosci 1996; 19: 331-8.
[34] Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. J Clin Invest 2001; 107: 7-11.
[35] Chen F, Castranova V, Shi X, Demers LM. New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. Clin Chem 1999; 45: 7-17.
[36] Uto T, Fujii M, Hou DX. 6-(Methylsulfinyl)hexyl isothiocyanate suppresses inducible nitric oxide synthase expression through the inhibition of Janus kinase 2-mediated JNK pathway in lipopolysaccharide-activated murine macrophages. Biochem Pharmacol 2005; 70: 1211-21.
[37] Xu Z, Wang BR, Wang X, Kuang F, Duan XL, Jiao XY, Ju G. ERK1/2 and p38 mitogen-activated protein kinase mediate iNOS-induced spinal neuron degeneration after acute traumatic spinal cord injury. Life Sci 2006; 79: 1895-1905.
[38] Lee YB, Yune TY, Baik SY, Shin YH, Du S, Rhim H, Lee EB, Kim YC, Shin ML, Markelonis GJ, Oh TH. Role of tumor necrosis factor-alpha in neuronal and glial apoptosis after spinal cord injury. Exp Neurol 2000; 166: 190-5.
[39] Yang L, Blumbergs PC, Jones NR, Manavis J, Sarvestani GT, Ghabriel MN. Early expression and cellular localization of proinflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α in human traumatic spinal cord injury. Spine 2004; 29: 966-971.
[40] Wang CX, Nuttin B, Heremans H, Dom R, Gybels J. Production of tumor necrosis factor in spinal cord following traumatic injury in rats. J Neuroimmunol 1996; 69: 151-6.