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

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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 pro-inflammatory 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-
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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. Paravertebral 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 tetramethyl benzidine and 0.1 mM peroxide (H₂O₂). Optical density (OD) was measured using spectrophotometer at 460 nm. MPO activity is the quantity of enzyme required to degrade 1 mmol of H₂O₂ 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 difluoride 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;
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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 neutrophils. 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 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<0.05), however, significant decrease in MPO activity is observed in AS SCI group (P<0.01).
It was observed that expression of phosphorylated NF-κB 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-κB that helped us to conclude that treatment of SCI using Angelica Sinensis can result in inhibition of NF-κB pathway (Figure 3).

The effect of Angelica Sinensis on expression of proinflammatory cytokines like 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...
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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.

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Disclosure of conflict of interest

None.

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