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Original Research Article

Dipsacus asperoides (Xue Duan) inhibits spinal cord injury-induced inflammatory responses in rats

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Abstract

Purpose: *To investigate the effect of Dipsacus asperoides (Xue Duan), a traditional Chinese medicine, on rats with spinal cord injury (SCI).*
Methods: *In this study a total of 40 adult rats were used after inducing SCI where Xue Duan was applied on experimental group and phosphate-buffered saline (PBS) was administered in corresponding control groups. Intraperitoneal administration of both compounds for a period of four weeks (28 days) was carried out at a dose of 10 mg/kg/day. Bright field microscopy was performed on the tissues.*
Results: *Bright Field microscopy of tissue sections showed significant reduction in cavity area that resulted from injury, that is from 0.19 ± 0.05 mm^2 to 0.09 ± 0.03 mm^2 ($p < 0.01$) in untreated and treated groups respectively. Similarly western blotting results showed a decrease in the expression of NF-kB p65 and I-kBα ($p < 0.01$). These two compounds are important in increasing secondary pathophysiology in SCI. The results for MPO activity also revealed significantly reduced infiltration of leukocytes to the injury site ($p < 0.01$).*
Conclusion: *This study reveals the positive effect of the plant material in reducing inflammation in rats with traumatic SCI.*

Keywords: *IKK/NF-kB pathway, MPO activity, Spinal cord injury, Inflammation, Xue Duan, Dipsacus asperoides*

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INTRODUCTION

Xu Duan, (*Dipsacus asperoides*) has been used in traditional Chinese medicine and it exhibits anti-inflammatory and antibacterial properties. It is a perennial plant widely distributed in mountains of South West China [1]. It has been used for the treatment of various disorders including liver and kidney dysfunctions. Various characteristics of this herb include strengthening of bones, facilitating regeneration of flesh and prevention of miscarriage. Additionally, this herb has been used to treat bone fractures, trauma as well as for relieving pain and swelling [2,3].

A number of events follow spinal cord injury at cellular as well as molecular level and these events can be divided in primary and secondary phases of spinal injury. Secondary phase increases the pathology of SCI because of the activation of inflammatory responses [4-6]. The expression of NF-kB and other transcription factors like cRel, Rel B, Rel A/p65, p50 and p52 are increased as a result of the activation of inflammatory responses, however, preventing activation of NF-kB can attenuate secondary damage after SCI [7-10]. It has been shown in different reports that a direct inhibition of I-kB kinase (IKK) can result in regulation of inhibition of products of NF-kB genes [11,12]. If IKK/NF-kB

pathway is targeted successfully then it can result in prevention of infiltration of inflammatory cells and apoptosis after SCI and also prove useful in restoring locomotors function in rats after SCI [13,14].

Spinal cord injury causes sensory and motor dysfunction in patients due to mechanical injury causes tissue damage resulting in morphological changes like edema, ischemia and hemorrhage [15,16]. It has been reported that in central nervous system (CNS) the regulation of pro-inflammatory cytokines is due to NF-kB which is a major transcription factor [17,18].

In this study the effect of *Dipsacus asperoides* was studied in order to evaluate the inhibition of IKK/NF-kB pathway and attenuation of apoptosis after spinal cord injury in rats.

EXPERIMENTAL

Dipsacus asperoides (Xue Duan) was obtained from Hangzhou Botanical Technology Co., Ltd. China. Phosphate buffer saline (PBS) was used as a control. In all 40 SD adult female rats were used. They were divided into two main groups designated as XD (Xue Duan) and PBS groups (n = 20 for each group). These were then sub-divided into four groups five rats where (n = 5) for each subgroup. After performing laminectomy Xue Duan was administered in all the experimental groups while PBS was administered in corresponding control groups for a total duration of four weeks. Sub-groups were created to study the effect of XD on rats weekly basis, and in each subgroup treatment was stopped after end of the week for analysis of effect of XD used while in other groups treatment was continued. XD and PBS were administered in the same amount at the same time interval so that comparison with control can be drawn.

Laminectomy at T9-T10 was performed after administering 10 % chloral hydrate anesthesia. At T9 a 10 g NYU impactor rod was dropped from 12.5 mm height to induce a partial SCI and post injury care was taken to minimize the sufferings of subjects while bladder was manually emptied twice daily. XD was administered at a dose of 10 mg/kg/day while PBS was administered only as a control at the same time interval in all corresponding groups. The study was approved by the Ethics Committee of Wuhan University, China and care was taken to minimize the sufferings of experimental animals used in the study.

After animals were anesthetized with 10 % chloral hydrate a transcardial perfusion was made using 4 % paraformaldehyde. For all animals T9-T10 spinal cord portion was removed and immersed for 24 h in a fixative while tissues were sectioned and immersed in paraffin for 24 h. General purpose histology was carried out on all sections. Tissue samples were collected on microscopic slides after removing paraffin and then slides were re-hydrated with graded ethanol before staining with hematoxylin eosin (HE). After staining these were viewed under microscope to see any difference of treatment on tissue sections taken.

Western blotting was used to study the expression of NF-kB p65 and phosphorylated I-kBα as previously described with slight modification [19]. 10 mm spinal cord segments were taken and total proteins were extracted from them. The segments used contained injury epicenter. Total Protein Extraction kit (Applygen Technologies Inc., Beijing, China). Concentration of proteins was determined using BCA protein Assay Kit (Applygen Technologies Inc., Beijing, China). Sample buffer was used for dilution of samples and after boiling for 5 min a 50 µg protein from each sample was loaded on 4 - 20 % polyacrylamide gel followed by electrophoretic separation and transferred to polyvinylidene diflouride membrane. Membrane was then incubated with specific primary antibodies after blocking, mouse anti rat NF-kB p65 monoclonal antibody (1:1000; Santa Cruz Biotechnology Santa Cruz, CA, USA) was used, along with monoclonal rabbit anti rat phosphorylated I-kBα (Ser 32) antibody (1: 500; Cell Signalling Technology, Danvers, MA, USA). Then visualization of reactive protein bands was carried out using ECL western blotting kit (Applygen Technologies Inc., Beijing, China) and horseradish peroxidase- conjugated anti rabbit antibodies (1: 2000; Jackson, West Grove, PA, USA) according to manufacturer's instructions. For detecting loading control actin in samples polyclonal rabbit anti-actin antibody (1:500; Santa Cruz Biotechnology., Santa Cruz, USA) was used. X-ray visualization of membranes was carried out from 10 s to 1 min. Protein bands were digitized and optical density (OD) was determined using Gel-Pro analyzer 4.0 software. An important parameter of inflammatory responses is infiltration of leukocytes to injury site which was studied using myeloperoxidase (MPO) activity in all groups. [19]

Statistical analysis

SPSS software (SPSS, Chicago, IL, USA) was used to carry out statistical analysis. All the

experiments were performed in triplicate and the values recorded as mean ± SEM. Analysis was performed using one-way ANOVA followed by Bonferroni post hoc analysis. *P* < 0.01 was considered significant value.

RESULTS

It was observed that in the treated group, the cavity area reduced significantly from 0.19 ± 0.05 to 0.09 ± 0.03 mm^2 compared to the untreated group where only PBS was used ($p < 0.01$), as shown in Figure 1.

The results of western blotting showed a considerable effect of treatment on IKK/NF-kB pathway. After 24 h of SCI an increase in NF-kB p65 was observed in all groups. However as treatment continued the expression of NF-kB p65 was attenuated in treated groups as compared to control groups in fourth sub - group where treatment continued till 28 days after spinal cord injury. Statistically significant difference was observed in treated and untreated groups ($p < 0.01$) (Figure 2).

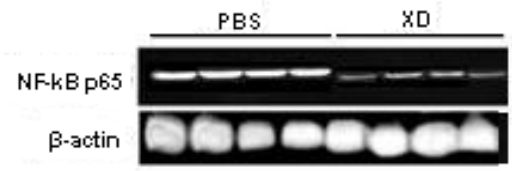

Figure 2: Western blotting for NF-kB p65. Representative western blots showing a significant attenuation in the expression of NF-kB p65 after treatment with XD and increased expression can be seen in untreated rats where $p^{*}<0.01$ and $p^{\circ}<0.01$ for both groups

A study of XD on expression of phosphorylated I-kBα also revealed an attenuation in its expression as observed by western blotting after continuous treatment for 4 weeks compared to control PBS groups. As was the case with NF-kB p65 increase in expression was observed after 24 h of SCI which then subsequently decreased ($p < 0.01$) (Figure 3).

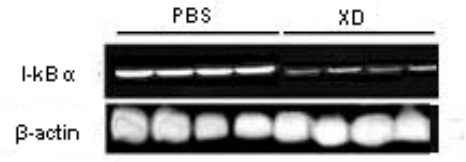

Figure 3: Western Blot showing expression of I-KBα**.** Western blots show decreased expression of I-kBα in treated groups as compared to PBS control after 28 days of treatment. In both treated and untreated groups statistically significant difference was observed where $p^{\circ}<0.01$ for XD and $p^{*}<0.01$ for PBS groups

The effect of administration of XD on infiltration of leukocytes was studied by estimating levels of MPO activity in all groups. It was observed that a decreased infiltration of neutrophiles occurs in groups where treated with Xue Duan. Figure 4 shows the difference in MPO activity in both treated and control groups where considerable down-regulation of neutrophils in groups treated with XD can be observed with statistically significant difference among both groups ($p < 0.01$).

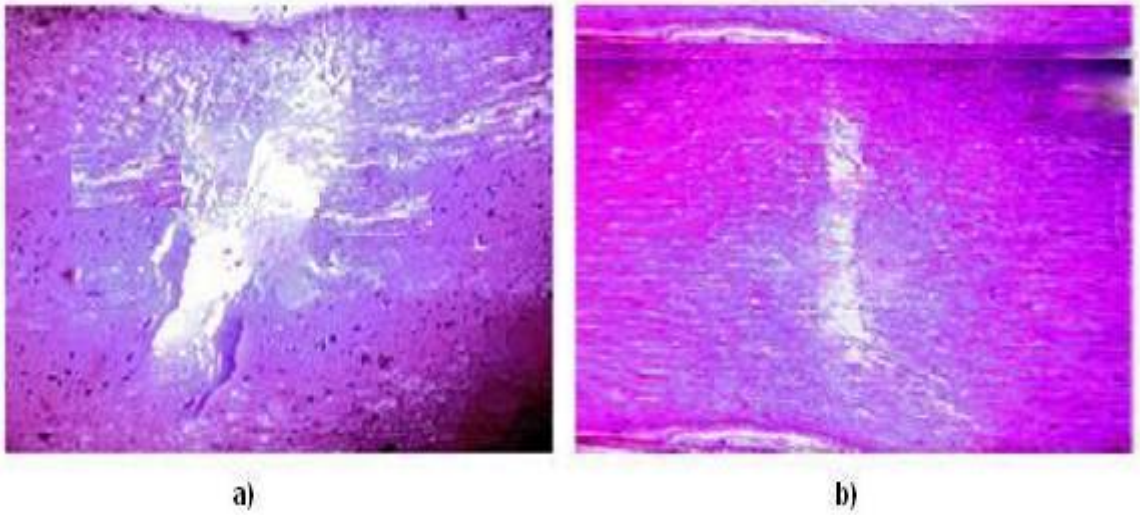

Figure 1: HE stained tissues sections in experimental and control groups after four weeks of treatment. **(a):** Cavity area shown by bright field microscopy in PBS control group (fourth sub - group). **(b):** Significant reduction in the cavity area of spinal sections after 28 days of administration of XD

DISCUSSION

Dipsacus asperoides (Xue Duan) is a part of a traditional Chinese medicine used for the treatment of bone fracture, pain relief as well as trauma. It also has anti-inflammatory as well as antibacterial properties [2,3]. Therefore, we decided to explore the effect of this herb on spinal cord injury in rats by studying its effect on inflammatory pathways.

There are two distinct phases of spinal cord injury, a primary phase which is characterized by mechanical injury and a secondary phase where inflammatory responses play their role. For functional recovery of patients these responses prove to be a major hurdle [20].

A main reason for secondary phase of spinal cord injury to be a major cause of concern is IKK/NF-kB pathway that plays a major role during this phase [21]. If this pathway is successfully targeted then pathogenesis after SCI can be reduced as in this case the major catalytic sub-unit IKKβ can be inhibited selectively and this can lead to reduction in the infiltration of inflammatory cells and apoptosis [9,22]. In this case a major transcription regulator is NF-kB which is also activated during secondary phase of spinal cord injury [23]. IKKβ is the main catalytic sub-unit of IKK and NF-kB is activated after phosphorylation of I-kB which is characterized as inhibitory protein [24]. One of the major proteins that belong to Rel family include NF-kB and other proteins belonging to this family are RelB, cRel, p52 and p50. As this pathway plays a major role in pathophysiology after SCI therefore we studied the expression of I-kBα and NF-kB p65. Treatment with Xue Duan was able to attenuate the expression of these proteins in SCI rats as is evident from western blotting pictures and statistical analysis.

Development of SCI in rats is also accompanied by infiltration of neutrophiles that move towards the injury site and invade it. Reactive mediators are then released which increase the permeability of vessels and damage the endothelial cells. Immune cells also move to the injury site and it causes damage to spinal injury site [25]. Infiltration of neutrophiles was evaluated by studying the MPO activity and it was observed that the treatment with Xue Duan decreases infiltration of neutrophiles to the injury site after SCI in rats.

CONCLUSION

The findings of this study demonstrate that the use of Xue duan can successfully treat SCI in rats. Therapeutic effect of this herb is first report for use in SCI rats to the best of our knowledge and it can be explored further for a better understanding of inhibition of inflammatory factors in SCI.

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