Tropical Journal of Pharmaceutical Research June 2017; 16 (6): 1377-1382
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v16i6.23

Original Research Article

Effects of the root extract of Dipsacus asperoides (Caprifoliaceae) on locomotor function and inflammation following spinal cord injury in rats

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Sent for review: 19 January 2017 *Revised accepted: 17 May 2017*

Abstract

Purpose: *The study was performed to determine the effect of Dipsacus asperoides (roots), commonly known as Xu Duan, on rats with spinal cord injury (SCI).*
Methods: *All the animals were separated into 3 groups: normal control; a group that received Dipsacus asperoides treatment after spinal injury, and a group that received phosphate-buffered saline after SCI. These groups allowed for determination of the effect of Dipsacus asperoides treatment on SCI-injured rats. Evaluation of locomotor function restoration based on Basso–Beattie–Bresnahan score was carried out while expressions of I-kBα and NF-kB p65 were estimated. Evaluation of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 levels was also carried by Western blotting and densitometry.*
Results: *The results showed that 10-mg/kg/day dose of Dipsacus asperoides restored locomotor function in rats after a period of 4 weeks. The treatment also decreased expressions of I-kBα, NF-kB p65 and Bax, but significantly increased expression of Bcl-2 ($p < 0.01$) in treated rats, compared to untreated rats.*
Conclusion: *The results indicate that treatment with Dipsacus asperoides extract effectively mitigates spinal cord injury by attenuating inflammation and apoptosis in SCI rats.*

Keywords: *Spinal cord injury, IKK-NFkB pathway, Bax, Bcl-2, Xu Duan, Apoptosis*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Dipsacus asperoides, commonly known as Xu Duan, is a perennial plant that is widely distributed in mountainous areas of South West China. It is used as a traditional medicine in China, and it has been shown to exhibit antimicrobial and anti-inflammatory characteristics [1]. Various disorders have been treated by this plant, including liver and kidney disorders. This herb also exhibits several properties with beneficial effects on the human body, including prevention of miscarriage, regeneration of flesh, and strengthening of bones. Other characteristics include relief from trauma and swelling, and better treatment of bone fractures [2,3].

Spinal cord injury (SCI) is divided into two phases, the primary and secondary phases. The

mechanical injury that results immediately after trauma is characterized as the primary phase. Events following mechanical injury at the molecular and cellular level are characterized as the secondary phase. The severity of symptoms and the time required for an effective recovery depends on the damage that has occurred. If nerve roots are damaged, this may cause paralysis in severe cases [4,5]. Inflammatory responses play a major role when the secondary phase develops after primary mechanical injury [6]. Different genes are expressed that mediate inflammatory responses by transcribing a family of transcription factors include p52, p50, RelB, RelA/p65 and cRel [7,8]. Inhibition of the NF-kB pathway works as a treatment for SCI due to inhibition of various gene products that are mediated through the NF-kB pathway [9,10].

After SCI, oxidative stress and inflammation occur, inducing apoptosis in neurons and oligodendrocytes [11,12]. This is accompanied by the release of reactive oxygen species and proinflammatory cytokines from microglia, which can result in the development of neurodegenerative disorders [13-16]. Therefore, reducing inflammation and oxidative stress and inactivating microglia can have therapeutic potential if achieved successfully. This study was designed to explore the therapeutic potential of *Dipsacus asperoides* extract in rats suffering from SCI. Restoration of locomotor function, infiltration of neutrophils at the site of injury, and other factors related to inflammation and apoptosis were studied.

EXPERIMENTAL

Dipsacus asperoides (Product Code BT 1301) was obtained from Hangzhou Botanical Technology, Co., Ltd., China. All the male Sprague–Dawley rats (230–250 g) were separated into 3 groups (n = 20): Normal control; SCI/XD group receives *Dipsacus asperoides* intraperitoneally after SCI; and SCI/PBS group receives PBS after SCI to allow observation of the effects of treatment. These three major groups were then sub-divided into four sub-groups each (n = 5). The experiment was stopped in one sub-group of each major group every week for 4 weeks. *Dipsacus asperoides* was administered using intraperitoneal injection of 10 mg/kg in the XD/SCI group, while PBS was administered at the same time interval and same dose in the SCI/PBS group. Treatment started on day 1 post-injury and continued for 4 weeks (28 days) in total. The powdered root extract of *Dipsacus asperoides*is was dissolved in sterile phosphate buffered saline (PBS) to prepare a stock solution which was sterilized by passing it througha 0.2 µm Millipore filter [17].

The experimental study was approved by the Ethics Committee of Nanjing Medical University, China (approval number: YX41322) and the animals used in this study were maintained in accordance with international guidelines on the care and use of laboratory animals [18]. In all rats, injury was induced using the NYU impactor rod according to previous reports [19]. Animals were anesthetized using 40 mg/kg dose of sodium pentobarbital solution injected intraperitoneally. Laminectomy was done by exposing muscles of paravertebra through incision at the back.

The Basso–Beattie–Bresnahan (BBB) score was determined to evaluate locomotor function in the rats every 7 days from day 1 until day 28. A score of zero indicated the absence of locomotor activity, and a score of 21 indicated normal locomotor function [20].

To evaluate the levels of NF-kB p65 and I-kBα through Western blotting, the techniques described in previous studies were used, with slight modifications [21].

Total protein was extracted after selecting a 10-mm size injury epicenter from the injured spinal cord segments using the Total Protein Extraction Kit (Applygen Technologies, Inc., Beijing, China). The BCA Protein Extraction Kit (Applygen Technologies, Inc., Beijing, China) was used to extract total proteins following the manufacturer's instructions. After obtaining 50 µg of protein from each sample, a dilution was made using sample buffer, electrophoresis was carried out on 4–20 % polyacrylamide gel, and then samples were transferred onto a polyvinylidine difluoride membrane. The primary antibodies used for incubating the membrane included a specific monoclonal antibody, mouse anti-rat NF-kB p65 (1:1000), and a monoclonal rabbit anti-rat phosphorylated I-kBα antibody (1 : 500). To visualize the bands, horseradish peroxidase conjugated anti-rabbit, anti-mouse IgG antibodies (1:2000) and ECL Western blotting kit (Applygen Technologies, Inc., Beijing, China) were used.

Polyclonal rabbit anti-actin antibody (1 : 500; Santa Cruz Biotechnology, Inc.) was used to visualize the bands of actin, which was used as loading control. To measure the intensity of bands, the optical density (OD) of each was recorded using the Gel-Pro Analyzer software. X-ray films were exposed for a period of 10 s to 1 min for this purpose. To study the possible effect of *Dipsacus asperoides* on apoptosis, an

evaluation of the level of Bax and Bcl-2 was carried out according to previously published reports [21,22].

Statistical analysis

All experiments were performed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and Student t-test. $P < 0.01$ was considered statistically significant.

RESULTS

Treatment with *Dipsacus asperoides* resulted in improvement of locomotor function in rats. Partial-weight ambulation was observed after 2 weeks, and a statistically significant improvement in the BBB score occurred at 4 weeks after treatment compared to the control, as shown in Figure 1.

Attenuated expression of phosphorylated NF-kB and I-kBα was observed in the SCI/XD group compared to the SCI/PBS group. This effect on expression was statistically significant ($p < 0.01$) in both cases. Clear bands were seen in the Western blot, and the corresponding densitometric analysis, shown in Figure 2, confirmed this result.

A base level of proapoptotic protein Bax expression occurred in the normal control, and an increased level of Bcl-2 was observed in the normal control. Treatment with *Dipsacus asperoides* resulted in a decreased level of Bax and an increased level of Bcl-2 in the SCI/XD group. These expression differences were statistically significant ($p < 0.01$), as shown in Figure 3.

The results of Western blotting and densitometry analysis indicated that treatment with *Dipsacus asperoides* significantly attenuated the expressions of phosphorylated I-kBα and NF-kB compared to the SCI/PBS control (Figure 2).

The effect of *Dipsacus asperoides* treatment on expressions of Bcl-2 and Bax protein was observed. The expression of Bax increased in the SCI/PBS control relative to the normal control. However, expression of Bax protein was significantly ($p<0.01$) reduces in the SCI/XD group than SCI/PBS group. Moreover, expression of Bcl-2 was significantly enhanced in the SCI/XD group than SCI/PBS (Figure 3).

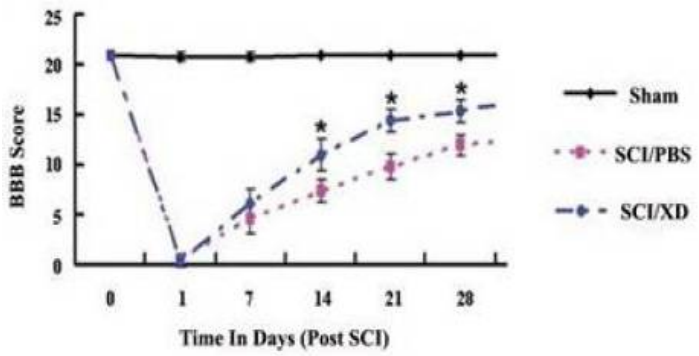

Figure 1: Comparison of BBB scores among the three groups. BBB score remained unchanged in the normal control, whereas it increased in the SCI/XD group compared to the SCI/PBS group; *$p < 0.01$

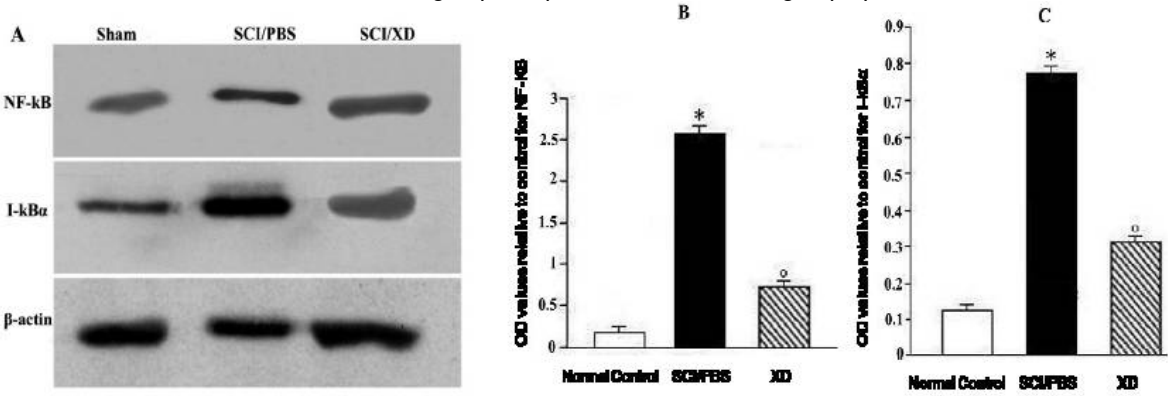

Figure 2: Effect of *Dipsacus asperoides* on expressions of NF-kB and I-kBα. **(A)** Representative Western blot showing the increased intensity of Western blot bands representing protein expression of NF-kB and I-kBα in the SCI/PBS group compared to the SCI/XD group. **(B)** Densitometric analysis showing NF-kB expression differences among all groups; *$p < 0.01$ and $^{o}p < 0.01$ **(C)** Densitometric analysis showing I-kBα expression differences among all groups; *$p < 0.01$ and $^{o}p < 0.01$

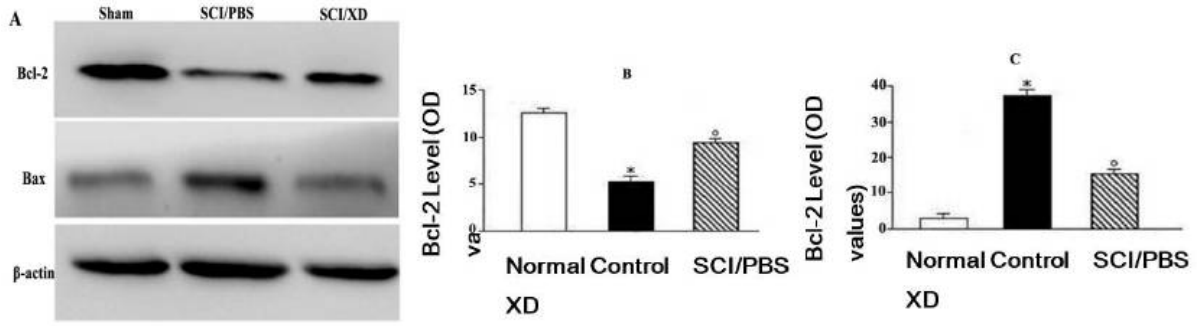

Figure 3: Effect of *Dipsacus asperoides* treatment on expressions of antiapoptotic and proapoptotic proteins. **(A)** Representative Western blot showing increased expression of Bcl-2 and decreased expression of Bax in the SCI/XD group compared to the SCI/PBS group. **(B)** Densitometric analysis showing Bcl-2 expression differences among all groups. Increased expression in the SCI/XD group compared to the SCI/PBS group indicates that *Dipsacus asperoides* treatment had a positive effect on SCI; $*p < 0.01$ and $^{o}p < 0.01$. **(C)** Densitometric analysis showing Bax expression differences among all groups. The SCI/XD group shows decreased expression compared to the SCI/PBS group; $*p < 0.01$ and $^{o}p < 0.01$

DISCUSSION

Dipsacus asperoides was traditionally used as medicine in China for the management of different disorders, including trauma and bone fractures, and has been reported to exhibit antibacterial and anti-inflammatory activities [2,3]. For this reason, the effects of *Dipsacus asperoides* treatment on the inhibition of inflammatory pathways in SCI were explored.

Significant improvement in locomotor function was observed in SCI/XD rats compared to SCI/PBS after 4 weeks of treatment. This improvement can be attributed to the positive effect that *Dipsacus asperoides* exerts on neuronal cells that are damaged in SCI. In different pathological conditions including electrical stimulation, cerebral ischemia, brain injury, neuroprotective genes can be induced *in vivo* [23].

Apoptosis is another important factor that results in demyelination and death of neurons after SCI in rats [24,25]. The level of apoptosis can be evaluated by observing the expression of Bax and Bcl-2. In this study, the results showed decreased expression of Bax in SCI/XD rats, while the expression of Bcl-2 increased compared to SCI/PBS rats.

Research has shown that secondary injury develops after SCI, regulated by the expression of the IKK–NF–kB pathway [26]. Therefore, successful targeting of this pathway can improve the outcomes of SCI, as reported in previous studies [9]. A catalytic subunit of IKK, namely IKKβ, can be targeted for successful control of inflammation and apoptosis, the two most important factors contributing to the pathogenesis of SCI [27].

This evaluation of locomotor function restoration and expression of NF-kB, I-kBα, Bax, and Bcl-2 showed a positive effect of *Dipsacus asperoides* treatment on the expression of these factors, with the most prominent and statistically significant result obtained at 4 weeks after treatment.

CONCLUSION

The findings of the study show that a 10-mg/kg/day dose of the root extract of *Dipsacus asperoides* (traditional Chinese medicine) can successfully treat SCI in rats. The neuroprotective effect exerted by this medicinal agent is a possible explanation for this effect. However, further studies are required to fully unravel the mechanism of neuroprotective activity.

DECLARATIONS

Acknowledgement

We acknowledge the help and facilities provided for this research by the staff of the Department of Orthopedics, Suzhou Science & Technology Town Hospital.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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