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Original Article

Triptolide improves locomotor function after spinal cord injury (SCI) inrats

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Abstract: In this study a possible therapeutic effect of Triptolide (TPL) was explored on SCI rats. A dose of 5 mg/kg/day was administered intraperitoneally and control groups were given PBS in the same amount at the same time interval in rats. A total of three main groups (n=20 each) were used and each group was divided in four sub-groups (n=5 each). Rats in all sub-groups were treated for a period of one week. Possible effect of TPL on IKK/NF-kB pathway was studied and levels of Bax and Bcl-2 evaluated. Activated caspase-3 level was tested in all the groups while TUNEL staining for the presence of apoptotic cells was also performed. Statistical analysis was performed in all experiments where one way ANOVA was used and a $P < 0.05$ was considered significant. Presence of a less number of apoptotic cells and lower levels of antiapoptotic protein Bcl-2 showed positive effect of the use of TPL on SCI rats. Elevated levels and statistically significant difference of the amount of proapoptotic protein Bax and activated caspase-3 was observed in untreated group as compared to treated and control groups. These results led to a conclusion that a dose of 5 mg/kg/day TPL injected intraperitoneally has a neuroprotective effect on SCI rats.

Keywords: SCI, neuroprotective effect, IKK/NF-kB pathway, caspase-3, Bax and Bcl-2

Introduction

In traumatic spinal cord injury death of a number of neurons occurs that can continue for many hours after the injury and it also causes problem for regeneration of neurons [1]. Primarily spinal cord injury can be categorized in two stages where a primary mechanical injury results due to trauma and it is then followed by secondary injury in which different molecular, cellular and biochemical events occur where inflammatory responses play a vital role in enhancing the damage caused at this stage. Different studies over the years have suggested that inflammatory responses after SCI cause damage because of demylination, glial scar formation and loss of neurons [2]. As macrophages cross the blood brain barrier (BBB) and microglia are activated after SCI they play a role as phagocytes in spinal cord which results in the release of nitrogen species, reactive oxygen species (ROS) and proinflammatory cytokines [3, 4].

Different events including ischemia, hemorrhage and edema mark those morphological changes that occur in spinal cord tissues after mechanical spinal injury and as a result sensory as well as motor dysfunction occurs in patients [5, 6]. Regulation and production of proinflammatory cytokines in central nervous system (CNS) is caused by a major transcription factor NF-kB [7, 8]. Apoptosis also occurs in SCI that adds to functional disability and it is regulated by Bcl-2 families and caspase-3 [9-11]. Inflammatory mediators are activated by microglia after primary injury [12]. As a result trophic factors are secreted that help in neuroprotection of CNS. All the factors including released proinflammatory cytokines and neurotoxic molecules result in spreading of secondary injury. Therefore, it has been reported that tissue

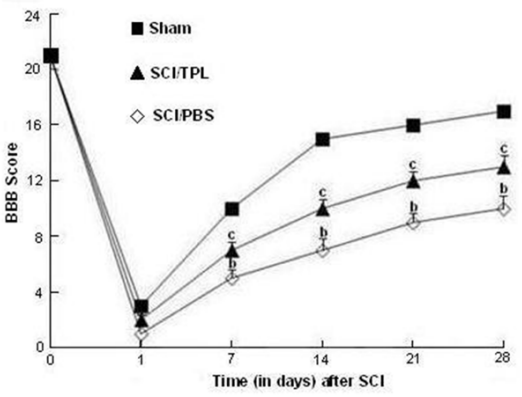

Figure 1. BBB Score as studied in all the groups. Improvement in locmotor function can be seen in treated as compared to untreated and control group of rats, where $p^c < 0.05$.

damage can be controlled and recovery after SCI can be facilitated if activation of microglia can be suppressed [13-15].

Since 1960s Triptergium is used in allopathic Chinese medicine and it has also been used in traditional Chinese medicine for treating inflammation and arthiritis [16]. It contains compounds like diterpenoid tripoxide (Triptolide) that is believed to have therapeutic potential against chronic nephritis, cancer, hepatitis and other diseases as well [17, 18]. Many studies have shown that due to anti inflammatory, anti-apoptotic and antiproliferative effects Triptolide (TPL) can be used for treating inflammatory joint diseases as well as treating cancer [19-23].

In this study we decided to explore the effect of TPL on functional outcome after spinal cord injury in rats. The effect of triptolide on inflammatory pathways and apoptosis was studied so that neuroprotective effect of this compound can be established.

Materials methods

In this study three main groups of Sprague Dawley rats (n=20 each, 230-250 g) were used. These main groups included a sham control group (n=20), an untreated group (SCI) where SCI was induced in rats and only phosphate buffer saline was administered without giving any treatment, it was labeled as SCI/PBS group. A third group of rats (n=20) was used denoted as SCI/TPL where Triptolide was administered intraperitoneally at a dose of 5 mg/kg/day. Each main group of rats was then sub-divided into four subgroups (n=5 each) where a weekly treatment was carried out. Each sub-group received treatment for the whole week and then treatment was stopped subsequently, after that week in that particular group while it continued in other groups until four weeks where all the four sub-groups were used. In corresponding control groups PBS was administered at the same time interval in same dose as TPL compound was administered.

All the experimental procedures were approved by the Ethics committee of Guangdong Academy of Medical Sciences, Guangzhou, Guangdong, China.

Spinal Cord Injury was induced in rats by using NYU impactor rod according to previously described reports [24]. At T9-T11 laminectomy was performed after anesthetizing animals by using 4% sodium pentobarbital intraperitoneally at 40 mg/kg body weight.

In all the rats locomotor function after SCI was studied using Basso-Beattie-Bresnahan (BBB) score. Here a score of 0 shows absence of locomotor function while a scale of 21 shows normal locomotor function [25].

For studying inhibition of inflammatory pathway, the level of NF-kB p65 and phosphorylated I-kBα was studied according to previously described reports with slight modifications [26]. Injury epicenter was selected for the extraction of total proteins using total Protein Extraction Kit (Applygen Technologies, Inc., Beijing, China). Then concentration of proteins was determined by using BCA protein Assay Kit (Applygen Technologies Inc., Beijing, China) using manufacturer's protocol. Samples were boiled for 5 minutes after diluting them in sample buffer and 50 μg protein from each sample was loaded on 4-20% polyacrylamide gel before electrophoretic separation and loading on polyvinylidene diflouride membrane. Specific antibodies were used for incubation of membranes after blocking that included, mouse anti-rat NF-kB p65 monoclonal antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, USA) while I-kBα Ser 32 monoclonal antibody was used (1:500, Cell Signalling Technology, Danvers, MA, USA).

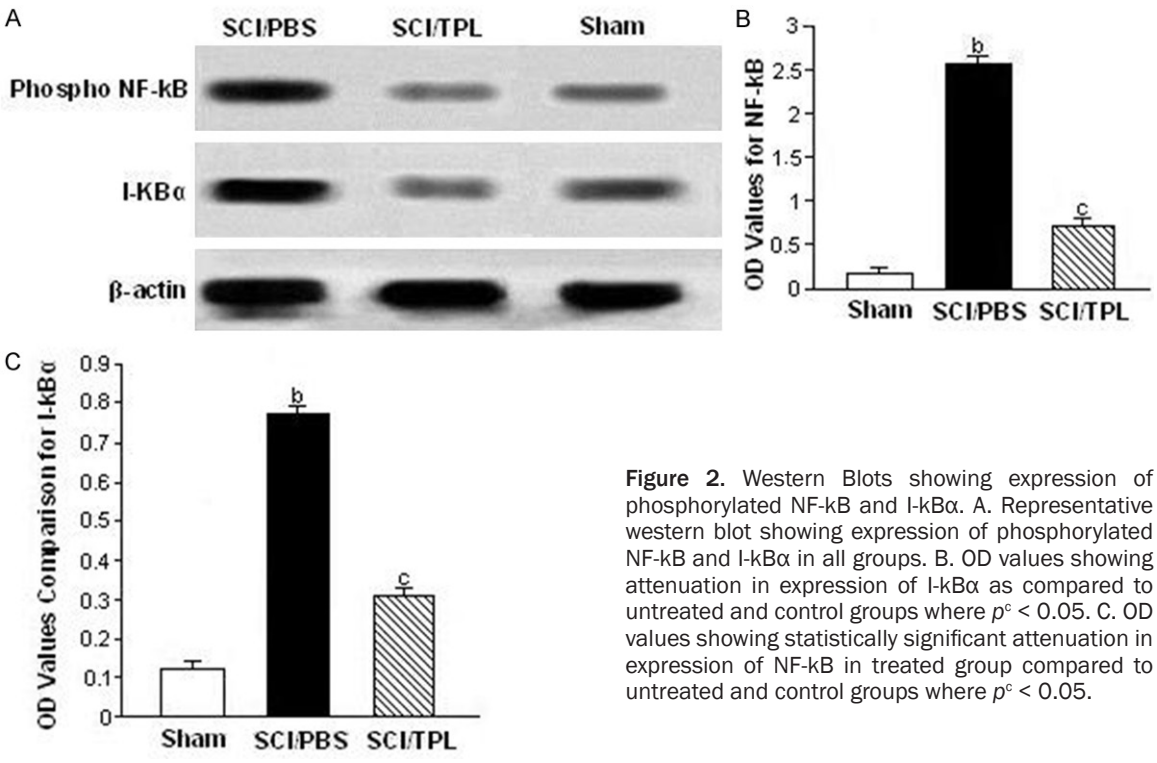

Figure 2. Western Blots showing expression of phosphorylated NF-kB and I-kBα. A. Representative western blot showing expression of phosphorylated NF-kB and I-kBα in all groups. B. OD values showing attenuation in expression of I-kBα as compared to untreated and control groups where $p^c < 0.05$. C. OD values showing statistically significant attenuation in expression of NF-kB in treated group compared to untreated and control groups where $p^c < 0.05$.

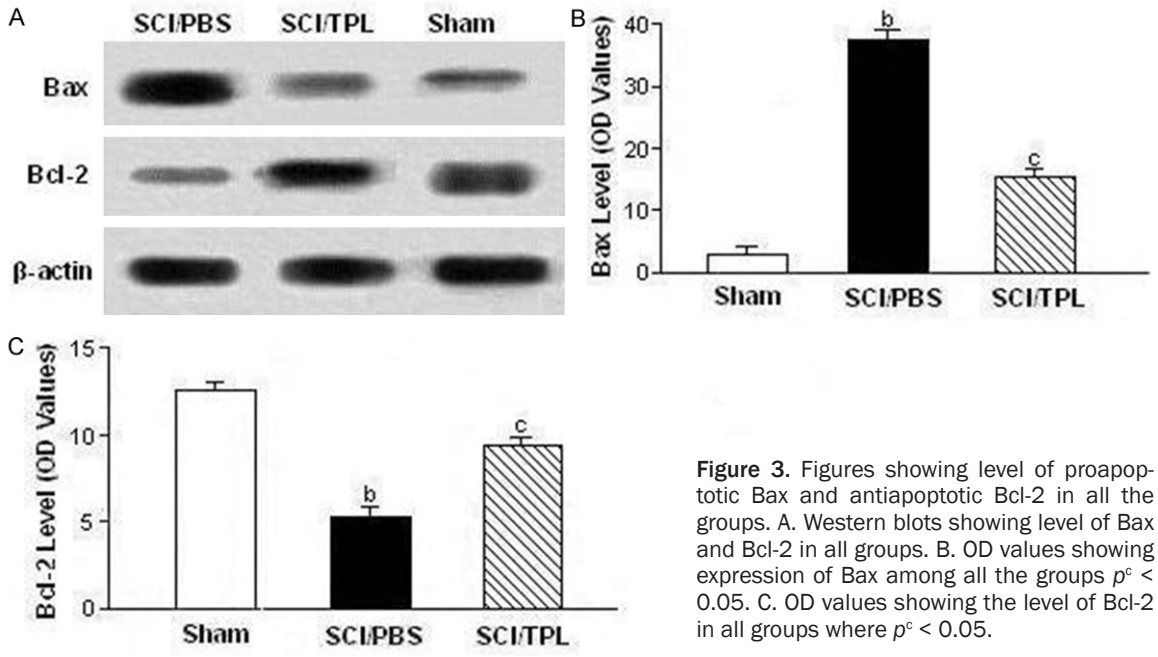

Figure 3. Figures showing level of proapoptotic Bax and antiapoptotic Bcl-2 in all the groups. A. Western blots showing level of Bax and Bcl-2 in all groups. B. OD values showing expression of Bax among all the groups $p^c < 0.05$. C. OD values showing the level of Bcl-2 in all groups where $p^c < 0.05$.

Levels of Bax, Bcl-2 and activated caspase-3 were also determined like previous studies with a slight modification [26, 27]. ECL western blotting kit (Applygen Technologies Inc, Beijing, China) and horseradish peroxidase conjugated anti mouse or anti rabbit IgG antibodies (1:2000; Jackson, West Grove, PA, USA) was used for visualizing protein bands in samples. Actin was used as a loading control and for its detection polyclonal rabbit anti-actin antibody (1:500, Santa Cruz Co., Santa Cruz, CA, USA) was used. All the membranes were exposed for

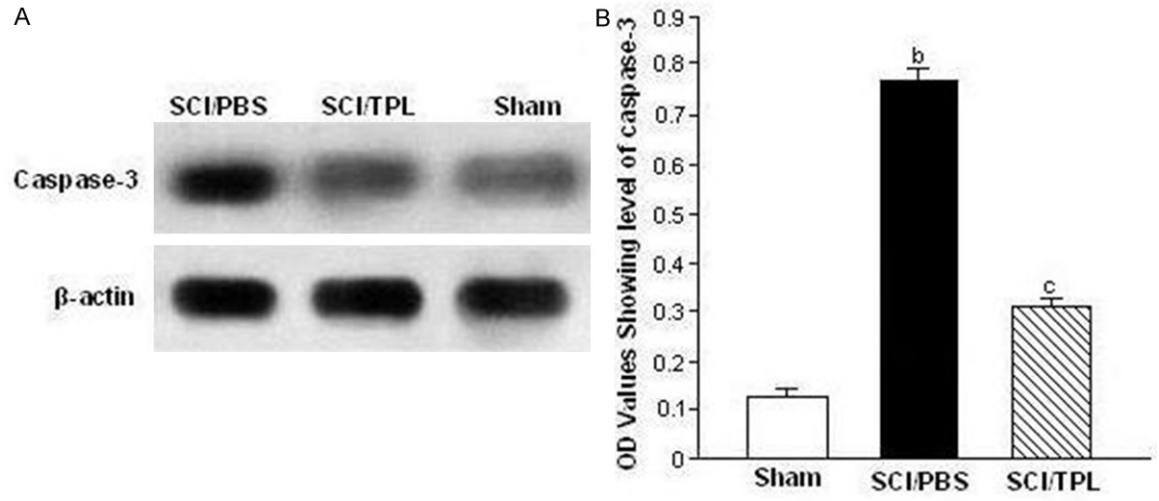

Figure 4. Figure showing the level of activated caspase-3 in rats with spinal cord injury. A. Shows representative western blots where increased intensity can be seen in untreated groups as compared to untreated rats and sham control. B. OD values showing the level of activated caspase-3 in all the groups where increased intensity can be seen in untreated groups that is statistically significant, where, $p^c < 0.05$.

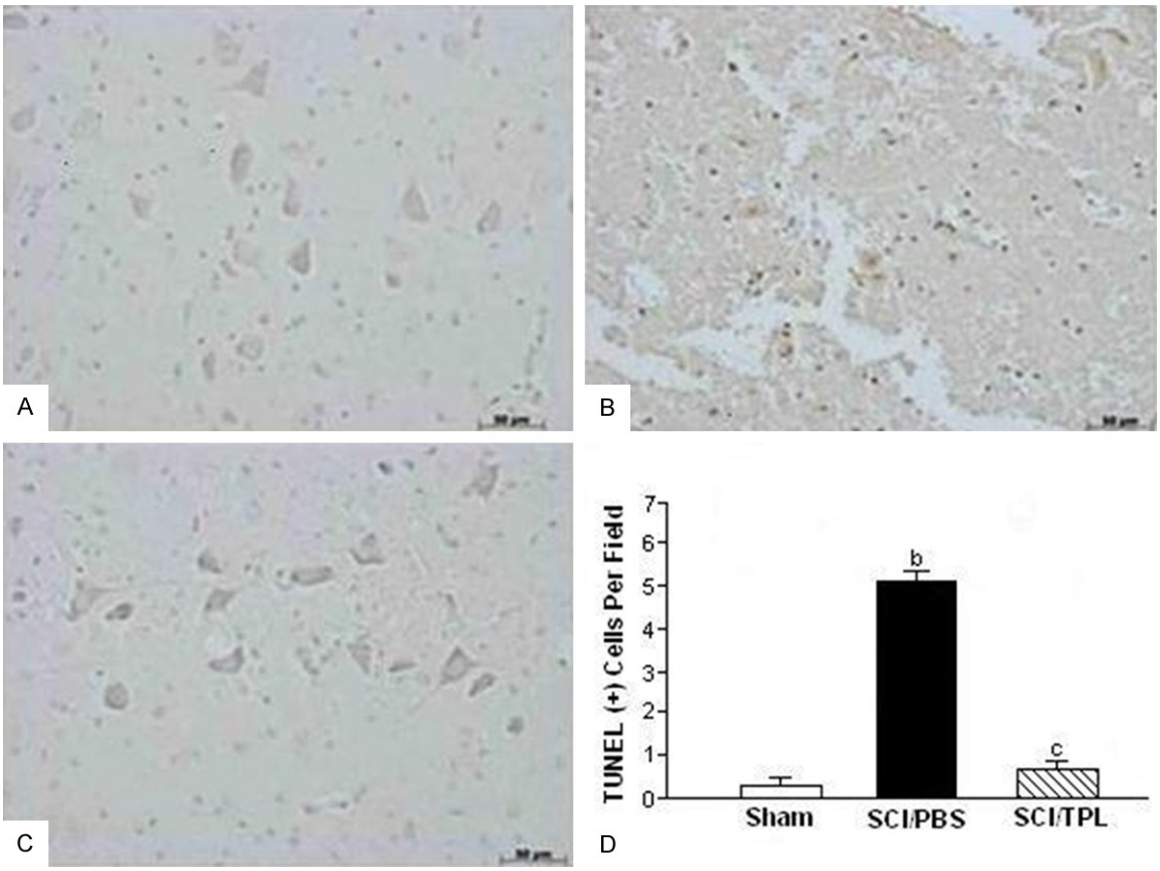

Figure 5. Figure showing results of TUNEL-like staining on all groups. A. Negligible amount of apoptotic cells can be seen in sham control animals. B. A greater number of apoptotic cells can be seen in SCI mice which are left untreated. C. A comparatively less number of cells can be seen in treated spinal cord injured rats. D. Statistically significant number of cells were present in untreated rats as compared to treated SCI rats, where $p^c < 0.05$.

a period of 10 s to 1 min to X-ray film and Gel Pro Analyzer 4.0 software was used to determine the optical density (OD) of protein bands. TUNEL Assay Kit (Apotag, HRP kit DBA, Milan, Italy) was used for performing Terminal Deoxynucleotidyltransferase-Mediated UTP End Labelling (TUNEL) Assay following manufacturer's instructions [10].

All the results were analyzed through statistical analysis by one way ANOVA followed by Bonferroni post hoc analysis and values were expressed as Mean ± SEM where p value less than 0.05 was considered significant.

Results

Study of locomotor function indicated that treatment with TPL resulted in partial weight ambulation of injured rats after two weeks and BBB score improved after 4 weeks of treatment of injured rats with 5 mg/kg/day TPL intraperitoneal injection as shown in **Figure 1**.

Results regarding attenuation of phosphorylated NF-kB and phosphorylated I-kBα showed that the expression of both these factors increased significantly in SCI/PBS group where no treatment was given and attenuated level of expression was observed in rats treated with TPL while in sham control this expression remained at basal level where $p^c < 0.05$ as shown in **Figure 2**.

The expression of proapoptotic protein Bax showed that its level increased in group of rats that were left untreated while a decreased expression was observed in group of rats treated with a dose of TPL, in sham control the level of this protein remained at basal level ($p^c < 0.05$). The expression of antiapoptotic protein Bcl-2 showed that treated group gave an increased expression of Bcl-2, a decreased level was observed in untreated SCI rats and sham control showed a normal basal level expression, results shown in **Figure 3**.

Activated caspase-3 was evaluated for studying the expression of proteins related with apoptosis and a decreased level was observed in treated groups as compared to untreated and control groups, shown in **Figure 4**.

TUNEL-like staining showed that a negligible number of apoptotic cells were observed in control group, while an increased number was seen in untreated rats and significantly decreased number of apoptotic cells was seen in group of rats treated with a dose of TPL, **Figure 5**.

Discussion

In spinal cord injury (SCI) inflammatory responses play a major role in complicating the patient outcome after primary mechanical injury to spinal cord of patients. It is this phase that is accompanied by damage of neurons, infiltration of neutrophiles, scar tissue formation and apoptosis [28]. This results in compromising the outcome of patients as severe effect is exerted on sensory and motor dysfunction of neurons, therefore, we evaluated the locomotor function activity on rats after SCI and treatment with TPL.

Neutrophils are the first cells that arrive at the injury site and release different reaction mediator components which not only damage the endothelial cells but also increase the vascular permeability. As a result more and more cells are attracted towards the injury site [29]. It has been observed in different studies that inhibition of IKK/NF-kB pathway can result in lowering the extent of damage caused by secondary injury after SCI and different Chinese medicinal compounds have been reported to exhibit such properties [30].

An important event causing demylination of neurons is apoptosis that is an important factor of SCI [31, 32]. In this study the presence of large number of apoptotic cells in TUNEL assay shows the extent to which apoptosis can occur in spinal cord injured rats. A decreased amount of cells, decreased expression of activated caspase-3 and Bax levels in treated groups shows possible therapeutic potential of triptolide in SCI.

Therefore, from this study it was concluded that a possible therapeutic effect is exerted by Triptolide on traumatic spinal cord injury where secondary damage is controlled based on the use of 5 mg/kg/day dose of the compound. All the results show statistically significant decrease in apoptotic proteins and improvement in locomotor function that can conclude possible neuroprotective effect of TPL on SCI.

Disclosure of conflict of interest

None.

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