Recombinant Human Erythropoietin Protects Against Experimental Spinal Cord Trauma Injury by Regulating Expression of the Proteins MKP-1 and p-ERK

H Huang¹, S Fan², X Ji¹, Y Zhang¹, F Bao¹ and G Zhang¹

¹Department of Orthopaedic Surgery, Yiwu Central Hospital, Yiwu, China; ²Department of Orthopaedic Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China

The present study explored the tissue-protective effect of erythropoietin in rats after experimental spinal cord injury (SCI) produced by dropping a weight onto surgically exposed spinal cord. Sixty rats were randomized to sham operation (spinal cord exposure; control), SCI plus intraperitoneal saline injection, or SCI plus intraperitoneal erythropoietin injection. Locomotor function was evaluated with Basso, Beattie and Bresnahan scores 1 day (24 h) and 7 days later, and rats were then killed for analysis of lesion site tissue. Compared with saline-treated SCI rats, erythropoietin-treated SCI rats showed significantly less locomotor dysfunction and faster locomotor recovery. Immuno-histochemistry showed that erythropoietin-treated SCI rats had a significantly lower phospho-extracellular signal-regulated kinase (p-ERK) protein expression and a significantly higher mitogen-activated protein kinase phosphatase-1 (MKP-1) protein expression than saline-treated SCI rats. Haematoxylin–eosin staining showed progressive disruption of dorsal white matter and neuron loss after SCI; lesions were less severe and there was more neuron regeneration in the erythropoietin group than in the saline group. It is concluded that erythropoietin reduces pathological changes and SCI severity via down-regulation of p-ERK and up-regulation of MKP-1.

KEY WORDS: ERYTHROPOIETIN; SPINAL CORD INJURY; IMMUNOHISTOCHEMISTRY; MITOGEN-ACTIVATED PROTEIN KINASE PHOSPHATASE-1; PHOSPHORYLATED EXTRACELLULAR SIGNAL-REGULATED KINASE; RATS

Introduction

Spinal cord injury (SCI) is a severe health problem worldwide that usually causes lifelong disability. Normally, SCI is divided into two phases: an acute phase, which is mostly due to mechanical damage, and a secondary phase. Primary injury results from direct mechanical impact to the spine, and the secondary phase injures the spinal cord through a process involving a complex
cascade of molecular events including disturbances of ionic homeostasis, local oedema, focal haemorrhage, excitotoxicity, the presence of free radicals and free fatty acids, and activation of an inflammatory response. Despite improvements in anaesthetic and surgical techniques, currently there are no efficacious therapies for SCI; an ideal treatment would be one that is administered systemically and has no significant side effects. Erythropoietin has been shown to play roles in the nervous system in both normal and pathological conditions. In vivo studies using experimental models of global and focal ischaemia, blunt trauma, immune-mediated inflammation, excitotoxin-elicited seizures, subarachnoid haemorrhage and toxin-induced parkinsonism suggest that erythropoietin might be beneficial in these situations, however the mechanism of erythropoietin’s neuroprotective effect is not fully known.

Mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) is a dual-specificity phosphatase involved in the regulation of cell survival, differentiation and apoptosis by inactivating MAPK by dephosphorylation. MAPks can be subdivided into at least three classes based on sequence homology: the extracellular signal-regulated kinases (ERK1/2); the Jun N-terminal kinases (JNKs); and the p38 kinases. Neurological insults, such as ischaemia and kainate-induced seizure, induce excessive release of excitatory amino acids and the subsequent neuronal cell death results in the phosphorylation of ERK (phosphorylated ERK, p-ERK) in vivo. In addition, MKP-1 may inhibit the MAPK/ERK pathway and protect the brain from ischaemic injury.

In the present study, SCI was induced in rats by a weight-drop method to investigate changes in the expression of MKP-1 and p-ERK in the injured spinal cord. The mechanism of the neuroprotective effect of erythropoietin against SCI was also studied in this animal model.

Materials and methods

MATERIALS

Male Sprague–Dawley rats were purchased from the Experimental Animal Centre of Zhejiang University (Hangzhou, China). Recombinant human erythropoietin was purchased from the China Shenyang Sunshine Pharmaceutical Company (Shenyang, China). Monoclonal MKP-1 and p-ERK antibodies were purchased from EMD Chemicals (Darmstadt, Germany).

INDUCTION OF SCI AND BEHAVIOURAL TESTING

Sixty male Sprague–Dawley rats with a body weight of approximately 210 g were randomly divided into three groups, each containing 20 rats: a sham operation control group; a SCI group, subjected to a contusion injury without subsequent erythropoietin treatment; and a group that received erythropoietin treatment after contusion injury. The rats were housed in an environmentally controlled animal laboratory and maintained on a 12 h light/12 h dark cycle with five rats per cage at ambient temperature (22 – 25 °C) and relative humidity 55 ± 10%.

Under sodium pentobarbital (40 mg/kg, intraperitoneally) anaesthesia, the vertebral column of the rats was exposed and laminectomy was performed at level T10. Contusion injury was then carried out in rats in the SCI and erythropoietin treatment groups, using a weight-drop device. This involved dropping a 10 g weight from a height of 50 mm onto the exposed spinal cord and the impounder was left for 20 s
before being withdrawn; this produced a moderate contusion. Immediately after the incision had been closed, rats in the SCI group received normal saline (by intraperitoneal injection) and rats in the erythropoietin treatment group received a single dose of erythropoietin (1000 IU/kg body weight, intraperitoneally). Animals in the control group were subjected to the same surgical procedure but no impact injury was imposed, and the spinal cord was left exposed for 5 min.

Hind-limb locomotor function was assessed using the open-field scoring method; individual rats were placed in a circular enclosure, allowed to acclimatize to it, and were then observed for 5 min by two examiners. Each rat was scored using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale\(^\text{12}\) 1 day (24 h) and 7 days after the operation. Rats were killed 1 day (24 h) and 7 days after the operation for histological and immunohistochemical examinations, respectively.

All procedures were conducted with the approval of the Zhejiang University Animal Care Committee. Ethical permission for this study was granted by the Ethical Committee of the School of Medicine, Zhejiang University (Hangzhou, China).

**IMMUNOHISTOCHEMISTRY AND HAEMATOXYLIN–EOSIN STAINING**

Five rats from each group were given a lethal dose of sodium pentobarbital 1 day (24 h) and 7 days after surgery. The thoracic cavity was opened and perfused intracardially with normal saline. After saline perfusion, the animals were perfused with 300 – 400 ml of fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, the lesion epicentre (about 4 mm) of the spinal cord of each rat was removed, fixed in the aforementioned fixative for 4 h, then placed in 30% phosphate-buffered sucrose until the tissue sank. Transverse sections of the spinal cord (8 µm) were cut on a freezing microtome. The sections were rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and mounted on 0.02% poly-l-lysine-coated slides.

The avidin–biotin–peroxidase complex method was used with 3,3′-diaminobenzidine hydrochloride (DAB) as the chromogen. Briefly, tissue sections were washed in PBS, incubated in 1% bovine serum albumin (BSA) for 30 min, then incubated overnight at 4 °C in the primary antibody (monoclonal MKP-1 or p-ERK antibody) plus 1% BSA in PBS. The dilution of the primary antibody was 1:100. Control sections were incubated in PBS alone. The next day, the sections were incubated in a biotinylated goat anti-mouse secondary antibody (diluted to 1:200 in PBS) and, subsequently, in an avidin–horseradish peroxidase solution. Immunolabelling was visualized with 0.05% DAB (Sigma, St Louis, MO, USA) plus 0.3% \(\text{H}_2\text{O}_2\) in PBS. The sections were then dehydrated through ethanol and xylene before being coverslipped with Permount™ (Sigma). Haematoxylin and eosin staining was used to determine morphological changes.

**IMAGE ANALYSIS**

For immunohistochemistry, injured spinal cord tissues were selected on each slide and examined at a magnification of ×400 with UTHSCSA ImageTool version 3.0 (University of Texas Medical School at San Antonio, San Antonio, TX, USA). The number of MKP-1- and p-ERK-positive cells per microscope field and their optical densities (at 650 nm) were measured.

**STATISTICAL ANALYSIS**

All data are presented as mean ± SD.
Statistical analysis was performed using SPSS® version 10.0 (SPSS®, St Louis, MO, USA) for Windows®. Between-group differences were evaluated using the two tailed t-test and P-values < 0.05 were considered statistically significant.

Results
LOCOMOTOR EFFECTS OF SCI AND ERYTHROPOIETIN
When observed during open-field walking, animals with SCI showed marked bilateral hind-limb paralysis with no movement at all or only slight movement of a joint from the first hours after injury. Animals in the sham operation control group walked normally after recovery from anaesthesia. When the animals were examined after the procedure, locomotor activity according to the BBB scale for the rats in the saline-treated SCI group recovered during the observation period to reach a plateau just below a score of 10 at 7 days after SCI. The erythropoietin-treated SCI group exhibited comparable yet significantly improved neurological function when compared with the saline group (P < 0.01), with a BBB score above 10 at 7 days after SCI (Fig. 1).

MORPHOLOGICAL CHANGES FOLLOWING SCI AND ERYTHROPOIETIN
Haematoxylin–eosin staining of injured thoracic spinal cord revealed the formation of a large cavity involving the dorsal and part of the lateral funiculi, and also the dorsal and central grey matter. The cavity extended for at least 2 mm in rostral and caudal directions. The central lesion area consisted of spared fibres, variable cysts and gliosis. Large cystic cavities that were primarily open with minimal gliosis and some macrophages were observed in regions where grey matter had previously existed. At 24 h after injury blood cells were found to have invaded the spinal cord, there was some neuron death and an increased number of gliocytes in the both saline and erythropoietin-treated SCI groups compared with the sham operation group (P < 0.05). The lesion was characterized by multifocal dorsal haemorrhage that, 24 h after injury, had evolved to a more advanced stage of tissue oedema in the both saline and erythropoietin-treated SCI groups compared with the control group (P < 0.05) (Fig. 2). By 7 days after injury the architecture of the

![Figure 1: Basso, Beattie and Bresnahan (BBB) locomotor rating scores (mean ± SD) in rats 1 and 7 days after being subjected to a sham operation (control), a weight-drop contusion [spinal cord injury (SCI)] followed by saline injection, or SCI followed by an injection of erythropoietin (EPO; 1000 IU/kg body weight). *P < 0.01 versus control, **P < 0.01 versus saline-treated SCI group.](image-url)
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dorsal white and central grey matter had become more disrupted, and gliocytes filled the injury area in the saline-treated SCI group; in erythropoietin-treated SCI rats there was a smaller cavity volume compared with the saline-treated SCI groups. There was some neuronal regeneration in the erythropoietin-treated rats but little regeneration was seen in the saline-treated SCI group 7 days after injury and, moreover, lesions in the saline-treated SCI group were more severe than in the erythropoietin-treated SCI group (Fig. 3).

**IMUNOHISTOCHEMISTRY FOR p-ERK AND MKP-1**

Light photomicrographs of immunohistochemical staining for p-ERK of spinal cords from the three groups of rats are shown in Fig. 4. More p-ERK protein was present at 7 days than at 24 h (1 day) after injury (Figs 4 and 5) in the saline-treated SCI group ($P < 0.01$), indicating that injury triggered the MAPK signal pathway. To confirm this observation, sections were analysed for MKP-1 and this was shown to be decreased in neuronal nuclei 24 h after injury in the saline-treated SCI group versus control ($P < 0.01$) (Fig. 6). By 7 days after contusion, inactivation of MKP-1 was complete in the saline-treated SCI group (Fig. 7); the lowest MKP-1 positive cell count (lowest MKP-1 protein expression) corresponded with the highest p-ERK positive cell count (highest p-ERK protein expression) in the saline-treated SCI group ($P < 0.01$ versus control; Table 1).
These high values for p-ERK protein expression were seen mainly in neuronal nuclei and cytoplasm; the neurons could be distinguished from glial cells by their relatively large nucleus, prominent cytoplasm and the pericellular space created by shrinkage. Moreover, compared with the saline-treated SCI group, the erythropoietin-treated SCI group showed a significantly lower p-ERK positive cell count and a significantly higher MKP-1 positive cell count 7 days after injury (P < 0.01; Table 1).
Understanding of the pathophysiology of SCI is of great value in planning clinical treatment strategies. Previous research has suggested that the prevention of secondary injury would reduce the spread of spinal cord tissue destruction and consequent functional impairment.\textsuperscript{2,13 – 15} The present study demonstrated a neurological benefit associated with erythropoietin injection after SCI; a single dose of 1000 IU/kg body weight erythropoietin in rats was associated with a markedly superior clinical course of recovery of motor function, as measured by BBB scores 1 and 7 days after SCI, despite the dramatic effects of SCI still observed at a histological level at 7 days. Considerable haemorrhage resulted from severe parenchymal disruption caused by the absorption of kinetic energy during the SCI induction procedure. In the later stages of the response to injury caused by contusion in mice, Yatsiv \textit{et al.}\textsuperscript{16} found that cellular debris and grossly disrupted axons elicited pronounced inflammatory and degenerative processes that initiated a secondary phase of

\begin{table}[h]
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\caption{Optical density at 650 nm (OD\textsubscript{650 nm}) and number of cells positive for p-ERK and MKP-1 in rats 1 and 7 days after being subjected to a sham operation (control), a weight-drop contusion (spinal cord injury [SCI]) followed by saline injection, or SCI followed by an injection of erythropoietin (EPO; 1000 IU/kg body weight)}
\begin{tabular}{|c|c|c|c|c|}
\hline
Group & p-ERK (OD\textsubscript{650 nm}) & p-ERK positive cell count (cells/mm\textsuperscript{2}) & MKP-1 (OD\textsubscript{650 nm}) & MKP-1 positive cell count (cells/mm\textsuperscript{2}) \\
\hline
1 day & & & & \\
Sham operation (control) & 96.4 ± 5.6 & 2.2 ± 0.5 & 168 ± 13.7 & 8.7 ± 2.3 \\
Saline-treated SCI & 176.0 ± 14.5\textsuperscript{a} & 6.5 ± 1.5\textsuperscript{a} & 105.8 ± 7.4\textsuperscript{a} & 3.9 ± 1.5\textsuperscript{a} \\
EPO-treated SCI & 146.5 ± 12.4\textsuperscript{b} & 4.9 ± 1.1\textsuperscript{b} & 135.3 ± 10.9\textsuperscript{b} & 5.4 ± 1.9\textsuperscript{b} \\
7 days & & & & \\
Sham operation (control) & 96.4 ± 5.6 & 2.3 ± 0.5 & 172 ± 14.3 & 8.5 ± 2.4 \\
Saline-treated SCI & 170.3 ± 13.9\textsuperscript{c} & 7.8 ± 1.6\textsuperscript{c} & 118.3 ± 7.9\textsuperscript{c} & 3.8 ± 1.6\textsuperscript{c} \\
EPO-treated SCI & 150.3 ± 13.0\textsuperscript{d} & 5.2 ± 1.2\textsuperscript{d} & 155.1 ± 13.2\textsuperscript{d} & 7.2 ± 2.0\textsuperscript{d} \\
\hline
\end{tabular}
\textsuperscript{a,p < 0.01 versus control; b,p < 0.01 versus saline-treated SCI; c,p < 0.01 versus control; d,p < 0.01 versus saline-treated SCI.}
\end{table}
injury, and that this effect could be reduced by erythropoietin treatment.

Another major finding of the present study is the observation that MKP-1 expression was higher and p-ERK expression was lower in the SCI group treated with erythropoietin than in the SCI group not treated with erythropoietin. MKP-1 is involved in the regulation of cell survival, differentiation and apoptosis by inactivating MAPKs by dephosphorylation. Activation of protein kinase C has been shown to trigger MKP-1 degradation through the ubiquitin–proteasome pathway, thereby contributing to persistent activation of ERK1/2 under conditions of glutamate-induced oxidative toxicity and glutamate-induced cell death in HT22 hippocampal cells and primary mouse cortical neurons. Other studies have demonstrated that the MAPK/ERK pathway has a pro-apoptotic effect on neuron death. The current study indicated that impact-induced cell death can be rescued by overexpression of MKP-1 after injection of a single dose of erythropoietin. These results suggest that erythropoietin has a protective effect in the impact SCI model and that this effect is mediated by the MAPK signalling pathway.

In conclusion, when the spinal cord was examined histologically and BBB scores were recorded 7 days after contusion, pathological changes and severe injury were markedly less severe in animals treated with erythropoietin than in untreated animals. The effect of erythropoietin was associated with down-regulation of p-ERK protein expression and up-regulation of MKP-1 protein expression, which suggests that erythropoietin and, possibly, its analogues may provide an effective immediate treatment following acute injury. Further study is needed to determine the extent of the time window for effective treatment and to identify the exact MAPK signalling pathway involved.

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Conflicts of interest
The authors have no conflicts of interest to declare in relation to this article.

References


Author’s address for correspondence

**Dr Shunwu Fan**

Department of Orthopaedic Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China.

E-mail: ajoy8888@163.com