Anti-apoptotic signal transduction mechanism of electroacupuncture in acute spinal cord injury

Quan Renfu,1 Chen Rongliang,1 Du Mengxuan,1 Zhang Liang,2 Xu Jinwei,1 Yang Zongbao,3 Yang Disheng4

ABSTRACT
Spinal cord injury (SCI) can be caused by a variety of pathogenic factors. In China, acupuncture is widely used to treat SCI. We previously found that acupuncture can reduce apoptosis and promote repair after SCI. However, the antiapoptotic mechanisms by which acupuncture exerts its effects on SCI remain unclear. Our aim was to investigate the role of the PI3K/Akt and extracellular signal-regulated kinases (ERK)1/2 signalling pathways in acupuncture treatment of acute SCI. Eighty pure-bred New Zealand rabbits were randomly divided into the following five groups (n=16 per group): control; model; elongated needle electroacupuncture (EA); EA+LY294002; and EA+PD98059. We established a spinal cord contusion model of SCI in all experimental groups except controls, in which only a laminectomy was performed. After SCI, three of the groups received EA once daily for 3 days. One hour before SCI, the two drug groups received LY294002 (Akt inhibitor; 10 μg, 20 μL) or PD98059 (ERK inhibitor; 3 μg, 20 μL) via intrathecal injection. At 48 h after SCI, animals were killed and spinal cord tissue samples were collected for transferase dUTP nick end labelling (TUNEL) assays, immunohistochemistry and western blot assays. EA significantly increased p-Akt and p-ERK1/2 expression, reduced cytochrome c and caspase-3 expression and inhibited neuronal apoptosis in the injured spinal cord segment. The opposite effects were seen after using Akt and ERK inhibitors. Acupuncture promotes the repair of SCI, possibly by activation of the PI3K/Akt and ERK1/2 signalling pathways and by inhibition of the mitochondrial apoptotic pathway.

INTRODUCTION
Spinal cord injury (SCI) is a severe neurological trauma that often leads to permanent paralysis. Acute SCI may be primary and secondary. Specifically, SCI induces primary mechanical damage and causes secondary damage to the spinal cord, mediated by a series of complex cellular and molecular processes. Apoptosis, or programmed cell death, is a controlled, non-inflammatory cell death that is initiated under certain physiological or pathological conditions. Its occurrence is a precisely regulated process involving gene regulation, signal transduction and, ultimately, execution of apoptosis, which eliminates unnecessary or abnormal cells. However, abnormal or excessive apoptosis often results in the deterioration of neurodegenerative disorders such as Alzheimer’s or Parkinson’s disease, or traumatic injury.1,2 Crowe et al3 first discovered that apoptosis occurred in the spinal cord (especially the white matter) within 6 h to 3 weeks after SCI, damaging motor and sensory functions. Apoptosis is regulated by the pro- and antiapoptotic members of the Bcl-2 protein family.4 With the activation of caspase-3 after SCI, this key downstream regulator can trigger and induce the caspase cascade through apoptosis, activating zymogen and mediating the apoptotic cascade. Traumatic brain injury, SCI and stroke are all accompanied by apoptosis.5 Therefore, research into the mechanisms of apoptosis is important for the prevention and treatment of neurodegenerative diseases.

The phosphatidylinositol 3-kinase (PI3-K)/serine-threonine protein kinase (Akt) and mitogen-activated protein kinase (MAPK) signalling pathways are involved in cell survival and apoptosis. Akt (also known as protein kinase B), is a key downstream effector in the PI3K signalling pathway and is located in the centre of the PI3K/Akt signalling pathway. The activated (phosphorylated)
form of Akt (p-Akt) can inhibit mitochondrial release of apoptotic factors and cytochrome c (cyt c), and also inactivates caspase-9 by phosphorylation of its Ser196 residue, thus blocking the apoptotic pathway.6,7 LY294002 is a PI3K/Akt specific inhibitor capable of inducing apoptosis. Crowder and Freeman8 found that nerve cells treated with nerve growth factor activated endogenous Akt protein kinase, whereas the specific PI3K kinase inhibitor LY294002 blocked nerve growth factor-induced survival of sympathetic neurons in vitro. These findings demonstrate that the PI3K/Akt signalling pathway plays an important role in promoting the survival of neurons. Extracellular signal-regulated kinases (ERK) include ERK1 and ERK2. After entering the nucleus, activated ERK1/2 acts on the transcriptional activator ELK-1, the genes c-Fos and c-Jun and inflammatory factors such as ATF (activating transcription factor), NF-κB (nuclear factor-kappaB) and AP-1 (activator protein 1), playing an important role in inhibiting apoptosis.9 PD98059 is a specific inhibitor of the MAPK signal transduction pathway, which can effectively block the upregulation of p35 to inhibit cytoplasm- or mitochondria-mediated cell degeneration and death.

Electroacupuncture (EA) has the dual effect of mechanical and electrostimulation. In recent years, experimental studies have shown that EA exerts a neuroprotective effect by enhancing neuronal activity and inhibiting apoptosis.10 Solá et al found that EA at the Jaji points at thoracic levels 7 and 8 significantly reduced SCI caused by myocardial ischaemia-reperfusion, possibly by the upregulation of Bcl-2 protein expression and downregulation of Bax protein expression, thus increasing the Bcl-2/Bax ratio and thereby inhibiting myocardial apoptosis.11 In addition, Bleicken et al12 and Li et al13 found that acupuncture significantly reduced neuronal apoptosis and promoted nerve repair and regeneration, playing a neuroprotective role in cerebral ischaemia. Collectively, the previous studies suggest that EA has a regulatory role in neuronal apoptosis. However, the detailed mechanism for the effect of EA on acute SCI remains unclear. In this study, we investigated the effect of EA on SCI repair and neuronal apoptosis through the PI3K/Akt and ERK1/2 signalling pathways.

MATERIALS AND METHODS

Animals and grouping

Eighty (40 male, 40 female) healthy, clean grade, pure-bred New Zealand rabbits, weighing 2.5–3.5 kg each, were provided by the Experimental Animal Center of Zhejiang Chinese Medical University (Zhangzhou, China). The animals were reared in separate cages (lot: SCXK (Zhejiang) 2008-0036) at controlled room temperature (20±2°C) and relative humidity (50%) under a 12:12 h light:dark cycle (light period, 07:00–19:00). All animals had free access to basic feed and tap water. After 3 weeks of quarantine, the animals were randomly grouped for experimentation. Animal experiments were approved by the experimental animal ethics committee of Zhejiang Chinese Medical University. Experimental protocols complied with the requirements of animal ethics issued by the Ministry of Science and Technology (People’s Republic of China).

The animals were randomly divided into five groups (n=16) using a random number table: (1) Control group—animals underwent surgical removal of the T13–L1 spinous processes and the complete vertebral plate to expose the spinal dura mater (0.8 mm in width) without touching the spinal cord and without EA; (2) Model group—a spinal cord concussion model was established successfully (see below), but no EA was carried out; (3) EA group—after successful model establishment, animals received EA once daily for 3 days; (4) EA+LY294002 group—animals received an intrathecal injection of LY294002 (10 μg, 20 μL; Sigma-Aldrich, St Louis, Missouri, USA) in the space between the fifth and sixth lumbar vertebrae (L5–6 interspace) 1 h before modelling and all other procedures were identical to those described for the EA group; (5) EA+PD98059 group—animals received an intrathecal injection of PD98059 (3 μg, 20 μL; Sigma-Aldrich) in the L5–6 interspace 1 h before modelling and all other procedures were identical to those described for the EA group.

Modelling method

A model of bladder dysfunction after SCI was established using a modification of Allen’s method.14 Rabbits were fasted for 8 h before experimentation and the surgery was performed on four of the groups. The animals were anaesthetised by injection of 20% urethane (1 g/kg) into the ear vein and fixed in the prone position. A median rostrocaudal incision (approximate length 2.5 cm) was made aseptically. The skin and subcutaneous tissues were dissected layer by layer, exposing the upper and lower space by the length of a vertebral body. The T13–L1 spinous processes and entire dorsal aspect of the vertebrae were removed to expose the spinal dura mater (width 0.8 mm).

A 10 g Kirschner wire was allowed to fall freely along a scaled catheter from a height of 80 mm, landing on a thin semicircle of plastic (diameter 4 mm, width 2 mm) that was placed over the exposed spinal cord. The semicircle was quickly removed after the injury, which caused an incomplete SCI in the spinal cord dorsal horn. The tissues and skin were then sutured layer by layer using 4/0 silk. The whole experiment was performed at 37±0.5°C. To prevent infection, animals were given daily intraperitoneal injections of penicillin (5 mg/kg) postoperatively and allowed to recover in separate cages with adequate food and water. Massage of the bladder was...
performed three times a day to help with urination until the establishment of reflex bladder emptying. Criteria for a successful model were as follows. After the injury, bleeding and oedema of the spinal cord occurred and the rabbits showed a tail-wagging reflex with retraction-like fluttering of the lower extremities and body. Upon the return of consciousness after anaesthesia, the animal had flaccid paralysis in both hind legs.

**EA method**

The four experimental groups underwent bilateral EA stimulation at points BL54 (Zhibian), ST28 (ShuiDao), CV6 (Qihai) and CV3 (Zhongji) as illustrated in figure 1. The four points were selected according to international standard acupuncture points as defined by the World Health Organization. BL54 is located in the lower hip at the joint of the outer and middle third of the connection between the greater trochanter and the sacrococcygeal vertebral junction. The other three acupuncture points are located in the abdomen. CV3 and CV6 are both in the midline at a point that is two- and seven-tenths, respectively, of the distance from the pubic symphysis to the umbilicus, while ST28 lies about 2 cm lateral to a midline point at four-tenths of this distance (CV4).

The skin at the selected acupuncture points was prepared and disinfected. Stainless steel acupuncture needles (Hwato, Suzhou, China), 0.25 mm in diameter, were inserted into the selected points to a depth of about 6 mm, then turned for 1 min and retained in position for 15 min. Concurrently, the ipsilateral BL54 and ST28 points were paired up and connected to a JL2B electrical pulse stimulator (Suzhou Medical Appliance Factory, Suzhou, China). The dilatational wave frequency was 20 Hz/40 Hz and the current intensity was set to produce local muscle spasm. The electrostimulation lasted for 15 min and the EA treatment alternated between the left and right sides.

**Intrathecal injection**

Subarachnoid puncture was performed horizontally in the L5–6 interspace. A sterilised PE-10 catheter was inserted 7–8 cm from L5 to 6 to reach the thoracic spinal cord, and withdrawal of cerebrospinal fluid was considered to indicate successful puncture. An equal volume of cerebrospinal fluid was slowly withdrawn before the test drug was injected. The animal was kept in a dorsal elevated position for 1 h after drug injection.

**Index detection**

**H&E staining**

Five tissue sections were randomly selected from each rabbit for H&E staining. The specimens were dewaxed and then washed with double-distilled water for 1 min. After 10 min of haematoxylin staining, the specimens were washed again with double-distilled water to remove excess dye, before being soaked in 1% hydrochloric acid alcohol for 1 min of colour separation (avoiding overseparation), followed by pro-blue treatment in an alkaline solution. After being washed with running water, the specimens were stained with eosin for 2 min, dehydrated in graded ethanol (50%, 70%, 80%, 90% and 100%), clarified in xylene and mounted with neutral gum. Structural and morphological changes in spinal cord tissues were examined under an optical microscope (Olympus, Kr).

**In situ terminal deoxynucleotidyl transferase dUTP nick end labelling assay of neuronal apoptosis**

Five tissue sections were randomly selected from each rabbit for the transferase dUTP nick end labelling assay of neuronal apoptosis.
extracted protein was quantified using a bicinchoninic acid (BCA) assay. The separation gel (15 μL) was prepared with 6% gel. After complete solidification, 4% stacking gel in a 4 mL system was added. For electrophoresis, sample wells were loaded with denatured protein samples (containing 100 μg of total protein). About 5 μL of protein marker was loaded at the other side of the gel for determination of protein molecular size. During electrophoresis, the system was maintained at a constant voltage of 80 V when samples were running in the stacking gel, and the voltage was adjusted to 120 V when samples entered the separation gel. The electrophoresis was terminated when the bromophenol blue indicator entered the agarose gel.

After Coomassie blue staining and bleaching, the gel was transferred to a polyvinylidene difluoride membrane and then stained with Coomassie brilliant blue reagent to examine the results of the membrane transfer. The membrane was removed and stained with Ponceau red, then rinsed with double-distilled water to remove the dye. The rinsed membrane was placed in a small plastic bag and a blocking solution (5% bovine serum albumin, diluted with Tris-buffered saline (TBS) containing 0.5% Tween 20) was added. The bag was oscillated at room temperature for 2 h. The membrane was then removed and transferred to a clean bag and incubated with the following primary antibodies, all at 1:500 dilution: goat anti-rabbit Akt and p-Akt polyclonal antibodies (Cell Signaling Technology, USA), goat anti-rabbit ERK1/2 and p-ERK1/2 polyclonal antibodies (Cell Signaling Technology), goat anti-rabbit polyclonal cytochrome c (cyt c; Santa Cruz, USA) and caspase-3 antibody (Santa Cruz). The bag was sealed and incubated at 4°C overnight.

The specimens were rinsed three times with TBS containing 0.5% Tween 20 at room temperature and then incubated with secondary antibody (1:500, horseradish peroxidase-conjugated mouse anti-goat IgG(H+L)). After bubbles were removed, the membrane was sealed and rocked at room temperature for another 2 h. The membrane was rinsed again three times (10 min each time) at room temperature with TBS containing 0.5% Tween 20. ECL solution was added to develop colour, followed by darkroom exposure, developing and fixing. A BI-2000 medical image analysis system (Chengdu TME Technology Co, Ltd, China) was used for image scanning and for the integrated optical density analysis of the protein bands. β-Actin was used as an internal control and the relative expression level (the ratio of integrated optical density of the target protein band to the β-actin band) was calculated to assess the target protein expression levels in different treatment groups.

Western blot assay of p-Akt, p-ERK1/2, cyt c and caspase-3 expression

All experimental specimens were stored at −70°C pending analysis. Total protein was extracted from the spinal cord segments using radioimmunoprecipitation assay buffer, according to the manufacturer’s instructions (Thermo Scientific, Rockford, Illinois, USA). The extracted protein was quantified using a bicinchoninic acid (BCA) assay. The separation gel (15 μL) was prepared with 6% gel. After complete solidification, 4% stacking gel in a 4 mL system was added. For electrophoresis, sample wells were loaded with denatured protein samples (containing 100 μg of total protein). About 5 μL of protein marker was loaded at the other side of the gel for determination of protein molecular size. During electrophoresis, the system was maintained at a constant voltage of 80 V when samples were running in the stacking gel, and the voltage was adjusted to 120 V when samples entered the separation gel. The electrophoresis was terminated when the bromophenol blue indicator entered the agarose gel.

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Statistical analysis

All data were processed using SPSS V.17.0 (SPSS Inc, USA). The results are presented as mean±SD. Data were compared using the analysis of variance and
least significant difference tests. A $p$ value $<0.05$ was considered statistically significant.

**RESULTS**

**Pathological characteristics of spinal cord tissues detected by H&E staining**

H&E staining showed that the control group of rabbits had normal spinal cord tissues at the sampling point, with no manifestations of haemorrhagic necrosis or oedema (figure 2). However, spinal cord tissue samples of the experimental groups all displayed different degrees of structural disorder (black arrows), accompanied by varying degrees of bleeding and inflammatory cell infiltration (blue arrows), with oedema and necrosis of nerve cells as well as vacuolation in the grey matter (red arrows). By comparison, spinal cord tissue samples of the EA group were characterised by a relatively intact structure with a greater number of surviving nerve cells (figure 2).

**Neural apoptosis detected by TUNEL assay**

TUNEL-positive cells (apoptotic neurons) were expressed at varying levels in the spinal cord tissues of different groups (figure 3). They were rarely found in normal spinal cord tissues of the control group, suggesting that normal spinal cord underwent a certain level of neuronal cell proliferation. By comparison, the number of TUNEL-positive cells increased in the injured spinal cord segment in the model group ($p<0.001$), indicating that apoptosis occurred frequently after SCI.

In the EA group, the number of TUNEL-positive cells in the injured spinal cord segment was lower than in the model group ($p<0.001$), indicating that EA inhibited the SCI-induced apoptotic activity in neurons. In the LY294002 and PD98059 groups, the number of TUNEL-positive cells in spinal cord tissues was greater than that of the EA group ($p=0.045$), indicating that the therapeutic effect of EA on the injured segment of spinal cord is Akt- and ERK1/2-dependent (figure 3).

**p-Akt and p-ERK1/2 distribution detected by immunohistochemistry**

In the control group, p-Akt and p-ERK1/2 inside and outside the nerve cells were both stained brown, showing that both molecules are expressed to a certain degree in normal spinal cord tissues. At 48 h after SCI, the numbers of p-Akt- and p-ERK1/2-positive cells were significantly lower in the model group than in the controls, but were restored towards normal in the EA group ($p=0.001$). This increased expression of p-Akt and p-ERK1/2 was inhibited by subarachnoid injection of LY294002 and PD98059 ($p=0.003$) and decreasing trends in the numbers of p-Akt- and p-ERK1/2-positive cells indicated that this effect of EA was blocked by LY294002 and PD98059 (figure 4).

**p-Akt and p-ERK1/2 expression detected by western blot assay**

Akt and ERK1/2 are active in their phosphorylated forms (p-Akt and p-ERK1/2, respectively). At 48 h after SCI, p-Akt and p-ERK1/2 protein expression levels were lower in the injured spinal cord segment of the model group than those in the normal control group ($p=0.001$). After EA, p-Akt and p-ERK1/2 protein expression levels were higher in the injured cord segment.

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**Figure 2**  Pathological characteristics of injured segments of the spinal cord in different groups of rabbits at 48 h after spinal contusion surgery (H&E staining, x100 magnification): (A) control group; (B) model group; (C) electroacupuncture (EA) group; (D) EA+LY294002 group; (E) EA+PD98059 group.
spinal cord segment than in the model group (p=0.002). With subarachnoid injection of LY294002 and PD98059, there was a lower level of p-Akt and p-ERK1/2 protein expression in the injured spinal cord segment than in those of the elongated needle group (p=0.001). These results indicate that LY294002 and PD98059 inhibited phosphorylation of Akt and ERK1/2, respectively, thereby suppressing the protective effect of p-Akt and p-ERK1/2 on acute SCI in elongated needle therapy (figure 5A).

Furthermore, the results suggest that elongated needle therapy significantly reduces cyt c expression levels in the injured spinal cord segment compared with the model group (p=0.001). After pre-injection of the Akt-specific inhibitor, LY294002, or ERK1/2-specific inhibitor, PD98059, the inhibitory effect of elongated needle therapy on the release of cyt c was significantly reduced (p=0.001; figure 5B).

Additionally, the results showed that at 48 h after SCI, caspase-3 was notably activated in the injured segment of spinal cord of the model group compared with that of the normal control group, showing a statistically significant difference (p=0.001). Elongated needle therapy significantly reduced caspase-3 expression in the injured spinal cord segment compared with the model group (p=0.001). The inhibitory

Figure 3 Neuronal apoptosis in injured segments of the spinal cord in different groups of rabbits 48 h after spinal contusion surgery (x ± SD, n=16 per group). (A) Black arrows indicate apoptotic neurons in the spinal cord detected by transferase dUTP nick end labelling (TUNEL) assay (×100 magnification): (a) control group; (b) model group; (c) electroacupuncture (EA) group; (d) EA+LY294002 group; and (e) EA+PD98059 group. (B) Percentage of TUNEL-positive cells (apoptotic neurons) in total cells in injured segments of the spinal cord in different groups of rabbits. $p<0.01$, compared with the control group; #p<0.01, compared with the model group; *p<0.05, compared with the EA group.
The results of this study show that after EA treatment, p-Akt and p-ERK1/2 protein expression was enhanced while mitochondrial cyt c and caspase-3 protein expression was reduced in the injured spinal cord segment, which suggests that EA promotes the repair of acute SCI. The beneficial effects of EA were reduced by Akt and ERK signalling pathway-specific inhibitors, suggesting that acupuncture may promote the repair of SCI via activation of the PI3K/Akt and ERK1/2 signalling pathways and inhibition of the mitochondrial apoptotic pathway.

Recently, there has been growing interest in the anti-apoptotic mechanisms of EA in the repair of acute SCI. Previous studies have shown that EA can inhibit neuronal apoptosis,10 reduce secondary injury of the spinal cord and promote the recovery of neurological functions after acute SCI. In our study, the result of morphological examinations and TUNEL assay showed...
that neuronal apoptosis was significantly enhanced after SCI and that this was notably reduced by EA.

Research indicates that PI3K/Akt and ERK1/2 are both involved in the protective mechanism in neurological injury. The PI3K/Akt pathway is thought to be an important cell survival signalling pathway closely related to nerve cell injuries. In 1999, Kitagawa et al first discovered that Akt was activated in injured nerve cells. Ouyang et al established a 15 min focal nerve cell injury model in which western blot assays detected the peak level of Akt-Ser-473 phosphorylation in hippocampal neurons at 24 h after ischaemia, with peak levels of cyt c and caspase-3 expression detected at 36–48 h after ischaemia. The findings of protein phosphorylation and single molecule expression in a specific order imply that Akt may be involved in the antiapoptotic mechanism.

Additionally, the MAPK signalling pathway is involved in the regulation of a variety of cellular responses and pathophysiological processes, mainly through the ERK, p38 and JNK pathways. Extensive studies have shown that apoptosis is regulated through extracellular signal transduction pathways as dominated by ERK. After the occurrence of nerve injury, ERK is rapidly activated in the injured area, which reduces apoptosis and damage to the body caused by nerve injury. ERK1 and ERK2 are abundant in the central nervous system and are involved in various pathophysiological processes in nerve tissues. When the spinal cord is injured, glutamate receptors in neurons are activated and the intracellular Ca2+ level is increased, thereby activating the ERK1/2 signalling pathway and triggering activation of a series of downstream genes (eg, Bcl-2 family). Additionally, the permeability of the mitochondrial membrane is reduced and the release of cyt c in the cytoplasm is decreased, exerting an antiapoptotic effect. In this study, p-Akt and p-ERK1/2 expression was significantly enhanced in the EA group compared with the model group, suggesting that EA may repair SCI by inhibiting apoptosis. This reparative effect was significantly weakened after the PI3K/Akt and ERK pathways were blocked, demonstrating that EA may regulate apoptosis-related gene expression, suppress the mitochondrial apoptotic pathway and promote the repair of acute SCI through the Akt and/or ERK signalling pathway.

CONCLUSIONS

EA promotes the repair of neurological function after acute SCI by inhibiting neuronal apoptosis and this antiapoptotic mechanism may be related to the activation of Akt and/or ERK1/2 signalling pathways, thus maximising the protection and recovery of structure and function of injured spinal cord tissues.

Summary points

- Acupuncture is widely used in the treatment of acute spinal cord injury (SCI) in China but the precise mechanisms of action underlying its neuroprotective effects are unknown.
- The phosphatidylinositol 3-kinase (PI3-K)/serine–threonine protein kinase (Akt) and mitogen-activated protein kinase (MAPK) signalling pathways are involved in cell survival and apoptosis in acute SCI.
- Electroacupuncture significantly increased p-Akt and p-ERK1/2 expression and inhibited neuronal apoptosis in the injured spinal cord segment in a rabbit model of SCI.

Contributors
QR conceived of the study, carried out the signal transduction mechanism studies, detection of apoptosis and drafted the manuscript. CR carried out the immunoassays and H&E staining, detection of apoptosis and drafted the manuscript. DM participated in the design of the study and performed the statistical analysis. ZL carried out the animal modelling and administration, helped to draft the manuscript. XJ carried out the ELISA and western blot tests. YZ carried out the treatment of acupuncture on animals. YD participated in the design and coordination of the study and helped to draft the manuscript.

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Competing interests
None.

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