Original Article

Protective effects of extract of *Ginkgo biloba* (EGb 761) on nerve cells after spinal cord injury in rats

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Study design: An experimental animal model was used to assess spinal cord injury following lateral hemitransection at thoracic spinal cord level.

Objective: To determine whether extract of *Ginkgo biloba* (EGb) could have a neuroprotective effect in spinal cord injury (SCI) in rats.

Setting: Department of Biological Sciences and Biotechnology, Tsinghua University, China.

Methods: A total of 72 adult rats were divided randomly into three groups: the EGb group, normal saline (NS) group, and sham operation group (sham group). After thoracic spinal cord hemitransection was performed at the level of the 9th thoracic vertebra (T9), rats in the EGb group were given 100 mg/kg EGb 761 daily, while rats in the NS group received NS. The rats in the sham group only underwent laminectomy without spinal cord hemitransection. At various time points after surgery, thoracic spinal cords were sampled and sliced for histochemistry, immunohistochemistry of inducible nitric oxide synthase (iNOS), and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of apoptotic cells.

Results: Myelin staining showed that the area of cavities was small and the demyelinated zones were limited at and around the injury site of the spinal cord in the EGb group, while the area of cavities was large and the demyelinated zones were serious in the NS group. Nissl staining showed that the ratio of bilateral ventral horn neurons (transection side/uninjured side) in the EGb group was higher than that in the NS group ($P < 0.05$). The apoptotic index and the percentage of iNOS-positive cells were lower in the EGb group than in the NS group. Furthermore, the percentage of iNOS-positive cells positively correlated with the apoptotic index ($r^2 = 0.729$, $P < 0.01$) after SCI.

Conclusion: This study demonstrated that EGb 761 could inhibit iNOS expression and have neuroprotective effect by preventing nerve cells from apoptosis after SCI in rats.

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Keywords: *Ginkgo biloba*; spinal cord injuries; apoptosis; inducible nitric oxide synthase; nerve cells

Introduction

Traumatic spinal cord injury (SCI) is a consequence of a primary mechanical insult and also a consequence of progressive secondary pathophysiological events including a number of cellular and biochemical cascades. These secondary injury processes are potential targets for therapies.^{1,2} A variety of agents, for example, neurotrophic factors, methylprednisolone, ganglioside, and NMDA antagonist gacyclidine have been investi-

gated for protecting the injured spinal cord from secondary pathological processes,^{3–6} but there is not a satisfactory therapeutic answer to spinal cord injury yet. Animal experiments have shown that extracts of *Ginkgo biloba* (EGb 761) can prevent neuronal damage in brain ischemia through inhibition of nitric oxide synthesis,⁷ and clinical studies have shown some beneficial effects of EGb 761 in the treatment of patients with Alzheimer's disease and other types of dementia.^{8,9} Recently, *in vitro* studies have shown that EGb 761 has a protective effect against neuronal apoptotic death,^{10–12} and EGb 761 might also influence caspase-3 activation.¹³ However, its potential use in patients with SCI is still vague. The

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purpose of the present study was to investigate the protective role of EGb 761 on nerve cells in secondary degeneration after SCI using histochemistry and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL), which is a specific method for visualizing apoptotic cells. In addition, inducible nitric oxide synthase (iNOS) immunohistochemistry was performed for evaluation of the correlation between iNOS expression and the apoptotic index (AI).

Materials and methods

Spinal cord injury model and drug application

A total of 48 adult female Sprague–Dawley (SD) rats (200–230 g) were used to prepare SCI models. After each rat was anesthetized by administering 10% chloral hydrate (0.35 ml/100 g) intraperitoneally (i.p.), a midline skin incision was made in the dorsal side, and the vertebral plates were exposed from T7 to T11. Then the lamina of T9 was carefully removed using a micro rongeur. After laminectomy, a lateral hemitransection was made at the T9 level with a No. 11 surgical blade. These animals were divided randomly into two groups: the EGb group and the normal saline (NS) group. After surgery, rats in the EGb group were given 100 mg/kg EGb 761 (W Schwabe Co., Karlsruhe, Germany) (dissolved in 2 ml NS) by intragastric administration daily till killed, a dose that has commonly been used by others in the investigation of CNS effects of EGb 761.¹⁴ The rats in the NS group were given 2 ml NS daily as control. The animals recovered in a clean cage, and bladders were evacuated twice a day until reflex bladder function was established. Another 24 adult female SD rats (200–230 g), which underwent laminectomy without spinal cord hemitransection, were used as the sham operation group (sham group).

All the animal tests were carried out in accordance with the US National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 1996 and approved by the Beijing Administration Committee of Experimental Animals.

Sample preparation and histochemistry

Six animals were killed by deep anesthesia at 1, 7, 14, and 21 days postoperation (PO) respectively in each group ($n = 6$, each time point). Transcardiac perfusion was performed with a left ventricular cannula while the right atrium was opened widely. First, circulating blood was washed out with 250 ml NS (37°C), and then 200 ml 4% neutral-buffered paraformaldehyde (4°C) was similarly used for fixation. The vertebral canal was dissected and a 20 mm-long section of the thoracic spinal cord was removed (10 mm away from the hemitransection site in both the rostral and caudal directions). The specimens were post fixed in 4% neutral-buffered paraformaldehyde (4°C) for 6 h, and then soaked in sucrose solution until subsidence. Then serial frozen sections with a thickness of 10 μ m were made, and in each group

three specimens were crosscut for cross-section while the other three were cut longitudinally for coronal section. The cross-sections were prepared from four locations: 3 and 7 mm rostral to the center of injury, 3 and 7 mm caudal to the center of injury.

The myelin structure was stained with Solochrome Cyamine (Beijing Chemical Factory, China) in order to manifest myelinoclasts zone. Nissl staining was made with Cresyl fast violet (Beijing Chemical Factory, China) to show Nissl body changes, and in order to count surviving cells and calculate the ratio of bilateral ventral horn neurons (ie injured side/uninjured side).

TUNEL examination

The Apop-Tag kit (Boster Co., Wuhan, China) was used for TUNEL, according to the supplier's recommendation. In brief, the frozen sections were digested with 20 μ g/ml proteinase K in 0.1 M Tris buffer (pH 7.5) for 15 min, and the endogenous peroxidase was blocked with 2% H₂O₂ for 5 min at room temperature. Then the sections were incubated with a reaction buffer containing TdT enzyme and dUTP-digoxigenin for at least 14 h at 4°C. After being incubated in biotinylated antidigoxigenin solution at 37°C for 30 min, the sections were incubated with streptavidin biotin complex solution at 37°C for 60 min. Finally, they were visualized with diaminobenzidine substrate working solution and then counterstained with hematoxylin. After each step, the sections were washed adequately with 0.1 M Tris buffer (pH 7.5). A negative control study was performed by replacing TdT enzyme with distilled water. The nuclei of positive cells were stained dark brown. In each section, eight high-power visual fields ($\times 400$) were selected (four in the gray matter, the other four in the white matter) to calculate the apoptotic index (AI, ie, the number of TUNEL-positive cells divided by the total number of cells).

Immunohistochemistry analysis

The primary anti-iNOS was rabbit multiclonal antibody (Sigma Co., USA), and the ABC kit was purchased from the Boster Company in Wuhan, China. The procedure of iNOS immunohistochemistry was as follows. The frozen sections were incubated in 3% hydrogen peroxide at room temperature for 10 min to inactivate endogenous peroxidase, then after the nonspecific reaction was blocked with normal goat serum at room temperature for 20 min, the sections were incubated with a reaction buffer containing primary antibody at a dilution of 1:100 for at least 14 h at 4°C. Afterwards the sections were incubated with biotinylated anti-rabbit IgG for 20 min at 37°C, then were incubated with streptavidin biotin complex solution at 37°C for 20 min. Each step was followed by adequately washing with phosphate buffered saline (PBS, 0.1 mol/l, pH 7.2–7.4), and PBS was used to replace the primary antibody for negative control. Finally, they were visualized with diaminobenzidine substrate working solution and then counterstained with hematoxylin. Brown granules were found in

the plasma of iNOS-positive cells. In each section, eight-high power visual fields ($\times 400$) were selected (four in gray matter, the other four in white matter) to calculate the percentage of iNOS-positive cells (the number of iNOS-positive cells divided by the total number of cells).

Data analysis

The cross-sections for histochemistry, TUNEL and immunohistochemistry were prepared from four locations as foregoing description. At each location, calculations were made for at least three sections. Averages and standard error of the means (SEM) are reported. The independence exponent *t*-test was used for comparisons of the ratio of bilateral ventral horn neurons, the percentage of iNOS-positive cells, and the apoptosis index (AI) between the EGb group and the NS group. One-way ANOVA was used for comparisons of above indexes among various time points post operation. The pearson correlation test was adopted for analysis of the relationship between the percentage of iNOS-positive cells and the apoptotic index. A value of $P < 0.05$ was considered significant, and $P < 0.01$ was considered very significant. Data were expressed as mean $\pm$ SEM.

Results

Histochemistry (Figures 1 and 2)

Myelin staining showed that the area of cavities was small and the demyelinated zones were limited at and around the injury site of the spinal cord in the EGb group (Figure 1a), while the area of cavities was large and the demyelinated zones were serious in the NS group (Figure 1b). Nissl staining showed that relatively more surviving neurons were found near the injury site as well as in the rostral and caudal zones of the spinal cord in the EGb group (Figure 1c and e), while there was a significant reduction in number of neurons in the NS group (Figure 1d, f–h). Quantitatively, although the number of neurons at the ventral horn decreased over time in both groups, the ratio of bilateral ventral horn neurons (ie transection side/uninjured side) in the EGb group was higher than that in the NS group at 7, 14, and 21 days after SCI ($P < 0.05$, Figure 2). In the sham group, there were no significant differences between the numbers of bilateral ventral horn neurons, and the ratios of bilateral ventral horn neurons did not differ significantly among various time points (1, 7, 14, and 21 days) postoperation (Figure 2).

TUNEL (Figures 3 and 4)

The apoptotic indexes (AI) at four time points post operation (PO) in the three groups are shown in Figure 3. TUNEL-positive cells were hardly detected in the sham group at four locations and all four time points. The pattern of changes in AI tended to be similar in both the EGb and NS groups. There were a few

Figure 1 Images of myelin staining (a, b) and Nissl staining (c–h) at 21 days after SCI for the EGb group (a, c, e) and the NS group (b, d, f–h). (a) The area of cavities was small and the demyelinated zones were limited in coronal sections of the EGb group. The cavity is indicated with the asterisk (the same below). (b) The area of cavities was large and the demyelinated zones were serious in the NS group. (c) Relatively more surviving neurons were found at the ventral horn near the injury site in coronal sections of the EGb group, while (d) shows that there was a significant reduction in the number of neurons in the NS group. (e) The numbers of neurons in the cross-sections 3 mm caudal to the injury center were similar on both sides in the EGb group, while (f) showed that neurons in the transection side decreased significantly compared with the uninjured side in the NS group also in the cross-sections 3 mm caudal to the injury center, and the spinal cord central canal dilatated markedly (indicated by an arrow). (g) Enlarged image of spinal cord ventral horn of the uninjured side in (f) showed the morphology of the most neurons was normal. (h) Enlarged image of spinal cord ventral horn of the transection side in (f) showed that the neurons swelled and the Nissl bodies were stained light (indicated by arrows). Bar = 20 μ m (a–f), 10 μ m (g, h)

Figure 2 Ratio of bilateral ventral horn neurons (injured side/uninjured side) at various time points (1, 7, 14, and 21 days) PO in each group. Error bars represent mean $\pm$ SEM, * $P < 0.05$

Figure 3 AI at various time points (1, 7, 14, and 21 days) PO in each group. Error bars represent mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$

TUNEL-positive cells scattered in gray matter and white matter 1 day after SCI, and AI in the EGb group was significantly lower compared to the NS group ($P < 0.05$). The number of apoptotic cells reached a peak at 7 days after SCI for both EGb and NS groups, and significant decrease of AI was observed in the EGb group compared to that in the NS group ($P < 0.01$). From 7 days after SCI, TUNEL-positive cells were found predominantly in white matter (Figure 4). At 14 days after SCI, the number of TUNEL-positive cells had decreased in both groups, and there was still a significant difference of AI between the two groups ($P < 0.01$). At 21 days after SCI, the number of TUNEL-positive cells had further decreased, and there was no significant difference of AI between the two groups.

Figure 4 Images of TUNEL staining in the cross-sections 3 mm caudal to the injury center in the EGb group (a) and the NS group (b) at 7 days after SCI. In both groups, TUNEL positive cells (cell nuclei were stained dark brown) were found predominantly in white matter. The number of positive cells was significantly lower in the EGb group compared to the NS group. Bar = 10 μ m (a, b)

Figure 5 Percentages of iNOS-positive cells at various time points (1, 7, 14, and 21 days) PO in each group. Error bars represent mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$

Immunohistochemistry (Figures 5 and 6)

iNOS-positive cells could be observed in gray matter and white matter, and included neurons, glial cells, ependymal cells, and endothelial cells. Percentages of iNOS-positive cells at four time points PO in three groups are shown in Figure 5. The pattern of changes in iNOS-positive cells tended to be similar to the TUNEL-positive reaction. Few iNOS-positive cells were detected in the sham group at all four time points PO, and there were no significant differences in the percentages of iNOS-positive cells among the four time points PO. For both EGb and NS groups, the percentages of iNOS-positive cells increased at 1 days after SCI, and reached a peak at 7 days after SCI (Figure 6). At 14 days after SCI, the iNOS-positive cells decreased, reaching a minimum at 21 days after SCI. When the rats were treated with EGb 761, the percentage of iNOS-positive cells was significantly lower than that in animals treated with NS at each time point ($P < 0.01$ or $P < 0.05$). Furthermore, the pearson correlation test showed that the percentage of iNOS-positive cells positively correlated with the apoptotic index ($r^2 = 0.729$, $P < 0.01$) after SCI.

Figure 6 Images of iNOS staining in the cross-sections 3 mm caudal to the injury center in the EGb group (a, b) and NS group (c, d) at 7 days after SCI. In both groups, iNOS-positive cells were found in gray matter (a, c) and white matter (b, d). The number of positive cells was significantly lower in the EGb group than in the NS group. Bar = 5 μ m (a–d)

Discussion

In the first few hours after SCI, damage, hemorrhage and inflammation contribute to a large amount of cellular necrosis, which is followed by a long period (up to several weeks) of secondary cell denaturalization and apoptosis.^{15–18} Wallerian degeneration following SCI is long-term apoptotic death of oligodendrocytes in long tracts.¹⁹ Preventing or reducing this delayed apoptosis may improve neurological recovery or facilitate nerve regeneration.^{20,21} EGb 761 prevents neurons from dying through a variety of mechanisms, and has been applied for clinical cerebrovascular and neurodegenerative diseases. This raises the question of whether EGb 761 has a similar effect on SCI.

The present experiments evaluated the protective role of EGb 761 on nerve cells after SCI. As contusive spinal cord injuries are the most common SCI in clinical treatment, animal models of contusive SCI may be more appropriate for assessing acute management strategies. However, the difference among individual animals and poor reproducibility of contusive models would devalue the comparability, so in this study, we adopted spinal cord hemitransection models to observe nerve cell changes on both sides of each animal specimen, comparing the lesioned side to the uninjured side, thus avoiding individual difference and enhancing comparability.

Myelin staining showed that the area of cavities was small and the demyelinated zones were limited at and around the injury site of the spinal cord in the EGb group, while the area of cavities was large and the

demyelinated zones were serious in the NS group. Nissl staining showed that the ratio of bilateral ventral horn neurons (transection side/uninjured side) in the EGb group was higher than that in the NS group. These results suggested the protective effect of EGb 761 on nerve cells after SCI.

We also investigated the effect of EGb 761 on iNOS expression and cellular apoptosis after SCI with immunohistochemistry and TUNEL methods. The results demonstrated that iNOS expression was upregulated after SCI and positively correlated to the apoptotic index. Nitric oxide (NO) is produced when nitric oxide synthase (NOS) catalyzes L-arginine to generate citrulline, and studies^{22,23} have shown that overdose of NO produced by iNOS was found to induce cell apoptosis in the traumatic SCI models, so inhibition of iNOS may be an efficient therapy for secondary SCI. Our experimental results showed the percentage of iNOS-positive cells and apoptotic index of nerve cells in the EGb group was significantly lower than that in the NS group, which suggested that EGb 761 suppressed iNOS expression and then prevented nerve cell death. Of course, EGb 761 may also protect nerve cells through other pathways.^{9,10,13,24–26} (1) ginkgolide is a native specific antagonist to the platelet activating factor (PAF) and can notably mitigate tissue injuries resulting from PAF. Additionally, ginkgolide B can prevent neurons from gluneroxicity through reduction of the rise in $[Ca^{2+}]_i$; (2) ginkgo chromocor can efficiently eliminate oxygen-derived free radicals; (3) some of the constituents of EGb 761 can also suppress monoamine oxidase, maintain activity of ATPase and facilitate neuroregen-

eration. EGb 761 is an extract found in the leaves of ginkgo, an ancient Chinese tree that is widespread, and this extract is available for clinical application.

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References

- 1 Beattie MS. Inflammation and apoptosis: linked therapeutic targets in spinal cord injury. *Trends Mol Med* 2004; **10**: 580–583.
- 2 Chen WH, Tzeng SF. Pituitary adenylate cyclase-activating polypeptide prevents cell death in the spinal cord with traumatic injury. *Neurosci Lett* 2005; **384**: 117–121.
- 3 Fehlings MG, Bracken MB. Summary statement: the Sygen(GM-1 ganglioside) clinical trial in acute spinal cord injury. *Spine* 2001; **26**: S99–S100.
- 4 Koda M *et al*. Brain-derived neurotrophic factor suppresses delayed apoptosis of oligodendrocytes after spinal cord injury in rats. *J Neurotrauma* 2002; **19**: 777–785.
- 5 Li X, Oudega M, Dancausse HA, Levi ADO. The effect of methylprednisolone on caspase-3 activation after rat spinal cord transection. *Restor Neurol Neurosci* 2000; **17**: 203–209.
- 6 Gaviria M, Privat A, d'Arbigny P, Kamenka JM, Haton H, Ohanna F. Neuroprotective effects of a novel NMDA antagonist, Gacyclidine, after experimental contusive spinal cord injury in adult rats. *Brain Res* 2000; **874**: 200–209.
- 7 Calapi G *et al*. Neuroprotective effects of *Ginkgo biloba* extract in brain ischemia are mediated by inhibition of nitric oxide synthesis. *Life Sci* 2000; **67**: 2673–2683.
- 8 Oken BS, Storzbach DM, Kaye JA. The efficacy of *Ginkgo biloba* on cognitive function in Alzheimer's disease. *Arch Neurol* 1998; **55**: 1409–1415.
- 9 Zimmermann M, Colciaghi F, Cattabeni F, Di LM. *Ginkgo biloba* extract: From molecular mechanisms to the treatment of Alzheimer's disease. *Cell Mol Biol* 2002; **48**: 613–623.
- 10 Ahlemeyer B, Krieglstein J. Pharmacological studies supporting the therapeutic use of *Ginkgo biloba* extract for Alzheimer's disease. *Pharmacopsychiatry* 2003; **36**: S8–S14.
- 11 Gong QH, Wu Q, Huang XN, Sun AS, Shi JS. Protective effects of *Ginkgo biloba* leaf extract on aluminum-induced brain dysfunction in rats. *Life Sci* 2005; **77**: 140–148.
- 12 Luo Y *et al*. Inhibition of amyloid-beta aggregation and caspase-3 activation by the *Ginkgo biloba* extract EGb761. *PNAS* 2002; **99**: 12197–12202.
- 13 Massieu L, Morán J, Christen Y. Effect of *Ginkgo biloba* (EGb 761) on staurosporine-induced neuronal death and caspase activity in cortical cultured neurons. *Brain Res* 2004; **1002**: 76–85.
- 14 MacLennan KM, Darlington CL, Smith PF. The CNS effects of *Ginkgo biloba* extracts and ginkgolide B. *Prog Neurobiol* 2002; **67**: 235–257.
- 15 Zurita M, Vaquero J, Oya S, Morales C. Effects of dexamethasone on apoptosis-related cell death after spinal cord injury. *J Neurosurg* 2002; **96**(Suppl): 83–89.
- 16 Liu XZ *et al*. Neuronal and glial apoptosis after traumatic spinal cord injury. *J Neurosci* 1997; **17**: 5395–5406.
- 17 Lu J, Ashwell KW, Waite P. Advances in secondary spinal cord injury: role of apoptosis. *Spine* 2000; **25**: 1859–1866.
- 18 Martin LJ, Chen K, Liu ZP. Adult motor neuron apoptosis is mediated by nitric oxide and Fas death receptor linked by DNA damage and p53 activation. *J Neurosci* 2005; **25**: 6449–6459.
- 19 Kim DH, Vaccaro AR, Henderson FC, Benzel EC. Molecular biology of cervical myelopathy and spinal cord injury: role of oligodendrocyte apoptosis. *Spine J* 2003; **3**: 510–519.
- 20 Horiuchi H, Ogata T, Morino T, Chuai M, Yamamoto H. Continuous intrathecal infusion of SB203580, a selective inhibitor of p38 mitogen-activated protein kinase, reduces the damage of hind-limb function after thoracic spinal cord injury in rat. *Neurosci Res* 2003; **47**: 209–217.
- 21 Waldmeier PC. Prospects for antiapoptotic drug therapy of neurodegenerative diseases. *Prog Neuropsychopharmacol Biol Psychiatry* 2003; **27**: 303–321.
- 22 Satake K *et al*. Nitric oxide via macrophage iNOS induces apoptosis following traumatic spinal cord injury. *Mol Brain Res* 2000; **85**: 114–122.
- 23 Yu YM, Matsuyama Y, Nakashima S, Yanase M, Kiuchi K, Ishiguro N. Effects of MPSS and a potent iNOS inhibitor on traumatic spinal cord injury. *Neuroreport* 2004; **15**: 2103–2107.
- 24 Christen Y, Maixent JM. What is *Ginkgo biloba* extract EGb 761? An overview – from molecular biology to clinical medicine. *Cell Mol Biol* 2002; **48**: 601–611.
- 25 Zhu L, Wu J, Liao H, Gao J, Zhao XN, Zhang Z. Antagonistic effects of extract from leaves of *Ginkgo biloba* on glutamate neurotoxicity. *Zhongguo Yao Li Xue Bao* 1997; **18**: 344–347.
- 26 Hsu SH, Chang CJ, Tang CM, Lin FT. *In vitro* and *in vivo* effects of *Ginkgo biloba* extract EGb 761 on seeded Schwann cells within poly(DL-lactic acid-co-glycolic acid) conduits for peripheral nerve regeneration. *J Biomater Appl* 2004; **19**: 163–182.