Extract of *Ginkgo biloba* promotes neuronal regeneration in the hippocampus after exposure to acrylamide

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Abstract

Previous studies have demonstrated a neuroprotective effect of extract of *Ginkgo biloba* against neuronal damage, but have mainly focused on antioxidation of extract of *Ginkgo biloba*. To date, limited studies have determined whether extract of *Ginkgo biloba* has a protective effect on neuronal damage. In the present study, acrylamide and 30, 60, and 120 mg/kg extract of *Ginkgo biloba* were administered for 4 weeks by gavage to establish mouse models. Our results showed that 30, 60, and 120 mg/kg extract of *Ginkgo biloba* effectively alleviated the abnormal gait of poisoned mice, and up-regulated protein expression levels of doublecortin (DCX), brain-derived neurotrophic factor, and growth associated protein-43 (GAP-43) in the hippocampus. Simultaneously, DCX- and GAP-43-immunoreactive cells increased. These findings suggest that extract of *Ginkgo biloba* can mitigate neurotoxicity induced by acrylamide, and thereby promote neuronal regeneration in the hippocampus of acrylamide-treated mice.

Key Words: nerve regeneration; brain injury; extract of *Ginkgo biloba*; acrylamide; doublecortin; brain-derived neurotrophic factor; growth associated protein-43; neurons; damage; hippocampus; mice; neural regeneration

Introduction

Acrylamide (ACR) is a white crystal chemical that is a common raw material of polyacrylamide product. In many industries around the world, ACR is used for water purification, the inner coating of pipelines, and pulp processing (Rosen et al., 2002; Dybing et al., 2003). Moreover, foods rich in starch can produce ACR after high temperature cooking (above 120°C) (Ma et al., 2011; Krishna et al., 2015; Sen et al., 2015). ACR produces defective neurological hallmarks such as skeletal muscle weakness and ataxia. Quantitative morphometric and electrophysiological analyses show that nerve terminals are the primary sites of ACR action

Graphical Abstract

Protective effect of extract of *Ginkgo biloba* (EGb) on toxic neurons

24% flavone, 6% lactone and organic acid

Anti-inflammatory and antiplatelet effects relieve nerve cell injury and brain ischemia

Neurotoxicity

Neuroethology

Pathology

Morphological abnormalities

Neuronal apoptosis and decrease

DCX

BDNF

GAP-43

EGb promotes neuronal regeneration

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After behavioral testing, four mice from each group were deeply anesthetized with 4% chloral hydrate by peritoneal injection and perfused with 0.9% saline followed by 4% paraformaldehyde in 0.01 M phosphate buffer solution (pH 7.4). Brains were immediately removed, post-fixed overnight in paraformaldehyde, washed with running tap water overnight, dehydrated, embedded in paraffin, and sliced into 4 µm-thick coronal sections with a microtome. Sections were mounted onto glass slides, hydrated in graded alcoholic solutions, incubated in 3% hydrogen peroxide to block endogenous peroxidase activity, and then incubated with blocking solution of normal horse serum. The primary antibodies, GAP-43, and DCX (Song et al., 2013). Therefore, we investigated the effects of extract of *Ginkgo biloba* (EGb) on neuronal regeneration in the hippocampus of mice treated with ACR. Accordingly, we demonstrate that expression levels of DCX, BDNF, and GAP-43 are strongly interconnected.

In recent years, numerous herbal medicines have attracted the attention of many researchers for the treatment of neurological diseases. For example, *Radix Puerariae* and *Rhizoma Acori Tatarinowii* exert neuroprotective effects (Zhu et al., 2016). EGb shows protective effects against senile dementia (Stackman et al., 2003; Tan et al., 2015) and cardiovascular disease (Schneider et al., 2010). Likewise, an effect of EGb has been demonstrated on anxiety-like behavior and locomotor activity (Ribeiro et al., 2016), modulation of inflammatory mediators and the cholinergic system (Kim et al., 2016), and in dementia treatment (Hashiguchi et al., 2015).

In particular, EGb enhances regeneration of injured peripheral nerves. Nonetheless, previous studies on the effect of EGb on brain damage have mainly focused on oxidative damage (Aydin et al., 2016; Sener et al., 2017) and neuronal damage (Massieu et al., 2004; Eckert et al., 2005), with neuronal regeneration insufficiently researched. Newborn neurons are labeled by DCX in the brain, and there is a strong relationship between neuronal regeneration and BDNF, GAP-43, and DCX (Song et al., 2013). Therefore, in this study, we investigated the protective effect of EGb on neuronal regeneration in the hippocampus of mice treated with ACR. Our study may provide a scientific foundation for the use of EGb in preventing and treating ACR neurotoxicity.

**Materials and Methods**

**Animal model preparation**

Forty male Kunming mice weighing 22–26 g (at the start of the experiment) were purchased from the Animal Experimental Center of Guangdong Province of China (certification No. SYXX (Yue) 2013-0002). All animals were housed in an animal room on 12-hour dark/light cycles, and the first trace of the previous animal (Neto et al., 2013).

**Immunohistochemistry**

After behavioral testing, four mice from each group were deeply anesthetized with 4% chloral hydrate by peritoneal injection and perfused with 0.9% saline followed by 4% paraformaldehyde in 0.01 M phosphate buffer solution (pH 7.4). Brains were immediately removed, post-fixed overnight in paraformaldehyde, washed with running tap water overnight, dehydrated, embedded in paraffin, and sliced into 4 µm-thick coronal sections with a microtome. Sections were mounted onto glass slides, hydrated in graded ethanol, immersed in 0.01 M citrate buffer (0.01 M; pH 6.0), and heated for 20 minutes in a microwave oven at 90°C for antigen retrieval. After cooling to room temperature, sec-
tions were treated with 3% hydrogen peroxide at 37°C for 15 minutes to inactivate endogenous peroxide enzyme. Sections were blocked with 1% bovine serum albumin (in 0.5% Triton-X-100) for 30 minutes at 37°C, and then incubated at 4°C overnight with primary antibodies: rabbit polyclonal anti-DCX (1:600; Abcam, Cambridge, MA, USA) and rabbit monoclonal anti-GAP43 (1:300; Abcam). On the second day, sections were rinsed three times in 0.01 M PBS and incubated for 40 minutes at 37°C with secondary antibodies: horseradish peroxidase AffiniPure goat-anti-rabbit IgG (1:500; EARTHOX, San Francisco, CA, USA). The peroxidase reaction was performed using diaminobenzidine (Boster, Wuhan, China) for 2–8 minutes. Color change was observed under a microscope (BX-51; Olympus, Tokyo, Japan). Sections were dehydrated and mounted in neutral resin after counterstaining with hematoxylin. Hippocampal sections were examined under a microscope.

**Imaging analysis**

Brain sections were examined and photographed under a microscope. Brains from four mice were chosen from each group. The total number of cells from six coronal sections per hippocampus, spanning −1.8 to −3.2 mm posterior to bregma, were used as the sample volume for cell counting. Four high-power (400×) fields were taken of the hippocampal dentate gyrus from each section. Image Pro-Plus 6.0 software (Media Cybernetics, Rockville, MD, US) was used to count immunopositive cells and measure optical density.

**Western blot assay**

Hippocampi of four mice from each group were homogenized in phenylmethyl sulfonylfluoride radioimmuno precipitation assay lysis buffer and centrifuged for 15 minutes at 4°C. Supernatant protein was removed and the concentration determined using the bicinchoninic acid Protein Assay Kit (Beyotime, Shanghai, China). Protein samples were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis under the same conditions, and transferred onto polyvinylidene fluoride membranes at 300 mA for 2 hours. Membranes were blocked with 5% nonfat milk for 1 hour at room temperature, and then incubated at 4°C overnight with primary antibodies: rabbit polyclonal anti-DCX (1:600; Abcam), rabbit monoclonal anti-GAP-43 (1:300; Abcam), and rabbit monoclonal anti-BDNF (1:250; Abcam). On the second day, membranes were rinsed four times with Tris-buffered saline with Tween, and incubated for 1 hour at room temperature with secondary antibodies: horseradish peroxidase AffiniPure goat-anti rabbit IgG (1:10,000; EARTHOX). Membranes were washed four times with Tris-buffered saline with Tween and detected by enhanced chemiluminescence (super ECL Assay Kit, EARTHOX). Blots were incubated with β-tubulin (1:1,000; Millipore, Billerica, MA, USA) as a loading control. All western blot analyses were made in triplicate. The optical density of each labeled band was measured using Quantity One software (Bio-Rad, CA, USA).

**Results**

**EGb effect on motor function in mice exposed to ACR**

Table 1 Effect of different EGb doses on abnormal gait in mice treated with ACR

|                          | Control group | ACR group | EGb (mg/kg) |
|--------------------------|---------------|-----------|-------------|
|                          | 30            | 60        | 120         |
| Hind limb foot splay (cm)| 3.57±0.41     | 4.41±0.88 | 3.38±0.85   |
| Gait score               | 1.00±0.00     | 3.75±0.46 | 1.50±0.53   |
| Numbers of going through grid (number/5 minutes) | 263.3±35.16  | 90.33±22.01 | 151.33±31.01 |
| Numbers of standing (number/5 minutes)         | 30.33±6.36   | 3.33±1.53  | 5.63±3.52   |

Data are expressed as mean ± SD (n = 8, one-way analysis of variance followed by Dunnett’s post hoc test). *P < 0.05, **P < 0.01, vs. control group; #P < 0.05, ##P < 0.01, vs. ACR group. Control group: Did not undergo any procedure; ACR group: administrated saline in the morning and 20 mg/kg/d ACR by gavage; 30, 60, and 120 mg/kg EGb groups; administrated 30, 60, and 120 mg/kg/d EGb in the morning and 20 mg/kg/d ACR in the afternoon for four weeks by gavage. EGb: extract of Ginkgo biloba; ACR: acrylamide.
Figure 1 EGb effect on DCX and GAP-43 immunoreactivity in the hippocampus of mice treated with ACR.
Light microscopic detection of hippocampal DCX (A and C, brown, × 400) and GAP-43 (B and D, brown, × 400) -immunoreactive cells by immunohistochemistry in different groups. (A1, B1) Control group; (A2, B2) ACR group; (A3, B3) 30 mg/kg EGb group; (A4, B4) 60 mg/kg EGb group; and (A5, B5) 120 mg/kg EGb group. Histograms in C and D show statistical analysis on numbers/mm² of DCX- and GAP-43-immunoreactive cells in the hippocampus of the ACR and 30, 60, and 120 mg/kg EGb groups. Data are expressed as the mean ± SD (n = 8, one-way analysis of variance followed by Dunnett's post hoc test). *P < 0.05, **P < 0.01, vs. control group; †P < 0.05, ‡P < 0.01, vs. ACR group. Control group: Did not undergo any procedure; ACR group: administrated saline in the morning and 20 mg/kg/d ACR by gavage; 30, 60, and 120 mg/kg EGb groups: administrated 30, 60, and 120 mg/kg/d EGb in the morning and 20 mg/kg/d ACR in the afternoon by gavage for 4 weeks. EGb: Extract of Ginkgo biloba; ACR: acrylamide; DCX: doublecortin; GAP-43: growth associated protein-43.

Figure 2 EGb effect on protein expression of DCX, BDNF, and GAP-43 in mice treated with ACR.
(A) Protein blots of DCX, BDNF, and GAP-43. Histograms were plotted based on comparison of relative optical density for expression of DCX (B), BDNF (C), and GAP-43 (D) proteins in the ACR and 30, 60, and 120 mg/kg EGb groups. Data are expressed as the mean ± SD (n = 8, one-way analysis of variance followed by Dunnett's post hoc test). *P < 0.05, **P < 0.01, vs. control group; †P < 0.05, ‡P < 0.01, vs. ACR group. Control group: Did not undergo any procedure; ACR group: administrated saline in the morning and 20 mg/kg/d ACR by gavage; 30, 60, and 120 mg/kg EGb groups: administrated 30, 60, and 120 mg/kg/d EGb in the morning and 20 mg/kg/d ACR in the afternoon by gavage for 4 weeks. EGb: Extract of Ginkgo biloba; ACR: acrylamide; DCX: doublecortin; BDNF: brain-derived neurotrophic factor; GAP-43: growth associated protein-43.
of grid crossing, significant differences were observed ($P < 0.01$; Table 1) between control and ACR groups. Specifically, the 30 mg/kg and 60 mg/kg Egb groups showed significant increases ($P < 0.05$; Table 1) compared with the ACR group. However, there was no difference between the 120 mg/kg Egb group and ACR group ($P > 0.05$; Table 1). For frequency of rearing, the number was significantly less in the ACR group ($P < 0.01$; Table 1) compared with the control group. Accordingly, there was a significant difference between the 60 mg/kg Egb group and ACR group ($P < 0.05$; Table 1).

**Egb effect on DCX and GAP-43 immunoreactivities**

Egb administration increased DCX immunoreactivity in the hippocampus of mice. DCX-immunoreactive cells were mainly distributed in the subgranular layer of the dentate gyrus of the mouse hippocampus. DCX is a neuronal precursor marker, which can be used to study neuronal proliferation, migration, and differentiation (Sánchez-Farías et al., 2015). Hence, we determined if Egb has beneficial effects on DCX immunoreactivity. DCX immunoreactivity decreased in the ACR group. DCX-immunoreactive cells were round or oval. Synaptic morphological and structural changes were found in the mouse hippocampus. Terminal branches were swollen and the number of synapses decreased. However, after 4 weeks of Egb administration, DCX immunoreactivity nearly returned to normal levels. Synapses maintained good morphology, with promotion of neurite outgrowth and increased synapse number in DCX-immunoreactive cells (Figure 1A). Quantitative analysis showed a significantly decreased number of DCX-immunoreactive cells in the ACR group compared with the control group ($P < 0.05$; Figure 1C). After 4 weeks of Egb administration, ACR and Egb significantly increased DCX immunoreactivity ($P < 0.05$; Figure 1C). Therefore, Egb supplementation may be useful against neuronal damage induced by ACR.

Egb administration increased GAP-43 immunoreactivity in the hippocampus. GAP-43 is a specific phosphoric acid protein that is highly expressed in active brain regions. During neural development, GAP-43 plays an important role in synaptogenesis, as well as in synaptic connections between nerve cells (Li et al., 2002). As a crucial molecule for neuronal growth, GAP-43 has a significant effect on neuronal development (Phatak et al., 2015). We found that ACR administration decreased GAP-43 immunoreactivity in the hippocampus in the ACR group compared with the control group. However, GAP-43 immunoreactivity increased at different concentrations of Egb administrated (Figure 1D). Quantitative analyses revealed significant differences in ACR mice compared with control mice ($P < 0.01$; Figure 1D). In addition, there were significant differences between the 30 mg/kg and 60 mg/kg Egb groups, and ACR group ($P < 0.05$, Figure 1D). Additionally, there were significant differences between the 120 mg/kg Egb group and ACR group ($P < 0.01$; Figure 1D).

**Western blot assay for DCX, BDNF, and GAP-43 protein expression in the hippocampus**

BDNF plays a crucial role in neuronal differentiation, growth, and development. Lack of central BDNF interferes with neuronal differentiation in the hippocampus of adult mice (Schmitz et al., 2014). Consistent with previous results, ACR treatment reduced protein expression of DCX, BDNF, and GAP-43 (Figure 2). However, in the 30, 60, and 120 mg/kg Egb groups, protein expression increased to differing levels. Quantitative analyses showed that DCX, BDNF, and GAP-43 showed significant differences between the ACR and control group ($P < 0.05$; Figure 2B-D). In contrast, DCX protein expression increased in the 120 mg/kg Egb group compared with the ACR group ($P < 0.05$; Figure 2B). A more significant increase was observed in the 60 mg/kg Egb group compared with the ACR group ($P < 0.01$; Figure 2B). Similarly, there was a significant difference in BDNF protein expression between the 30 mg/kg Egb group and ACR group ($P < 0.05$; Figure 2C), with more marked differences between the 60 and 120 mg/kg Egb groups and ACR group (both $P < 0.01$; Figure 2C). GAP-43 protein expression was significantly higher in the 60 and 120 mg/kg Egb groups compared with the ACR group ($P < 0.05$; Figure 2D).

**Discussion**

ACR is a highly hydrophilic chemical that shows medium toxicity to nerves, and which can damage the nervous system via many routes. Previous studies have shown that ACR produces similar neurotoxicity at low and high doses, with low doses only requiring longer exposure (Erkekoglu et al., 2014). Furthermore, symptoms of peripheral and central nervous system damage appear in ACR poisoned mice (Pennisi et al., 2013; Mehri et al., 2015). ACR can lead to pathological lesions and remodeling in nerve terminals with the presence of distal axonal swellings and degeneration (LoPachin et al., 2015). Symptoms of ACR-mouse neurotoxicity include severe gait abnormalities. In the present study, ACR-treated mice showed symptoms of paralyzed hind limbs, which could not support their body weight, and decreased movement capacity. We proposed that ACR might destroy the central nervous system through inhibition of signal transmission between synapses, or alternatively, changes in function of the efferent system. At present, there are no specific drugs to relieve ACR toxicity, therefore many studies have focused on the active ingredients in herbal plants. Egb is a traditional Chinese medicine that contains flavonoids and other active components with important medical properties. Egb shows a protective effect against cerebral ischemia injury, neuroplasticity, and neurodegenerative diseases (Müller et al., 2012; Zhang et al., 2012, 2017). Thus, we used Egb to relieve ACR poisoning.

To investigate the effect of ACR poisoning on movement disorders, and in turn the therapeutic effect of Egb, we performed gait analysis and the open-field test. We found that ACR-treated mice presented with tremors during walking and weakness or paralysis in posterior limbs, consistent with a previous study (DeGrandchamp et al., 1990). We also observed that particular neurotoxicity symptoms were produced in a certain way when ACR enters the body, such as...
behavioral changes and abnormal gait. Nevertheless, not all our experimental findings agree with this assumption. For example, in the open-field test, administration of 120 mg/kg EGb had no effect on number of grid crossings, while 30 mg/kg and 120 mg/kg EGb had no effect on frequency of rearing. These results suggest that the therapeutic effect of EGb has a strict concentration range. We will address the most effective concentration of EGb for a therapeutic effect in future experiments.

DCX is associated with the normal brain development processes of neuronal cell birth and migration (Rao et al., 2004; Reiner et al., 2013; Yoo et al., 2016). BDNF plays an important role in prevention of neurobiological changes and neuronal protection (Garraway et al., 2016; Gonzalez et al., 2016; Shrivastava et al., 2016; Cheah et al., 2017). GAP-43 is essential for promoting denervation-induced sprouting, maintaining normal climbing fiber structure, and remodeling axon terminals (Wang et al., 2001; Erkekoglu et al., 2014; Hou and Kang, 2016). We investigated the mechanism of ACR neurotoxicity by analyzing structural changes in the hippocampus. We found decreased DCX, BDNF, and GAP-43 expression in the hippocampus of ACR-treated mice. These results are consistent with other studies (Ogawa et al., 2012; Song et al., 2013), and suggest that neuronal regeneration is blocked by ACR administration. Altogether, this indicates that the brain is particularly vulnerable to the neurotoxic effects of ACR, which are associated with behavioral changes in mice. Encouragingly, we found increased DCX expression in the hippocampus in all three EGb-treated groups, as well as increased BDNF and GAP-43 expression levels. Thus, inhibition of neuronal regeneration is associated with decreased BDNF and GAP-43 expression in the mouse hippocampus, while EGb caused an increase in BDNF and GAP-43 expression, which promotes neural growth.

DCX is a microtubule-associated protein expressed by neuronal precursor cells, and is associated with neuronal regeneration (Ryu et al., 2016). Nerve injury and neurotoxicity prevent DCX expression (Ko et al., 1999). However, nerve injury can cause increased DCX expression (Ma et al., 2015). Therefore, it is likely that different protection mechanisms deal with distinct types of damage. Some injuries stimulate the brain’s repair system and others influence neurotrophic factors or neuronal survival. Hence, treatment measures should be selected according to different damage mechanisms. Our results show that DCX expression can be reduced by ACR administration. Simultaneously, BDNF and GAP-43 expression are decreased, which means that neurogenesis is blocked in the mouse hippocampus. However, EGb administration reversed the damage induced by ACR. These observations confirm that EGb has a protective effect by promoting neuronal regeneration.

It is well known that EGb is particularly effective on promoting brain blood circulation and antioxidants (Rojas et al., 2012). Here, the mechanisms of how ACR injures the central nervous system, and how EGb influences DCX, BDNF, and GAP-43 expression are not clear. Thus, we still do not know how EGb influences ACR neurotoxicity, whether ACR is cleared in brain blood, or EGb only increases DCX, BDNF, and GAP-43 expression, or both. This needs further research to address these issues. Nonetheless, our findings demonstrate that there is a strong relationship between increased DCX, BDNF, and GAP-43 expression and protection afforded by EGb.

In conclusion, EGb administration improves ACR-induced neuronal damage, mainly by promoting neuronal regeneration, which is shown by increased DCX, BDNF, and GAP-43 expression. Thus, EGb has a therapeutic effect on ACR neurotoxicity. EGb may promote neuronal regeneration in the hippocampus of ACR-treated mice, and can be exploited to improve ACR damage, although its mechanism still needs further investigation.

Author contributions: WHL and XYM designed the study, performed experiments and wrote the paper. YBF, SML and JL participated in the experimental implementation and data analysis. HQL and LL prepared animal models. GYL and SMF supervised the study and modified the paper. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

Research ethics: The study was approved by the Animal Ethics Committee of Guangdong Pharmaceutical University of China (gdpu2016022). The experimental procedure followed the the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1986).

Data sharing statement: The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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