

Geniposide prevents rotenone-induced apoptosis in primary cultured neurons

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doi: 10.4103/1673-5374.167760
<http://www.nrronline.org/>

Accepted: 2015-06-28

Abstract

Geniposide, a monomer extracted from gardenia and widely used in Chinese medicine, is a novel agonist at the glucagon-like peptide-1 receptor. This receptor is involved in neuroprotection. In the present study, we sought to identify an anti-apoptotic mechanism for the treatment of neurodegenerative diseases. Primary cultured neurons were treated with different concentrations of rotenone for 48 hours. Morphological observation, cell counting kit-8 assay, lactate dehydrogenase detection and western blot assay demonstrated that 0.5 nM rotenone increased lactate dehydrogenase release, decreased the expression of procaspase-3 and Bcl-2, and increased cleaved caspase-3 expression in normal neurons. All these effects were prevented by geniposide. Our results indicate that geniposide diminished rotenone-induced injury in primary neurons by suppressing apoptosis. This may be one of the molecular mechanisms underlying the efficacy of geniposide in the treatment of neurodegenerative diseases.

Key Words: nerve regeneration; geniposide; rotenone; cell apoptosis; Alzheimer's disease; caspase-3; Bcl-2; neural regeneration

Funding: This research was supported by grants from the Shanxi Science and Technology Department in China, No. 2011081060 and Shanxi Scholarship Council of China, No. 2011-44.

Li L, Zhao J, Liu K, Li GL, Han YQ, Liu YZ (2015) Geniposide prevents rotenone-induced apoptosis in primary cultured neurons. *Neural Regen Res* 10(10):1617-1621.

Introduction

There are a number of similarities between the pathologies of diabetes and Alzheimer's disease, indicating that the diseases should respond to similar treatment (Li and Hölscher, 2007). The protective effect of glucagon-like peptide (GLP)-1 on the brain has been shown *in vitro* (Wang et al., 2013) and *in vivo* (Li et al., 2012), and GLP-1 analogues have been used to treat diabetes since 2005 (Lin, 2008). Geniposide is a major active ingredient in the fruits of *Gardenia jasminoides* Ellis (Wang et al., 2014), which is known for its antitumor (Koo et al., 2004), anti-inflammatory (Koo et al., 2006), antithrombotic (Suzuki et al., 2001), immunosuppressant (Chang et al., 2005), neuroprotective (Liu et al., 2009), and hypoglycemic (Kojima et al., 2011) properties. Using high throughput screening, Liu et al. (2006) confirmed that geniposide is an agonist for GLP-1 receptor.

A number of studies have focused on the neuroprotective action of geniposide. Liu et al. (2007, 2009) explored this in PC12 cells and showed that it regulates the expression of anti-oxidant proteins such as heme oxygenase-1 (HO-1) and Bcl-2 by activating the transcription factor p90RSK *via* the mitogen-activated protein kinase signaling pathway. The same report also implicated the phosphatidylinositol 3-kinase signaling pathway in the neuroprotective effect of geniposide against the oxidative damage induced by H₂O₂. Similarly, geniposide was shown to protect PC12 cells from CoCl₂ in mitochondria-mediated apoptosis (Guo et al., 2009) and

from oxidative damage induced by 3-morpholinopropanolamine hydrochloride, by enhancing the expression of heme oxygenase 1 *via* the cyclic adenosine monophosphate-protein kinase A-response element binding protein signaling pathway (Yin et al., 2010). Geniposide activates the GLP-1 receptor and protects against β -amyloid-induced neurotoxicity, regulating the expression of insulin-degrading enzyme in cortical neurons (Yin et al., 2012). The molecule can also protect SH-SY5Y cells against formaldehyde stress by modulating the expression of Bcl-2, P53, caspase 3 and caspase 9, and by increasing the activity of intracellular superoxide dismutase and glutathione peroxidase (Sun et al., 2013). It is unclear whether anti-apoptosis is one of the molecular mechanisms by which geniposide exerts a protective effect against neurodegenerative disease. Here, we investigated this hypothesis by exploring whether geniposide prevents rotenone-induced apoptosis in primary cultured neurons.

Materials and Methods

Animals

Swiss mouse pups (1–3 days old) were obtained from the Animal Center at Shanxi Medical University in China (license No. SCXK (Jin) 2009-0001). All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications, No. 80–23, revised 1978) and approved by the Animal Ethics Committee in Shanxi

Medical University in China.

Primary culture of mouse neurons

Tissue preparation was performed on ice. Neonatal mice were sacrificed by decapitation. Cortical and hippocampal tissues were isolated and washed in Ca^{2+} - and Mg^{2+} -free Hanks balanced salt solution. The tissue was cut into small pieces and incubated with 0.25% trypsin for 10 minutes at 37°C. The trypsin was inactivated using fetal bovine serum to prevent excessive dissociation. The dissolved tissues were centrifuged at 1,000 r/min for 5 minutes, and the cells were taken and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin (Trans, Beijing, China) in a humidified atmosphere at 37°C, 5% CO_2 , for 6 hours. They were then transferred to neurobasal medium (Gibco, Carlsbad, CA, USA) containing 2% B27 supplement, 1% L-glutamine and 1% penicillin and streptomycin. Neurons were cultured and grown for 7 days until they reached 70–80% confluence.

Estimation of effective rotenone concentration by cell counting kit-8 (CCK-8) assay

Primary neuronal cells were seeded in 96-well cell culture plates (1×10^6 cells per well) at 37°C for 7 days. Cells were incubated with various concentrations of rotenone (0.5, 1, 3, and 5 nM; Solarbio, Beijing, China) for 2 days, to identify the lowest concentration at which cellular survival was inhibited. CCK-8 solution (10 μL per well; Dojindo Laboratories, Tokyo, Japan) was added directly to the cell suspension and followed by a 2-hour incubation at 37°C. Cell morphology was observed under a light microscope (Olympus, Tokyo, Japan). Optical density was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Estimation of geniposide concentrations that offer protection for cultured neurons by lactate dehydrogenase (LDH)

Cultured neurons were incubated for 7 days, then pre-treated with various concentrations of geniposide in normal saline (0, 10, 50, 100, and 200 μM ; Marker Inc., Tianjin, China), to identify the optimal neuroprotective concentration after 2 hours of incubation. Neurons were then exposed to rotenone (0.5 nM) for 48 hours. The viability of cultured neurons was measured by LDH assay (Jiancheng Company, Nanjing, Jiangsu Province, China), according to the manufacturer's instructions. LDH activity was quantified by measuring optical density at a wavelength of 440 nm with a 722 Visible Spectrophotometer (Shanghai Optical Instrument Factory, Shanghai, China). Released LDH activity was expressed as a percentage of total LDH activity, which was determined after freeze-thaw lysis of the cells.

Western blot assay of apoptotic signaling protein levels in neurons

After incubation for 7 days, cultured neurons were pre-treated with geniposide (50 μM) for 2 hours followed by rotenone (0.5 nM) for 48 hours. The expression of apoptosis-re-

lated proteins (procaspase-3, cleaved caspase-3 and Bcl-2) in the neurons was measured by western blot assay. Cells were plated in culture dishes at a concentration of 1×10^6 cells per dish, and grown for 7 days until they reached 70–80% confluence. They were then collected in ice-cold radio-immunoprecipitation assay buffer (containing Tris pH 7.4, NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 5 mM ethylenediamine tetraacetic acid, 1 mM sodium orthovanadate, 10 mM sodium fluoride) and 1 nM phenylmethanesulfonyl fluoride (Beyotime Company, Shanghai, China). Samples were centrifuged at 12,000 r/min for 5 minutes at 4°C, and the supernatant was taken. The concentration of total cell proteins was determined using the Bicinchoninic Acid Protein Assay kit (Beyotime Company). Equal volumes of loading buffer were added to each sample. Samples (30 μg protein) were run in a 15% Tris-Tricine gel and transferred electrophoretically onto polyvinylidene difluoride membranes. The membranes were blocked with 5% bovine serum albumin in phosphate buffered saline supplemented with 0.05% Tween-20 at room temperature for 2 hours, and then incubated overnight with rabbit anti-procaspase-3 polyclonal antibody (1:500; Abcam, Cambridge, UK), rabbit anti-cleaved caspase-3 polyclonal antibody (1:1,000; Cell Signaling Technology, Boston, MA, USA), rabbit anti-Bcl-2 polyclonal antibody (1:500; Abcam) and rabbit anti-rat β -actin monoclonal antibody (1:500; Abcam). After washing with phosphate buffered saline supplemented with Tween 20, the membranes were incubated with goat anti-rabbit IgG-horseradish peroxidase (1:5,000; Abcam) for 2 hours at room temperature. The bands were visualized using the enhanced chemiluminescence system (Transgen, Beijing, China). The relative amounts of proteins on the blots were scanned and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA), which was used to count relative optical density: a optical density ratio of target protein to β -actin.

Statistical analysis

All data were expressed as the mean \pm SEM. Differences between groups were assessed by one-way analysis of variance. Dunnett's *t*-test was used for pairwise comparison between groups. Statistical analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA) and displayed using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). All experiments were performed independently in triplicate. $P < 0.05$ was considered statistically significant.

Results

Neurotoxicity induced by rotenone in primary cultured neurons

Evident morphological changes such as cell loss, shrinkage, intensification of refraction, floatation, and string-of-beads appearance were seen in the cultured hippocampal and cortical neurons exposed to rotenone (5 nM) for 48 hours, compared with control cells. The CCK-8 assay showed that different concentrations of rotenone decreased the number of cultured neurons in a dose-dependent manner ($P < 0.01$

or $P < 0.05$). The lowest concentration that induced changes in cellular viability was 0.5 nM (Figure 1).

LDH release in cultured neurons treated with different concentrations of geniposide

Geniposide did not induce a significant change in the percentage of total LDH activity at any concentration compared with the control group ($P > 0.05$; Figure 2).

Protective effect of geniposide against rotenone toxicity

Release of LDH was significantly greater in cultured neurons exposed to 0.5 nM rotenone than in control neurons ($P < 0.01$). This increase was prevented by pretreatment with different concentrations of geniposide ($P < 0.01$; Figure 3).

Geniposide inhibited apoptotic signals induced by rotenone

Western blot assay revealed that procaspase-3 was decreased and cleaved caspase-3 was increased 48 hours after exposure to rotenone (0.5 nM) ($P < 0.01$). These effects were prevented by pretreatment with 50 μ M geniposide ($P < 0.01$ or $P < 0.05$ vs. rotenone). The rotenone-induced decrease in Bcl-2 expression was also prevented by pretreatment with 50 μ M geniposide ($P < 0.05$). No significant differences were observed between control and 50 μ M geniposide-treated neurons in any of the three proteins ($P > 0.05$; Figure 4).

Discussion

The complexity of Chinese medicine results, in part, from the fact that a single Chinese herb may contain a large number of compounds, among which are the key component(s) and the effective monomer(s). Each of these compounds may interact, making it difficult to identify the specific actions of each component. An increasing number of studies indicate that the pharmacokinetics and therapeutic effects of a monomer differ between its pure form and when it is part of the herbal extract. Geniposide, a monomer extracted from *Gardenia jasminoides Ellis*, has an extensive range of biological effects, and interest in its molecular mechanism is growing. GLP-1 is neuroprotective and activates the GLP-1 receptor in the brain (Gao et al., 2011). Using high throughput screening, Liu et al. (2006) identified geniposide as a novel agonist at this receptor. We therefore hypothesized that geniposide exerts its neuroprotective effects by acting at brain GLP-1 receptors. Geniposide has been suggested as a treatment for Alzheimer's and Parkinson's diseases. Our previous study indicated that geniposide may exert its neuroprotective effect via the PI3K signaling pathway after activating the GLP-1 receptor in the brain (Gao et al., 2014). In the present study, we have demonstrated the anti-apoptotic and neuroprotective effect of geniposide in cultured neurons. Excessive neuronal apoptosis leads to a variety of diseases including Alzheimer's and Parkinson's diseases (Taylor et al., 2008). *In vitro* and *in vivo* studies of Parkinson's disease models have revealed that rotenone induces Parkinson's disease-like behavioral and neuropathological changes, including the induction of apoptosis and acceleration of α -synuclein for-

mation (Bové et al., 2005; Olanow, 2007).

As a mitochondrial complex I inhibitor, rotenone exposure may mimic certain aspects of Parkinson's disease. Non-familial (sporadic) Parkinson's disease is characterized by a 15–30% reduction in mitochondrial complex I activity. Neurodegenerative processes involving aggregation of β -amyloid and hyperphosphorylated tau are often coupled with impairment of mitochondrial complex I. Similarly, rotenone induces β -amyloid aggregation, as well as increasing tau hyperphosphorylation (Chaves, 2010). In the present study, we established a cellular model of neuronal apoptosis using rotenone-treated primary cultured neurons, to test the hypothesis that geniposide may protect neurons from apoptosis in neurodegenerative diseases. Rotenone is a naturally occurring organic pesticide known to inhibit complex I of the mitochondrial electron transport chain (Hartley et al., 1994). Epidemiological studies have suggested a positive correlation between rotenone exposure and Parkinson's disease in human populations (Tanner et al., 2011). Furthermore, chronic rotenone exposure induces a Parkinsonian phenotype in rodents (Cannon et al., 2009). Rotenone can induce the formation of reactive oxygen species, indicating a potential mechanistic link between oxidative stress and Parkinson's disease (Li et al., 2003). In addition, rotenone was used to induce neurodegenerative disease in cultured neurons (Worth et al., 2014).

Our findings indicate that rotenone-induced apoptosis is associated with the expression and activation of caspase-3 and Bcl-2. Caspase-3 is considered to be the central protein in the execution of apoptosis, and its activated form (cleaved caspase-3) is a valuable tool by which to identify apoptotic cells, even before the morphological features of apoptosis are present. Many proteins contribute to the regulation of apoptosis; among them, there are the Bcl-2 family proteins, which include proapoptotic proteins such as Bax, and anti-apoptotic proteins such as Bcl-2 and Bcl-xL (Cunha et al., 2013). The present study shows that geniposide pretreatment inhibits the expression of cleaved caspase-3 and prevents the reduction in procaspase-3 induced by rotenone. Bcl-2 preserves mitochondrial integrity by preventing loss of mitochondrial membrane potential and/or release of pro-apoptotic proteins such as cytochrome C into the cytosol (Harada and Grant, 2003). Our data suggest that geniposide prevents rotenone-induced downregulation of Bcl-2.

In conclusion, the GLP-1 receptor agonist geniposide protects cultured neurons from rotenone-induced damage. This may be one of the molecular mechanisms underlying the action of geniposide, and our results suggest that GLP-1 may be a promising novel target in the treatment of neurodegenerative diseases.

Author contributions: LL and YZL were responsible for the study proposal, design, grant support and wrote the paper. GLL analyzed western blot assay. JZ and KL performed all of experimental process. YQH determined the survival of cultured neurons. All authors approved the final version of the paper.

Conflicts of interest: None declared.

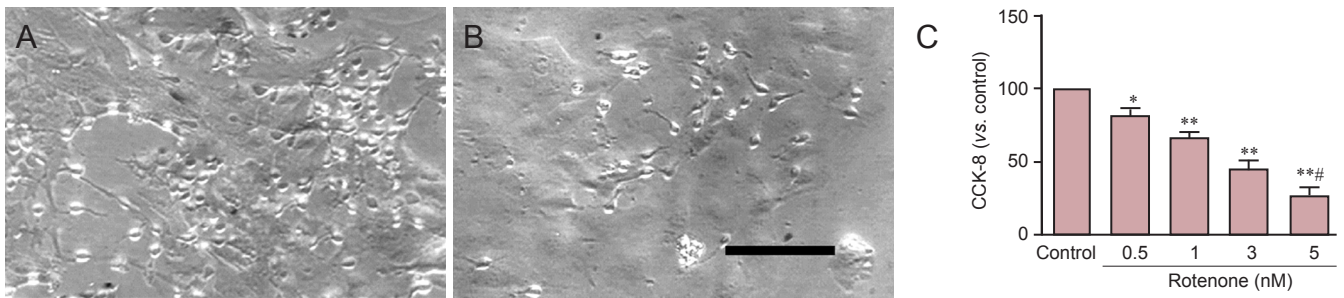


Figure 1 Survival of cultured neurons treated with various concentrations of rotenone.

(A, B) Hippocampal and cortical control (A) and rotenone-treated (B) neurons. There were notably fewer neurons after rotenone (5 nM) exposure (optical microscopy). Scale bar: 500 μ m. (C) Cell viability was analyzed using CCK-8 assay. Optical density (OD) of control group was set to 100% and the data were normalized to control group. Experiments were performed in triplicate. Neurons were incubated for 7 days before exposure to rotenone (0, 0.5, 1, 3, 5 nM) for 48 hours in the culture medium. Data are expressed as the mean \pm SEM. Differences between groups were assessed by one-way analysis of variance. Dunnett's *t*-test pairwise comparison was used to compare groups. * $P < 0.05$, ** $P < 0.01$, vs. control neurons; # $P < 0.05$, vs. 0.5 nM rotenone treated neurons.

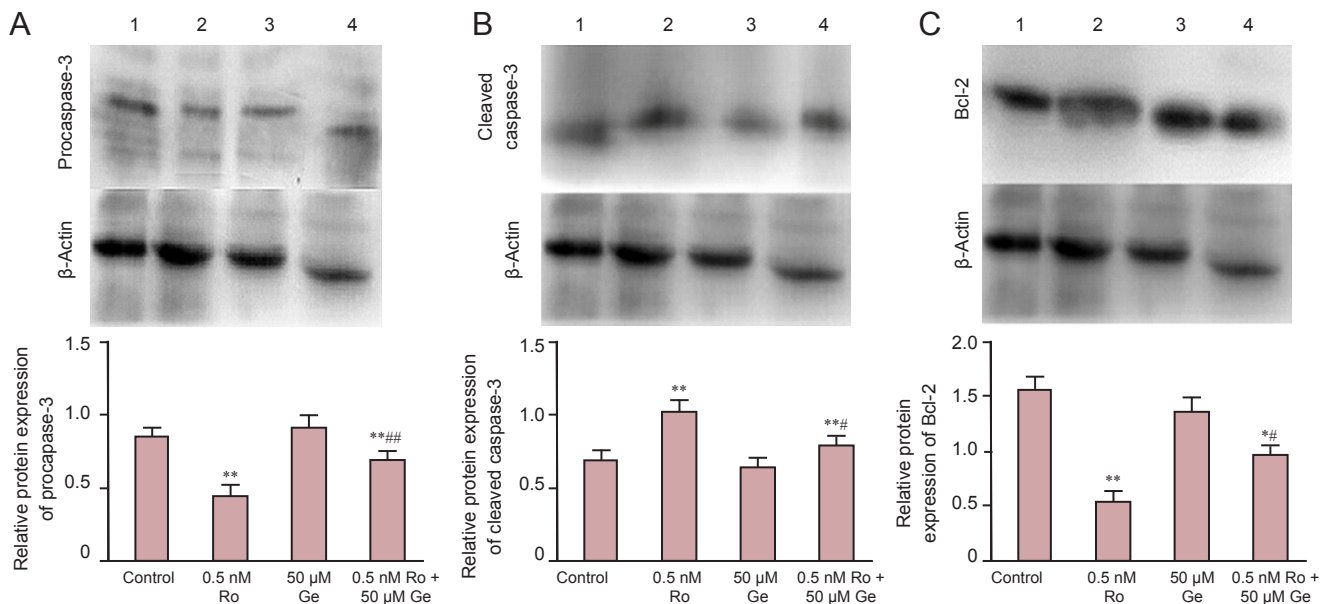


Figure 4 Effects of geniposide (50 μ M) on apoptosis-related protein expression in rotenone-treated neurons (western blot assay).

(A) Procaspase-3, (B) cleaved caspase-3, (C) Bcl-2. Lane 1: Control; lane 2: 0.5 nM rotenone; lane 3: 50 μ M geniposide; lane 4: 50 μ M geniposide pretreatment and 0.5 nM rotenone. The optical density ratio of target protein to β -actin protein was determined. Data are expressed as the mean \pm SEM. Differences between groups were assessed by one-way analysis of variance. Dunnett's *t*-test was used for pairwise comparison between groups. * $P < 0.05$, ** $P < 0.01$, vs. control neurons; # $P < 0.05$, ### $P < 0.01$, vs. 0.5 nM Ro-treated neurons. Ro: Rotenone; Ge: geniposide.

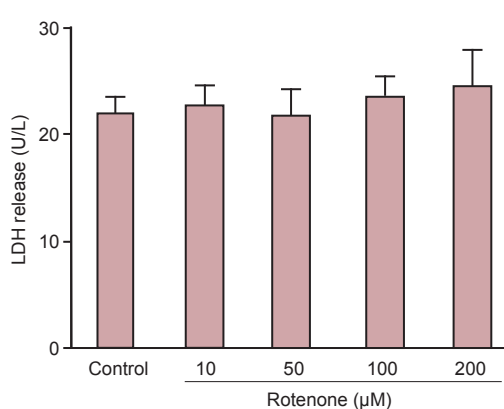


Figure 2 LDH release in cultured neurons treated by different concentrations of geniposide.

Data are expressed as the mean \pm SEM. Differences between groups were assessed by one-way analysis of variance. Dunnett's *t*-test pairwise comparison method was used to compare groups. LDH: Lactate dehydrogenase.

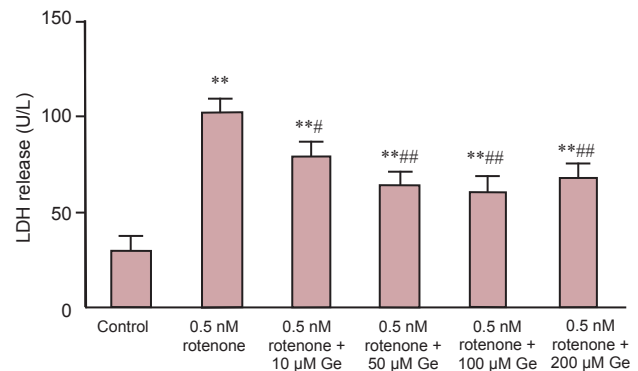


Figure 3 Effects of geniposide on LDH release in rotenone-treated neurons.

Data are expressed as the mean \pm SEM. Differences between groups were assessed by one-way analysis of variance. Dunnett's *t*-test was used for pairwise comparison between groups. ** $P < 0.01$, vs. control neurons; # $P < 0.05$, ### $P < 0.01$, vs. 0.5 nM rotenone-treated neurons. Geniposide inhibited rotenone-induced LDH release. Ge: Geniposide; LDH: lactate dehydrogenase.

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