

Dried Rehmannia root protects against glutamate-induced cytotoxicity to PC12 cells through energy metabolism-related pathways

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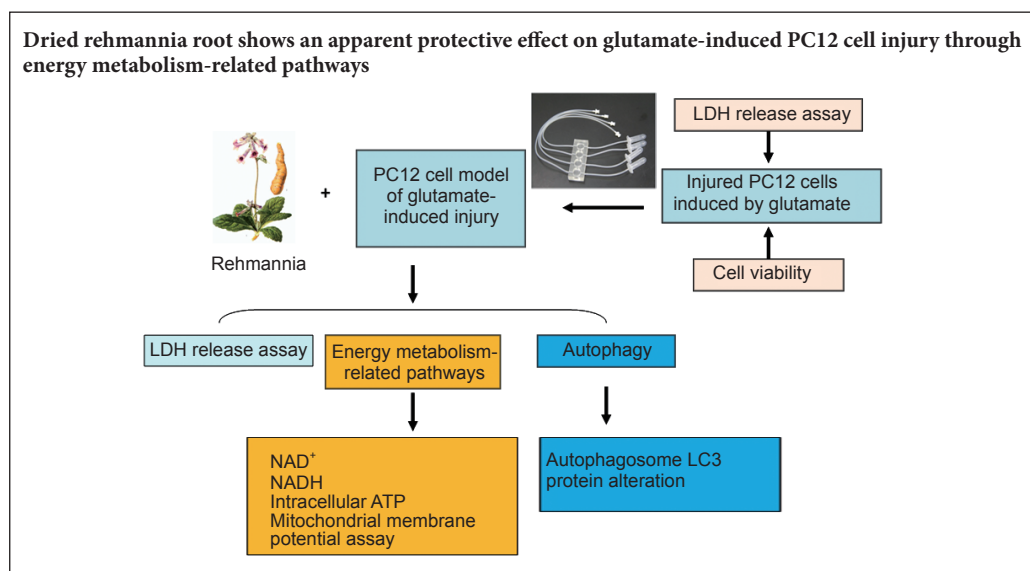
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Graphical Abstract



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Abstract

Rehmannia has been shown to be clinically effective in treating neurodegenerative diseases; however, the neuroprotective mechanisms remain unclear. In this study, we established a model of neurodegenerative disease using PC12 cytotoxic injury induced by glutamate. The cells were treated with 20 mM glutamate in the absence or presence of water extracts of dried Rehmannia root of varying concentrations (70%, 50% and 30%). The different concentrations of Rehmannia water extract significantly increased the activity of glutamate-injured cells, reduced the release of lactate dehydrogenase, inhibited apoptosis, increased the concentrations of NADH, NAD and ATP in cells, ameliorated mitochondrial membrane potential, and reduced the levels of light chain 3. Taken together, our findings demonstrate that Rehmannia water extracts exert a cytoprotective effect against glutamate-induced PC12 cell injury *via* energy metabolism-related pathways.

Key Words: nerve regeneration; Rehmannia water extracts; glutamate; PC12 cells; autophagy; energy metabolism; neural regeneration

Introduction

Neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, are a group of degenerative disorders of the central nervous system that primarily affect the elderly and severely reducing their quality of life. Glutamate is the principal excitatory neurotransmitter in the central nervous system, and modulates numerous physiological and pathophysiological processes (Durand et al., 2008). Excessive

release of glutamate can directly lead to excitotoxicity in neurons through both ligand-gated ion channels, including the N-methyl-D-aspartate and α -amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors, and G-protein-coupled metabotropic glutamate receptors. Glutamate-mediated excitotoxicity is thought to play a role in many neurological diseases, such as brain trauma (Brittain et al., 2011), stroke (Brennan-Minnella et al., 2013), Alzheimer's disease (Degos

et al., 2013) and Parkinson's disease (Hsieh et al., 2012).

The positive clinical outcomes and substantial efficacy of traditional Chinese medicines have brought hope for the treatment of Parkinson's disease. *Rehmannia*, known as di-huang and disui, refers to the root of *Rehmannia glutinosa*, a herb of the Scrophulariaceae family. *Rehmannia* is far more frequently prescribed in China than in other countries, and it tastes sweet, bitter and cold, can clear heat, cool blood, and nourish *Yin* fluid (Cui et al., 2000; Zhao et al., 2007). *Rehmannia* root is effective for treating a variety of nervous system diseases (Jiang et al., 2015), such as Alzheimer's disease, cerebral ischemia and Parkinson's disease (Wei, 2016). *Rehmannia* root improves endogenous antioxidant enzyme activities, increases free radical scavenging, inhibits the proliferation of microglial cells, reduces the excessive expression of inflammatory factors, and improves neural plasticity (Teng et al., 2014). This formulation has satisfactory clinical efficacy in the treatment of Parkinson's disease (He, 2005; Ge et al., 2012). However, the mechanisms underlying the therapeutic effects of *Rehmannia* are unclear.

Autophagy and energy metabolism-related signaling pathways play important roles in nerve injury (Koike et al., 2008; Wen et al., 2008; Cui et al., 2012). There are no studies showing that *Rehmannia* root promotes nerve repair by regulating the cellular autophagy pathway. In the present study, we investigated the cytoprotective effects of *Rehmannia* root against glutamate-induced cytotoxicity in PC12 cells using a perfused three-dimensional (3D) cell culture system. We also examined the cell and molecular mechanisms underlying the neuroprotective actions of the traditional Chinese medicine.

Materials and Methods

Ultra high performance liquid chromatography (UHPLC) of different *Rehmannia* extracts

Radix *Rehmanniae* (Huichuntang Co., Ltd., Anhui Province, China) was weighed, extracted with water, and then subjected to three serial extractions with AB-8 resin, consisting of 30%, 50% and 70% ethanol elutions (Table 1). The different extracts were weighed, transferred to 10 mL volumetric flasks, ultrasonicated with methanol for 30 minutes, and centrifuged (15,000 r/min) twice for 5 minutes each. The resulting supernatant was filtered with a 0.45- μ m microporous membrane.

Reference solution preparation

Catalpol, 10.3 mg, was weighed and transferred to a 10-mL volumetric flask, according to the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2015) and dissolved in a 0.1% acetonitrile-phosphate solution. Then, 1 mL of this liquid

was diluted to 100 mL. This stock solution was stored at 4°C.

Chromatographic conditions

A Shimadzu UHPLC system (Japan) was equipped with a Solvent Delivery Pump (Shimadzu LC-30AD), a vacuum degasser (DGU-20A), a Shimadzu UV-VIS spectrophotometric detector (SPD-20A) and ChemStation software (Shimadzu). A Kromasil analytical column was used (5 μ m, 150 \times 4.6 mm). The mobile phase for UHPLC analysis, consisting of 0.1% acetonitrile-phosphate, was passed under vacuum through a 0.22- μ m filter membrane and degassed by exposure to ultrasonic waves before use. UHPLC analysis with ultraviolet detection at 210 nm was performed at a flow rate of 1 mL/min. The column temperature was 35°C. The sample injection volume was 5 μ L or 40 μ L. The total run time was 55 minutes.

Cell culture

Rat PC12 (pheochromocytoma) cells were purchased from ATCC (Manassas, VA, USA). PC12 cells of passages 6–14 were cultured in 100-mm dishes in RPMI-1640 medium (Gibco, Waltham, MA USA) supplemented with 10% fetal bovine serum (Gibco) at 37°C in a humidified atmosphere of 5% CO₂.

Microbioreactors for parallel screening

Cells seeded on coverslips were placed in 4-well TissueFlex® (Zyoxel Ltd., Oxford, UK) microbioreactors (Figure 1) for parallel screening. Briefly, cells were plated at 2.4 \times 10⁶ cells/cm² onto the coverslip in the wells of the microbioreactor, which was coated with 0.01% poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA), and cultured under perfusion conditions at 37°C. Culture medium (RPMI-1640 containing fetal bovine serum) was supplied continuously by a multi-channel peristaltic pump (TYD01-01, LeadFluid Technology Co., Ltd., Baoding, Hebei, China) at 0.5 μ L/min. After 48 hours, the morphology of the cells was observed with an inverted microscope (Leica, Solms, Germany).

Cell viability analysis

Cell viability was measured with a quantitative colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich). Briefly, PC12 cells were seeded onto 96-well plates at 1.0 \times 10⁴ cells/well. After treatment with different concentrations of glutamate (0, 1, 5, 10 or 20 mM) for 24 hours, cells were incubated with MTT solution (0.5 mg/mL) for 4 hours at 37°C in the dark. A 100-

Table 1 *Rehmannia* extracts from different parts and amount of dry product samples

Extracted parts	Amount of samples (mg)
30% ethanol elution content	136.13
50% ethanol elution content	135.87
70% ethanol elution content	134.60

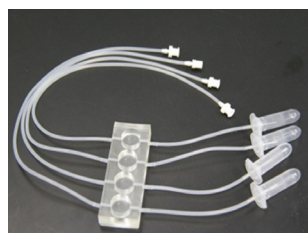


Figure 1 The multiple parallel perfused microbioreactor (TissueFlex®) used for the experiments.

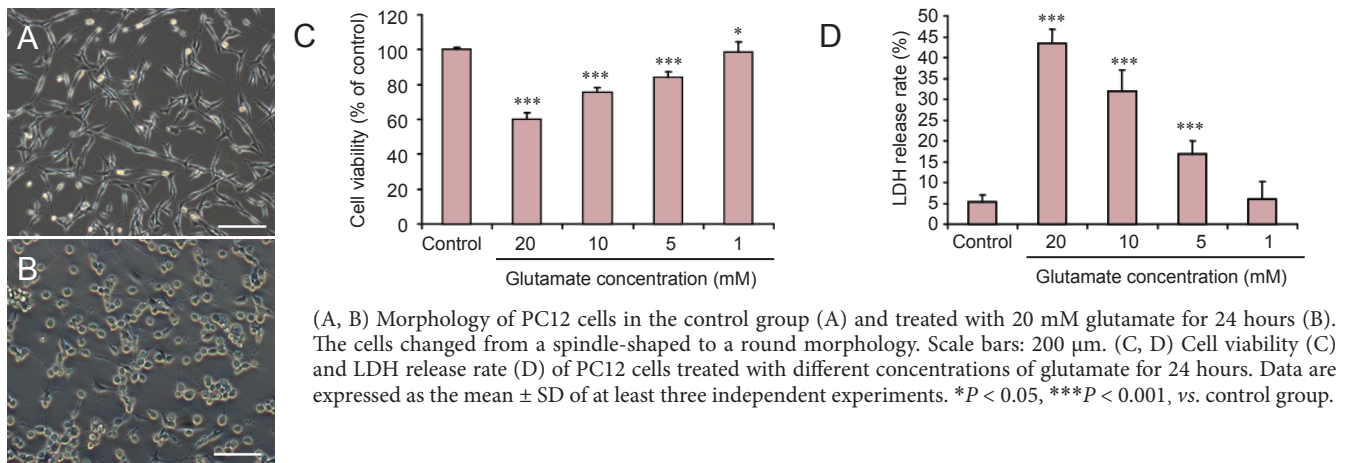


Figure 2 Morphology, viability and lactate dehydrogenase (LDH) release rate of PC12 cells before and after treatment with glutamate.

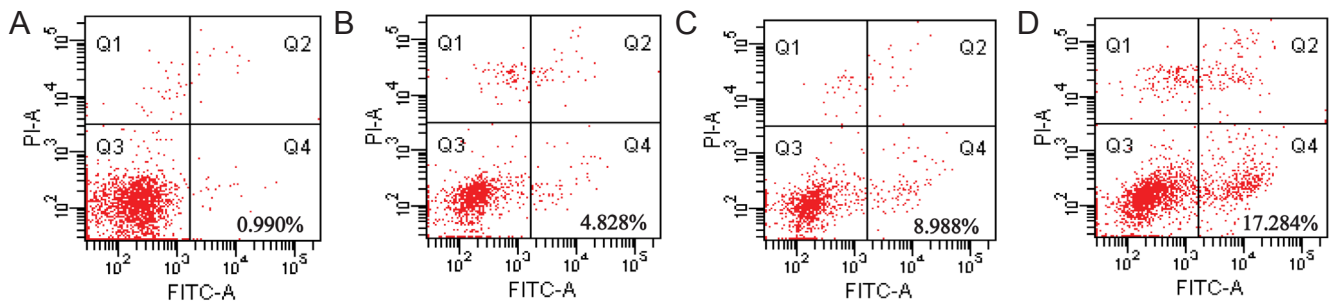


Figure 3 Different concentrations of water extract of dried Rehmannia root reduced the apoptosis rate.

PC12 cells were exposed to 20 mM glutamate in the three-dimensional perfusion microbio-reactor. (A) Water extract processed with AB-8 resin, consisting of 70% ethanol elution; (B) water extract processed with AB-8 resin, consisting of 50% ethanol elution; (C) water extract processed with AB-8 resin, consisting of 30% ethanol elution; (D) water extract.

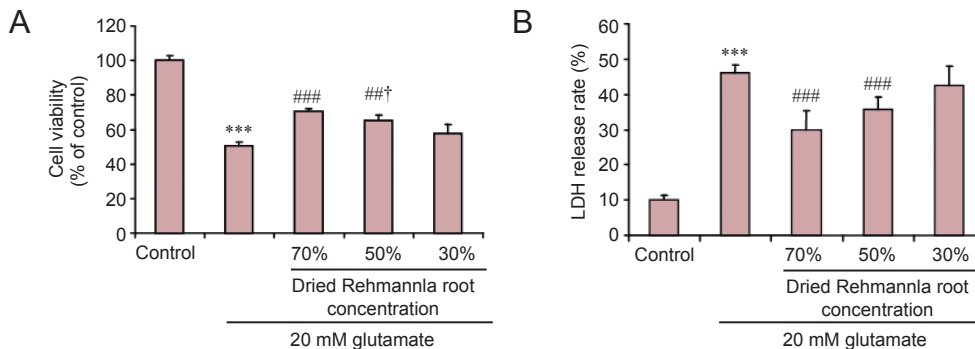


Figure 4 Effects of dried Rehmannia root on PC12 cell injury induced by 20 mM glutamate.

(A) PC12 cell injury was induced by glutamate for 24 hours. Concentrations of 70%, 50% and 30% of dried Rehmannia root were tested for their protective effect against glutamate injury. (B) LDH release rate in the glutamate group was 46.51%, which was significantly different compared with the control group. The 70% and 50% concentrations of dried Rehmannia root had significant protective effects against glutamate cytotoxicity. The 30% concentration of dried Rehmannia root had no significant protective effect. Data are expressed as the mean \pm SD of at least three independent experiments. *** P < 0.001, vs. control group; ### P < 0.01, #### P < 0.001, vs. 20 mM glutamate group; † P < 0.05, vs. 30% concentration group. LDH: Lactate dehydrogenase; MTT: 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

μ L volume of dimethyl sulfoxide was used to dissolve the crystals. The absorbance was measured spectrophotometrically using a microplate reader (BioTek, Vermont, USA) at 540 nm. The viability of treated cells was reported as a percentage of that of control cells.

Lactate dehydrogenase (LDH) release assay

LDH released from PC12 cells was assessed using the Cyto-

Tox-ONE Homogeneous Membrane Integrity Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cells were grown to 70% confluence in 96-well culture plates and exposed to 0, 1, 5, 10 or 20 mM glutamate for 24 hours. Then, 50 μ L of the culture supernatant was transferred to a separate assay plate. The LDH assay was allowed to proceed at room temperature for 10 minutes prior to the addition of 25 μ L stop solution containing 10%



Figure 5 Effects of dried Rehmannia root on energy metabolism in glutamate-treated PC12 cells.

(A) NADH, (B) NAD⁺, (C) ratio of NAD⁺/NADH. The three different concentrations of dried Rehmannia root alleviated glutamate-induced cytotoxicity to varying degrees. The 70% and 50% concentrations of dried Rehmannia root had significant effects compared with the glutamate group. The 30% concentration of dried Rehmannia root had no significant effect. The assay was done on three separate mitochondrial isolations. Data are expressed as the mean ± SD of at least three independent experiments. ****P* < 0.001, vs. control group; ###*P* < 0.001, vs. 20 mM glutamate group.

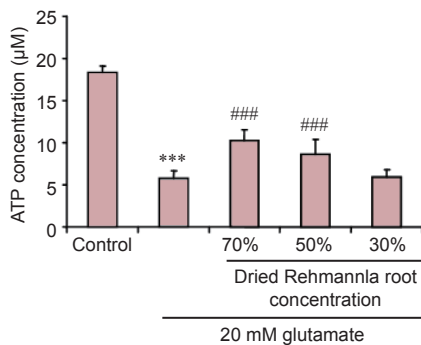


Figure 6 Effects of dried Rehmannia root on adenosine triphosphate (ATP) concentration after glutamate-induced PC12 cell injury (fluorescence assay).

After PC12 cells were treated with glutamate, the cellular ATP concentration significantly decreased. All three concentrations of dried Rehmannia root improved ATP levels to varying degrees. The 70% and 50% concentrations had significant effects compared with the glutamate group. The assay was done on three separate mitochondrial isolations. Data are expressed as the mean ± SD of at least three independent experiments. ****P* < 0.001, vs. control group; ###*P* < 0.001, vs. 20 mM glutamate group.

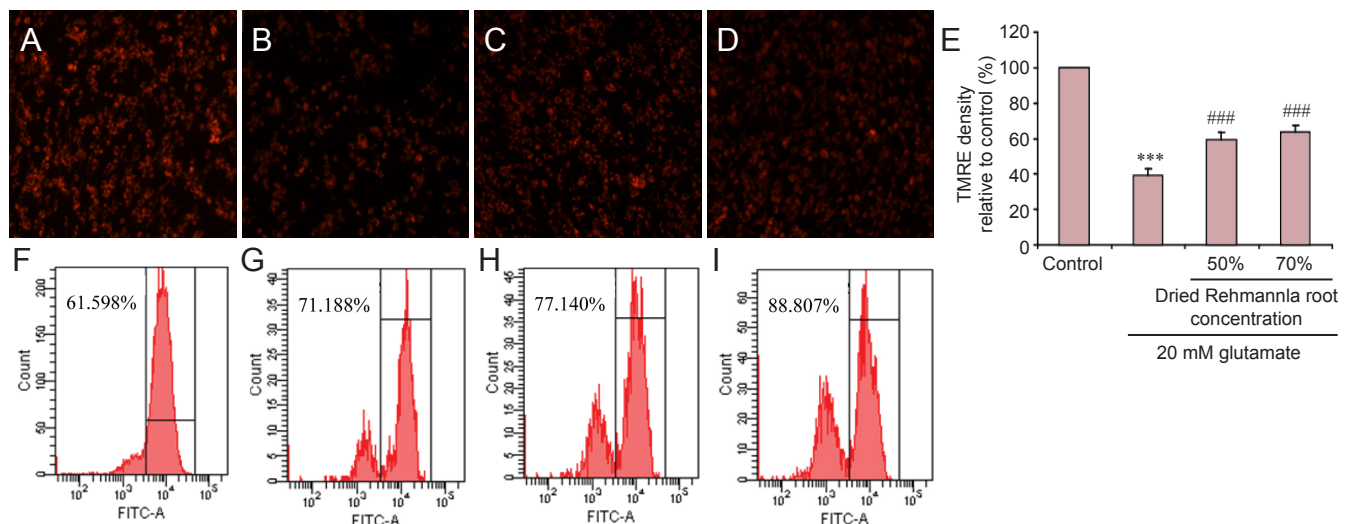


Figure 7 Mitochondrial membrane potential detected using the TMRE fluorescent probe under the fluorescence microscope.

(A–D) Glutamate group: PC12 cells were treated with 20 mM glutamate. The 70% and 50% concentrations of dried Rehmannia root increased TMRE density. MitoTracker derivatives of the orange-fluorescent tetramethylrosamine dye were used. Scale bar: 100 μm. (E) Quantitative analysis of TMRE density values. Data are expressed as the mean ± SD of at least three independent experiments. ****P* < 0.001, vs. control group; ###*P* < 0.001, vs. 20 mM glutamate group. (F–I) The various concentrations of the water extract of dried Rehmannia root ameliorated mitochondrial membrane potential. Flow cytometry was used to assess mitochondrial cardiolipid oxidation. Representative results are from three independent experiments. (A, F) Control group; (B, G) glutamate-treated group; (C, H) 70% concentration of dried Rehmannia root and 20 mM glutamate group; (D, I) 50% Concentration of dried Rehmannia root group and 20 mM glutamate group.

sodium dodecyl sulfate per well. The contents of the wells were mixed by shaking the plates for 10 seconds prior to measuring resorufin fluorescence (560 nm excitation/590 nm emission).

Adenosine triphosphate (ATP) assay

Intracellular ATP levels were quantified using an ATP Bioluminescence Assay Kit (Roche Applied Science, Basel, Switzerland) following the standard protocol. PC12 cells

exposed to 20 mM glutamate were treated with 0% (control), 70%, 50% or 30% dried *Rehmannia* root extract. After one wash with phosphate-buffered saline (PBS), the cells were lysed with the Cell Lysis Reagent (Beyotime Biotechnology, Shanghai, China), which was mixed with 50 μ L Luciferase Reagent (Biovision, San Francisco, CA, USA). A plate reader (Biotek, Winooski, Vermont, USA) was used to detect the chemiluminescence of the samples. The ATP concentrations of the samples were calculated using an ATP standard curve, and normalized to the protein concentrations of the samples, which were determined using the bicinchoninic acid assay (Zhang et al., 2016).

Mitochondrial membrane potential (MMP) assay

MMP and superoxide levels were assessed after treating PC12 cells, plated at a seeding density of $1 \times 10^5/\text{cm}^2$, with 20 mM glutamate. PC12 cells treated with 0%, 70%, 50% or 30% dried *Rehmannia* root for 24 hours (the 0% glutamate group served as the control). Cells were then incubated for 20 minutes at 37°C with 20 nM tetramethylrhodamine methyl ester (Life Technologies, Carlsbad, CA, USA) or 2.5 μ M MitoSox (Life Technologies) (Pan et al., 2015). Six wells per treatment were analyzed, and the entire experiment was repeated on another batch of cells. Fluorescence intensity was analyzed using a CyAn ADP Analyzer (Beckman Coulter, Brea, CA, USA).

For measuring NAD^+ , mitochondria were isolated from confluent PC12 cells grown in T75 flasks, lysed in extraction buffer from a commercial NADH/NAD determination kit (Biovision), and NAD was measured following the manufacturer's protocol. The assay was performed in triplicate using three separate mitochondrial isolations.

Annexin V/propidium iodide (PI) assay using flow cytometry

Flow cytometry was performed to determine the levels of early-stage apoptosis, late-stage apoptosis and necrosis using the ApoScreen Annexin V kit (SouthernBiotech, Birmingham, AL, USA) according to the manufacturer's protocol. Briefly, PC12 cells exposed to various concentrations of glutamate were digested with 0.25% trypsin, washed with cold PBS, and resuspended in cold $1 \times$ binding buffer at concentrations between 1.0×10^6 and 1.0×10^7 cells/mL. A 5- μ L aliquot of labeled Annexin V was added to 100 μ L of the cell suspension. The number of stained cells was assessed immediately on a flow cytometer (FACS Aria II, BD Biosciences, Franklin Lakes, NJ, USA).

Immunofluorescence staining

PC12 cells cultured on slides were rinsed once with PBS, fixed with 4% paraformaldehyde overnight, and then incubated with a rabbit anti-light chain 3 (LC3) polyclonal (1:100; Novus Biologicals, Los Angeles, CA, USA) primary antibody for 90 minutes at room temperature. The cells were then washed with PBS and incubated with rabbit FITC-conjugated secondary antibody (1:70; Abcam, Cambridge, MA, USA) for 60 minutes at 37°C. Nuclei were stained with Hoechst

33258 (Sigma-Aldrich) for 5 minutes at room temperature, and then mounted with glycerol. Images were captured on an Axioskop 2 fluorescence microscope (Zeiss, Oberkochen, Germany).

Western blot assay

Protein concentrations were determined with the Bradford method (Redmile-Gordon et al., 2013). Proteins were separated by 12% SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes (Bio Basic, New York, USA). The membranes were then reacted with rabbit anti-LC3 polyclonal antibody or rabbit anti-P-AKT polyclonal antibody (both 1:1,000; Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1,000; Santa Cruz Biotechnology, Dallas, TX, USA) at 37°C for 12 hours. The signals were detected with enhanced chemiluminescence detection kits (GE Healthcare, Chicago, IL, USA). The gray scales of the bands were quantified by scanning densitometry using Quantity One 4.5.0 software (Bio-Rad, Hercules, CA, USA).

Autophagy inhibitor treatment

To examine whether the changes in expression of LC3 induced by the dried *Rehmannia* root extract were associated with an effect on autophagy, PC12 cells were treated with 3-MA (an autophagy inhibitor, 5 mM, Sigma-Aldrich) simultaneously with the dried *Rehmannia* root for 24 hours.

Statistical analysis

Data are expressed as the mean \pm SD for all cells from at least three independent experiments. The significance was determined with one-way analysis of variance followed by paired Student's *t*-test. *P* < 0.05 was accepted as statistically significant.

Results

UHPLC fingerprint analysis

From the UHPLC chromatogram, retention time and area, there were no obvious chromatographic peaks at retention times of 3.6, 8.3, 9.0, 12.0, 14.0, 28.6, 29.5, 33.5 or 39.0 minutes in the ethanol extract. In comparison, peaks were observed in the water extract at retention times of 10.0, 26.0 and 34.0 minutes. A larger number of chromatographic peaks with greater areas were observed in the water extract, water extract passed through the AB-8 resin and eluted with water, and the water extract passed through the AB-8 resin and eluted with 30% ethanol. More chromatographic peaks with a greater area were observed in the water extract passed through the AB-8 resin and eluted with 50% ethanol compared with 70% ethanol. There were characteristic chromatographic peaks at retention times of 5.0, 5.7, 15.0, 16.2 and 18.6 minutes, compared with ethanol extraction at retention times of 20.7, 28.0 and 33.0 minutes.

Glutamate-induced PC12 cell injury

The morphology of PC12 cells changed after exposure to 20

mM glutamate for 24 hours (Figure 2A, B). MTT and LDH release assays showed that 20 mM glutamate decreased cell viability to $62.3 \pm 0.6\%$ of the control. Furthermore, glutamate induced PC12 damage in a dose-dependent manner. Different concentrations of glutamate caused PC12 cells to release different amounts of LDH, and at 20 mM, viability was approximately 50% after 24 hours of culture (Figure 2C, D).

Screening for the optimal concentration of dried Rehmannia root using the perfused microbio-reactor 3D cell culture system

Apoptosis is a major mode of cell death triggered by cytotoxic drug treatment (Smilansky et al., 2015). To evaluate the induction of apoptosis, the cells were stained with Annexin V-FITC and PI after treatment with different concentrations of water extract of dried Rehmannia root. The apoptosis rate of cells treated with 70%, 50% and 30% concentrations of the dried Rehmannia root extract was $0.990 \pm 0.20\%$ (Figure 3A), $4.828 \pm 0.16\%$ (Figure 3B) and $8.988 \pm 0.15\%$ (Figure 3C), respectively. The rate of apoptosis in PC12 cells treated with glutamate, but without Rehmannia root, was $17.284 \pm 2.20\%$ (Figure 3D). These findings suggest that 70% dried Rehmannia extract protects PC12 cells from glutamate-induced apoptosis in the 3D bioreactor system.

Effects of dried Rehmannia root on glutamate-induced cytotoxicity to PC12 cells

The effects of Rehmannia root on LDH release was assessed in injured PC12 cells treated with 20 mM glutamate for 24 hours. Cell viability was measured using the MTT assay. The LDH release rate in the 20 mM glutamate group was significantly different from the control group as well as the 70% and 50% Rehmannia groups, indicating that dried Rehmannia root has a significant cytoprotective effect against glutamate toxicity to PC12 cells. However, 30% Rehmannia had no significant protective effect (Figure 4A). MTT cell viability assay showed that 70% and 50% concentrations of dried Rehmannia root had significant cytoprotective effects, while the 30% concentration had no significant effect. The cell viability decreased as the concentration of Rehmannia decreased (Figure 4B).

Effects of dried Rehmannia root on energy metabolism in glutamate-treated PC12 cells

To assess whether glutamate-induced injury to PC12 cells affects energy metabolism, the levels of NAD^+ , NADH and NAD^+/NADH were evaluated. The NADH concentration decreased from $13.63 \mu\text{M}$ to $1.33 \mu\text{M}$, the NAD^+ concentration decreased from $0.69 \mu\text{M}$ to $0.37 \mu\text{M}$, and the NAD^+/NADH ratio decreased from 9.70% to 3.59% after treatment with 20 mM glutamate. All three concentrations of dried Rehmannia root ameliorated glutamate-induced cellular injury, and the 70% and 50% concentrations had significant effects (Figure 5).

Effects of dried Rehmannia root on ATP concentration in PC12 cells exposed to glutamate

To study whether dried Rehmannia root protects PC12 cells from glutamate-induced injury by impacting ATP levels, we

measured ATP concentrations. All three concentrations of dried Rehmannia root alleviated glutamate cytotoxicity to varying degrees. In addition, ATP levels were significantly higher after treatment with 70% and 50% concentrations of dried Rehmannia root compared with non-treated (0% Rehmannia) cells (Figure 6).

Effects of Rehmannia on MMP in glutamate-treated PC12 cells

We next examined the effects of Rehmannia on MMP. The various concentrations of Rehmannia enhanced MMP (Figure 7A–E). Flow cytometry assay confirmed these findings (Figure 7F–I).

Effects of Rehmannia on LC3 protein levels in glutamate-treated PC12 cells

Immunofluorescence and western blot assay were used to evaluate LC3 protein expression. To confirm whether the changes in expression of LC3 produced by Rehmannia were caused by an effect on autophagy, the autophagy inhibitor 3-MA was added simultaneously with the Rehmannia preparation for 24 hours. Immunofluorescence assay showed that LC3 protein expression was enhanced after treatment with glutamate (Figure 8B). After treatment with 70% dried Rehmannia root for 6 hours, LC3 protein expression was lower compared with the non-treated group (Figure 8C). Western blot assay showed that LC3 protein levels were significantly higher at 12 hours compared with the other time points.

Effects of the autophagy inhibitor 3-MA on apoptosis in PC12 cells

Next, we sought to confirm whether the autophagy pathway is involved in the cytoprotective effect of Rehmannia. PC12 cells were treated with the autophagy inhibitor 3-MA and/or Rehmannia, and apoptosis was assessed with fluo-3 AM (calcium fluorescent probe) and acridine orange staining. In PC12 cells treated with both the autophagy inhibitor 3-MA and Rehmannia root, apoptosis was reduced compared with cells treated with Rehmannia root alone (Figure 9).

Discussion

The positive clinical outcomes and substantial efficacy of traditional Chinese medicines have brought hope for the treatment of Parkinson's disease. Accumulating evidence indicates that traditional Chinese medicines not only alleviate Parkinsonian symptoms, but also reduce levodopa-induced dyskinesia and other complications (He et al., 2004). Current studies suggest that most Parkinsonian symptoms and levodopa-induced dyskinesia are the results of deficient stirring wind pattern (Sheng et al., 2011; Park, 2017). The Rehmannia formulation was developed based on clinical experiences. This formulation has satisfactory clinical efficacy in the treatment of Parkinson's disease and levodopa-induced dyskinesia (Ge et al., 2012; Qiu et al., 2014).

To assess the bioactivity of different Rehmannia extracts, UHPLC was used in this experiment using macroporous res-

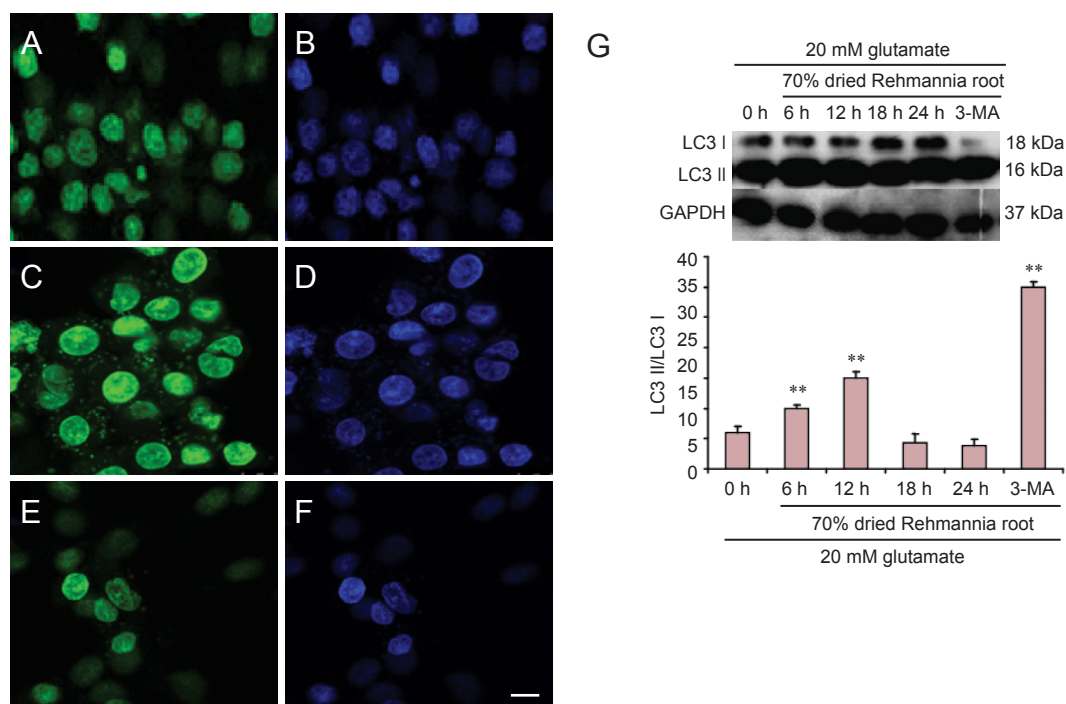


Figure 8 Autophagosomal LC3 protein and nuclei observed with laser confocal microscopy.

(A–F) Changes in autophagosomal LC3 protein and nuclei. (A, C, E) LC3 protein (green fluorescence); (B, D, F) nuclei (blue fluorescence). (A, B) Control group. (C, D) PC12 cells treated with glutamate. (E, F) 70% concentration of dried Rehmannia root with 20 mM glutamate. Scale bar: 100 μ m. (G) LC3 protein levels were assessed by western blot assay. LC3 II and LC3 I are different forms of LC3 protein, and are indicators of the degree of activation of LC3 protein. GAPDH was used as a loading control. The effect of dried Rehmannia root on autophagy in PC12 cells treated with 20 mM glutamate was observed. The expression levels of LC3 II and GAPDH were assessed by western blot assay at 24 hours. 3-MA was used to examine whether autophagy pathways play a role in the neuroprotection. Data are presented as the mean \pm SD ($n = 3$). $**P < 0.01$, vs. 0 h group. h: Hour(s); LC3: light chain 3; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

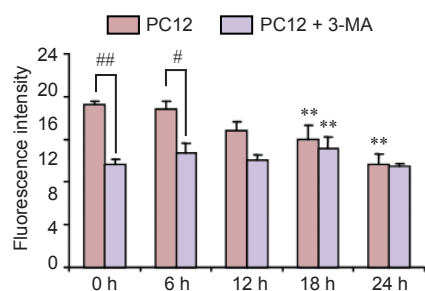


Figure 9 Effects of the autophagy inhibitor 3-MA on the cytoprotective action of dried Rehmannia root in PC12 cells.

The effect of 3-MA on apoptotic changes in PC12 cells after exposure to 20 mM glutamate for 24 hours. The cells were treated with the dried Rehmannia root for 0, 6, 12, 18 or 24 hours. Data are expressed as the mean \pm SD of at least three independent experiments. $**P < 0.01$, vs. control (20 mM glutamate group); $\#P < 0.05$, $##P < 0.01$. h: Hour(s).

in adsorption equipment, and the Rehmannia root extracts were eluted with different concentrations of ethanol. The UHPLC fingerprint was used for quality control (Liu et al., 2014).

A perfused 3D cell culture system was used for its advantages over static two-dimensional cell culture systems (Sadri et al., 2014; Li and Han, 2017), particularly in overcoming nutrient limitations by continuous replenishing of the culture medium (Wu et al., 2006; Wu et al., 2008). These bioreactors have been used successfully for engineered tissue

culture (Seidel et al., 2004) and for maintaining the viability of organs for a defined period of time. The innovative 3D cell culture system mimics the brain microcirculation and ensures adequate drug concentrations (Bijonowski et al., 2013; Pollock, 2013; Caralt, 2015; Coakley et al., 2016). Here, a model of glutamate-induced PC12 cell injury was established with the 3D cell culture system, and different Rehmannia compositions were perfused in parallel to screen for the optimal concentration (Coecke et al., 2002; Lemmo et al., 2014).

In the present study, PC12 cells were treated with glutamate to establish an excitotoxic injury model (Wang et al., 2014). The effects of different concentrations of glutamate on PC12 cells were assessed with cell viability and LDH experiments (Wei et al., 2014). A glutamate concentration of 20 mM was found to be effective, consistent with other studies (Zhao et al., 2015; Jia et al., 2016).

The 70% Rehmannia extract had the best cytoprotective effect in the bioreactor, based on apoptotic rates. The cell viability and LDH release assays confirmed that 70% Rehmannia had the greatest cytoprotective effect in our model.

Mitochondrial ATP is the main energy source for intracellular metabolic pathways (Schapira, 2006; Hagl et al., 2015; Shindo et al., 2015). Mitochondria synthesize ATP from ADP in the matrix using the energy provided by the proton electrochemical gradient (Capaldi et al., 1994; Nijtmans et al., 1995; Zeviani and Di Donato, 2004). Energy metabolism is critical for numerous processes, including apoptosis (Law

et al., 2017; Zhao et al., 2017). We examined LDH release, NAD⁺ and ATP concentrations, as well as mitochondrial membrane potential, and found that all three concentrations of Rehmannia root affected these biochemical parameters in the 3D system (Gao et al., 2016; Hu et al., 2016).

To further assess the changes in the autophagy pathway, PC12 cells were treated with the 70% concentration of Rehmannia root for different time periods. Also known as LC3-associated phagocytosis, the intersection of autophagy and phagocytosis was initially described as a pathway that limited the proliferation of engulfed pathogens by expediting phagosome maturation (Ejlervskov et al., 2013; Mehta et al., 2014). The expression of LC3 protein was examined by western blot assay, which showed that 70% Rehmannia root suppressed autophagy in a time-dependent manner (Liu et al., 2017). In cells co-treated with the autophagy inhibitor 3-MA and Rehmannia root, apoptosis was reduced compared with cells treated with Rehmannia root alone, suggesting that autophagy might be involved in mediating the cytoprotective action of the traditional Chinese medicine. Therefore, Rehmannia might have therapeutic potential for the treatment of nervous system diseases (Ahn et al., 2015; Jiang et al., 2015).

It has been reported that Akt is necessary for neuronal viability and for restoring neurological function following trauma or ischemia (Maiese, 2016). In our previous studies, levels of phosphorylated Akt were increased in glutamate-treated PC12 cells, suggesting that Akt is neuroprotective during PC12 cell injury (Lin et al., 2014; Wei et al., 2016). Moreover, Akt is also a critical autophagy regulator, with activated Akt signaling suppressing autophagy (Lu et al., 2017).

Taken together, our findings suggest that dried Rehmannia root has a significant cytoprotective effect against glutamate-induced injury in PC12 cells. This cytoprotective effect appears to involve energy metabolism-related pathways. Further studies using in vivo experiments are needed to validate the current results. Additional studies are also required to clarify the role of autophagic signaling pathways in the neuroprotective functions of Rehmannia root.

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