

# Preconditioning with Gua Lou Gui Zhi decoction enhances H<sub>2</sub>O<sub>2</sub>-induced Nrf2/HO-1 activation in PC12 cells

JINGJIE MAO<sup>1,2</sup>, ZUANFANG LI<sup>2</sup>, RUHUI LIN<sup>1,2</sup>, XIAOQIN ZHU<sup>2</sup>,  
JIUMAO LIN<sup>1,2</sup>, JUN PENG<sup>1,2</sup> and LIDIAN CHEN<sup>3</sup>

<sup>1</sup>Academy of Integrative Medicine; <sup>2</sup>Fujian Key Laboratory of Integrative Medicine on Geriatrics;

<sup>3</sup>College of Rehabilitation Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350122, P.R. China

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**Abstract.** Spasticity is common in various central neurological conditions, including after a stroke. Such spasticity may cause additional problems, and often becomes a primary concern for afflicted individuals. A number of studies have identified nuclear factor (erythroid-derived 2)-like 2 (Nrf2) as a key regulator in the adaptive survival response to oxidative stress. Elevated expression of Nrf2, combined with heme oxygenase 1 (HO-1) resistance, in the central nervous system is known to elicit key internal and external oxidation protection. Gua Lou Gui Zhi decoction (GLGZD) is a popular traditional Chinese formula with a long history of clinical use in China for the treatment of muscular spasticity following a stroke, epilepsy or a spinal cord injury. However, the mechanism underlying the efficacy of the medicine remains unclear. In the present study, the antioxidative effects of GLGZD were evaluated and the underlying molecular mechanisms were investigated, using hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced rat pheochromocytoma cells (PC12 cells) as an *in vitro* oxidative stress model of neural cells. Upon application of different concentrations of GLGZD, a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay and ATP measurement were conducted to assess the impact on PC12 cell proliferation. In addition, inverted microscopy observations, and the MTT and ATP assessments, revealed that GLGZD attenuated H<sub>2</sub>O<sub>2</sub>-induced oxidative damage and signaling repression in PC12 cells. Furthermore, the mRNA and protein expression levels of Nrf2

and HO-1, which are associated with oxidative stress, were analyzed using reverse transcription quantitative polymerase chain reaction (PCR) and confocal microscopy. Confocal microscopy observations, as well as the quantitative PCR assay, revealed that GLGZD exerted a neuroprotective function against H<sub>2</sub>O<sub>2</sub>-induced oxidative damage in PC12 cells. Therefore, the results demonstrated that GLGZD protected PC12 cells injured by H<sub>2</sub>O<sub>2</sub>, which may be associated with the upregulation of Nrf2 and HO-1 mRNA and protein expression levels in PC12 cells.

## Introduction

Approximately 80% of patients with cerebral apoplexy sequelae are reported to have a certain degree of limb spasm (1), with a 38% higher incidence one year following a stroke (2). Although spasms can help certain patients with venous reflux and relieve edema, spasms hinder functional recovery in the majority of patients. If active treatment is not administered, permanent increased muscle tension and joint contracture can seriously impact on patient quality of life. Therefore, seizures that occur following a stroke remain an obstacle in stroke rehabilitation therapy (3).

Gua Lou Gui Zhi decoction (GLGZD) was originally prescribed during the Eastern Han Dynasty around 210 AD, as written in a famous traditional Chinese medicine (TCM) book, 'Essentials from the Golden Cabinet' (4). As outlined by Zhongjing Zhang in this book, the decoction comprised *Radix Trichosanthis*, *Ramulus Cinnamomi*, *Paeonia lactiflora*, *Glycyrrhiza*, *Zingiber officinale* Roscoe and *Fructus Jujubae*. As evidenced by the age of the documented prescription of GLGZD, this decoction has been used clinically for a number of years in China for the treatment of muscular spasticity following a stroke, epilepsy or a spinal cord injury (5,6). Previous studies have demonstrated that GLGZD is effective in the treatment of post-stroke spasticity, improving the Fugl-Meyer assessment and Barthel index scores (7,8). However, the mechanisms underlying the efficacy of GLGZD remain unclear.

Rat pheochromocytoma (PC12) cells have a similar morphology and function to sympathetic neurons in the presence of nerve growth factor (NGF), and subsequently have been widely utilized as a model for the study of neuronal ion

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**Correspondence to:** Professor Lidian Chen, College of Rehabilitation Medicine, Fujian University of Traditional Chinese Medicine, 1 Qiuyangxi Road, Fuzhou, Fujian 350122, P.R. China  
E-mail: cld@fjtc.edu.cn

**Abbreviations:** GLGZD, Gua Lou Gui Zhi decoction; Nrf2, nuclear factor (erythroid-derived 2)-like 2; HO, heme oxygenase; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; PCR, polymerase chain reaction; NGF, nerve growth factor; ARE, antioxidant response element; TCM, traditional Chinese medicine

**Key words:** spasticity, stroke, oxidative stress, nuclear factor (erythroid-derived 2)-like 2, PC12, Gua Lou Gui Zhi decoction

channels and receptors, and responses to stress and injury (9). Oxidative stress is an important pathological consequence of stroke and ischemia-reperfusion injury. In addition, oxidative stress has been reported to be a core pathological component of stroke and brain ischemia-reperfusion injury, since oxidative stress elevates and contributes to cerebral ischemia reperfusion injury (10-12). A previous study found that within the brain microvasculature following ischemia-reperfusion injury, hydrogen peroxide ( $H_2O_2$ ) is produced in excess, leading to an increased susceptibility of nerve cells to damage and the development of various neuropathies (13). The use of PC12 cells as a model of cerebral ischemia is similar to using primary neurons as an *in vitro* and *in vivo* model of a cerebral ischemia neural pathology (14). Therefore, the present study utilized  $H_2O_2$  treatment of PC12 cells to stimulate oxidative stress in the neurons.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor that is involved in cellular protection against oxidative stress through the antioxidant response element (ARE)-directed induction of several antioxidant enzymes, including heme oxygenase (HO)-1 (15). HO-1 is a novel enzyme with potent anti-inflammatory, antioxidant and antiproliferative effects (16,17). Nrf2 and HO-1 proteins have been shown to play important neuroprotective roles in oxidative damage. Therefore, to clarify the potential mechanisms underlying the antispasticity effects mediated by GLGZD following stroke, the present study evaluated the antioxidant effects of GLGZD and investigated the underlying molecular mechanisms using PC12 cells injured by  $H_2O_2$  *in vitro*. In the investigations of these mechanisms, the effects of GLGZD on the mRNA and protein expression levels of Nrf2 and HO-1 in PC12 cells injured by  $H_2O_2$  treatment were analyzed.

## Materials and methods

**Preparation of the ethanol extract from GLGZD.** GLGZD used in the study consisted of extracts of *Trichosanthis Radix* (9 g), *Ramulus Cinnamomi* (9 g), *Paeonia lactiflora* (9 g), *Glycyrrhiza* (6 g), *Zingiber officinale* Roscoe (9 g) and *Fructus Jujubae* (9 g), all in a ratio of 3:3:3:2:3:3. Dried crude medicinal plants were supplied by Guo Yi Tang Chinese Herbal Medicine Store (Fuzhou, Fujian, China) for the preparation of the GLGZD ethanol extract. A total of 300 g GLGZD was extracted with 3,000 ml ethanol (75%) using a reflux method, after which the solution was filtered. The ethanol solvent was evaporated on a rotary evaporator (model RE-2000; Yarong Biochemistry Instrument Co., Shanghai, China), and the GLGZD extract was obtained using a spraying desiccation method with a spray dryer (model B-290; Büchi Labortechnik AG, Flawil, Switzerland). The stock was stored at  $-20^{\circ}C$  and working concentrations of GLGZD were established by dissolving the extract in culture media to a concentration of 8 mg/ml. Hydrogen peroxide ( $H_2O_2$ ) was a product of Junsei Chemical Co., Ltd. (Tokyo, Japan).

**Cell culture and NGF-induced differentiation of PC12 cells.** A PC12 rat pheochromocytoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum,

100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, which were all purchased from Gibco Life Technologies (Carlsbad, CA, USA), in a  $37^{\circ}C$  humidified incubator with 5%  $CO_2$  (Thermo Fisher Scientific, Waltham, MA, USA). After 24 h of incubation, differentiation was induced by treatment with 50 ng/ml NGF (NGF 2.5S; EMD Millipore, Madison, WI, USA) every other day for 6 days.

**Drug treatment.** All the experiments were performed 24 h following cell seeding onto plates at a density of  $1 \times 10^5$  cells/ml.  $H_2O_2$  was freshly prepared prior to each experiment. Cells were preincubated with GLGZD at the various concentrations (0.5 and 1.0 mg/ml) for 24 h prior to exposure to 300  $\mu$ M  $H_2O_2$  for 4 h. The control and  $H_2O_2$  treatment groups were cultured in an equal volume of RPMI 1640 medium without or with  $H_2O_2$ , respectively. Subsequently, assays for cell survival and other cellular properties were performed as follows.

**Analysis of cell viability.** Cell survival was observed using an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). Cell viability was simultaneously evaluated with a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay obtained from Sigma-Aldrich (St. Louis, MO, USA). PC12 cells were seeded into 96-well plates at a concentration of  $1 \times 10^5$  cells/ml, and subsequently treated with or without 300  $\mu$ M  $H_2O_2$  for 4 h following the addition of GLGZD at 0.5 and 1 mg/ml for 24 h at  $37^{\circ}C$ . After incubation for 3 h with 20  $\mu$ l MTT (5 mg/ml), the cells were lysed in dimethyl sulfoxide (Sigma-Aldrich), and the amount of MTT formazan was qualified by determining the absorbance at 570 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was expressed as a percentage of the control culture value.

**Cellular ATP level determination.** PC12 cells were plated in a six-well cell culture plate overnight and incubated with GLGZD ethanol extract (0.5 and 1 mg/ml) for 24 h prior to exposure to, or absence of,  $H_2O_2$  (300  $\mu$ M) for 4 h. The levels of ATP in the cells were measured using a firefly luciferase-based ATP assay kit (Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's instructions. PC12 cell culture and GLGZD exposure were performed as aforementioned. After rinsing with phosphate-buffered saline (PBS), the cells were disrupted in 200  $\mu$ l lysis buffer, and centrifuged at  $12,000 \times g$  at  $4^{\circ}C$  for 5 min, from which the supernatant was collected. In a 1.5-ml tube, a 100- $\mu$ l sample of the supernatant was mixed with 100  $\mu$ l ATP detection working solution. The luminance (RLU) was immediately measured using a Turner BioSystems luminometer (Promega Corporation, Madison, WI, USA). Standard curves for quantification were generated using known amounts of an ATP standard, and the protein concentration of each treatment group was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Total ATP levels were expressed as normalized luminance (NRLU) in nmol/mg protein (18).

**Reverse transcription quantitative polymerase chain reaction (PCR) for Nrf2 and HO-1 mRNA expression.** Total RNA was isolated from the PC12 cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the

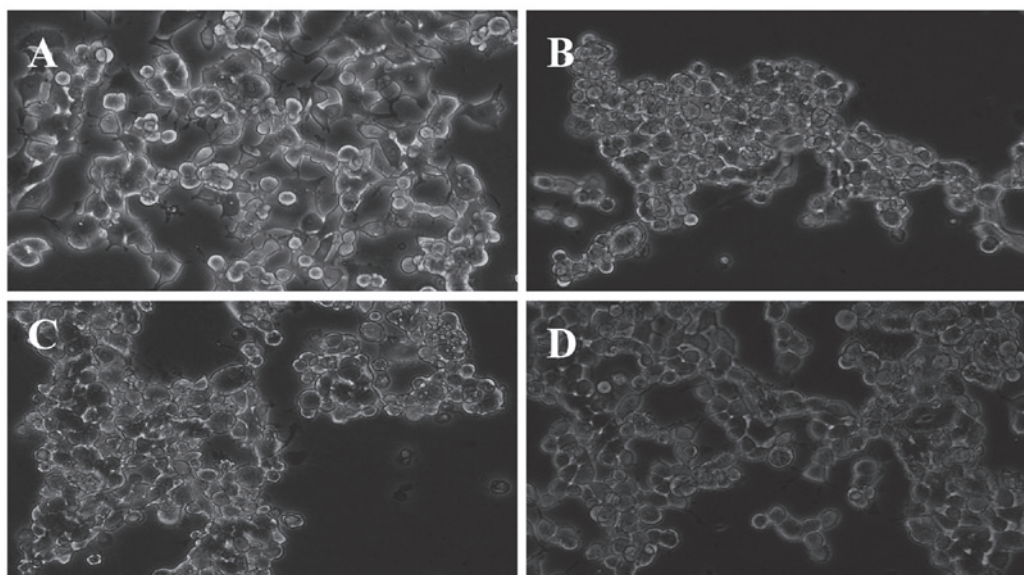


Figure 1. Effects of Gua Lou Gui Zhi decoction (GLGZD) on the morphological characteristics of hydrogen peroxide ( $\text{H}_2\text{O}_2$ )-treated PC12 cells. (A) PC12 cells without treatment (control group); (B) PC12 cell injury group exposed to  $300 \mu\text{M}$   $\text{H}_2\text{O}_2$  for 4 h; (C and D) PC12 cells treated with GLGZD at 0.5 and 1.0 mg/ml, respectively for 24 h, prior to exposure to  $300 \mu\text{M}$   $\text{H}_2\text{O}_2$  for 4 h. Photographs of the cells were captured with an inverted microscope (magnification,  $\times 40$ ).

manufacturer's instructions. In total,  $1 \mu\text{g}$  RNA was reverse transcribed into cDNA using a RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas GmbH, St. Leon-Rot, Germany). The total volume of the reverse transcription reaction mixture was  $20 \mu\text{l}$  cDNA, then  $2 \mu\text{l}$  cDNA was used for each amplification. cDNA was amplified by quantitative PCR ( $50^\circ\text{C}$  for 2 min,  $95^\circ\text{C}$  for 10 min followed by 40 cycles of  $95^\circ\text{C}$  for 15 sec and  $60^\circ\text{C}$  for 1 min) using an ABI Prism 7500 Sequence Detector (Applied Biosystems Life Technologies, Foster City, CA, USA) with SYBR Green Real-Time PCR Master Mix Reagent (Takara Bio, Inc., Otsu, Japan). Data from the reaction were collected and analyzed using Sequence Detector Software (SDS version 1.6; PE Applied Biosystems, Foster City, CA USA). Relative quantification of gene expression was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  data analysis method and normalized against GAPDH for each sample. The sequences of the primers used for PCR were as follows: Nrf2 forward, 5'-CCATTTACGGAGACCCAC-3' and reverse, 5'-TGAGCG GCAACTTTATTC-3'; HO-1 forward, 5'-GAAACAAGC AGAACCCAGTC-3' and reverse, 5'-AGAGGTCACCCA GGTAGCG-3'; GAPDH forward, 5'-ACGGATTTGGTCGTA TTGGGC-3' and reverse, 5'-CTCGCTCCTGGAAGATGG TGAT-3'. GAPDH was selected as the housekeeping gene.

**Fluorescent immunohistochemistry.** Differentiated PC12 cells were seeded into confocal-specific sterile glass-bottom cell culture Petri dishes (15 mm diameter; Nest Biotechnology Co., Ltd., Wuxi, China) and cultured for 24 h. The cells were treated with different concentrations of GLGZD (0.5 and 1.0 mg/ml), or received no treatment, for 24 h, followed by incubation with  $300 \mu\text{M}$   $\text{H}_2\text{O}_2$  for 4 h. Subsequently, the cells were fixed with 4% paraformaldehyde in PBS (pH 7.4; Gibco Life Technologies) for 30 min and permeabilized in 0.1% Triton X-100 in PBS for 10 min at room temperature. Nonspecific binding was blocked with 5% goat serum in PBS for 30 min. Next, the cells were incubated at  $4^\circ\text{C}$  overnight

with the following primary antibodies: Rabbit anti-Nrf2 (1:100 polyclonal; cat. no. SC-13032; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) and rabbit anti-HO-1 (1:200 polyclonal; cat. no. SC-10789; Santa Cruz Biotechnology, Inc.). Following incubation with the primary antibodies, the cells were washed three times in cold PBS and incubated with a goat anti-rabbit secondary antibody conjugated to a fluorescent marker (1:100, Alexa 488; Abcam, Cambridge, MA, USA). The cells were mounted with 4,6-diamidino-2-phenylindole (1:1,000; Beyotime Institute of Biotechnology) to stain the nuclei, and the samples were analyzed using an LSM 710 laser scanning confocal microscope under  $40\times$  water objective (Carl Zeiss AG, Oberkochen, Germany).

**Statistical analysis.** Data were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Quantitative data are expressed as the mean  $\pm$  standard deviation of at least triplicate experiments. One-way analysis of variance was used for data comparisons between multiple groups, where  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effects of GLGZD on the viability of  $\text{H}_2\text{O}_2$ -treated PC12 cells.** Neuroprotective effects of GLGZD were further confirmed by the morphological observations (Fig. 1). Following incubation with  $50 \mu\text{g/ml}$  NGF for 6 days, the morphological observations indicated that the majority of the NGF-differentiated PC12 cells had ceased division and extended their neuritic processes (Fig. 1A). Exposure to  $\text{H}_2\text{O}_2$  for 4 h resulted in heterogeneity in their shape, a loss of connections between neural cells and extensive detachment from the culture plate. As shown in Fig. 1B, the PC12 cell number markedly decreased, with the majority of the cells darkened or shrunken, and a number of the treated cells were even fragmented and yielding debris after 4 h

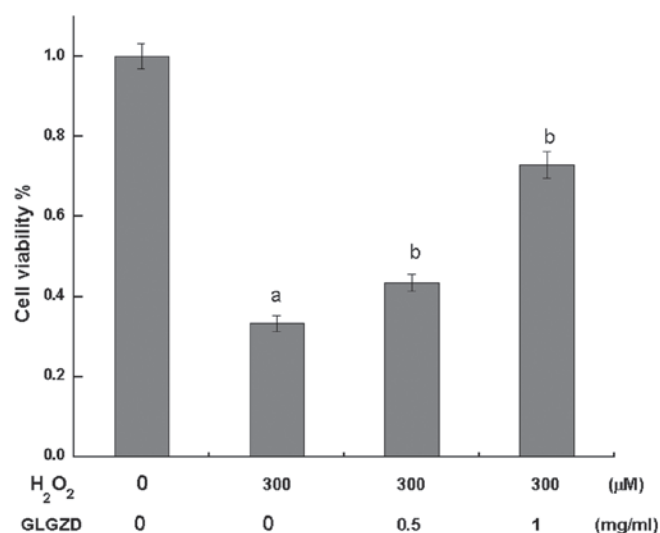


Figure 2. Effects of GLGZD on the cell viability of H<sub>2</sub>O<sub>2</sub>-treated PC12 cells. Cell survival rate was analyzed using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay. Data are expressed as the mean  $\pm$  standard deviation of three independent experiments completed in triplicate. <sup>a</sup>P<0.05, vs. untreated control cells; <sup>b</sup>P<0.05, vs. cells treated with H<sub>2</sub>O<sub>2</sub> alone. GLGZD, Gua Lou Gui Zhi decoction; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

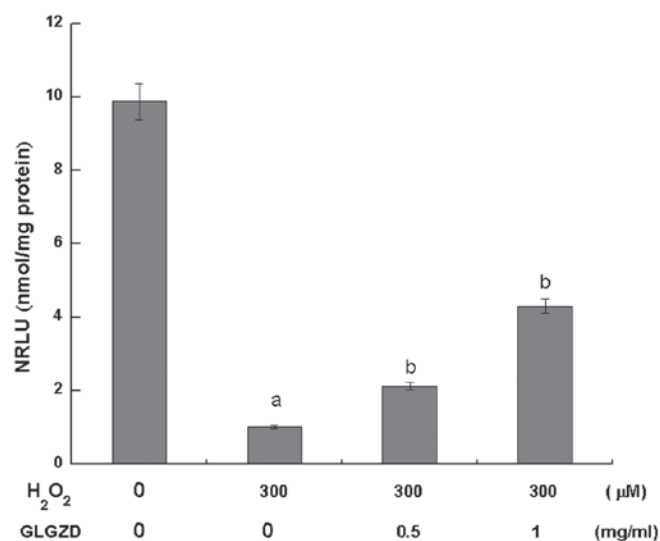


Figure 3. Effects of GLGZD on the ATP levels in H<sub>2</sub>O<sub>2</sub>-treated PC12 cells. Total ATP levels were expressed as the NRLU of firefly luciferase (nmol/mg protein). Data are expressed as the mean  $\pm$  standard deviation of three independent experiments performed in triplicate. <sup>a</sup>P<0.05, vs. untreated control cells; <sup>b</sup>P<0.05, vs. cells treated with H<sub>2</sub>O<sub>2</sub> alone. GLGZD, Gua Lou Gui Zhi decoction; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; NRLU, normalized luminance.

exposure to 300  $\mu$ M H<sub>2</sub>O<sub>2</sub>. Pretreatment with GLGZD was shown to alleviate the damage induced by H<sub>2</sub>O<sub>2</sub>. Based on the morphological changes, 1 mg/ml GLGZD (Fig. 1D) exerted a greater protective effect compared with 0.5 mg/ml GLGZD (Fig. 1C). Since the PC12 cells were very sensitive to H<sub>2</sub>O<sub>2</sub>, pretreatment with GLGZD (0.5 and 1.0 mg/ml) for 24 h dose-dependently increased cell viability compared with cells treated with H<sub>2</sub>O<sub>2</sub> alone, as determined by the MTT assay (Fig. 2).

**Changes in the cellular ATP levels.** After 4 h of H<sub>2</sub>O<sub>2</sub> exposure, the ATP levels in the two GLGZD treatment groups were significantly higher (P<0.05) compared with the group of cells treated with H<sub>2</sub>O<sub>2</sub> alone. However, the ATP levels were significantly lower in these groups when compared with the control group (Fig. 3).

**GLGZD protects PC12 cells against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress injury.** To determine the mechanism by which GLGZD attenuated H<sub>2</sub>O<sub>2</sub>-induced cell damage, the mRNA expression levels of Nrf2 and HO-1 were determined by quantitative PCR analysis (Fig. 4). Amplification curves of Nrf2 and HO-1 mRNA expression in the PC12 cells are shown in Fig. 4A. In SYBR green real-time quantitative PCR-based melt-curve analysis each gene of real-time fluorescent quantitative PCR is a single peak, the dissolution of instructions are specific amplification (Fig. 4B). Statistically significant decreases in the mRNA expression levels of Nrf2 and HO-1 were observed in the cells treated with H<sub>2</sub>O<sub>2</sub> alone, as compared with the control cells. Conversely, the mRNA expression levels of Nrf2 and HO-1 were significantly increased following treatment with 0.5 or 1.0 mg/ml GLGZD, as compared with the cells treated with H<sub>2</sub>O<sub>2</sub> alone. Simultaneously, antibodies specific to the activated forms



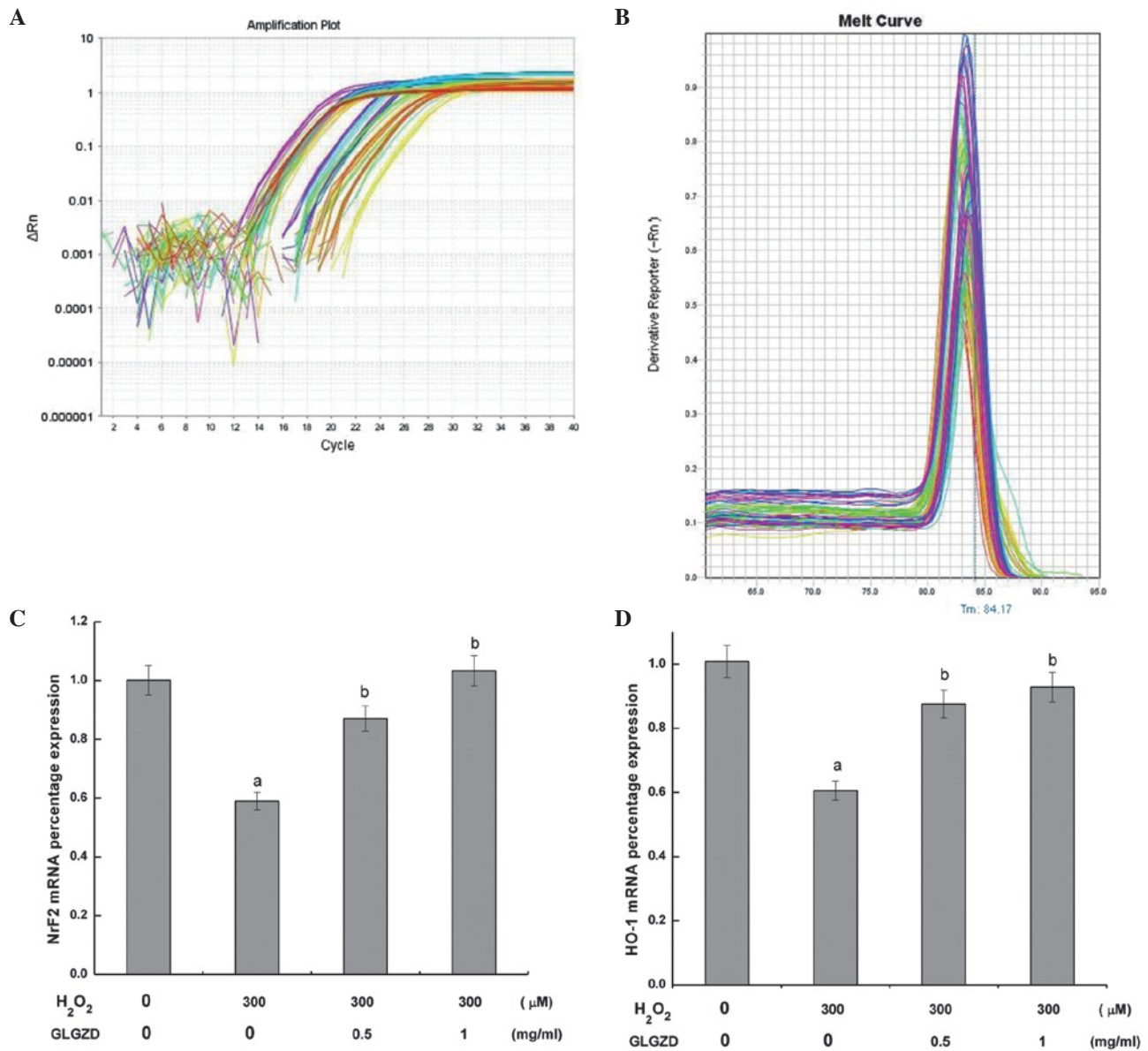


Figure 4. Effects of GLGZD on the mRNA expression levels of Nrf2 and HO-1 in H<sub>2</sub>O<sub>2</sub>-treated PC12 cells, as determined by quantitative polymerase chain reaction (PCR). (A) Amplification curves of Nrf2 and HO-1 mRNA expression in the PC12 cells and (B) SYBR green-based melt-curve analysis. Similar curves were obtained from each of the two tested sets of primers specific for each targeted organism. Each gene in the fluorescent quantitative PCR is shown as a single peak, and the dissolution of instructions are specific amplification. (C) The Nrf2 mRNA expression was analyzed as the percentage of total PC12 cells. (D) The HO-1 mRNA expression was analyzed as the percentage of total PC12 cells. Data are expressed as the mean  $\pm$  standard deviation of three independent experiments performed in triplicate. <sup>a</sup>P<0.05, vs. untreated control cells; <sup>b</sup>P<0.05, vs. cells treated with H<sub>2</sub>O<sub>2</sub> alone. GLGZD, Gua Lou Gui Zhi decoction; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; Nrf2, nuclear factor (erythroid-derived 2)-like 2; HO, heme oxygenase.

of Nrf2 and HO-1 were utilized to assess the protein expression levels, since Nrf2 and HO-1 can protect nerves against oxidative injury (16). Using confocal fluorescent microscopy, fluorescent labeling of the antibodies targeting the Nrf2 and HO-1 proteins was observed to be markedly weaker in the group of cells treated with H<sub>2</sub>O<sub>2</sub> alone when compared with the control and GLGZD treatment groups. Alternatively, the GLGZD treatment groups exhibited a higher fluorescence intensity compared with the H<sub>2</sub>O<sub>2</sub> treatment group (Figs. 5 and 6).

## Discussion

TCMs, including Chinese herbs, such as Buyang Huanwu decoction (19), Guizhi Fuling capsules (20), Xiaoxuming decoc-

tion (21), Danggui-shaoyao-san (22) and Yi-Gan-San (23), use compounds extracted from natural products and have a relatively safe side-effect profile compared with modern chemotherapeutics. In addition, such traditional therapies have been used for millennia as alternative treatments for various diseases and maladies. GLGZD is a classical TCM that was first prescribed in the first century during the Eastern Han Dynasty. Supporting the historic utilization and efficacy of this traditional medicine, GLGZD was previously demonstrated to exhibit considerable therapeutic effects on spasticity in stroke patients. The anti-inflammatory and antiapoptotic mechanisms of GLGZD have been shown to reduce the neurotoxicity of excitatory amino acids, which lowers limb spasticity following a stroke (24-26). In the assessment of cell

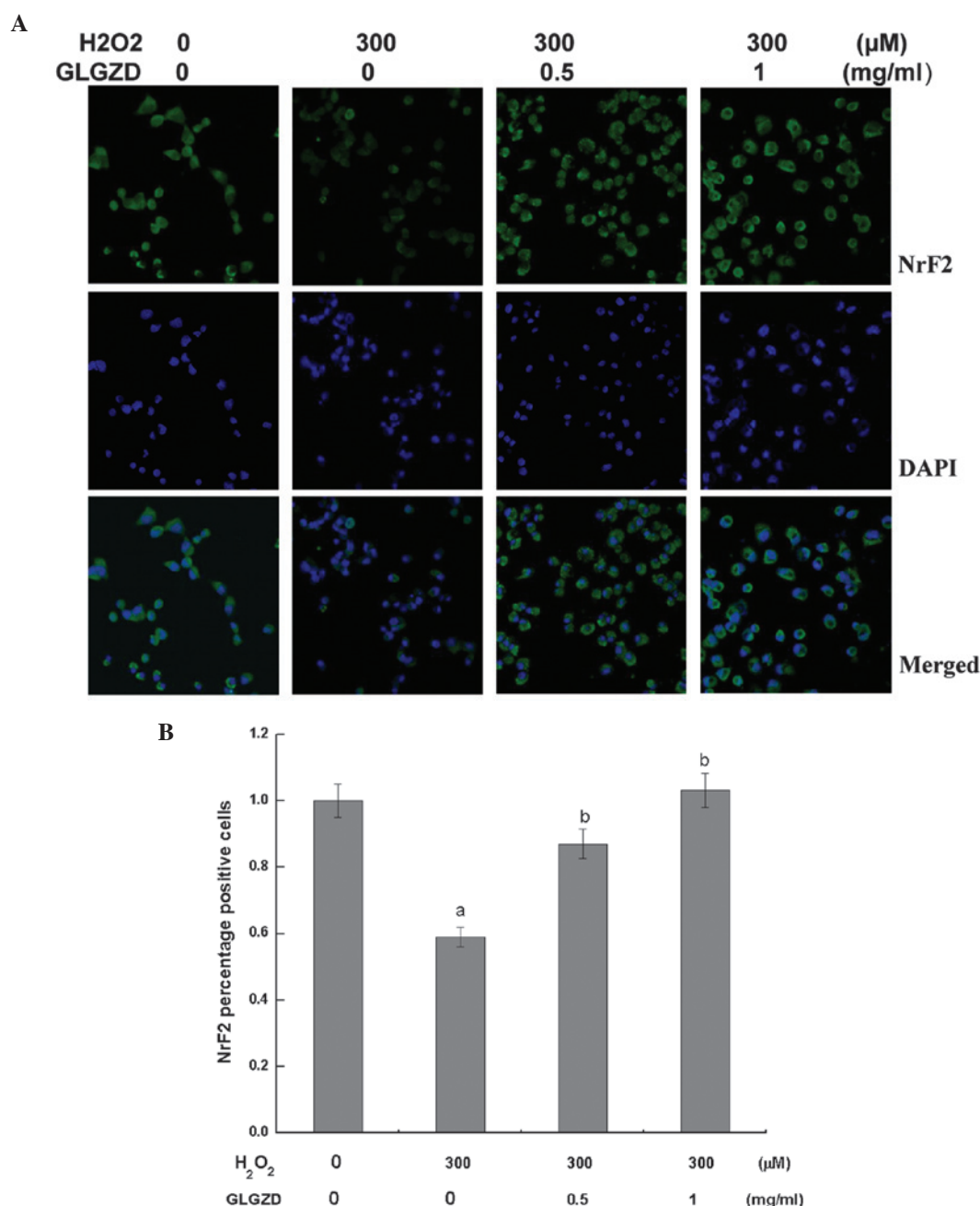


Figure 5. Representative effects of GLGZD on the expression of Nrf2 protein in PC12 cells. (A) Nrf2 was labeled with immunofluorescence staining (green), and nuclear counterstaining signals with DAPI (blue) are shown (magnification, x40). (B) Quantity of Nrf2-positive neurons was expressed as the percentage of cells colocalizing the Nrf2 subunit with DAPI. Data are expressed as the mean  $\pm$  standard deviation of three independent experiments completed in triplicate. <sup>a</sup>P<0.05, vs. untreated control cells; <sup>b</sup>P<0.05, vs. cells treated with H<sub>2</sub>O<sub>2</sub> alone. GLGZD, Gua Lou Gui Zhi decoction; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; Nrf2, nuclear factor (erythroid-derived 2)-like 2; DAPI, 4,6-diamidino-2-phenylindole.

viability, a tetrazolium salt, MTT, is cleaved to form formazan by the mitochondrial respiratory chain enzyme, succinate dehydrogenase, in living cells. In the present study, an MTT assay was performed to assess the cell viability, and the results demonstrated that following PC12 cell differentiation, pretreatment with GLGZD at 0.5 or 1 mg/ml for 24 h clearly protected the cells from damage caused by H<sub>2</sub>O<sub>2</sub>, and improved the overall cell survival rate (Fig. 2). In addition, morphological observations using inverted microscopy revealed that GLGZD treatment improved H<sub>2</sub>O<sub>2</sub>-induced cell narrowing, with the cells appearing round or polygonal, with an increased cell diopler, cell connectivity and adherent conditions (Fig. 1).

Measurement of ATP levels is a classic method for the assessment of cell mitochondrial function and condition, and the results of the present study revealed that GLGZD pretreatment in the PC12 cells significantly elevated the levels of ATP in the injury model group, indicating that GLGZD is able to reduce the extent of H<sub>2</sub>O<sub>2</sub> damage inflicted on the mitochondria in PC12 cells (Fig. 3).

Activation of the Nrf2/ARE signaling pathway has been shown to exert a protective effect on cerebral ischemia reperfusion injury, and high levels of Nrf2 and HO-1 resistance in the central nervous system are known to invoke key internal and external oxidation protection (27). Dietz *et al* reported that

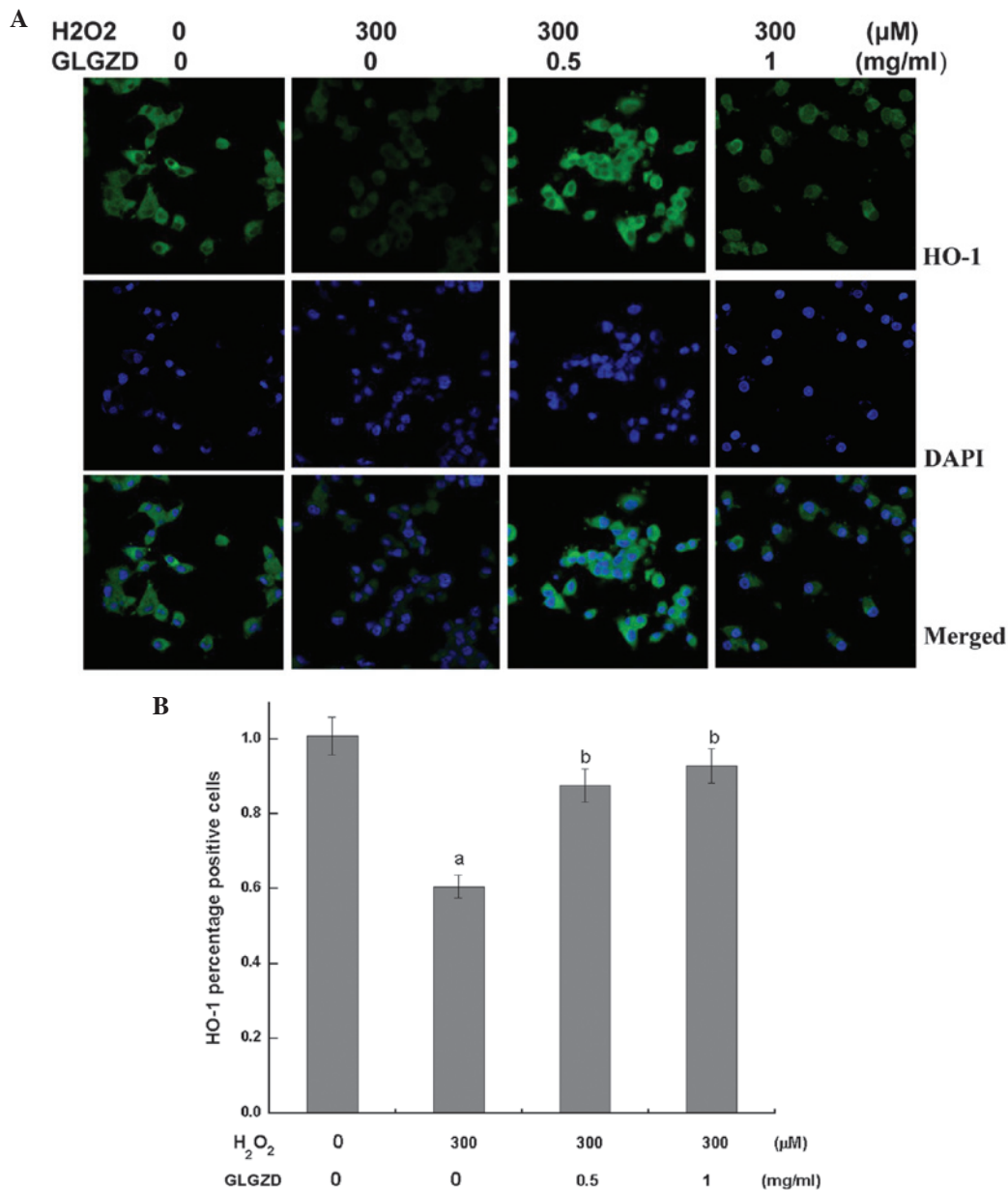


Figure 6. Representative effects of GLGZD on the expression of HO-1 protein in PC12 cells. (A) HO-1 was labeled with immunofluorescence staining (green), and nuclear counterstaining with DAPI (blue) is shown (magnification, x40). (B) Quantity of HO-1-positive neurons was expressed as the percentage of cells colocalizing the HO-1 subunit with DAPI. Data are expressed as the mean  $\pm$  standard deviation of three independent experiments performed in triplicate. \*P<0.05, vs. untreated control cells; <sup>b</sup>P<0.05, vs. cells treated with H<sub>2</sub>O<sub>2</sub> alone. GLGZD, Gua Lou Gui Zhi decoction; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HO, heme oxygenase; DAPI, 4,6-diamidino-2-phenylindole.

Nrf2, through interacting with the ARE, altered the expression of coding antioxidants and detoxifying enzymes, which subsequently protected cells against oxidative stress (28). Currently, the Nrf2/ARE pathway appears to be the most important endogenous antioxidant stress pathway (29). Nrf2 regulates the cell oxidation stress reaction of important transcription factors through interacting with oxidation reaction components, such as the ARE, which subsequently affects the coding of antioxidants, and the pathways activated can trigger the downstream expression of HO-1 and other protective genes, fully enhancing the cell protection activities (30,31). In the present study, the reverse transcription quantitative PCR results revealed that preconditioning with different concentrations of GLGZD upregulated the mRNA expression levels of Nrf2 and HO-1 in

H<sub>2</sub>O<sub>2</sub>-damaged PC12 cells (Fig. 4). In addition, the confocal laser scanning microscopy data indicated that preconditioning with GLGZD enhanced the protein expression levels of Nrf2 and HO-1 in H<sub>2</sub>O<sub>2</sub>-injured PC12 cells (Figs. 5 and 6).

In conclusion, the results of the present study demonstrated that GLGZD is able to protect PC12 cells from H<sub>2</sub>O<sub>2</sub>-induced damage, which may be associated with the observed upregulation of the gene and protein expression levels of Nrf2 and HO-1. Therefore, prompt antioxidant therapy may be a therapeutic option to target one of the important mechanisms underlying spasticity following a stroke. To the best of our knowledge, the present study has reported for the first time that GLGZD exerts a neuroprotective effect against H<sub>2</sub>O<sub>2</sub>-induced nerve cell damage. These

results provide evidence that GLGZD may be a potential therapeutic agent for the treatment of post-stroke neuronal cell death and spasticity.

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