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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Neuroprotective effects of ganoderic acid extract against epilepsy in primary hippocampal neurons

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Abstract

The purpose of this study was to investigate the effects of ganoderic acid extract (GAE) on magnesium-free induced epilepsy in primary hippocampal neurons. Neuroprotective effects of GAE were studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry as well as real time PCR (RT-PCR). GAE improved the cell viability of hippocampal neurons in the dose and time-dependent relationships, the best optimal dose is 200 μg/mL and action time 24 hours. Treatment of hippocampal neurons with GAE in the presence of magnesium-free significantly reduced the number of apoptotic cells, and decreased the expressions of Caspase-3 and PDCD5 mRNA. Collectively, our results showed that the neuroprotective potential of GAE against magnesium-free induced epilepsy in primary hippocampal neurons.

Keywords: Neuroprotective; Ganoderic acid extract; Hippocampal neurons; Epilepsy

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Introduction

Epilepsy is the most common neurologic disorder affecting people of all ages. Up to 10% of the population will suffer one seizure during their lifetimes, and around 50 million people worldwide have epilepsy (Russell et al., 2013). Experimental animals and human data have shown that certain seizures cause damage to brain (Henshall and Murphy, 2008). Neuronal damages in patients with epilepsy and in status epilepticus-induced animal models are associated with numerous apoptotic neurons. Various apoptosis-related proteins and enzymes are abnormally expressed following status epilepticus, which ultimately results in hippocampal apoptosis, such as immediate early genes, p53, Caspase and Bcl-2 families (Liou et al., 2003).

The magnesium-free model of epilepsy was developed several decades ago (Sombati and Delorenzo, 1995). The mechanism of induction and maintenance of epilepsy in the magnesium-free model of seizures has been extensively studied (Gutierrez et al., 1999; Isaev et al., 2012), and this model of epilepsy has clinical relevance as magnesium deficits can increase seizure susceptibility to stimuli or even cause seizures in humans. So now the magnesium-free model of epilepsy has been widely used as a model to test antiepileptic drugs (Albus et al., 2008).

Because conventional antiepileptic drugs often offer unsatisfactory seizure control and can have severe side effects, traditional Chinese medicine has become a hot topic in epilepsy research (Wang et al., 2005; Hijikata et al., 2006, Li et al., 2009; Wu et al., 2012). *Ganoderma lucidum* is a medicinal mushroom that has been used as a Chinese traditional folk remedy for more than 4000 years. *Ganoderma lucidum* has long been known to present a wide spectrum of biological effects including prevention of chronic diseases (Li et al., 2007), immunoregulatory (Ma et al., 2008) and anti-tumor activities (Jang et al., 2010). It has also been suggested that *Ganoderma lucidum* has neuroprotective effect on the nervous system (Zhu et al. 2005), and pre-administration of *Ganoderma lucidum* spore may have an important role to protect hippocampus demonstrated from impairment induced by pentylenetetrazol in

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epileptic rat (Zhao et al., 2008). Ganoderic acid extracted from *Ganoderma lucidum* have been reported to be responsible for many of the pharmaceutical activities of *Ganoderma lucidum* (Min et al., 2000; Dudhgaonkar et al., 2009). However, there have been no reports available about use of ganoderic acid extract (GAE) in treating neurodegenerative diseases and its neuronal effects have not been sufficiently explored.
Therefore, the objective of this study was to investigate the effects of GAE on induced epileptogenesis by culturing hippocampal neurons in magnesium-free medium. We investigated the neuroprotective effect of GAE on the cell viability and apoptosis of hippocampal neurons, expression of Caspase-3 and PDCD5 mRNA, to provide possible therapeutic applications for prevention and treatment of epilepsy.

Materials and Methods

Materials

Ganoderic acid extract (GAE) was purchased from PharmaTech (Cayman) Inc (Shanghai, China), Wistar rats were provided by the Experimental Animal Center of Jiamusi University (certification number: SYXK-2011-0004). Neurobasal medium and fetal bovine Sera (FBS) were purchased from GIBCO (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neuron specific enolase (NSE) and propidium iodide (PI) assay kit were obtained from Sigma (St. Louis, MO, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). All other common chemicals were from Sigma Aldrich (St. Louis, MO, USA).

Preparation of primary hippocampal cultures

Primary hippocampal neurons were prepared from the Wistar rat within 24 hours after birth as described previously by Mangan and Kapur (Mangan and Kapur, 2004). Hippocampi were dissected and digested with D-Hank's balanced salt solution containing 0.125% trypsin for 8 min at room temperature. Tissues were dissociated by repeated trituration. Then, the cells were seeded at a density of 3.5×10^8 cells/mL on poly- L-lysine (10μg/ml)-coated plates and maintained in growth medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% room air. After 6h, the medium was replaced with neurobasal medium with 2% B27 supplement and cultured under humidified air containing 5% CO_2 at 37°C. All experiments were performed in 9-day-old cultures. The purity of hippocampal neurons was identified by neuron specific enolase (NSE) antibody; more than 90% were used in the studies. The cell model of epilepsy was established by exposing hippocampal neurons to magnesium-free media for 3h on 9 days of culture.

Cell viability in hippocampal neurons

Hippocampal neurons were treated with magnesium-free for 3h without GAE treatment as the model group. The cell viability of GAE on hippocampal neurons was assessed by MTT method (Zhao et al., 2012). Briefly, cells were incubated on a microtiter plate in the absence or presence of various concentrations (50, 100, 150 and 200μg/mL) of GAE for eight replicates and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24h. Cells were randomly divided into four groups: control group, model group, GAE group (200μg/mL) and Sodium Valproate (SV) group(100mg/mL) (Stephen et al., 2001). Cells were incubated on a microtiter plate in GAE (200μg/mL) for eight replicates and incubated at 37°C in a humidied atmosphere of 5% CO_2 for a series of time points (12, 24, 48 and 72h). The supernatants were discarded, washed with phosphate buffered saline (PBS) twice and MTT reagent (5 mg/mL in PBS) was added to each well, after incubated in the dark for 4h, remove the supernatants, then 200μL DMSO was added and incubated for another 30min. The optical density (OD) was measured using a microplate reader (Thermo Molecular Devices Co., Union City, USA) with absorbance set at 490 nm.

Flow cytometric analysis of apoptosis

Apoptotic cells were counted by flow cytometry. Approximately 1.0×10^6 cells from each sample were collected and fixed at 4°C for 4h with cold 80% ethanol. Thereafter, the cell pellets were resuspended in 100mL of phosphatidylcholine buffer (1.611% $Na_2HPO_4.12H_2O$, 0.074% sodium citrate, pH7.8) for 15min at room temperature, and 1.0mL of propidium iodide (PI) solution (50μg of PI, 4mM of sodium citrate, 1mg/mL of RNase A and 1% of Triton X-100) was added for incubation away from light for 30 min at 37°C. Cells were stained with annexin V/ propidium iodide (PI). Apoptotic cells were detected by a flow cytometer using the FACSCalibur™ detector (Becton Dickinson, USA) at 488 nm excitation wave length. Data were analyzed with WinMDI2.9 Software. Each experiment type was repeated 3 times and no obvious deviations were observed.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from hippocampal neurons by using TRIzol reagent according to the manufacturer's protocol, and cDNA libraries were generated by reverse transcription using Oligo d (T) primers. In accord with previous report (Chen et al., 2006), the primers used for PCR amplification were as follows: The primers used for amplification of PDCD5 were 5′- TACAGATGGCACGGTATGGA -3′(forward) and 5′- CTCCTGGGTCTGTCTAAGC -3′(reverse);

Caspase-3 were 5′- AGTTGGACCCACCTTGTGAG - 3′(forward) and 5′- AGTCTGCAGCTCCTCCACAT - 3′(reverse). The PCR reaction was initiated by 2min incubation at 95°C, terminated after a 10min extension at 72°C, 40 cycles for denaturation at 94◦C for 20s, annealing at 60°C for 20s, and extension at 72°C for 30s. PCR products were separated via 1.0% agarose gel electrophoresis and visualized by ethidium bromide staining (Chen et al., 2006).

Statistical analysis

All results are expressed as mean values ± standard deviations (SDs) (n = 3). The significance of difference was calculated by one-way analysis of variance via SPSS (Release 12.1; SPSS Inc., Chicago, IL), and values P<0.05 were considered to be significant.

Results and Discussion

Effect of GAE on cell viability in hippocampal neurons

To clarify the protective effect of GAE, magnesium-free induced hippocampal neurons were evaluated using an established MTT assay method. Hippocampal neurons were pretreated with different concentrations of GAE, with the explosion of magnesium-free. In this study, magnesium-free was used to induce hippocampal neurons, with the decrease of cell viability to 42 ± 6.4%. After a further incubation for 24 h in the presence or absence of GAE, cell viability was measured by MTT assay. We found that the cell activity of GAE was in the dose-dependence relationship, using magnesium-free induced hippocampal neurons. As shown in Figure 1A, GAE at the concentration of 200μg/mL is sufficient to increased cell viability. After treatment of hippocampal neuron with the concentrations of GAE (200μg/mL) for 12, 24, 48 and 72 h, there was significant protective of cell viability observed in time-dependent relationship (Figure 1B). We choose the best action time is 24 hours and optimal dose 200 μg/mL. It was revealed that the viability of hippocampal neurons was significantly decreased (P<0.01) associated with the magnesium-free solution, while, the viability of hippocampal neurons cultured with GAE seems to be relatively increased. Taken together, these results demonstrated that GAE can alleviate the damage to hippocampal neurons in magnesium-free induced epilepsy, exhibiting a protective effect on neurons. Previous studies showed that *Ganoderma lucidum* has a protective effect against neuronal cell damage, as well as antioxidant effects (Zhou et al., 2010; Zhou et al., 2011).

Cell apoptosis analysis

Apoptosis is a normal component of the development and health of multicellular organisms and a cell-intrinsic programmed suicide mechanism that results in the controlled breakdown of the cell into apoptotic bodies (Campisi, 2003). Based on previous results of cell viability, we evaluated the rates of cell death in order to estimate the ability of GAE. Results are shown in Figures 2 and 3. Following culture with magnesium-free, the apoptotic cells were increased (0.25 ± 0.07% into 22.26 ± 0.84%), the result suggested

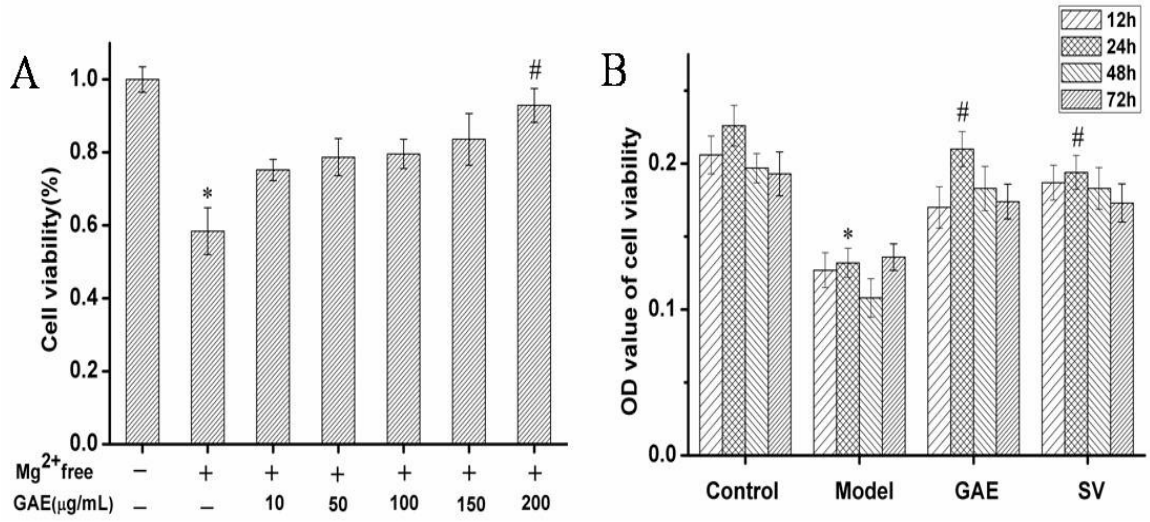

Fig. 1: Effect of ganoderic acid extract on cell viability in hippocampal neurons exposed to magnesium-free. (A) Cells were exposed to magnesium-free in the absence or presence of GAE (50, 100, 150 and 200 μg/mL) for 24h. (B) Cells were incubated in GAE (200 μg/mL) for a series of time points (12, 24, 48 and 72 h). Cell viability was assessed by MTT assay and the results are expressed as means ±SD (n=3). * P < 0.01, compared with the control group. # P < 0.01, compared with the model group.

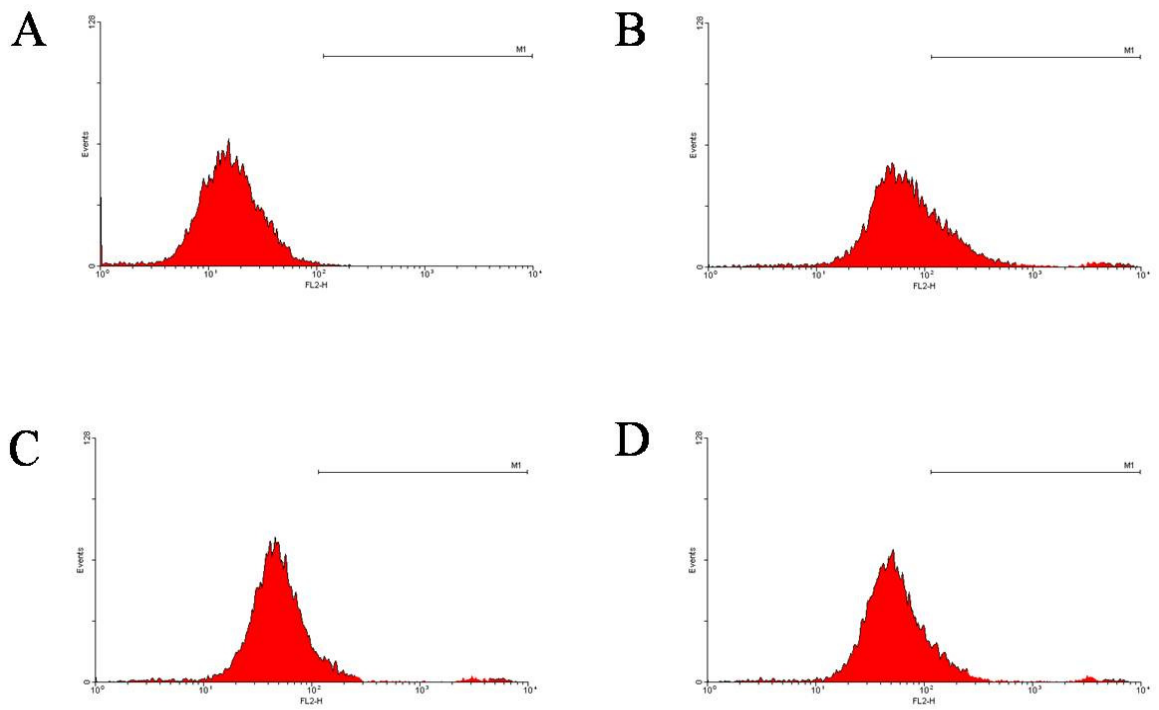

Fig. 2: Effects of GAE on the apoptosis of hippocampal neurons assayed by flow cytometry. (A) control group; (B) model group; (C) ganoderic acid extract (GAE) group; (D) sodium valproate (SV) group.

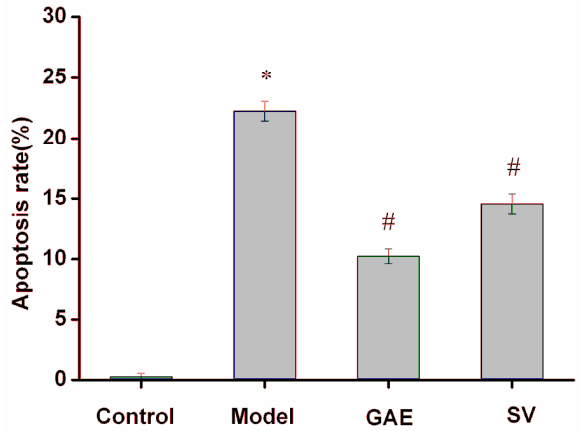

Fig. 3: Quantitative analysis of the apoptosis in hippocampal neurons. * P<0.01, compared with the control group. # P<0.01, compared with the model group

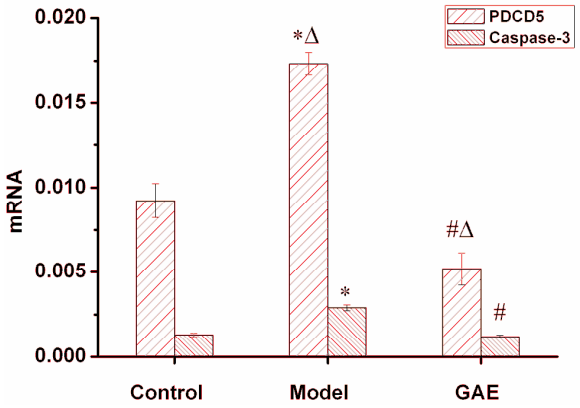

Fig. 4: The mRNA expression of Caspase-3 and PDCD5 was determined by RT-PCR. The results are expressed as means ±SD (n=3). *P<0.01, compared with the control group. #P<0.01, compared with the model group. ΔP<0.05, compared with the model group

that magnesium-free was able to induce apoptosis of hippocampal neurons. After treatment with GAE for 24h, and the apoptotic cells were decreased (10.22 ± 0.62%). Flow cytometry analysis revealed that GAE significantly inhibited cell apoptosis (P<0.01).

Effect of GAE on mRNA expressions of Caspase-3 and PDCD5

So far, it was found that apoptotic pathways are all related with the activation of the Caspase proteases, and Caspase-3 is the most important executioner of apoptosis (Kim 2005, Hsu et al. 2011); the programmed cell death 5 (PDCD5) is a novel protein related to regulation of cell apoptosis, by modulating Caspase-3 activaion (Chen et al., 2001; Xu et al., 2001). Since GAE treatment inhibited apoptosis in hippocampal neurons, we investigated whether this phenomenon was the result of the regulation of Caspases-3 and PDCD5. Caspase-3 and PDCD5 mRNAs were evaluated using

RT-PCR analysis. As shown in Figure 4, Caspase-3 and PDCD5 mRNA expressions in the model of epilepsy were increased comparison to control ($P<0.01$). Following 24 h treatment of hippocampal neurons with appropriate concentrations of GAE, Caspase-3 and PDCD5 mRNA expressions were decreased ($P<0.01$), the expression is positively correlated with each other ($P<0.05$). It indicates that the GAE may interfer with the expressions of Caspase-3 and PDCD5 mRNA, and this effect will reduce the number of apoptotic cells, consistent with the above experimental data.

Conclusion

GAE has neuroprotective effects against magnesium-free induced epilepsy in primary hippocampal neurons by multiple lines of evidence. The data revealed that GAE protected hippocampal neurons by improving cell viability, decreased the number of apoptotic cells, and decreased the expression of Caspase-3 and PDCD5 mRNA. The anti-apoptotic properties of GAE might play a major role in rendering a protective action against magnesium-free induced epilepsy in primary hippocampal neurons. This study reports for the first time that GAE could ameliorate magnesium-free induced epilepsy in hippocampal neurons and exert neuroprotective activity. Further studies on the detailed mechanism and in animal models of epilepsy should be conducted.

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