Department of Epidemiology[1], School of Public Health, Harbin Medical University, Harbin; Department of Child Development and Behavior[2], the Third Affiliated Hospital; School of Public Health[3], Jiamusi University, Jiamusi, P. R. China; School of Life Sciences[4], Institute of Biomedical and Environmental Science and Technology, University of Bedfordshire, Luton, UK

Ganoderic acid A potentiates the antioxidant effect and protection of mitochondrial membranes and reduces the apoptosis rate in primary hippocampal neurons in magnesium free medium

Z. M. Jiang[1, 2], H. B. Qiu [1, 3*], S. Q. Wang[2], J. Guo[2], Z. W. Yang[2], S. B. Zhou[4]

Received June 26, 2017, accepted September 14, 2017

**Corresponding author: Hong-Bin Qiu, 188 Xuefu Street, Jiamusi, Heilongjiang Province, P.R. China hbqiu66@yahoo.com*

Pharmazie 73: 87–91 (2018) *doi: 10.1691/ph.2018.7108*

Ganoderma lucidum extracts have shown antiepileptic effects in *in vivo and in vitro* studies. In this work, primary hippocampal neurons cultured in magnesium-free medium were used to study the neuroprotective effects of ganoderic acid A and B (GA-A and GA-B) on superoxide dismutase (SOD) activity and mitochondrial membrane potential, to improve our understanding of their antiepileptic effect. The activity of SOD was determined by the xanthine oxidase assay, the variations of mitochondrial membrane potential and cell apoptosis were measured by JC-1 fluorescent staining and flow cytometry. It was found that the SOD activity and mitochondrial membrane potential (118.84 U/mg protein and 244.08 $\Delta\psi_m$) of the epileptic hippocampal neurons were significantly lower than control values (135.95 U/mg protein and 409.81 $\Delta\psi_m$), associated with an increase of cell apoptosis (31.88% vs. 8.84%). These circumstances can be improved by treatment of GA-A/GA-B (for SOD, 127.15±3.82 / 120.52±4.30 U/mg protein; for membrane potential ($\Delta\psi_m$), 372.35 / 347.28; and for cell apoptosis (%), 14.93 / 20.52). Results indicated that GA-A significantly improved SOD activity, while both GA-A/GA-B tranquillized the mitochondrial membrane potential of hippocampal neurons, and thereby protected these neurons by inhibiting apoptosis.

1. Introduction

Epilepsy is one of the most common neurological diseases affecting people of all ages (Silberberg et al. 2015). Most epileptic cases arise from excessive and abnormal synchronization of the brain, with recurrent seizures (Russell et al. 2013). The disease is usually controlled by daily anti-epileptic drugs (Mintzer et al. 2015). Unfortunately, more than 1/3 patients are not effectively controlled by the anti-epileptic drugs available (Perucca et al. 2007), which may exhibit side effects e.g. other nervous system problems (Perucca et al. 2013). Further, these drugs can only control the seizures, with no effect on pathological changes and the process of epilepsy. They have spurred the development of medications towards a better treatment and minimum, or less side effects.

Certain seizures cause damage to the hippocampal neurons in experimental animals and humans (Henshall et al. 2016). These are associated with an increase in free radical generation which may result in mitochondrial dysfunction and numerous apoptotic processes (Waldbaum and Patel 2010; Zsurka and Kunz 2015). Various apoptosis related proteins or cytoplasmic organoids have been found to be the causes of hippocampal apoptosis, such as, decreasing of the superoxide dismutase (SOD) activity and damage of mitochondria (Waldbaum and Patel 2010; Zsurka and Kunz 2015). SOD, a key enzyme in converting the dismutation of superoxide radicals into hydrogen peroxide, is an important antioxidant in nearly all cells exposed to oxygen. Its activity will be decreased dramatically due to a large quantity of SOD consumed during free radical scavenging. Therefore, SOD level reflects the antioxidant capacity of cells as well as indirectly reflect the hippocampal neuronal damage (Zsurka and Kunz 2015). In various epilepsy models, the hippocampal damage usually has the typical features of apoptosis, with the cell biological function reduced by oxidative stress (Henshall 2007). Results did indicate that lower SOD activity was found in the damaged hippocampal neurons in patients with status epilepticus (Henshall and Murphy 2008). Furthermore, recent studies have revealed that mitochondria are the primary site of reactive oxygen species making them uniquely vulnerable to oxidative damage in the hippocampus that consequently may affect neuronal excitability and seizure susceptibility (Waldbaum and Patel 2010). In a sense, the role of mitochondrial dysfunction, e.g. the damage of the energy supply system, and oxidative stress, are known to incite chronic epilepsy as well as precede neuronal cell death (Waldbaum and Patel 2010; Zsurka and Kunz 2015).

Ganoderma lucidum is one of the precious medicinal fungi. *Ganoderma lucidum* spores could decrease hippocampal epileptiform activities, via attenuating the apoptosis induced by epilepsy (Wang et al. 2013, 2014). Ganoderic acids (GAs) are the major chemical constituents of *Ganoderma lucidum* spores, and responsible for many pharmacological effects (e.g. immunomodulation and antioxidant protective system) (Koyama et al. 1997; Liu et al. 2015). Our previous studies showed neuroprotective effects of raw GAs extracts in epileptiform primary hippocampal neurons (Liu et al. 2013; Yang et al. 2016). Other GAs, e.g. GA-T1, T2 or GA-MK, also targeted on the antioxidant defense system and induced apoptosis in HeLa cervical cancer cells (Liu et al. 2015). However, among dozens of isolated GAs, ganoderic acid A and B are the best characterized. There is no report on their neuroprotective effect, as well as the correlative effects on SOD and the mitochondrial membrane.

Epileptic animal models have been frequently used to test antiepileptic drugs and explore their mechanism of action (Raol and Brooks-Kayal 2012; Phulen and Bhattacharyya 2014). An in vitro model of epileptic hippocampal neurons, the magnesium-free cell culture model of epilepsy, has been developed decades ago (Sombati and Delorenzo 1995; Mangan and Kapur 2004) and has widely been used in epileptic research (Churn et al. 2000; Liu et al. 2013; Wang et al. 2014), with the clinical relevance that magnesium deficits can increase seizure susceptibility to stimuli or even cause seizures in humans (Yang et al. 2016). Neurons cultured in a medium without Mg^{2+} for 3 h generate a neuronal firing frequency of 5-17 Hz, and more than 90% of the neurons

continue to undergo spontaneous epileptiform discharges up to 24 h. This in vitro epileptic model has been widely used for investigation of the biochemistry, electrophysiology and molecular biology of the changes that occur under experimental conditions (Mangan and Kapur 2004; Avoli et al. 2002). Therefore, this model was used to study the effects of GA-A and GA-B on SOD activity and mitochondrial membrane potential of epileptiform hippocampal neurons, by xanthine oxidase assay and JC-1 fluorescent staining flow cytometry. These results will be valuable to the understanding on the anti-epileptic mechanism of GAs, and have the potential to open up new therapeutic approaches for epilepsy.

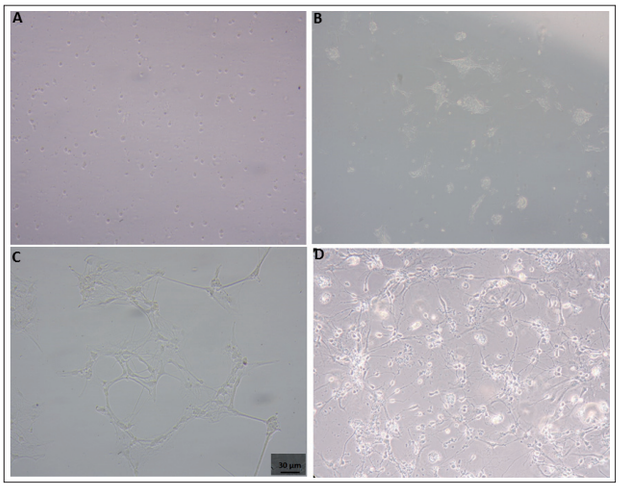

Fig. 1: Hippocampal neurons (x200) cultured at 24 h, the cells were adhered with spindle-shape or irregularly shape (A); day 3, the cell connected network of synapse (B); day 5, the neurons were bigger and obvious and long (C); and day 9, the neurons were mature and unevenly distributed (D).

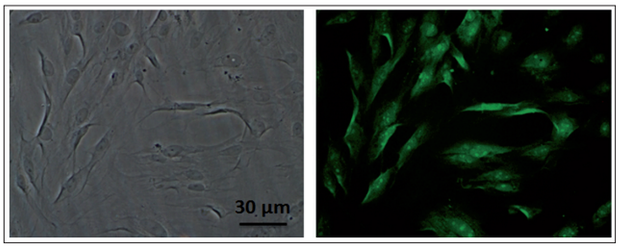

Fig. 2: Immunofluorescence stain of NSE in hippocampal neurons (×400). Cytoplasm neurites stained green, the mature bodies were plump, triangular or round, at the same time, their neurites were dense, thick and interweaved into a network. Cytoplasm and neurites stained green, revealing the presence of NSE. Conversely, the nuclei were colourless.

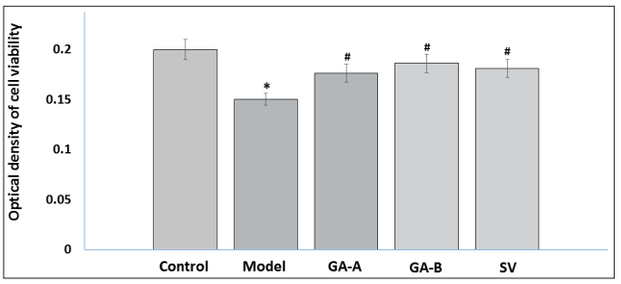

Fig. 4: Cells were incubated with ganoderic acids A and B (GA-A and GA-B, 50 μg/mL) for 24 h. Sodium valproate (SV, 100 mg/mL) is used as a positive contrast. Cell viability was assessed by MTT assay and the results are expressed as means ± SD (n=5). OD is absorbance. * $P < 0.01$, compared with the control group. # $P < 0.01$, compared with the model group.

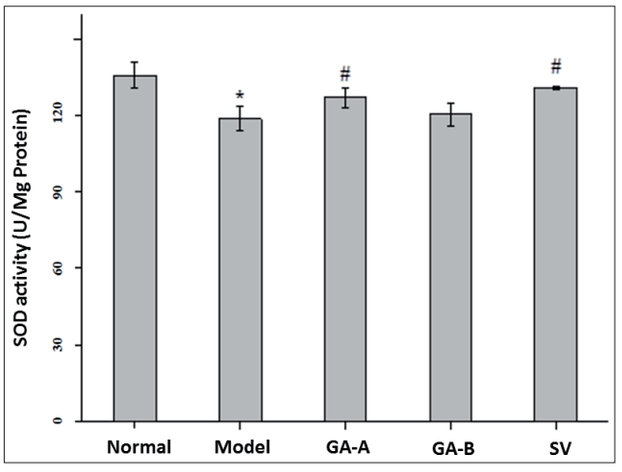

Fig. 5: SOD activities of compounds compared with sodium valproate (SV). Values of each curve are means ± SD (n = 5). * $P < 0.01$, compared with the control group. # $P < 0.01$, compared with the model group.

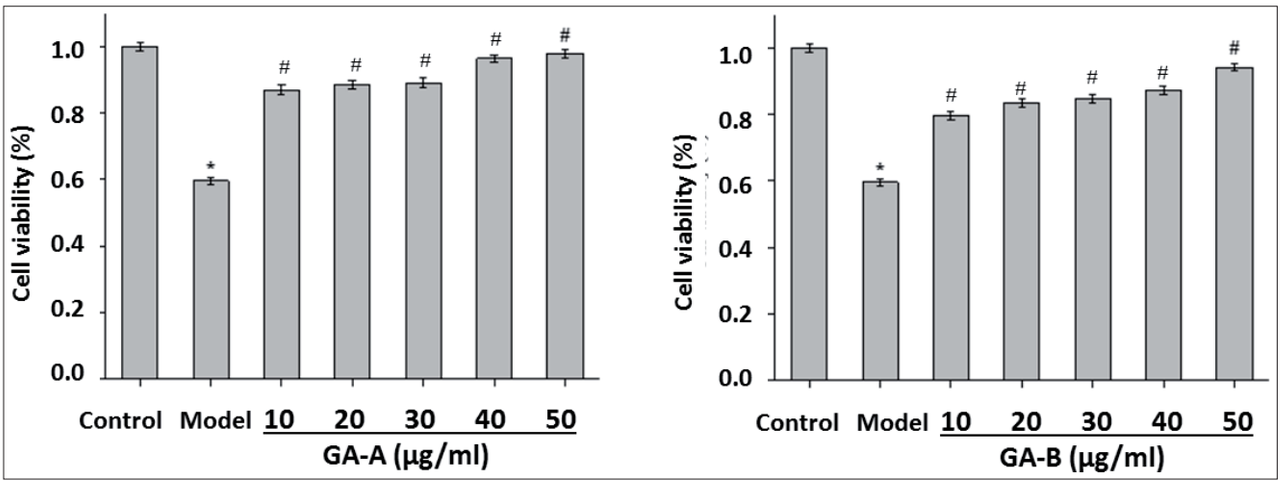

Fig. 3: Cells were cultured in a magnesium-free medium in the absence or presence of ganoderic acids A and B (GA-A and GA-B) (10, 20, 30, 40, 50 μg/ml) for 24h. Cell viability was assessed by MTT assay and the results are expressed as means ± SD (n=5). * $P < 0.01$, compared with the control group. # $P < 0.01$, compared with the model group.

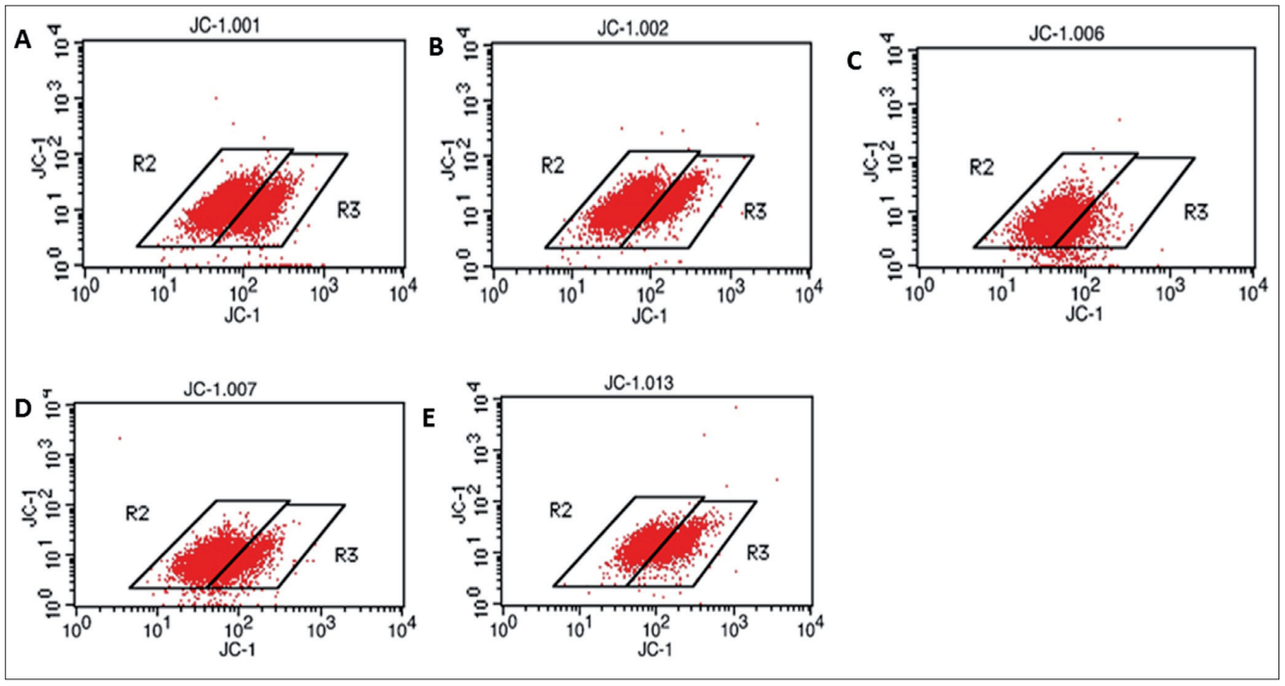

Fig. 5: SOD activities of compounds compared with sodium valproate (SV). Values of each curve are means ± SD (n = 5). * P < 0.01, compared with the control group. # P < 0.01, compared with the model group.

2. Investigations, results and discussion

2.1. Morphology observation

Cultured hippocampal neurons were observed at 24 h, 3 days, 5 days, and 9 days. The cells were adhered with spindle-shape or irregularly shape synapses at 24 h (Fig. 1A). Then the cell number increased, with the connected network of synapse at day 3 (Fig. 1B); the neurons were bigger and obvious, the neurites were dense, thick and long at day 5 (Fig. 1C). The neurons were mature and unevenly distributed at day 9 (Fig. 1D).

2.2. Effect on cell viability

Hippocampal neurons were detected by NSE immunofluorescence staining. It was found that the mature bodies were plump, triangular or round, at the same time, their neurites were dense, thick and interweaved into a network (Fig. 2). Cytoplasm and neurites stained green, revealing the presence of NSE. Conversely, the nuclei were colourless. On day 9, the hippocampal neurons differentiated completely, and the purity of hippocampal neurons was higher than 96%.

To clarify the protective effect of GA-A and GA-B, hippocampal neurons cultured in magnesium-free medium were evaluated using an established MTT assay method. In this work, magnesium-free medium was used to induce hippocampal neurons, with a decrease of cell viability to 40.5±2.2% (P < 0.01) (Fig. 3). After a further incubation of 24 h in the presence or absence of GA-A and GA-B (10, 20, 30, 40, 50 µg/ml), we found that the cell activities of hippocampal neurons with GA-A and GA-B were increased with dosages, using magnesium-free induced hippocampal neurons. As shown in Fig. 3, GA-A and GA-B at all concentrations (10-50 µg/ml) increased cell viability. The viability of hippocampal neurons cultured with added GA-A and GA-B were increased significantly at 24 h (Fig. 4) of incubation, and equal to the first-line antiepileptic drug of sodium valproate, compared to other time points (data not shown). Thus, an action time of 24 h and a dose of 50 µg/ml were adopted for the subsequent experiments. Taken together, these results demonstrated that GA-A and GA-B can alleviate the damage to hippocampal neurons cultured in a magnesium-free environment, exhibiting a protective effect on neurons. It is consistent with previous experiments that *Ganoderma lucidum* (Wang et al. 2013, 2014; Zhou et al. 2010, 2012) and our raw GA extracts (Liu et al. 2013; Yang et al. 2016) have a neuroprotective effect in hippocampal neurons, with a significant correlation between antioxidant effects.

2.3. Effect on the SOD activity

As described in the introduction, GA-A and GA-B from *Ganoderma lucidum* are both anticipated to prevent damage to hippocampal neurons, because of their chemical structure and pharmacological properties (Shaio 2003; Boh et al. 2007). However, its neuronal effects have not been sufficiently explored. In recent years, it was found that SOD activity could reflect the damage of hippocampal neurons (Henshall and Murphy 2008; Chen et al. 2009). In this regard, the xanthine oxidase assay was utilized to provide comprehensive analysis effects of GA-A and GA-B on SOD activity, in order to understand the protective effect of GA-A and GA-B on epileptiform hippocampal neurons. The experimental results of each group are summarized in Fig. 5. In contrast to the control group, SOD activity was significantly decreased in epileptiform hippocampal neurons, from 135.95±5.30 to 118.84±4.70 U/mg protein. The difference was significant (P < 0.01). With the treatment of GA-A, the SOD activity of hippocampal neurons (127.15±3.82 U/mg protein) was significantly higher than that of the epileptic cell model, showing a significant difference (P < 0.01), but, not in the treatment of GA-B (120.52±4.30 U/mg protein). The change in SOD activity does not appear to be that marked, this might be caused by not excluding the dead cells, whose mitochondria may still affect artifactually SOD determination. Consequently SOD levels might be increased compared to present results if the dead cells were removed. The correlation coefficient was positive, which is 0.9314 between SOD activity and cell viability in current condition. In contrast, the SOD activity of hippocampal neurons with sodium valproate was greatly changed, which was 130.94±0.49 U/mg protein. Thus, GA-A, but not GA-B might significantly improve the SOD activity of epileptiform discharge hippocampal neurons.

2.4. Effect on the mitochondrial membrane potential

It was found that the role of mitochondria in programmed cell death came into the spotlight after the discovery of localized anti-apoptotic

factors' mainly on the mitochondrial membrane (Zhou et al. 2010). The participation of mitochondria in apoptosis has also been substantiated by a large number of reports describing proapoptotic mitochondrial alterations, such as the production of reactive oxygen species (Zhou et al. 2012). Our results revealed that the mitochondrial membrane potential of the epileptiform hippocampal neurons (model group) was significantly lower than that of the control group ($\Delta\psi_m$, 244.08±23.61 vs. 409.81±34.21), associated with apoptosis rate increasing significantly (31.88±1.05 vs. 8.84±0.74) (Table and Fig. 6). The difference had significant difference ($P < 0.01$). With the treatment of GA-A / GA-B, the value of mitochondrial membrane potential was increased to 372.35±22.37 / 347.28±25.13, respectively; and the apoptosis rate was significantly reduced (14.93±1.72 / 20.52±1.55). There were significant differences between the two groups and the model group ($P < 0.01$). This indicated that GA-A and GA-B can regulate and improve the function of mitochondria, tranquillize the mitochondrial membrane potential, and then restraint the apoptosis of epileptiform hippocampal neurons. Several studies have already shown that the importance of preservation of the mitochondrial membrane potential is critical to mitochondrial events of apoptosis (Lakhani et al. 2006). However, ganoderic acids Mf and S induce mitochondria mediated apoptosis via decreasing the mitochondrial membrane potential in human cervical carcinoma HeLa cells (Liu and Zhong 2006). Status epilepticus results in oxidative damage involving calcium overload and induction of apoptosis (Waldbaum and Patel 2010; Zsurka and Kunz 2015). Our previous studies did show that extracts of *Ganoderma lucidum* reduce the calcium overload in epileptic neurons (Wang et al. 2013). Subsequently, it would be worth to test the effect of GAs on the calcium turnover, which not only affect mitochondrial membrane potential but also apoptosis.

Table: Mitochondrial membrane potential (MMP, $\Delta\psi_m$) and apoptosis rate of each group

Group	MMP ($\Delta\psi_m$)	Apoptosis rate (%)
Control	409.81±34.21	8.84±0.74
Model	244.08±23.61*	31.88±1.05*
GA-A	372.35±22.37#	14.93±1.72#
GA-B	347.28±25.13#	20.52±1.55#
Sodium valproate	384.19±25.32#	47.13±2.01#

Note: * $P < 0.01$, compared with the control group. # $P < 0.01$, compared with the model group.

In summary, experimental data from animal and human studies have shown that certain seizures cause damage to the hippocampal neurons, associated with numerous apoptotic cells. Various apoptosis-related proteins and/cytoplasmic organoids have been found to be the cause of hippocampal apoptosis. The mitochondrial damage in the epileptiform hippocampal neurons may be caused by lipid peroxidation induced by oxygen free radicals. Our experiments indicated that ganoderic acids regulated mitochondrial lipid peroxidation and stabilized the mitochondrial membrane potential, to maintain the normal structure of mitochondria. In addition, the apoptosis rate has a close relationship with SOD activity and mitochondrial membrane potential, which indicated that the mitochondrial pathway is an important pathway to the apoptosis of epileptiform hippocampal neurons. Altogether, ganoderic acid A can significantly improve SOD activity, and both ganoderic acid A and B stabilize the mitochondrial membrane potential in hippocampal neurons, and thereby protect the hippocampal neurons by inhibiting apoptosis.

3. Experimental

3.1. Animals and materials

Newborn *Wistar* rats (24 hours old) were provided by the Experimental Animal Center of Jiamusi University, whose Ethical Committee also provided guideline and approval. The rats were handled in compliance with the principles of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH, 1986). All efforts were made to minimize both the number of animals used and any suffering that they might experience. GA-A and B (HPLC grade) were provided from WuXi App Tec Inc. (Shanghai, China). Mitochondrial membrane potential assay kit with JC-1 and SOD activity assay kit were obtained from the Beyotime Institute of Biotechnology (Jiangsu, China). Neurobasal medium, B27 supplement and fetal bovine sera (FBS) were purchased from GIBCO (Grand Island, NY, USA). Neuron specific enolase (NSE) assay kit was obtained from Boster Biological Technology Ltd. (Wuhan, China). All other general chemicals were bought from Sigma (St. Louis, MO, USA).

3.2. Cell culture

In accordance with our previous work (Wang et al. 2013, 2014; Liu et al. 2013; Yang 2016), all surgery was performed under sodium pentobarbital anesthesia. Primary hippocampal neurons were harvested from the newborn *Wistar* rats. Setup details were in accordance with our previous studies (Wang et al. 2013, 2014; Liu et al. 2013; Yang 2016). Briefly, hippocampal tissues were harvested under sterile conditions. They were collected into D-Hanks solution and washed three times at 4 °C. Under a microscope, they were cut into 1 mm^3 pieces and then they were incubated with 0.125% trypsin solution of 5 times volume of hippocampal tissues in a 37 °C incubator (containing 5% CO_2), with gentle shaking of the preparation every 5 min. 20 min later, an equivalent volume of maintaining medium [Neurobasal medium (Cat. No. 21103049, Gibco), 2% B27 supplement (Cat. No. 17504044, Gibco) and 0.5 mmol/L glutamine] was added and the preparation incubated for a further 5 minutes to stop the trypsin digestion. The cells were then centrifuged at 1000 rpm for 5 minutes. Supernatant was removed and maintaining medium added to the cells which were then filtered through a 200 mM mesh. The filtered solution with cells was adjusted to a 5×10^5/ml cell suspension by using maintaining medium. Cells (of density 5 x 10^5/ml) were respectively transferred into a 25 cm^3 culture plate, each well of 6- and 96-well plates, and incubated in a 5 % CO_2 incubator (37 °C). When the cultured cells grew against the wall of culture plate well, the whole culture medium (neurobasal medium, 2% B27 supplement, 0.5 mmol/L glutamine and 10% FBS) was replaced by a nutrient maintaining medium. Half the culture plate medium volume was replaced every other day. Images of cultured hippocampal neurons were recorded at 24 h, 3, 5 and 9 days respectively as previous studies (Wang et al. 2013, 2014; Liu et al. 2013; Yang 2016). Hippocampal neurons cultured for 9 days were used and identified by detection of NSE with antibodies (Wang et al. 2013, 2014). When the purity of the neurons was up to 96%, the cells were used for further assessment. Epileptic model of hippocampal neurons was set up using a conventional method (Wang et al. 2013, 2014). Briefly, hippocampal neurons were cultured in nutrient maintaining medium. At day 9, the nutrient maintaining medium was replaced with extracellular medium without Mg^{2+} (145 mmol NaCl, 2.5 mmol KCl, 2 mmol $CaCl_2$, 10 mmol HEPES, 10 mmol glucose, 0.002 mmol glycine, pH 7.2, 290610 mOsm) and treated for 3 h. Then, the normal extracellular culture medium (145 mmol NaCl, 2.5 mmol KCl, 2 mmol $CaCl_2$, 1 mmol $MgCl_2$, 10 mmol HEPES, 10 mmol glucose, 0.002 mmol glycine, pH 7.2, 290610 mOsm) was replaced and the cells incubated for a further 3 h.

3.3. Cell viability in hippocampal neurons

Epileptic hippocampal neurons were treated as the model group. The cell viabilities of GA-A and GA-B on hippocampal neurons were assessed by the MTT method (Guo et al. 2013). Briefly, cells were plated in the 96-well culture plates in the absence, or presence of various concentrations (10, 20, 30, 40, 50 μg/ml, five wells per concentration) of GA-A, or GA-B respectively, incubated at 37 °C, with 5% CO_2, with continuous observation for 24 h (Wang et al. 2013, 2014). Then, neurons were randomly divided into five groups: 1) normal hippocampal neurons (control group); 2) epileptic hippocampal neurons group (model group, see details in section 2.2); 3)/4) GA-A/GA-B groups, cells were treated with Mg^{2+} free extracellular medium for 3 h, then cultured with normal medium containing GA-A/GA-B (50 μg/ml); 5) Sodium valproate (SV) group, cells were treated with Mg^{2+} free extracellular medium for 3 h, then cultured with normal medium containing sodium valproate (a first-line drug for epilepsy, 100 mg/mL). The time dependencies of agents were observed at 12, 24, 48 and 72 h, respectively. MTT solution (5 mg/mL) was then added to each well. After 4 h incubation, the formazan precipitate was dissolved in 200 μL dimethyl sulfoxide, and then the absorbance was measured at 490 nm in a microplate reader (Thermo Molecular Devices Co., Union City, CA, USA). All assays were repeated three times.

3.4. Determination of SOD activity

Measurement of the effects of GA-A or GA-B on SOD activity was performed using the xanthine oxidase assay according to the manufacturer's directions. Briefly, cells were digested and centrifuged at 1000 x g for 10 min at 4 °C. The supernatants were discarded, washed with phosphate buffered saline (PBS, pH=7.4) twice, lysed cells in ice cold 0.1 M Tris/HCl, pH 7.4 containing 0.5 % Triton X-100, 5 mM β-ME, 0.1 mg/ml PMSF. The crude cell lysate was centrifuged at 14 000 g for 5 min at 4 °C and the cell debris was discarded. The absorbance was measured at 550 nm using a microplate reader (Thermo Molecular Devices Co., Union City, USA).

3.5. Flow cytometric analysis

Mitochondrial membrane potential ($\Delta\psi_m$) and apoptosis were counted by flow cytometry. Approximately 1.0×10^6 cells from each sample were collected. After indicated treatments (Sombati and Delorenzo 1995), cells were incubated with JC-1 staining solution (10 μg/ml) for 20 min at 37 °C in the dark. JC-1 is capable of selectively entering mitochondria and emits green/red fluorescence when $\Delta\psi_m$ is relatively low/high. For apoptosis, 1.0 ml of propidium iodide solution (50 μg of propidium iodide, 4 mM of sodium citrate, 1 mg/ml of RNase A and 1% of Triton X-100) was added to the incubating cells away from light for 30 min at 37 °C. The fluorescence of separated cells was detected with a flow cytometer using the FACSCalibur™ detector (Becton

Dickinson, USA) at 488 nm excitation wave length. Apoptotic cells were analyzed with a FACSCalibur® flow cytometer (BD Biosciences, Burlington, MA, USA) (Rao and Brooks-Kayal 2012). Data was analyzed with WinMDI2.9 software. Each assay was repeated 3 times and no obvious deviations were observed.

3.6. Images and statistical analysis

Images of hippocampal neurons were taken by the confocal laser scanning (CLS) method (Wang et al. 2013), and Image-Pro Plus software (Media Cybernetics, Silver Spring, MA, USA) was used for image analysis (Wang et al. 2013). Data was expressed as mean values±standard deviations (SD) (n = 5). The significance of difference was calculated by one-way analysis of variance via SPSS software (Release 12.1; SPSS Inc., Chicago, IL), and values $p < 0.05$ were considered to be significant.

Acknowledgement: Authors thank Mr. Patrick Kelly (University of Bedfordshire, UK) for proofreading and comments.

Conflicts of interest: None declared

References

Avoli M, D'Antuono M, Louvel J, Kohling R, Biagini G (2002) Network and pharmacological mechanisms leading to epileptiform synchronization in the limbic system in vitro. Prog Neurobiol 68: 167–207.

Boh B, Berovic M, Zhang J, Zhi-Bin L (2007) Ganoderma lucidum and its pharmaceutically active compounds. Biotechnol Annu Rev 13: 265-301.

Chen K, Zhang Q, Wang J, Liu F, Mi M, Xu H (2009) Taurine protects transformed rat retinal ganglion cells from hypoxia-induced apoptosis by preventing mitochondrial dysfunction. Brain Res 1279: 131-138.

Churn SB, Sombati S, Jakoi ER (2000) Inhibition of calcium/calmodulin kinase II alpha subunit expression results in epileptiform activity in cultured hippocampal neurons. Proc Natl Acad Sci USA 97: 5604–5609.

Guo X, Shen X, Long J, Han J, Che Q (2013) Structural identification of the metabolites of ganoderic acid B from Ganoderma lucidum in rats based on liquid chromatography coupled with electrospray ionization hybrid ion trap and time-of-flight mass spectrometry. Biomed Chromatogr 27: 1177-1187.

Henshall DC, Hamer HM, Pasterkamp RJ, Goldstein DB, Kjems J, Prehn JH (2016) MicroRNAs in epilepsy: pathophysiology and clinical utility. Lancet Neurol 15: 1368-1376.

Henshall DC, Murphy BM (2008) Modulators of neuronal cell death in epilepsy. Curr Opin Pharmacol 8: 75-81.

Henshall DC (2007) Apoptosis signalling pathways in seizure-induced neuronal death and epilepsy. Biochem Soc Trans 35: 421-423.

Koyama K, Imaizumi T, Akiba M, Kinoshita K, Takahashi K, Suzuki A (1997) Antinociceptive components of Ganoderma lucidum. Planta Med 63: 224-227.

Lakhani SA, Masud A, Kuida K, Porter GA,Jr, Booth CJ, Mehal WZ, Inayat I. Flarell RA (2006) Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science 311: 847-851.

Liu JX, Liu XG, Wang L, Wu F, Yang ZW (2013) Neuroprotective effects of ganoderic acid extract against epilepsy in primary hippocampal neurons. Res Opin Anim Vet Sci 13: 420-425.

Liu RM, Li YB, Liang XF, Liu HZ, Xiao JH, Zhong JJ (2015) Structurally related ganoderic acids induce apoptosis in human cervical cancer HeLa cells: Involvement of oxidative stress and antioxidant protective system. Chem Biol Interact 240: 134-144.

Liu RM, Zhong JJ (2012) Ganoderic acid Mf and S induce mitochondria mediated apoptosis in human cervical carcinoma HeLa cells. Phytomedicine 19:569.

Loscher W, Klitgaard H, Twyman RE, Schmidt D (2013) New avenues for anti-epileptic drug discovery and development. Nat Rev Drug Discov 12: 757-776.

Mangan PS, Kapur J (2004) Factors underlying bursting behavior in a network of cultured hippocampal neurons exposed to zero magnesium. J Neurophysiol 91: 946–957.

Mintzer S, French JA, Perucca E, Cramer JA, Messenheimer JA, Blum DE (2015) Is a separate monotherapy indication warranted for antiepileptic drugs? Lancet Neurol 14: 1229-1240.

NIH Publications No. 80–23, revised 1986

Perucca E, French J, Bialer M (2007) Development of new antiepileptic drugs: challenges, incentives, and recent advances. Lancet Neurol 6: 793-804.

Phulen S, Bhattacharyya A (2014) Models of epilepsy used in antiepileptic drug discovery: a review. Int J Pharm Pharm Sci 6: 1-7.

Rao YH, Brooks-Kayal AR (2012) Experimental models of seizures and epilepsies. Prog Mol Biol Transl Sci 105: 57-82.

Russell JF, Fu YH, Ptacek LJ (2013) Episodic neurologic disorders: syndromes, genes, and mechanisms. Annu Rev Neurosci 36: 25-50.

Shiao MS (2003) Natural products of the medicinal fungus Ganoderma lucidum: occurrence, biological activities, and pharmacological functions. Chem Rec 3: 172-180.

Silberberg D, Anand NP, Michels K, Kalaria RN (2015) Brain and other nervous system disorders across the lifespan - global challenges and opportunities. Nature 527: S151-4.

Sombati S, Delorenzo RJ (1995) Recurrent spontaneous seizure activity in hippocampal neuronal networks in culture. J Neurophysiol 73: 1706-1711.

Waldbaum S, Patel M (2010) Mitochondrial dysfunction and oxidative stress: a contributing link to acquired epilepsy? J Bioenerg Biomembr 42: 449-455.

Wang SQ, Li XJ, Qiu HB, Jiang ZM, Simon M, Ma XR (2014) Anti-epileptic effect of Ganoderma lucidum polysaccharides by inhibition of intracellular calcium accumulation and stimulation of expression of CaMKII alpha in epileptic hippocampal neurons. PLoS One 9: e102161.

Wang SQ, Li XJ, Zhou S, Sun DX, Wang H, Cheng PF (2013) Intervention effects of ganoderma lucidum spores on epileptiform discharge hippocampal neurons and expression of neurotrophin-4 and N-cadherin. PLoS One 8: e61687.

Yang ZW, Wu F, Zhang SL (2016) Effects of ganoderic acids on epileptiform discharge hippocampal neurons: insights from alterations of BDNF,TRPC3 and apoptosis. Pharmazie 71: 340-344.

Zhou Y, Qu ZQ, Zeng YS, Lin YK, Li Y, Chung P (2012) Neuroprotective effect of preadministration with Ganoderma lucidum spore on rat hippocampus. Exp Toxicol Pathol 64: 673-680.

Zhou ZY, Tang YP, Xiang J, Wua P, Jin HM, Wang Z (2010) Neuroprotective effects of water-soluble Ganoderma lucidum polysaccharides on cerebral ischemic injury in rats. J Ethnopharmacol 131: 154-164.

Zsurka G, Kunz WS (2015) Mitochondrial dysfunction and seizures: the neuronal energy crisis. Lancet Neurol 14: 956-966.