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Effects of Ganoderma Acid Extract on the SOD and Mitochondrion in Zero-Mg^{2+} Induced Epileptic Hippocampal Neurons

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Ganoderma lucidum **has been used as a traditional medicinal herb for centuries in China, and its acid extract (ganoderic acid extract, GAE) has shown some antiepileptic effects in the animal models. This was the study of the effects of GAE on the superoxide dismutase (SOD) and mitochondria in cultured primary hippocampal neurons, treated by the Mg^{2+} free medium, in order to aid our understanding of interactions involving GAE with the epileptiform discharge hippocampal neurons and warrant the experimental aspects for the novel anti-epilepsy agents. The SOD vigour was determined by the xanthine oxidase assay, the variations of mitochondrial membrane potential and cell apoptosis were measured by the JC-1 fluorescent staining and flow cytometry. Our results revealed that the SOD activity and mitochondrial membrane potential (117.68 U/mg pro and 244.08) of the epileptiform discharge hippocampal neurons were significantly lower than the normal ones (135.95 U/mg pro and 409.81), associated with the obvious increase of cell apoptosis (61.39 vs. 28.84). These circumstances can be reversed, with the treatment of GAE (123.86 U/mg pro, 386.92 and 14.37). It indicated that GAE can significantly improve the SOD activity and mitochondrial dysfunction, stabilize the mitochondrial membrane potential, and thereby protect the hippocampal neurons by inhibiting the apoptosis.**

Key words: Ganoderic acid extract; Epileptiform hippocampal neurons; SOD; Mitochondrial membrane potential; Cell apoptosis.

Epilepsy, a condition caused by disorderly discharging of cerebral neurons, is the most common transient dysfunctions of the brain affecting people of all ages[1]. Up to 10% of the population will suffer one seizure during their lifetimes, and around 50 million people worldwide have epilepsy[2]. The disease is usually controlled, but can not be cured with drugs, although surgery may be considered in some cases. Experimental animals and human data have shown that certain seizures cause damage to the hippocampal neurons, associated with numerous apoptotic cells[3]. Various apoptosis-related proteins and / cytoplasmic organoids have been found to be the cause of hippocampal apoptosis, such as the superoxide dismutase (SOD) & mitochondrion[4-6].

Superoxide dismutase (SOD, EC 1.15.1.1) has been identified as an important antioxidant defense in nearly all cells exposed to oxygen, which could catalyze the dismutation of superoxide (O^{2-}) into oxygen and hydrogen peroxide[7]. In term of oxidative stress, the function level of SOD reflects the antioxidant capacity of cells, which playing a vital role to keep the balance of oxidation and antioxidant[8]. In various epilepsy models, the

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hippocampal damage usually has the typical features of apoptosis, with the cell biological function reducing by the oxidative stress[9]. It indicated that the SOD activity could indirectly reflect the damage of hippocampal neurons in patients with status epilepticus[4-6]. Recent studies have revealed that mitochondria play a central role in many metabolic tasks, such as signaling through mitochondrial reactive oxygen species, apoptosis-programmed cell death, also play an important role in the apoptosis[10]. In a sense, the mitochondria can determine the cell survival and death. Mitochondrial dysfunction, the damage of energy supply system and the release of membrane inclusions (cytochrome C, caspase-3, et. ac.) seem the starting of apoptotic cascade, and then lead to the neuronal apoptosis[4-6].

The potential for unsatisfactory seizure control and severe side effects of conventional antiepileptic drugs has remained an important concern to both clinicians and patients over the years. For the development of more effective and less toxic agents, herbal remedies have become a hot topic in the design of anti-epilepsy drugs[11-14]. The fungus *Ganoderma lucidum* has been used as a Chinese traditional folk for centuries. Its fruiting body has neuroprotective effect on the nervous system[15-18], and pre-administration of *Ganoderma lucidum* spore can protect hippocampus demonstrated from impairment induced by pentylenetetrazol in epileptic rat[19, 20]. Ganoderic acid extract (GAE) has been reported to be responsible for many of the pharmaceutical activities of *Ganoderma lucidum*[21, 22]. However, there have been fewer reports about use of GAE in treating neurodegenerative diseases and its neuronal effects have not been sufficiently explored[23].

The magnesium-free model of epilepsy was developed several decades ago, with the clinical relevance that magnesium deficits can increase seizure susceptibility to stimuli or even cause seizures in humans[24]. It has been widely used to test the antiepileptic drugs[25]. Here, the magnesium-free model of epilepsy was used to study the effects of GAE on the superoxide dismutase (SOD) and mitochondria of the epileptiform hippocampal neurons, throughout the xanthine oxidase assay and the JC-1 fluorescent staining flow cytometry. The results will provide possible therapeutic applications for prevention and treatment of epilepsy.

MATERIALS AND METHODS

Newborn Wistar rats (up to 24 hours old) were provided by the Experimental Animal Center of Jiamusi University (certification number: SYXK-2011-0004). Ganoderic acid extract (GAE, higher than 95% pure) was purchased from WuXi AppTec Inc. (Shanghai, China). Mitochondrial membrane potential assay kit with JC-1 and SOD activity assay kit were from 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 common chemicals were from Sigma (St. Louis, MO, USA).

Cell culture of hippocampal neurons

In accord with the previous literatures[20, 23,26], primary hippocampal neurons were harvested from the newborn Wistar rats. D-Hank's balanced salt solution containing 0.125 % trypsin was used for the digestion (8 min, at room temperature). The cells were seeded at a density of 3.5×10^8 cells/ml on poly-L-lysine (10 µg/ml)-coated plates and incubated in a 37 °C incubator (containing 5 % CO_2). After 6h, the medium was replaced with neurobasal medium and 2 % B27 supplement. Hippocampal neurons cultured for 9 days were used for the purity evaluation by the neuron specific enolase (NSE) antibody. When the purity of the neurons was up to 96 %, the cells were used for the further assessment.

The epileptic cell model (named as model group) was set up using a conventional method[20, 23]. Hippocampal neurons, cultured for 9 days, were exposed to the Mg^{2+} free media for 3h, and then replaced with the normal culture medium. GAE treatment (GAE group) was based on the establishment of epileptic cell model: the culture medium was replaced with an Mg^{2+} free medium for 3 hours, and then a normal culture medium containing 200 µg/ml of GAE replaced this and neurons were cultured for a further 24 hours[20, 23].

Determination of SOD activity

Measurement of the effects of GAE on the superoxide dismutase (SOD) was performed

using the xanthine oxidase assay according to the manufacturer's directions[27,28]. 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) 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. Centrifuge the crude cell lysate at 14000 x g for 5 minutes at 4°C and discard the cell debris. The optical density (OD) was measured using a microplate reader (Thermo Molecular Devices Co., Union City, USA) with absorbance set at 450 nm.

Flow cytometric analysis

Mitochondrial membrane potential ($\Delta\psi_m$) and apoptosis were counted by the flow cytometry. Approximately 1.0×10^6 cells from each sample were collected. After indicated treatments[23, 29], 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 the apoptosis, 1.0 ml of propidium iodide (PI) solution (50 µg of PI, 4 mM of sodium citrate, 1 mg/ml of RNase A and 1 % of Triton X-100) was added for the incubation 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. Data were analyzed with WinMDI2.9 software. Each experiment type was repeated 3 times and no obvious deviations were observed.

Statistical analysis

Data 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

GAE effects on the SOD activities

As described in the Introduction section, ganoderic acid extract (GAE) from *Ganoderma lucidum* is expected to be related with the protection of hippocampal neurons, because of the structure and pharmacological properties[21-23]. However, its neuronal effects have not been sufficiently explored. In recent years, it was found that the SOD activity could reflect the damage of hippocampal neurons[4-6]. In this regard, the xanthine oxidase assay was utilized to provide comprehensive analysis effects of GAE on the SOD, in order to understand the protective effect of GAE on epileptiform hippocampal neurons. The experimental results of each group are summarized in Figure 1. In contrast to the normal, SOD activity was significantly decreased in epileptiform discharge hippocampal neurons, from 135.95 ± 2.10 to 117.68 ± 2.50 U/mg pro. The difference had significance of statistics ($P < 0.05$). With the treatment of GAE, the SOD activity of hippocampal neurons (123.86 ± 2.20 U/mg pro) was significant higher than that of epileptic cell model, showing significant difference ($P < 0.01$).

Mitochondrial membrane potential and apoptosis rate

It was found that the role of mitochondria in programmed cell death come into the spotlight after the discovery that antiapoptotic members localize mainly to the mitochondrial membrane[30, 31]. 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[31, 32]. Our results revealed that the mitochondrial membrane potential of the epileptiform discharge hippocampal neurons was significantly lower than that of the normal group ($\Delta\psi_m$, 244.08 ± 23.61 vs. 409.81 ± 34.21), associated with apoptosis rate increasing significantly (61.39 ± 1.05 vs. 28.84 ± 0.74) (Table 1 and Figure 2). The difference had significance of statistics ($P < 0.01$). With the treatment of GAE, the value of mitochondrial membrane potential was increased to 386.92 ± 21.35, and the apoptosis rate was significantly reduced (14.37 ± 1.62). There were significant differences between the two groups (P

Table 1. Mitochondrial membrane potential ($\Delta\Psi_m$) and apoptosis rate of each group

Group	$\Delta\Psi_m$	Apoptosis rate (R3)
Normal group	409.81 ± 34.21	28.84 ± 0.74
Model group	244.08 ± 23.61 *	61.39 ± 1.05 *
GAE group	386.92 ± 21.35 *	14.37 ± 1.62 *

The symbols * indicate significant differences ($p < 0.01$) with respect to normal group.

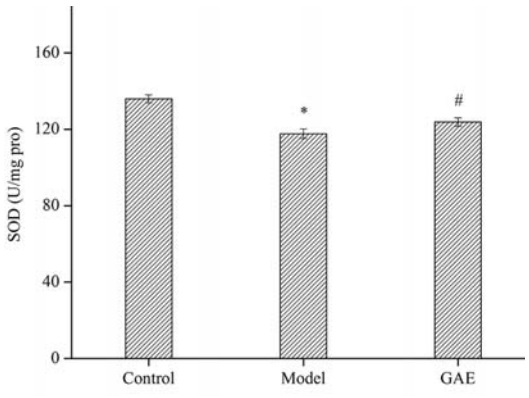

Fig 1. GAE effects on the SOD activities for each group The symbols * and # indicate significant differences ($p < 0.01$) with respect to normal group.

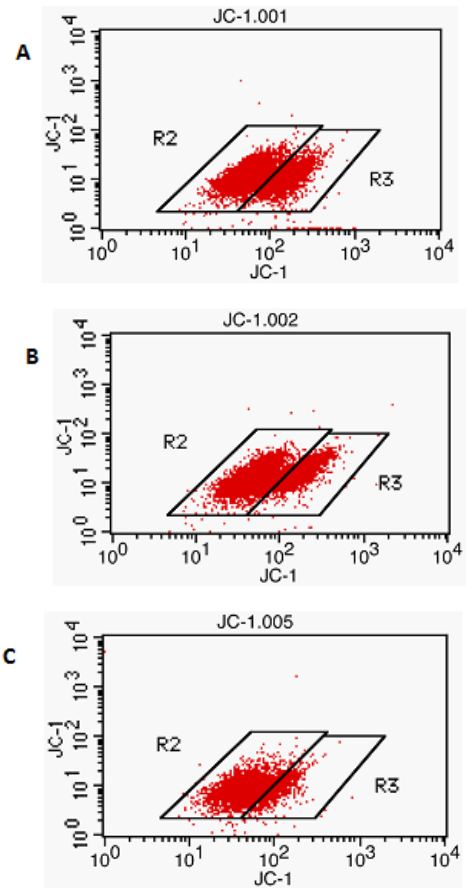

Fig 2. Mitochondrial membrane potential and apoptosis rate: A) Normal group, B) Model group and C) GAE group

< 0.01). It indicated that GAE can regulate and improve the function of mitochondria, tranquillize the mitochondrial membrane potential, and then restraint the apoptosis of epileptiform hippocampal neurons.

Taken together, the experiments indicated that the mitochondrial damage in the epileptiform hippocampal neurons may be caused by the lipid peroxidation induced by the oxygen free radicals. GAE can regulate the mitochondrial lipid peroxidation and stabilize the mitochondrial membrane potential, to maintain the normal structure and function of mitochondria. In addition, the apoptosis rate has the close relationship with the SOD activity and mitochondrial membrane potential (Figures 1 and 2), which indicated that the mitochondria pathway is an important pathway to the apoptosis of epileptiform hippocampal neurons. Altogether, GAE can significantly improve the SOD activity and mitochondrial dysfunction in hippocampal neurons, and thereby protect the hippocampal neurons by inhibiting the apoptosis.

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