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Beneficial effects of chlorogenic acid on alcohol-induced damage in PC12 cells

Shi-Qi Fang[a,1], Yong-Tang Wang[b,1], Jing-Xiang Wei[a], Ya-Hai Shu[a], Lan Xiao[a], Xiu-Min Lu[a,*]

[a] College of Pharmacy and Bioengineering, Chongqing University of Technology, Chongqing 400054, China
[b] State Key Laboratory of Trauma, Burns and Combined Injury, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing 400042, China

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ABSTRACT

As one of the most commonly abused psychotropic substances, ethanol exposure has deleterious effects on the central nervous system (CNS). The most detrimental results of ethanol exposure during development are the loss of neurons in brain regions such as the hippocampus and neocortex, which may be related to the apoptosis and necrosis mediated by oxidative stress. Recent studies indicated that a number of natural drugs from plants play an important role in protection of nerve cells from damage. Among these, it has been reported that chlorogenic acid (CA) has neuroprotective effects against oxidative stress. Thus, it may play some beneficial effects on ethanol-induced neurotoxicity. However, the effects of CA on ethanol-induced nerve damage remain unclear. In order to investigate the protective effects of CA on alcohol-induced apoptosis in rat pheochromocytoma PC12 cells, in the present study, cell viability and the optimal dosage of CA were first quantified by MTT assay. Then, the cell apoptosis and cell cycle were respectively investigated by Hoechst 33258 staining and flow cytometer (FCM). To further clarify the possible mechanism, followed with the test of mitochondria transmembrane potential with Rhodamine 123 (Rho 123) staining, the expression of Bcl-2, Capase-3 and growth associated protein-43 (GAP-43) were analyzed by immunofluorescence assay separately. The results showed that treatment with 500 mM alcohol decreased the cell viability and then significantly induced apoptosis in PC12 cells. However, when pretreated with different concentrations of CA (1, 5, 10, 50 μM), cell viability increased in different degree. Comparatively, CA with the concentration of 10 μM most effectively promoted the proliferation of damaged cells, increased the distribution ratio of the cells at the G2/M and S phases, and enhanced mitochondria transmembrane potential. This appears to be in agreement with up-regulation of the expression of Bcl-2 and GAP-43, and down-regulation of the expression of Capsae-3. Taken together, CA can increase cell viability and promote cell differentiation by preventing alcohol-induced cell from apoptosis. The mechanism may be related to the enhancement of the expression of GAP-43 and the inhibition of mitochondrial apoptotic pathway including promotion of mitochondria transmembrane potential, up-regulation of the expression of Bcl-2, and down-regulation of the expression of Capsae-3.

1. Introduction

Alcohol, also called ethanol, is a neurotropic molecule which has deleterious effects on the central nervous system (CNS). Alcohol abuse has become a public health issue, and has been shown to cause aberrations in synaptic plasticity and related neuronal function associated with several neurological disorders [1,2]. The morbidity is only less than cardiovascular disease and tumor. The harm of the alcoholism to human is more and more serious, especially in Europe and the United States. According to a survey from WHO, there are about 3.3 million deaths in 2012 estimated to have been caused by achohol consumption, of which approximately 132,000 directly related neuropsychiatric disorders. Growing evidences indicate that the processes underlying alcohol-induced neurotoxicity appear to occur in conjunction with both apoptosis and necrosis in vitro [3,4], and oxidative stress can induce both types of cell death, seems to account for the cytotoxic induced by alcohol [5]. As a result, apoptosis and necrosis

* Corresponding author.
E-mail address: luxm@cqut.edu.cn (X.-M. Lu).
[1] These authors contributed equally to this work.

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induced by oxidative stress may play an important role in alcohol-induced damage in CNS.

Natural herbal medicine is derived from the Chinese medicine system with low price, toxic and side effect. Besides many drugs, such as Naloxone and Meclofenoxate, it has been found that the neural therapy effect of herbal musk, angelica, and fennel longan, but they are not specialized in repairing nerve damage induced by alcohol. Therefore, it is urgent to find drugs for treatment of alcoholism with low cost and side effect. For this reason, we focus on CA, a potent phenolic antioxidant condensated from cinnamic acid and quinic acid. The chemical structure of CA contains active hydroxyl, which generates hydrogen radicals with antioxidant effects to eliminate the hydroxyl radical and superoxide anion radical (Fig. 1). In this way can CA protect tissue against oxidative damage [6–9]. Recent years, growing evidences indicate that great progresses have been developed in the effects of CA on CNS degenerative disorders. In a model of experimental neuropathic pain, CA has been proved to ameliorate mechanical and cold hyperalgesia, which may be useful for novel treatments of neuropathicpain [10,11]. Furthermore, studies have shown that CA has the protective effect on cardiovascular and central nervous system, and can inhibit matrix metalloproteinase-2 and 9 to reduce brain injury and edema [12]. As a result, CA may play some beneficial effects on ethanol-induced neurotoxicity. However, the effects of CA on ethanol-induced nerve damage remains unclear, and the molecular mechanisms underlying this effect remain to be clarified.

This research intends to investigate the protective effect of CA on alcohol-induced apoptosis of PC12 cells and to further clarify the possible mechanism, which may provide a theoretical basis of CA for the clinical treatment for nerve injury induced by alcohol.

2. Materials and methods

2.1. Cell culture

PC12 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM medium (Gibco BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 humidified incubator. When at 50–60% confluences, the cells were seeded on poly-l-ornithine-coated coverslips and maintained in 5% CO_2 at 37 °C. When reached logarithmic growth phase, the cells were treated with different amounts of chemicals as indicated, and divided into three groups, including normal control group (Control) without treatment, alcohol injury group treated with 500 mM alcohol, and CA treated group with 500 mM alcohol and CA (the optimal dosage assessed by MTT assay), then further cultured for 24 h. CA was purchased from Sigma-Aldrich Co., LLC. (St. Louis, MO) and was dissolved at a concentration of 0.1 M in DMSO as a stock solution stored at −70 °C.

2.2. MTT assay

The protective effect of CA on alcohol-induced reduction of PC12 cell viability, and the optimal dosage of CA were first detected and quantified by MTT assay. PC12 cells were seeded at a density of 1×10^4 cells/ml in 96-well culture plates. After adhering to the wall, cells were incubated with various concentrations of CA (1, 5, 10, 50 μM) and 500 mM of ethanol for 24 h separately. Control wells consisted of cells incubated with medium only, and the cells with 500 mM ethanol acted as the negative control. In order to keep the ethanol concentration constant, a methodology to deliver ethanol to cultured cells was developed according to the description of Adickes et al. [13]. After treatment above, cells were incubated with 20 μL MTT (5 mg/mL, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, St Louis, MO, USA). After 4 h at 37 °C, the supernatant was removed, and 150 μL DMSO was added. When the blue crystal was dissolved, the optical density (OD) was detected at a 570 nm wavelength using a 96-well multiscanner autoreader (Bio-Rad, USA), and the cell survival rate of each group was standardized to control with the OD ratio of each group and control.

2.3. Cell cycle analysis

Cells cultured with 500 mM ethanol only, and 500 mM ethanol combined with the optimal dosage 10 μM CA for 24 h were harvested by centrifugation and washed twice with ice-cold PBS before fixation in 70% ethanol overnight at 4 °C. Then, the cells were washed twice with PBS and resuspended in 50 μg/ml RNase (Sigma) for 30 min at 37 °C, and incubated with 25 μg/ml propidium iodide (PI, Sigma-Aldrich) for 30 min at 4 °C in the dark. The cells were analyzed using a flow cytometer (FACS-caliber, Becton Dickinson, San Jose, CA, USA) and all histograms were analyzed by ModFit software. The untreated PC12 cells were also investigated as a positive control.

2.4. Hoechst 33258 staining

Treated as previous description, the PC12 cells were then centrifuged at 800 × g for 5 min, and fixed in 4% formalin for 10 min. After washing twice with PBS, the cells were stained with Hoechst 33258 (20 μg/ml, Sigma) in PBS containing 80% (v/v) glycerol, mounted on a slide, and observed with a fluorescent microscope (BX51, Olympus, Japan).

2.5. Cell apoptosis analysis

Apoptotic rate was quantified by Annexin V-PI apoptosis assay kit (BD Biosciences), and cell apoptosis analysis was fulfilled with a flow cytometer. Cells treated with CA for 24 h were harvested by centrifugation and washed twice with ice-cold PBS. The cells were fixed in ice-cold 70% ethanol and frozen at −20 °C. Then, the cells were washed twice with PBS and resuspended in 50 μg/ml RNase

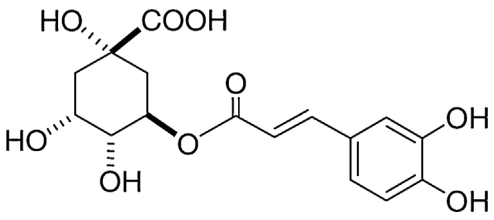

Fig. 1. Chemical structure of chlorogenic acid.