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Research Article

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Protective effect of cyclovirobuxine D derivatives on alcohol induced injury in PC12 cells

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ABSTRACT

To explore the impact of the new derivatives cyclovirobuxine D (CBV-D) model of alcohol-induced injury in PC12 cells. The model of alcohol-induced injury in PC12 cells, new derivatives of the cyclovirobuxine D PC12 cells by MTT assay, Hoechst 33258 staining and lactate dehydrogenase (LDH) activity was measured. The new D derivatives cyclovirobuxine inhibit the damage caused by alcohol PC12 cells, apoptosis rate, while LDH release was reducing. cyclovirobuxine D derivatives on PC12 cell damage caused by excitatory amino acids have a protective effect.

Key words: Cyclovirobuxine D, PC12 cells, Proliferation, Apoptosis

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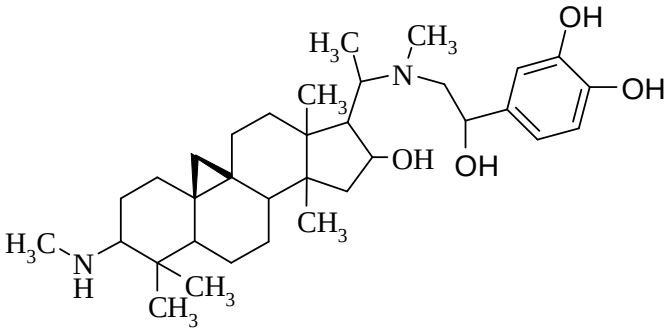
INTRODUCTION

Cyclovirobuxine D (CVB-D) also is named Huang Yang which is one kind of alkaloids that were extracted from boxwood Buxus microphylla plants and some relative plants, belongs to pregnane derivate[1,2]. CVB-D is deemed to possess the function of protecting neurons according to inhibiting lipid from peroxidating, eliminate free radicals[3]. Some studies revealed that CVB-D could improve and protect the lesions of acute cerebral ischemia and hypoxia. Vitro study also demonstrated CVB-D could relieve the neuron injures in hypoxia cultures and excitatory amino acid impact, possess the neurons protection function[4].

PC12 cells are the rat adrenal pheochromocytoma cell line[5]. Differentiated PC12 cells have the typical neurosecretory cell morphological and functional characteristic. Catecholamines possess the good physiological effect, play an important role in signal transduction of brain and neurons, meantime its could use as medicine, with an antioxidant, antimutagenesis and anti-aging effect[6,7]. For this purpose, we modified the structure of CVB-D, added the antimutagenesis perssad which has neuron protection effect to the CVB-D, compounded the new CVB-D derivatives. The preliminary screening discovered that CVB-D derivative HD-X has the protection effect on PC12 cells. We utilize the PC12 cells injury modal induced by alcohol, study the new CVB-D derivative HD-X's protection effect on PC12 cells.

HD-X was synthesized at the Institute of Chemistry and Biology at Henan University. The purity was more than 98% by HPLC analysis. The compound was dissolved in dimethysulfoxide （DMSO，Solarbio Science & technology Co, Ltd.). Its structure is illustrated in figure 1.



（figure 1）9B,19-hydroxy-16A-ring-4,4,13,14-tetramethyl-3-methyl-amino-17-{1-methyl-1-[2-(hydroxy-3,4-dihydroxy-phenyl) ethyl] amino}ethyl pregnane

EXPERIMENTAL SECTION

Cell culture and Reagents PC12 cells were grown at 37℃ in a humidified CO_2(5%) incubator with completed DMEM medium (Gibco BRL, USA) supplemented with 10% (V/V) fresh fetal bovine serum(Hyclone laboratory). Cell passaged and cultured for 24h followed by treated with different concentrations ethanol(0 mM, 100 mM, 200 mM, 300 mM) for indicated time.

PC12 cells obtained from the Cell Bank of the Chinese Academy of Sciences, Shanghai, were cultured in DMEM medium (Gibco BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone laboratory), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were plated at 50–60% confluence. To induce neurite outgrowth, the PC12 cells were plated on poly-l-ornithine-coated coverslips and maintained in 5% CO_2 at 37°C. When the cells reached approximately 50%~70% confluence, they were treated with different amounts of chemicals as indicated.

Logarithmic growth phase of the cell experiment is divided into four groups: ① normal control group (Control): no treatment; ②alcohol injury group: 2×10^{-1}mol·L^{-1} alcohol; ③alcohol damage+HD-X1 derivative group: 1×10^{-5}mol·L^{-1} HD-X pretreatment 30min, then with 2×10^{-1}mol·L^{-1} alcohol; ④alcohol damage+HD-X2 derivative group: 1×10^{-4}mol·L^{-1} HD-X pretreatment 30min, then with 2×10^{-1}mol·L^{-1} alcohol; then cultured 24 h.

MTT assay

PC12 cells were seeded at a density of 1×10^4 cells/ml in 96-well culture plates, and 24 h later they were treated with the indicated concentrations of ethanol for 12 h, 24 h, 36 h and 48 h. Control wells consisted of cells incubated with medium only. After treatment, cells were incubated with 20μL MTT (5mg / mL, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, St Louis, MO, USA). After 4 h at 37℃, the supernatant was removed, and 150μL 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).

Hoechst 33258 / PI staining

PC12 cells were seeded at a density of 1×10^4 cells/mL on the glass cover slides of a 35-mm chamber. After being treated with QNT4 for 24 h, the cells were washed twice with PBS and incubated with 5 μg/mL Hoechst 33258 (Sigma, St Louis, MO, USA) for 10 min at 37 °C in the dark. The cells were then washed and fixed with 4% paraformaldehyde in PBS for 5 min at 4 °C. Nuclear morphology was then examined under a fluorescent microscope (BX51, Olympus, Japan).

LDH activity was measured

24h after collection alcoholic injury broth in 722 spectrophotometer, LDH activity was measured under 340nm wavelength.

LDH release inhibition rate = ($LDH_{alcohol\ group}$-$LDH_{derivatives\ group}$) / ($LDH_{alcohol\ group}$-$LDH_{control\ group}$) × 100%.

Western blotting

Cells were lysed by incubating in RIPA lysis buffer [50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/L sodium orthovanadate, 10 μL/mL protease inhibitor cocktail, 1 mmol/L phenylmethylsulfonyl fluoride] for 20 minutes at 4°C. Protein concentrations were determined with the Bio-Rad assay system (Bio-Rad, Hercules, CA). Total proteins were fractionated by SDS-PAGE and transferred onto Immobilon-P transfer membranes (Millipore Corp.). The membranes were blocked for 1 h with 5% nonfat milk

or bovine serum albumin (BSA) in PBS with 0.1% Tween-20. Blots were incubated with primary antibody overnight at 4°C followed by secondary antibodies for 1 hour each at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence (Pierce). The membranes were then incubated with stripping buffer (1mM Glycine, 1% SDS) for 30 minutes at 37°C, re-blocked, and re-probed with β-actin as a loading control.

Statistical analysis

Data are shown as Mean±S.E. ANOVOL was used for analyze the multi-factors comparison, followed by Dunnett's test. For single comparison, the significance between control and treatment groups was determined by t-test. A value of $P<0.05$ was considered as statistically significant.

RESULTS

Effect on cell viability

There CBV-D derivative in the protection of the experimental group, PC12 cells MTT uptake capacity than the model group (P <0. 05 or P <0. 01), increased cell survival, suggesting HD-X derivatives alcohol-induced injury model of PC12 cells have a protective effect. Table 1.

Table 1 HD-X impact for alcohol-induced PC12 cell injury survival

group	Dose /（mol/L）	MTT	
		A_{570}	Inhibition rate %
Control group	0	0. 97 ±0.18**	--
Alcohol group	2×10^{-1}	0.58±0.07*	43.23
Alcohol +HD-X1	2×10-1+1×10^{-5}	0.73±0.13**	32.16
Alcohol +HD-X2	2×10-1+1×10^{-4}	0.80±0.09***	19.63

*Note: * was contrast with control group P <0 05; ** and *** was contrast with alcohol group P <0 01.*

Morphological observation

PC12 cells alcohol group, Hoechst33258 staining apoptotic morphological changes, manifested as chromatin condensation, nuclear fragmentation into pieces and other typical features of apoptosis changes in CBV-D derivatives have protected the experimental group This shows the number of apoptotic cells was significantly decreased (Figure 2).

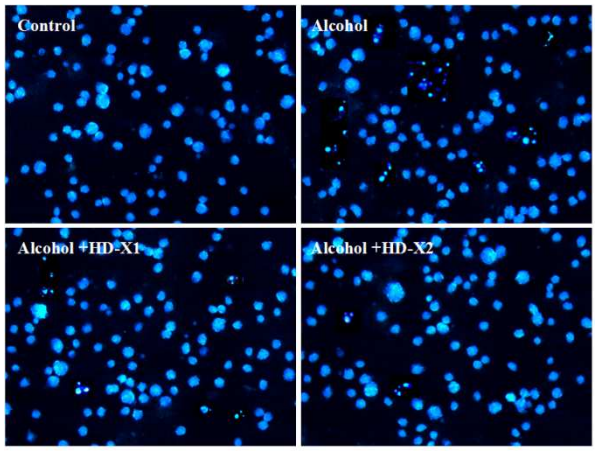


Figure 2 HD-X morphology observed protective effect of alcohol-induced injury in PC12 cells

Table2 HD-X on cell LDH release after the alcohol-induced injury in PC12 cells($\overline{X}$ ±s)

Group	Dose (mol / L)	LDH（U/L）
Control group	0	22.36±3. 76
Alcohol group	2×10^{-1}	46.78±7. 87*
Alcohol +HD-X1	$2\times10^{-1}+1\times10^{-5}$	35.57±6. 24**
Alcohol +HD-X2	$2\times10^{-1}+1\times10^{-4}$	30.46±6. 97**

*Note: *was contrast with control group P <0 01; **was contrast with alcohol group P <0 05.*

Impact on the LDH release

PC12 cells were released after alcohol damage to the culture medium LDH increased, and the normal control group was statistically significant (P<0 01.); While adding two dose groups with CBV-D derivatives found in the culture

medium lower levels of LDH (P<0. 05 or P<0. 01), exposing the decrease, which means that CBV-D derivatives of alcohol-induced damage in PC12 cells have a protective effect. Table 2.

Effects of HD-X on Cytochrome C expressions in PC12 cells

We observed a HD-X-induced decrease of the mitochondrial membrane potential in PC12 cells, followed by increased cytochrome c release from the mitochondria into the cytosol.

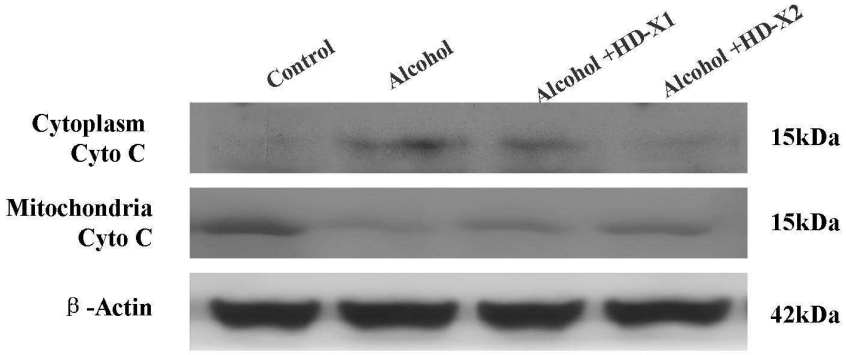


Figure3. Effects of HD-X on the release of Cytochrome c from mitochondria to the cytosol

DISCUSSION

Numerous studies[8-10] have demonstrated that the alcohol involved in the induction of human diseases : neurodegenerative diseases, diabetes , atherosclerosis and metabolic disorders associated with the zinc and the like. Research shows[11] that alcohol may increase the production of free radicals by affecting the function of neurotransmitter receptors, interfering neurotrophic factor signaling pathways, activation of endogenous molecular mechanisms of apoptosis signaling pathways, and promote nerve cell damage[12]. PC12 cells rat adrenal medulla pheochromocytoma clonal cell lines, compared with the nerve cells in primary culture model , with the general characteristics of neuroendocrine cells, is an internationally recognized basic in vitro neurobiological. [13] Neurochemical and neurological diseases ideal model system for the study of neurological disorders, such as Alzheimer 's disease, Parkinson 's disease, which are widely used as neural cell differentiation, ion channels, receptors, and neurotransmitter release experiments material is one of the most important research neurotoxicity cell lines[14,15].

The experimental results show that alcohol on PC12 cells inhibited cell proliferation and induce apoptosis , after giving advance cyclovirobuxine D of new derivatives of alcohol on PC12 cell proliferation inhibition reduced apoptosis rate decline. Meanwhile, LDH is a cytoplasmic membrane marker enzyme[16,17], reflecting the common biochemical markers of cell death , the culture medium LDH leakage rate was significantly higher in the alcohol group , after giving advance cyclovirobuxine D derivatives, cell supernatants the LDH levels significantly reduced , suggesting that the new cyclovirobuxine D derivatives reduce alcohol- induced neuronal injury , neurons have a protective effect . In the mitochondrial apoptotic pathway[18], the release of cytochrome c is a critical event because cytochrome c forms a complex with procaspase-9 in the cytoplasm (resulting in the activation of procaspase-9), which will eventually lead to the activation of caspases 3 and the induction of apoptosis. Protection mechanism may inhibit the release of cytochrome C related.

CONCLUSION

Taken together, we conclude that the new cyclovirobuxine D derivatives on PC12 cell injury induced by alcohol has a significant protective effect, The growth inhibition was in large part mediated via apoptosis-associated mitochondrial dysfunction.

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