1,8-Cineol Attenuated $AB_{25\text{-}35}$-Induced PC12 Cell Injury through Reducing Caspase 3 Expression and NO Production

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ABSTRACT

Objective To investigate the effect of 1,8-cineol on caspase 3 expression and NO production induced by $A\beta_{25\text{-}35}$ in PC12 cells.

Methods PC12 cells were cultured *in vitro*, and cell injury was induced by $A\beta_{25\text{-}35}$ with a concentration of 20 μM. 1,8-cineol (1, 3, 10 μM) was pretreated before $A\beta_{25\text{-}35}$ treatment. PC12 cell viability was evaluated by MTT detection assay. Caspase 3 protein expression was detected by Western blotting. The level of NO production in PC12 cells was measured using ELISA detection assay kit.

Results In cultured PC12 cells *in vitro*, MTT results showed that 20 μM of $A\beta_{25\text{-}35}$ reduced cell viability significantly compared with control group. The cell viability was increased by pretreatment with 1,8-cineol with concentrations of 3 and 10 μM compared with $A\beta_{25\text{-}35}$ only group. Western blotting results showed compared with control group, caspase 3 expression was increased significantly in 20 μM $A\beta_{25\text{-}35}$ group. Compared with $A\beta_{25\text{-}35}$ group, 1,8-cineol of 3 and 10 μM group reduced caspase 3 protein expression significantly. The level of NO production in PC12 cells was increased significantly, which was decreased by pretreatment with 3 and 10 μM of 1,8-cineol.

Conclusions: Our results revealed a protective effect of 1,8-cineol on $A\beta_{25\text{-}35}$ induced PC12 cell injury through inhibition of caspase 3 expression and NO production.

Keywords Amyloid beta, cineol, caspase 3

INTRODUCTION

Alzheimer's disease (AD) is an age-related and progressive neurodegenerative disorder characterized by cognitive impairment. Amyloid beta (Aβ) which aggregate into oligomers are found in the brains of patients with AD [1]. Aβ deposition leads to diversities of toxic mechanisms including oxidative stress, mitochondrial diffusion, and excitotoxicity [2]. It could cause neuron loss and synaptic lesion. Aβ-induced neurotoxicity has emerged a possible therapeutic approach to slow the progression of AD [3]. However, there are still lack of effective drugs for treatment. Recent studies of small molecules acting on neuronal cells has been proposed as an alternative for the treatment of AD [4].

1,8-cineol, also known as eucalyptol, is a major monoterpene principally from Eucalyptus essential oils [5]. 1,8-

cineol has been proven clinical efficacy on therapeutic benefits in inflammatory airway diseases such as chronic obstructive pulmonary disease (COPD) [6]. Our previous study indicated anti-inflammatory action of 1,8-cineol through TLR4 expression inhibition [7]. Little is known about the effect of 1,8-cineol in Aβ-induced neuronal injury. Thus, the present study was to investigate the role of 1,8-cineol against $A\beta_{25-35}$-induced PC12 cell injury and possible mechanisms.

MATERIALS AND METHODS

REAGENTS

1,8-cineol and $A\beta_{25-35}$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Thermo scientific. (Hyclone, USA). NO detection assay was purchased from Beyotime Biotechnology (Shanghai, China). Trypsin was purchased from Sangon Biotech (Shanghai, China). Rabbit polyclonal antibodies against cleaved caspase 3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GAPDH antibody was purchased from Kangcheng Bioengineering Co., Ltd (Shanghai, China). Anti-rabbit IRDye700DX®-conjugated antibody or anti-mouse IRDye800DX®-conjugated antibody was purchased from Rockland (LICORE, USA).

DRUG ADMINISTRATION

PC12 cells were randomly divided into five groups, then incubated with $A\beta_{25-35}$ oligomers at concentration of 20 μM for 24 h. Soluble oligomeric forms of $A\beta_{25-35}$ were prepared as reported previously. The peptide was dissolved into sterile PBS and make a final concentration of 100 μM, incubated under 37 °C for 24 h.
To observe the effect of 1,8-cineol against $A\beta_{25-35}$ induced cell injury, PC12 cells were incubated with 1,8-cineol at concentrations of 1, 3, 10 μM for 1 h, then co-administrated with $A\beta_{25-35}$ for 24 h. 1,8-cineol was diluted with sterile PBS.

CELL CULTURE

PC12 cells were cultured in DMEM medium containing 10% fetal bovine serum at 37 °C in humidified air containing 5% CO_2. The cultured medium was changed each 24 hours and the cells in the exponential phase of growth were used in all experiments.

MTT DETECTION ASSAY

PC12 cell viability was detected by MTT reduction assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) was dissolved in dimethyl sulfoxide (DMSO) and added in the medium with a concentration of 0.5 mg/ml. The cells were kept in the incubator for 4 h at 37 °C. The medium was removed and 100 μl

DMSO was added to dissolve the formazan precipitates in 96-well plates. The absorbance was measured at a wavelength of 570 nm using multiscan spectrum. Cell viability was expressed as percentage of viable cells relative to that of non-treated cells in the control group.

WESTERN BLOT ANALYSIS

After treatment, PC12 cells were collected and total protein was extracted using protein extraction kit. Cell lysates were centrifuged at 12000 g for 30 min at 4°C.The protein concentration was determined by coomassie brilliant blue method. The protein samples were diluted with loading buffer and separated by 10% SDS-PAGE, then transferred onto a nitrocellulose membrane. The membrane was incubated with 7.5% defatted milk for 2 h, and then incubated with rabbit polyclonal anti-cleaved caspase 3 antibody and mouse monoclonal anti-GAPDH antibody overnight. The blots were incubated with with anti-rabbit IRDye700DX®-conjugated antibody or anti-mouse IRDye800DX®-conjugated antibody (1:5000, Rockland, USA). The membranes were washed and scanned by an Odyssey infrared imaging system. The protein bands were quantitatively evaluated

by Quantity One® analysis software. Protein expression was expressed as percentage of the control group.

DETERMINATION OF NO PRODUCTION

After treated with A$\beta_{25\text{-}35}$ and 1,8-cineol for 24 h, the cells were collected and crushed. The level of NO in PC12 cells was evaluated using nitric oxide assay kit. The detection procedure was performed according to manufacturers' instructions.

STATISTICAL ANALYSIS

All data are expressed as means ± SD. The significance of differences among groups was performed using one-way ANOVA followed by Dunnett's post hoc test (SPSS 15.0 for Windows, SPSS in., USA). $P < 0.05$ was considered statistically significant.

RESULTS

Protective effect of 1,8-cineol on Aβ25-35 -induced toxicity in PC12 cells

To investigate the effect of 1,8-cineol on neurotoxicity, PC12 cells were pretreated with different concentrations of (1, 3, 10 μM) for 1 h, and then exposed to 20 μM of A$\beta_{25\text{-}35}$ for 24 h. The cell viability was evaluated by MTT reduction assay. As Fig.1 shown, cell viability of the cells in A$\beta_{25\text{-}35}$ group was significantly decreased compared with the control group. As compared with untreated cells in A$\beta_{25\text{-}35}$ group, cell viability of the cells pretreated with 1, 8-cineol (3, 10 μM) was significantly reduced (Fig.1, $P < 0.01$). 1,8-cineol at the concentration of 1 μM also increased cell viability but not significant.

Effect of 1,8-cineol on Aβ25-35-induced caspase-3 expression in PC12 cells

The effect of 1,8-cineol on protein expression of caspase 3 was detected by Western blotting. As shown in Fig.2, after exposed to 20 μl A$\beta_{25\text{-}35}$ for 24 h, cleaved caspase 3 expression increased significantly compared to control group. As compared with untreated A$\beta_{25\text{-}35}$ group, the cleaved caspase 3 expression in 1,8-cineol (3, 10 μM) group significantly decreased. 1 μM of 1,8-cineol also reduced cleaved caspase 3 expression. No significance was found between 1 μM 1,8-cineol group and A$\beta_{25\text{-}35}$ group.

Effect of 1,8-cineol on Aβ25-35-induced NO content in PC12 cells

Varieties of evidences have reported that NO play an important role in neural cell injury in Alzheimer's disease. In the present study, we struggled to investigate whether 1,8-cineol could affect the elevated level of NO production in A$\beta_{25\text{-}35}$-treated PC12 cells. A$\beta_{25\text{-}35}$ 20 μM caused a higher level of NO production compared with control group. Pretreatment with 3, 10 μM of 1,8-cineol could significantly decreased the level of NO content as compared with A$\beta_{25\text{-}35}$ group (Fig.3, $P < 0.01$).

DISCUSSION

1,8-cineol is a natural component clinically applied as the active ingredient of soledum. As previously reported, 1,8-cineol is well established for the therapy of airway diseases such as chronic sinusitis and bronchitis [8]. Although clinical trials underline the beneficial effects of 1,8-cineol in treating respiratory diseases, the molecular mechanisms still remains unclear. 1,8-cineol inhibit nuclear NF-κB translocation, cytokine production and oxidative stress in many inflammatory conditions [9].

In the present study, A$\beta_{25\text{-}35}$ treatment at 20 μM decreased the cell viability of PC12 cells while pretreatment with 1,8-cineol reversed the effect at 3 and 10 μM. A previous study showed 1,8-cineol exerted protective effect against ischemic neuron injury at concentration of 10 μM [10]. The effective concentration is similar to that observed in our study. Here we showed that 1,8-cineol could protect PC12 neuronal cells against A$\beta_{25\text{-}35}$-induced toxicity.

Cell apoptosis may contribute to neuronal degeneration in the progression of AD [11]. It is well known that

apoptosis in neurons is a leading pathway for Aβ-induced toxicity. Thus prevention of Aβ-induced apoptosis is regarded as a potential and reasonable therapeutic strategy for AD therapy. Caspase 3 plays a pivotal role in the neuronal cell apoptosis [12]. In the study we found $A\beta_{25\text{-}35}$ treatment increased cleaved caspase 3 protein expression in agreement with previous studies [13]. 1,8-cineol pretreatment downregulated the expression of cleaved caspase 3 induced by $A\beta_{25\text{-}35}$. Thus our results indicated that the mitochondrial apoptosis pathway is involved with protective effect of 1,8-cineol against $A\beta_{25\text{-}35}$-induced PC12 cell apoptosis.

Low concentrations of NO protect neural cells from apoptosis, whereas excessive NO causes apoptosis [14]. And excessive ROS generation initiated the apoptotic cell death by regulating iNOS and increasing apoptosis-related protein expression [15]. It's reported NO could have vital functions in central nervous system. 1,8-cineol affected the eNOS phosphorylation and thus its activation [16]. In this study, we found that 1,8-cineol significantly reduced the production of NO in PC12 cells induced by $A\beta_{25\text{-}35}$.

In summary, we evaluated the protective potential of 1,8-cineol against $A\beta_{25\text{-}35}$-induced toxicity and investigated the underlying mechanisms. Our results indicated that 1,8-cineol inhibits $A\beta_{25\text{-}35}$-induced cytotoxicity by suppressing caspase 3 expression and NO production. Further study are needed to focus the mechanisms of 1,8-cineol in the pathogenesis of Alzheimer's disease.

Figures and legends

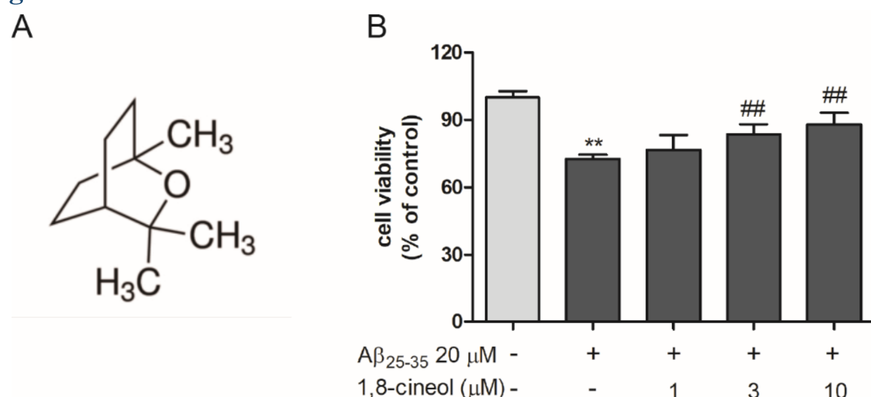

Fig.1 Chemical structure and the effect of 1,8-cineol on cell viability of PC12 cells treated by $A\beta_{25\text{-}35}$. PC12 cells were pretreated with 1,8-cineol, a monoterpene structure shown as A, with concentrations of 1, 3, 10 μM , and incubated with 20 μM $A\beta_{25\text{-}35}$ for 24 h. Cell viability was detected by MTT reduction assay and expressed as percentage of control group (B). $^{**}P < 0.01$ vs control group, $^{\#\#}P < 0.01$ vs $A\beta_{25\text{-}35}$-treated only group.

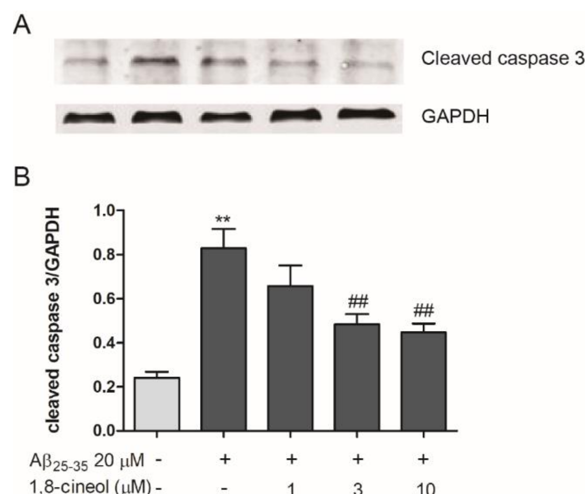

Fig.2 Effect of 1,8-cineol on caspase 3 protein expression in $A\beta_{25-35}$-induced PC12 cells Cells were pretreated with 1,8-cineol for 1 h with concentrations of 1, 3, 10 μM , and then incubated with 20 μM $A\beta_{25-35}$ for 24 h. Cleaved caspase 3 protein expression was detected by Western blotting (A) and the ratio of cleaved caspase 3 to GAPDH was calculated (B). $^{**}P < 0.01$ vs control group, $^{\#\#}P < 0.01$ vs $A\beta_{25-35}$-treated only group.

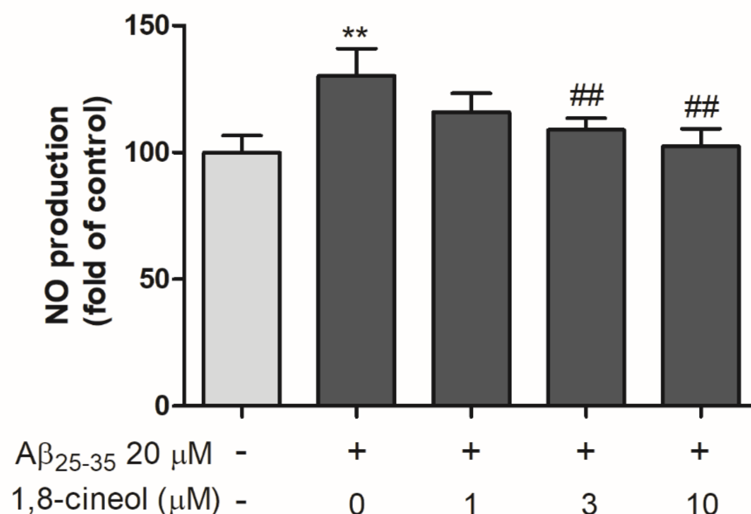

Fig.3 Effect of 1,8-cineol on NO production in $A\beta_{25-35}$-induced PC12 cells Cells were collected and the level of NO content was evaluated using nitric oxide assay kit. $^{**}P < 0.01$ vs control group, $^{\#\#}P < 0.01$ vs $A\beta_{25-35}$-treated only group.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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