



1,8-cineol attenuated A β ₂₅₋₃₅-induced neuron injury through inhibiting IL-6, IL-8 release and NF- κ B expression

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ABSTRACT:

Objective: To explore the protective effect of 1,8-cineol against Amyloid beta₂₅₋₃₅ (A β ₂₅₋₃₅)-induced cell injury in primary rat cortical neurons.

Methods: Primary rat cortical neurons were cultured *in vitro*, treated with different concentrations of A β ₂₅₋₃₅ (2.5, 5, 10, 20, 40 μ M) and 1,8-cineol (1, 3, 10 μ M). Cell viability of neuronal cells were detected by MTT assay and cell death was detected by lactate dehydrogenase release (LDH). The production of IL-6 and IL-8 in the supernatant was measured by ELISA assay kits. NF- κ B protein expression was detected by Western blotting.

Results: In primary cultured neurons, A β ₂₅₋₃₅ concentration dependently reduced cell viability and increased LDH release. 1,8-cineol with concentrations of 3 and 10 μ M protected neuronal cells against A β ₂₅₋₃₅ induced cell injury for 24 h. 3 and 10 μ M of 1,8-cineol also significantly decreased the levels of IL-6 and IL-8 cytokine production in the supernatant. Increased NF- κ B expression was also significantly reduced by 1,8-cineol treatment evaluated by Western blotting.

Conclusions: Our results revealed a protective effect of 1,8-cineol on A β ₂₅₋₃₅ induced neuron injury through inhibition of IL-6, IL-8 production and NF- κ B expression.

Keywords: Amyloid beta (A β), cineol, neuron, cytokine

INTRODUCTION

Amyloid beta (A β), which aggregate into oligomers in neurons, play a critical role in the

pathogenesis of Alzheimer's disease (AD). Increasing evidence demonstrated that A β toxicity induced neurotoxicity in the cerebral cortex and hippocampus *in vitro and in vivo*,

resulting in neuronal apoptosis and cognitive dysfunction (Sowade et al. 2017; Kim et al. 2014; Robert et al. 2015). Abundant senile plaques, mainly composed of A β peptide, were found in AD patients brains, contributing to inflammatory responses (Vukic et al. 2009). Though mechanisms are not fully understood, lots of studies tried to find the way to inhibit the A β neurotoxicity in the brain.

Deposits of A β in AD patients brain induced inflammation, leading to secretion of pro-inflammatory cytokines such as TNF- α and IL-8 (Hanzel et al. 2014). The inflammatory pathway was further accelerated by nuclear factor- κ B (NF- κ B) activation, which translocated from the cytoplasm to the nucleus binding to its specific target genes including those involved in the inflammatory response (Srinivasa et al. 2015).

1,8-cineol is a major monoterpene principally from Eucalyptus essential oils, which has been used to treat bronchitis and asthma (Worth and Dethlefsen 2012). It strongly inhibited the production of cytokines, leukotriene, and prostaglandins in asthma (Juergens et al. 2003). Recent study demonstrated that 1,8-cineol protected rat pheochromocytoma PC12 cells from A β toxicity (Khan et al. 2014). Our previous study indicated the role of 1,8-cineol as a strong inhibitor of TNF- α and IL-1 β cytokines by inhibiting TLR4 expression (Zhao et al. 2014). However, the effect of 1,8-cineol on A β toxicity in cortical neurons are still unknown. Thus, the present study was performed to investigate the protection of 1,8-cineol against A β ₂₅₋₃₅-induced cell injury in primary cultured rat cortical neurons.

Materials and methods

Reagents

A β ₂₅₋₃₅ and 1,8-cineol were purchased from Sigma-Aldrich (St. Louis, MO, USA). High glucose Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco (Temecula, CA, USA). Trypsin was purchased from Sangon

Biotech (Shanghai, China). LDH was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rabbit polyclonal antibodies against NF- κ B p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GAPDH antibody was purchased from Kangchen Bioengineering Co., Ltd (Shanghai, China). enzyme-linked immunosorbent assay (ELISA) kits for IL-6 and IL-8 were obtained from Neobioscience Biotechnology (Shenzhen, China).

Primary culture of cortical neurons

Primary cortical neurons were obtained from the cortical cortex of neonatal Sprague-Dawley rats within 24 h of birth (Zhang et al. 2013; Meloni et al. 2001). Briefly, rats were decapitated and the brains were rapidly removed with ice-cold Hank solution. The cerebral cortices were dissected and digested with 0.25% trypsin for 10 minutes at 37 °C. The dissociated cells were then immediately seeded onto 24 or 6-well plates pre-coated with poly-L-lysine (0.1 mg/mL). Cells were incubated in high-glucose DMEM medium supplemented with 10% fetal bovine serum (FBS), 10% horse serum, 1% penicillin and streptomycin, 2 mM glutamine, 0.01% N₂, and 0.04% B₂₇. Three days after plating, proliferation of non-neuronal cells were inhibited by adding cytosine arabinoside (10 μ M) for 24 h. Cultures were maintained at 37 °C under a humidified atmosphere with 5% CO₂. On day 10 *in vitro*, neuronal cells comprised about 95% of the primary cultured cells, were used for experiments.

All the animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Ethics Committee of Laboratory Animal Care and Welfare, Weifang Medical University.

Drug administration

The neuronal cells were randomly divided into six groups, then were treated with A β ₂₅₋₃₅ oligomers at concentrations of 0, 2.5, 5, 10, 20, 40 μ M for 24 h. The optimal concentration was used for subsequent experiments. Soluble oligomeric forms of A β ₂₅₋₃₅ were prepared as reported previously. A β ₂₅₋₃₅ was firstly dissolved into sterile ddH₂O to make a stock solution of 1 mM and stored at -20 °C. To prepare oligomers, the peptide was dissolved to a final concentration of 100 μ M, followed by incubation at 37 °C for 24 h to allow aggregation.

To investigate the effect of 1,8-cineol against A β ₂₅₋₃₅ toxicity on neuronal cells, primary cultured cortical neurons were pretreated with 1,8-cineol at concentrations of 1, 3, 10 μ M for 1 h, then co-treated with A β ₂₅₋₃₅ for 24 h. 1,8-cineol was diluted with sterile saline. The medium was collected and LDH release was detected. Plates were washed carefully with phosphate-buffered saline (PBS), and cell viability was measured by MTT reduction assay.

Assessment of cell viability and LDH assay

Cell viability was assessed by MTT reduction assay, performed as described previously. Briefly, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) was dissolved in dimethyl sulfoxide (DMSO) at 5 mg/ml as a stock solution. At the end of experiments, MTT was added in culture medium to reach a final concentration of 0.5 mg/ml at 37 °C for 4 h. Then, the medium was removed and 100 μ l DMSO was added to dissolve the formazan precipitates. The absorbance was measured at a wavelength of 570 nm in a ELISA microplate recorder.

LDH activity in the medium was determined according to the protocols of the LDH ELISA kit designed by manufacturer. An aliquot of media was mixed with NAD and lactate solution, and then measured at a wavelength of 450 nm in

the ELISA recorder. All the results are expressed as percentages of the control group.

Immunoblot examination

The primary neurons were cultured in 6-well plates and protein lysates were extracted after treatment. The neuronal cells were washed three times with 0.01 mM PBS, then radio immunoprecipitation assay (RIPA) buffer containing pepstatin 1, leupeptin 2, phenylmethyl -sulfonyl fluoride 1, aprotinin 80 were added into each well and cells were harvested. The lysates were centrifuged at 12,000 g for 30 min at 4°C. The protein samples diluted with loading buffer and separated by 10% SDS-PAGE then transferred onto nitrocellulose membranes (Invitrogen, USA). The membranes were then incubated with blocking buffer (Tris-buffered saline containing 7.5% defatted milk powder). Afterward the membranes were incubated with the rabbit polyclonal anti-NF- κ B (1:200) or mouse monoclonal anti-GAPDH antibody (1:5000) overnight, and then incubated with anti-rabbit IRDye700DX[®]-conjugated antibody or anti-mouse IRDye800DX[®]-conjugated antibody (1:5000, Rockland, USA). The signals were detected by an Odyssey infrared imaging system. Protein bands were quantitatively evaluated by densitometry using Quantity One[®] analysis software.

Determination of cytokine production

After drug treatment, the media were collected from neurons cultures with 1,8-cineol and A β ₂₅₋₃₅ administration for 24 h. The media were centrifuged at 2000 rpm for 10 min to remove cells and debris. The interleukin-6 and interleukin-8 in the media were examined using ELISA kits for IL-6 and IL-8 according to manufacturers' instructions.

Statistical Analysis

All data are expressed as means \pm SEM. Statistical comparisons of viability between different treatment groups were performed with One-way ANOVA, followed by Turkey's

post hoc test (SPSS 15.0 for Windows, SPSS inc., USA). $P < 0.05$ was considered statistically significant.

Results

A β_{25-35} induced toxicity on rat cortical neurons

To investigate the effect of A β_{25-35} on neuron viability, primary cultured cortical neurons were treated with various concentrations of A β_{25-35} (0, 2.5, 5, 10, 20, 40 μ M). Untreated group was added into same volume of vehicle as control. After 24 h, cell viability was measured using the MTT reduction assay. As shown in Fig.1A, the viability of cortical neurons was significantly dose-dependently reduced after treated with A β_{25-35} for 24 h. Compared with control group, the viability treated with 2.5 μ M of A β_{25-35} was lightly reduced but no significant difference was found. However, A β_{25-35} treatment also induced significant increase on LDH activities in a concentration -dependant manner in cultured cortical neurons (Fig.1B). Combined with the above results, 20 μ M of A β_{25-35} was selected as an optimal concentration for subsequent experiments, since the cell viability was 60-70% at the concentration ($66.5 \pm 6.7\%$, $P < 0.01$) compared with untreated neuronal cells.

Neuroprotective effects of 1,8-cineol on A β_{25-35} -induced toxicity

To evaluate the effect of 1,8-cineol on A β_{25-35} -induced neurotoxicity, primary cortical neurons were pretreated with varying concentrations of 1,8-cineol (1, 3, 10 μ M) for 1 h, and then exposed to 20 μ M of A β_{25-35} for 24 h. The possible effect of 1,8-cineol was evaluated based its effect on cell viability by MTT and LDH assays. Fig.1C and D shows that A β_{25-35} 20 μ M significantly decreased cell viability and increased the activity of LDH ($P < 0.01$), which was restored by 1,8-cineol at 3

and 10 μ M. 1 μ M of 1,8-cineol also increased cell viability and lightly decreased LDH activity but not significant.

Anti-inflammatory effect of 1,8-cineol on cytokine production

Biochemical and neuropathological studies highlighted that Neuroinflammation played an important role in the progression of Alzheimer's disease. Inflammation process once initiated, may contributed to neuronal dysfunction and cell death, establishing a vicious cycle. In the present study, A β_{25-35} 20 μ M caused a higher level of IL-6 and IL-8 production in the supernatant of cultured cortical neurons as compared with untreated neuronal cells ($P < 0.01$). 1,8-cineol pretreatment with concentrations of 3 and 10 μ M significantly decreased the level of IL-6 (Fig.2A, $P < 0.01$) and IL-8 (Fig.2B, $P < 0.05$) release in cortical neurons compared with neuronal cells treated with A β_{25-35} only.

1,8-cineol inhibited NF- κ B activation

To study the anti-inflammatory mechanism of 1,8-cineol, we determined and analyzed the protein level of NF- κ B p65 in cortical neurons. It has been reported that A β can activate NF- κ B in neurons cells, indicating an important role of this molecular pathway in the pathogenesis of AD. As previously demonstrated, 1,8-cineol regulated the function of NF- κ B in inflammatory conditions. In the present study, we verified whether in our experimental conditions, 1,8-cineol modulated A β_{25-35} induced NF- κ B activation. Western blot analysis showed a significant increase of NF- κ B p65 expression with A β_{25-35} incubation. Pretreatment with 1,8-cineol 10 μ M in cortical neurons followed by A β_{25-35} exposure led to a significant reduction of NF- κ B p65 expression after 24 h (Fig.3A and B, $P < 0.05$).

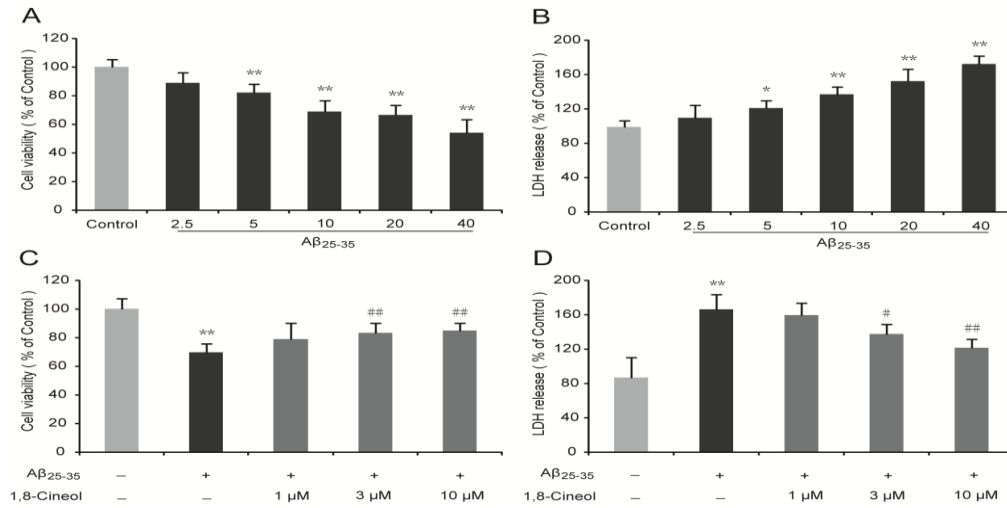


Figure 1: Effect of 1,8-cineol against Aβ₂₅₋₃₅-induced toxicity on primary cortical neurons. Neuronal cells were treated with increasing Aβ₂₅₋₃₅ concentrations (2.5 to 40 μM) for 24 h. Cell viability was assessed by measuring the MTT reduction (A), and LDH release was detected (B). 1 h before exposure to Aβ₂₅₋₃₅ (20 μM), cortical neurons were pre-incubated alone or with 1,8-cineol at concentrations of 1, 3, 10 μM. After 24 h, cell viability (C) and LDH release level (D) were tested. Values are expressed as percentage of the mean value of untreated cells at each time point. Results represents means ± SEM. **P* < 0.05, ***P* < 0.01 vs. the control group; #*P* < 0.05, ##*P* < 0.01 vs. cortical neurons treated with Aβ₂₅₋₃₅ only.

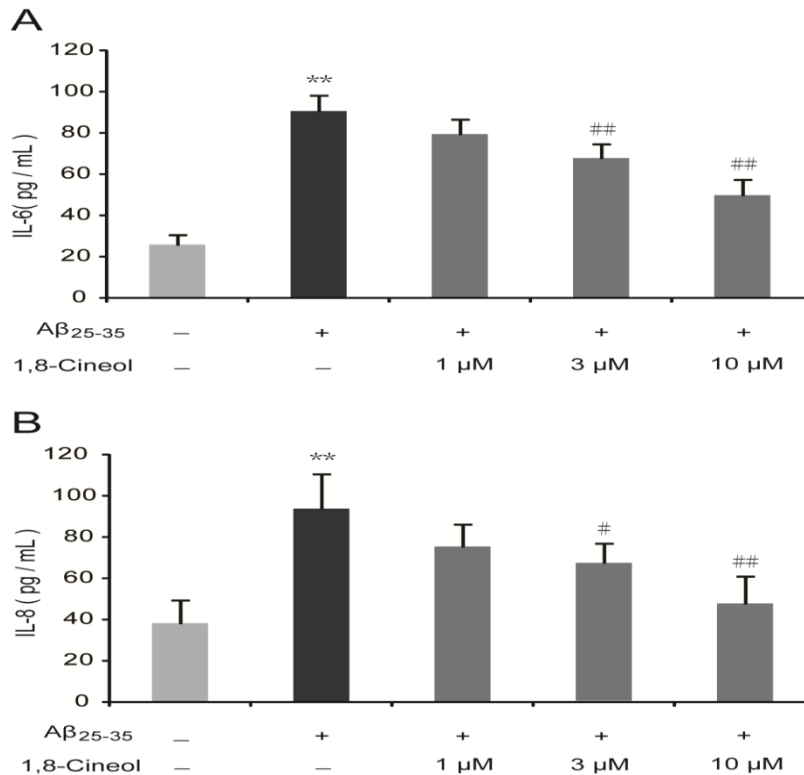


Figure 2: 1,8-cineol reduced IL-6, IL-8 release in the supernatant. Cortical neurons were pretreated with three concentration of 1,8-cineol (1, 3, 10 μM) for 1 h, then were further exposed to 20 μM of Aβ₂₅₋₃₅. After 24 h, the supernatant were collected. IL-6 (A) and IL-8 (B) release level in the supernatant were measured by ELISA. Values were expressed as means ± SEM. **P* < 0.05, ***P* < 0.01 vs. the control group; #*P* < 0.05, ##*P* < 0.01 vs. cortical neurons treated with Aβ₂₅₋₃₅ only.

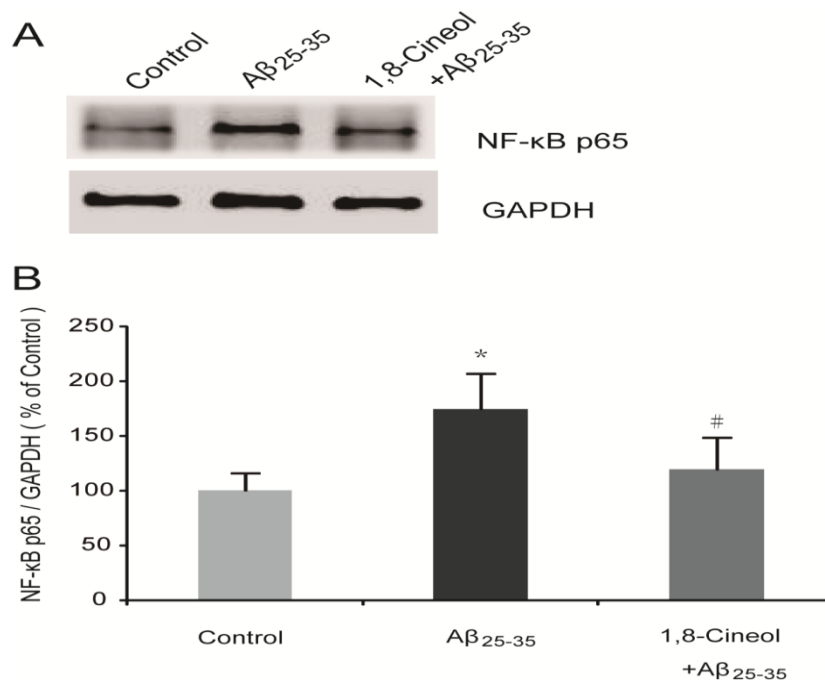


Figure 3: The effect of 1,8-cineol on NF-κB p65 expression. Cortical neurons were pretreated with 10 μM of 1,8-cineol for 1 h, and then exposed to 20 μM of Aβ₂₅₋₃₅ for 24 h. The proteins were collected and the expression of NF-κB p65 was detected using immunoblot analysis. **P* < 0.05 vs. the control group; #*P* < 0.05 vs. cortical neurons treated with Aβ₂₅₋₃₅ only.

Discussion

Alzheimer’s disease is a chronic neurological disorder which is the most frequent cause of cognitive decline affecting the people worldwide (Rafii and Aisen 2015). Aβ, a major component of senile plaques, is the defining feature of AD neuropathology. Numerous studies indicate that Aβ can induce inflammation and cell apoptosis *in vitro* and *in vivo* (Ghasemi et al. 2014; Choi et al. 2012; Du et al. 2014). Inflammatory processes contribute to CNS dysfunction, neuronal cell loss and injury, playing an important role in the AD pathogenesis. Inhibition of inflammation is now recognized as a potential target for the treatment and prevention of the disease.

In the present study, we assessed Aβ₂₅₋₃₅-induced toxicity in rat cortical neurons by MTT and LDH assay, and confirmed that Aβ₂₅₋₃₅ treatment at 20 μM for 24 h significantly reduced cell viability. Pretreatment with different concentrations of 1,8-cineol markedly attenuated decreased cell viability. The protective effect of 1,8-cineol was further

confirmed by LDH release detection. We also found that 1,8-cineol effectively reduced the production of pro-inflammatory cytokines IL-6 and IL-8 and regulated NF-κB activation induced by Aβ₂₅₋₃₅ treatment. All these findings demonstrated that 1,8-cineol could protect neuronal cells against Aβ₂₅₋₃₅-induced toxicity.

Multiple studies suggests that Aβ peptides play a pivotal role in inducing neuroinflammation in AD (Pimplikar 2014). Aβ-burdened neurons might be the initial cells triggering inflammation, resulting in the progression of the disease (Hanzel et al. 2014). Inflammatory cytokines like TNF-α, IL-8, IL-6 were significantly increased in the cortex and hippocampus in AD, which can also promote Aβ production by modulating γ-secretase activity (Liao et al. 2004). Consistent with these findings, in the present study, cortical neurons treated with Aβ displayed increased levels of TNF-α and IL-1β, which was restored by 1,8-cineol pretreatment. The mechanism underlying Aβ-induced inflammation is involved in several signal pathways. It has been

reported that NF- κ B activation and translocation was closely related with cytokine production induced by A β (Yu et al. 2012; He et al. 2011). Greiner found that 1,8-cineol inhibited proinflammatory target genes by inhibiting nuclear NF- κ B p65 translocation via I κ B α (Greiner et al. 2013). Our results indicated that 1,8-cineol significantly inhibited the upregulated NF- κ B expression induced by A β ₂₅₋₃₅ treatment. These findings support that NF- κ B activation is possibly involved in the neuroprotective effects of 1,8-cineol.

In summary, we demonstrated that 1,8-cineol exerted neuroprotection against A β -induced neurotoxicity by inhibiting IL-6, IL-8 production and regulating NF- κ B activation in neuronal cells. Future study are needed to focus on neuroprotective effect of 1,8-cineol *in vivo*. The more attention paid to clarify the further mechanisms of 1,8-cineol will help us to discover more potent candidates for the treatment of AD.

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