Biological evaluation of YHK （养生片仔癀）and Brainwell（养脑力）on β-amyloid aggregation and β-amyloid-induced cytotoxicity

1. Introduction

In the liver, the enzyme alcohol dehydrogenase oxidizes alcohol into acetaldehyde, which is then further converted into the harmless acetic acid (vinegar) by acetaldehyde dehydrogenase. The excessive aldehydic load and mitochondrial dysfunction lead to neurological diseases, including Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), dementia, ataxia, seizure, hypertension-induced encephalopathy and ischemic stroke. One of the more studied reactive aldehyde is 4-Hydroxynonenal (4-HNE). 4-HNE is one of the most abundant and reactive lipid peroxides. Increased hepatic 4-HNE contents present in both human alcoholics and alcohol-fed animals. Multiple studies report on the accumulation of 4-HNE protein adducts as an early event in the pathogenesis of AD. 4-HNE hastens Aβ protofibril and curved fibril formation. The accumulation of 4-HNE-modified amyloid β-peptides has been shown to inhibit the proteasome, and the resulting accumulation of ubiquitinated modified proteins leads to a pro-inflammatory response amplifying neurodegeneration. Thus, disease-modifying strategies targeting 4-HNE levels might benefit AD patients. By utilizing neural cell model, the project intends to study the potential role of YHK and Brainwell (BW) in treating and relieving AD or other brain disorders. The study will promote the pharmacologic study of YHK and Brainwell, provide new insights and directions for the YHK and Brainwell application.

2. Current study objectives

2.1 To investigate the potential inhibitory effect of YHK and Brainwell on Aβ aggregation and Aβ-induced cell toxicity.

Aβ1-42 peptide together with YHK and Brainwell will be dissolved in water and incubated according to previous report (Yang et al., 2005). The Aβ aggregation will be determined by measuring the fluorescence intensity of thioflavin (Th-T) assay (Hasegawa et al., 1999). In Aβ-induced cytotoxicity test, cultured PC12 in 96-well plate will be treated with aged Aβ. Followed by assay with 3-(4, 5-dimethylthiazol-2)-2,5 diphenyltetrazolium bromide (MTT). On the other hand, PC12 cells, in 96-well plates were pretreated with YHK before the addition of aged Aβ1-42. After incubation, the cell viability will be determined by MTT assay.

2.2 To investigate the effect of YHK and Brainwell on 4-HNE induced Aβ aggregation.

Aβ1-42 peptide will be incubated with different dosage of YHK and Brainwell together with 4-HNE/alcohol. The aggregation of Aβ will be by Th-T fluorescence analysis and observed by atomic force microscope. Cell viability will be measured by MTT colorimetric method.

3. Material and methods

3.1 Preparation of extracted samples

YHK (Kyotsujigyo Inc., Japan) and Brainwell (Kyotsujigyo Inc.) samples were grinded into powder, weighed 50mg into a 1.5-mL eppendorf, added with 1 mL of water, sonicated for 30 min, and centrifuged at 13,200 rpm for 10 min. The supernatants were collected and filter by PTFE syringe filter (0.22 μm). The stock solution was 50 mg/mL and stored at -20 ^{o}C.

3.2 Cell culture

PC12 cells originated from rat adrenal medulla were purchased from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 6% fetal bovine serum (FBS) and horse serum (HS), 100 units/mL of penicillin and 100 μg/mL of streptomycin, placed in a humidified CO_2 (7.5%) incubator at 37 ^{o}C. Fresh medium was applied every other day. The medium was changed by fresh DMEM with 1% FBS and HS, 100 units/mL of penicillin and 100 μg/mL of streptomycin 3 hours before extract treatment. In extracts treatment, extracts were diluted by DMEM with 1% FBS and HS, 100 units/mL of penicillin and 100 μg/mL of streptomycin.

3.3 Cell viability assay

Cell viability was assessed by MTT [3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] assay. Cells were plated in 96-well plate for 24 hours and treated with extracts at different concentrations for 48 hours. Then the cells were incubated with MTT for 3 hours at 37 ^{o}C. The medium was discarded and 100 μL of DMSO was added to each well. The plate was shaken in dark place for 15 min. After that, the absorbance under 570 nm was measured by a microplate reader (Thermo Scientific, Fremont, CA).

3.4 Determination of Aβ aggregation

Aβ1-42 peptide at final concentration of 10 μM together with extracts of YHK or Brainwell at different concentrations were dissolved in water and incubated for 4 days at 37 ℃ according to previous report (Yang et al., 2005). Ten μL sample was mixed with 190 μL thioflavin solution (ThT, 5 μM in 50 mM glycine, pH 8.5) to determine Aβ aggregation by measuring the fluorescence intensity at emission 435 nm and excitation 480 nm by EnVision™ Multilabel Reader (Hasegawa et al., 1999).

3.5 Determination of Aβ-induced cytotoxicity

In Aβ-induced cytotoxicity test, PC12 cells in 96-well plate were treated with aged Aβ (4 days at 37 ℃) for 24 hours. Followed by addition of MTT in PBS at final concentration of 0.5 mg/mL for 3 hours, the medium was aspirated, and the cultures were re-suspended by 150 μL DMSO to determine the cell viability by measuring the absorbance at 570 nm. On the other hand, PC12 cells in 96-well plates were pretreated with extracts of YHK or Brainwell at different concentrations for 24 hours or curcumin (30 μM;

positive control) for 3 hours before the addition of aged Aβ (10 μM) for 24 hours. After incubation, the cell viability was determined by MTT assay as same as above.

3.6 Other Assays

Statistical tests were performed using one-way analysis of variance; differences from basal or control values were classified as * where $p < 0.05$, ** where $p < 0.01$, and *** where $p < 0.001$.

4. Result interpretation

4.1 Effect of YHK or Brainwell extracts on the growth of PC12 cells.

Cell viability assay was firstly performed in PC12 cells to determine a safe concentration range (0-5 mg/mL) of each extract at which all extracts did not induce cell proliferation or death. Result showed that the cell viability was not significantly affected by YHK and Brainwell extracts at concentrations from 0-5 mg/mL (Fig. 1 & Fig. 2). Therefore, the concentrations, not more than 5 mg/mL, were selected for the following study.

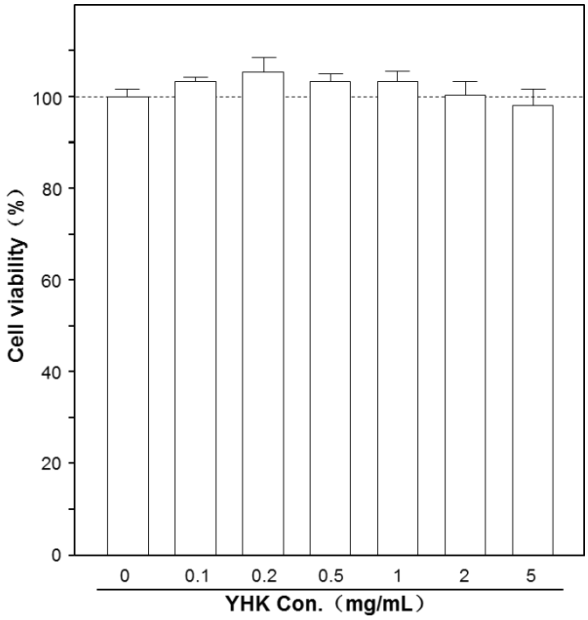

Fig.1 Effects of different concentrations of YHK on PC12 cell viability

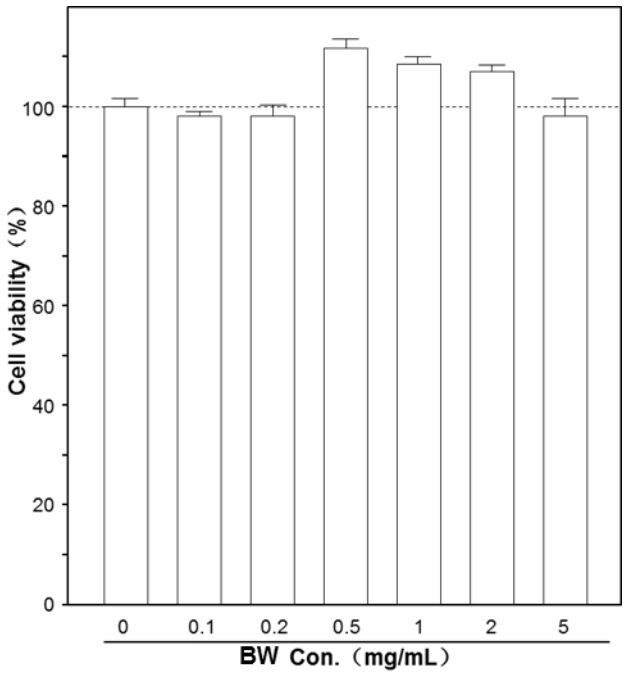

Fig.2 Effects of different concentrations of Brainwell on PC12 cell viability

4.2 Effect of EtOH or 4-HNE on the growth of PC12 cells.

Another cell viability assay was performed in PC12 cells to determine a safe concentration range of EtOH or 4-HNE at which they did not induce cell proliferation or death. Result showed that the cell viability was not significantly affected by EtOH and 4-HNE at concentrations from 0-50 Mm (EtOH) and 0-20 μM (Fig. 3 & Fig. 4). Therefore, 10, 50 mM of EtOH, and 20 μM of 4-HNE were selected for following study.

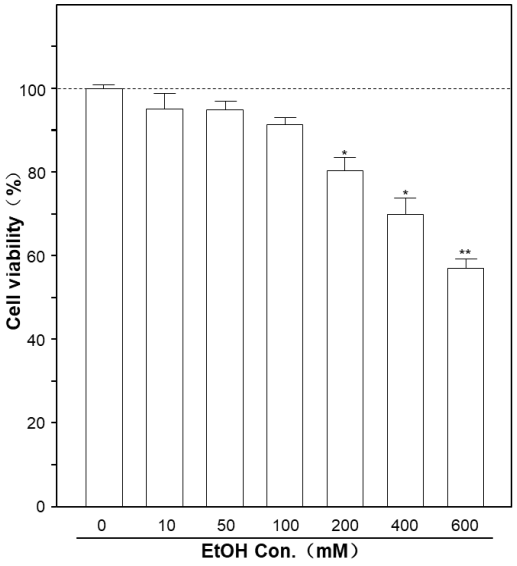

Fig. 3 Effects of different concentrations of EtOH on PC12 cell viability

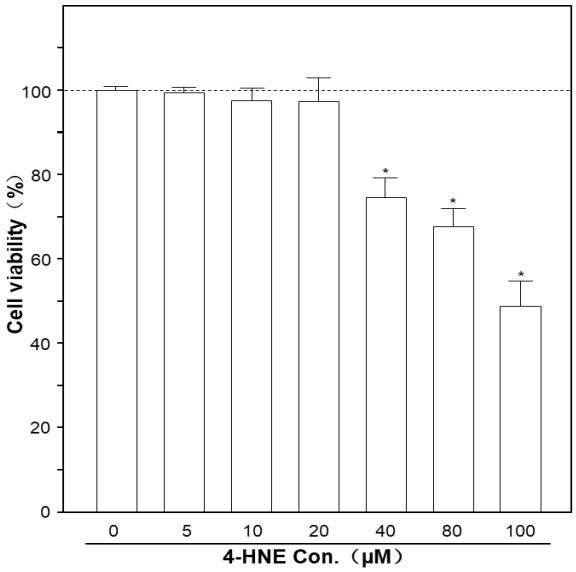

Fig. 4 Effects of different concentrations of 4-HNE on PC12 cell viability

4.3 Establishment of Aβ aggregation and Aβ-induced cytotoxicity models

Aβ is one of the critical proteins relating to the clinical pathology in AD. Aβ is aggregated to form senile plaque, resulting in neuronal cell death. Numerous researches were targeted on the activity in suppressing the aggregation of Aβ. Experimentally, Aβ aggregate was formed after 2 days of incubation of soluble Aβ at 37 °C. The number of Aβ aggregate was peaked at day 6 of aging. From the results, Aβ dissolved in either 10 or 50 mM ethanol did not aggregate efficiently (Fig. 5). Aβ dissolved in 10 μM 4-HNE showed heavier aggregation than control group (Aβ only). In the aggregation assay, curcumin inhibited Aβ aggregation by 60% (Fig. 5); this was in accord with previous study and served as a positive control (Yang et al., 2005).

The cytotoxic effect of aggregated Aβ was then measured in cultured PC12 cells. The application of aged Aβ in the cultures induced the cell death (Fig. 6). Ethanol (10 and 50 mM), 10 μM 4-HNE and 30 μM curcumin did not show evident effects on cell viability. When aged Aβ aggregates were added to 10 and 50 mM ethanol-treated PC12 cells, no toxicity was determined. Aβ aggregates in 10 μM 4-HNE-treated cultures could induce cell death. Curcumin is well known for inhibiting the formation of Aβ (Yang et al., 2005) and protecting Aβ-induced cytotoxicity (Reddy et al., 2016; Zhang et al., 2018). It is a widely used positive control for studying Aβ aggregation and Aβ-induced cytotoxicity. For a positive control, the treatment of curcumin inhibited Aβ-induced toxicity by 60% (Fig. 6). From these observations it was clear that 10 μM 4-HNE could induce the process of Aβ aggregation and Aβ-induced toxicity in PC12 cells.

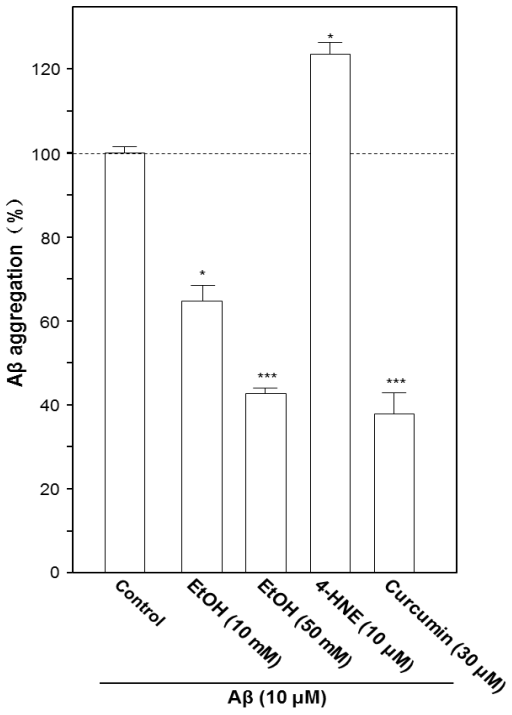

Fig.5 Effects of ethanol and 4-HNE on Aβ aggregation

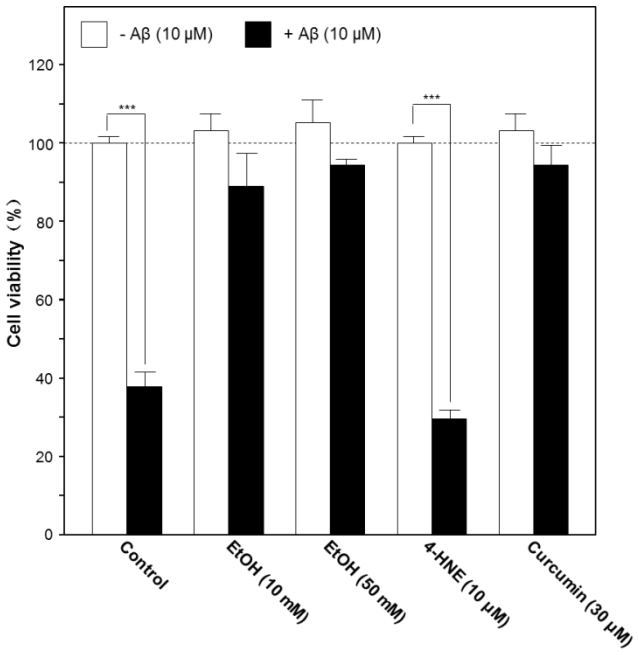

Fig.6 Effects of ethanol and 4-HNE on Aβ-induced cytotoxicity

4.4 Effects of YHK and Brainwell extracts on Aβ aggregation

Aβ was incubated with various drugs (4-HNE, curcumin, YHK and Brainwell extracts) at different concentrations as indicated in Fig. 5 for 6 days at 37 ℃. With the co-incubation of YHK or Brainwell extracts, at 0.1 and 0.5 mg/mL (a concentration chosen from previous study), no significant aggregation of Aβ was observed (Fig. 7). Co-incubation of Aβ with YHK or Brainwell extracts, at 5 mg/mL, reduced Aβ aggregation was determined (Fig. 7; *p < 0.05; **p < 0.01; ***p < 0.001; compared with control). In addition, YHK or Brainwell extracts at 5 mg/mL could alleviate 4-HNE-induced Aβ aggregation (Fig. 7; ##p < 0.01; compared with 4-HNE-induced group).

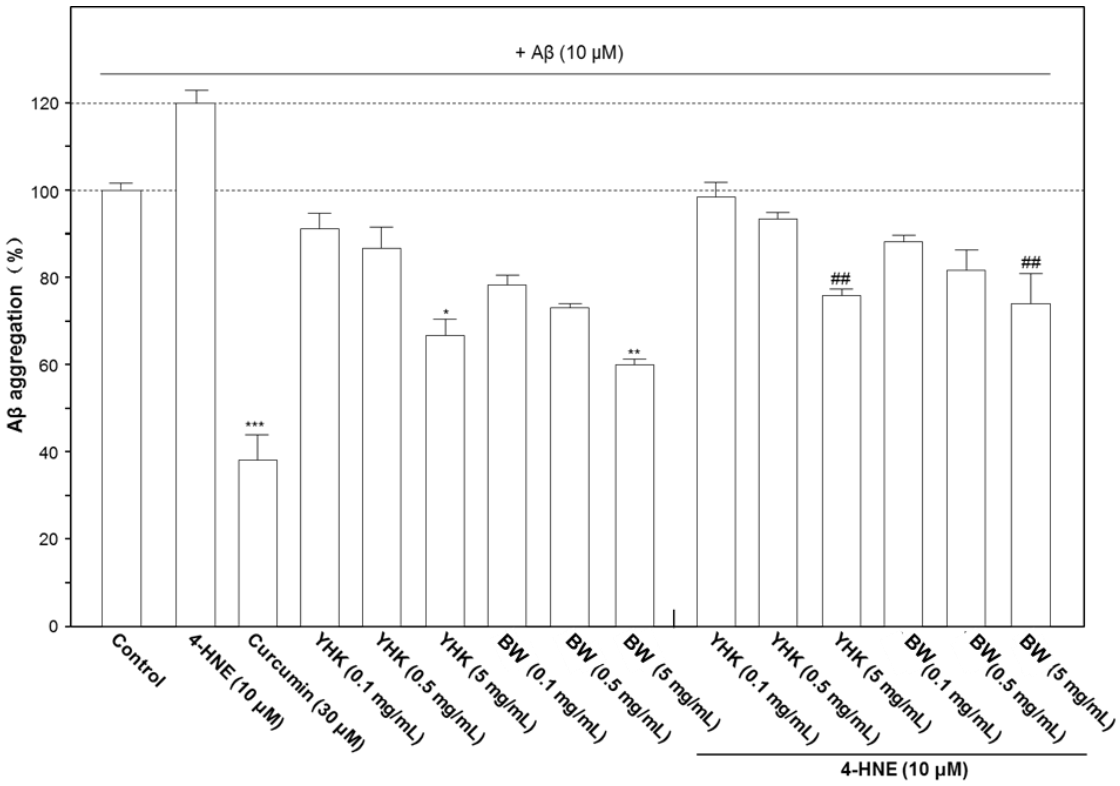

Fig.7 Effects of YHK and Brainwell extracts on Aβ aggregation

4.5 Effects of YHK and Brainwell extracts on Aβ-induced cytotoxicity in PC12 cells

Cultured PC12 cells were treated with various drugs (4-HNE, curcumin, YHK and Brainwell extracts) at different concentrations as indicated in Fig. 8, followed with application of aged Aβ. Cultures treated with YHK or Brainwell extracts, at 0.1 and 0.5 mg/mL, showed no significant effect on Aβ-induced cytotoxicity (Fig. 8). Cultures treated with YHK or Brainwell extracts, at 5 mg/mL, could reverse Aβ-induced cytotoxicity (Fig. 8; *p < 0.05; ***p < 0.001; compared with control). In addition, YHK or Brainwell extracts at 5 mg/mL could alleviate 4-HNE-aggravated Aβ-induced cytotoxicity (Fig. 8; #p < 0.05; compared with 4-HNE-treated group).

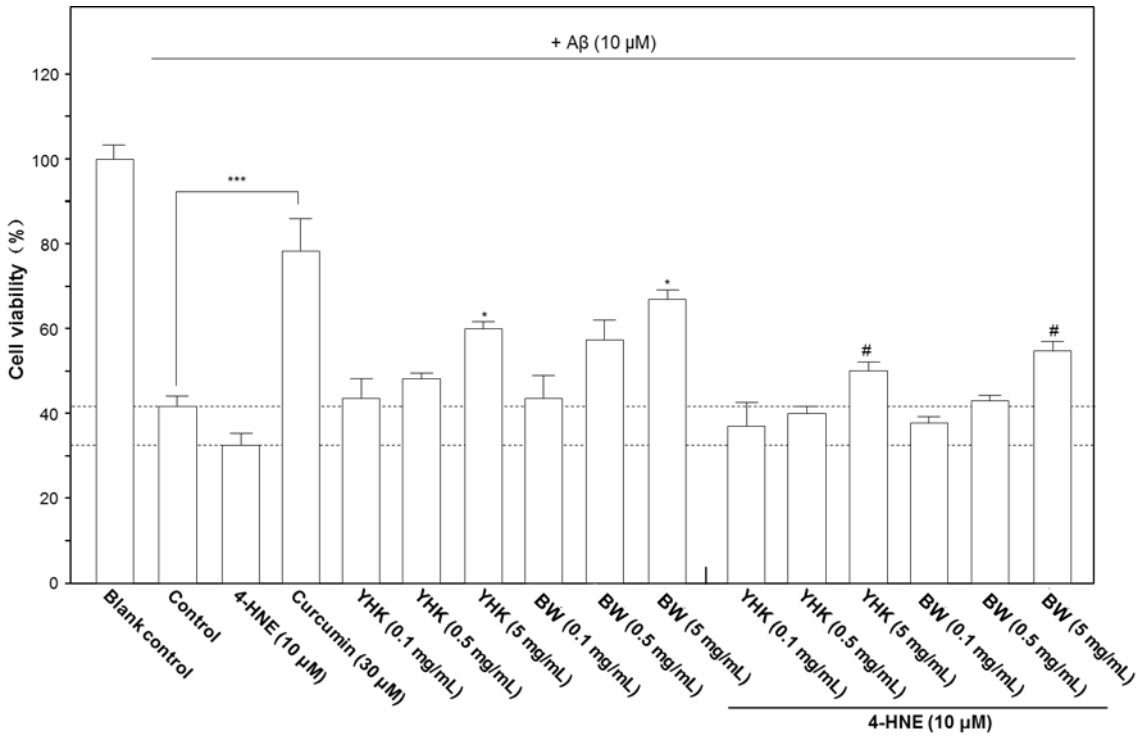

Fig.8 Effects of YHK and Brainwell extracts on Aβ-induced cytotoxicity

4.6 Effects of YHK and Brainwell extracts on reversion of Aβ aggregation

Aβ 1-42 (10 μM) was incubated 6 days at 37ºC. Then, add various drugs (4-HNE, curcumin, YHK and Brainwell extracts) at different concentrations in incubated Aβ for 3 days. With the co-incubation of YHK or Brainwell extracts, at 0.1 and 0.5 mg/mL (a concentration chosen from previous study), no significant reversion of Aβ aggregation was observed (Fig. 9). Co-incubation of Aβ with YHK or Brainwell extracts, at 5 mg/mL, reverse of Aβ aggregation was determined (Fig. 9; *$p < 0.05$; **$p < 0.01$; compared with control). The reversion of Aβ aggregation reached to the maximum at 3 days incubation of YHK or Brainwell extracts (Fig. 10).

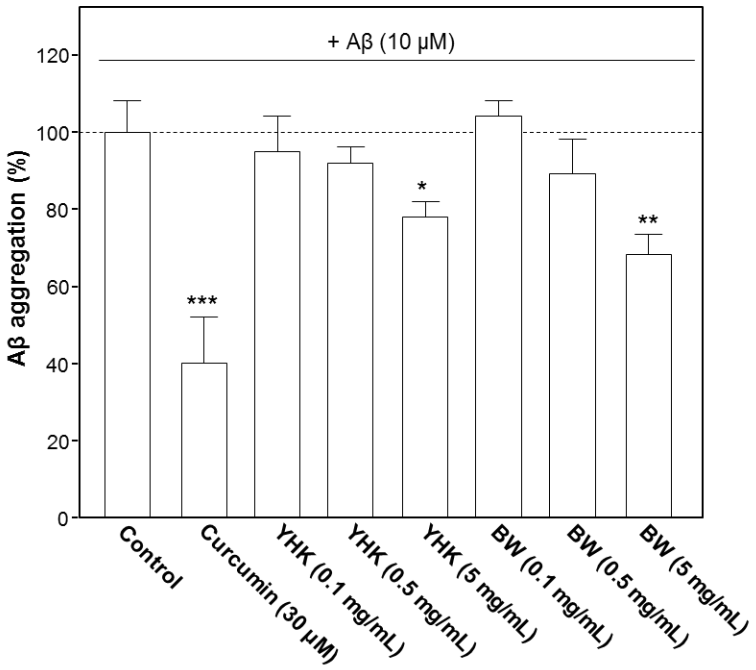

Fig.9 Effects of YHK and Brainwell extracts on reversion of Aβ aggregation

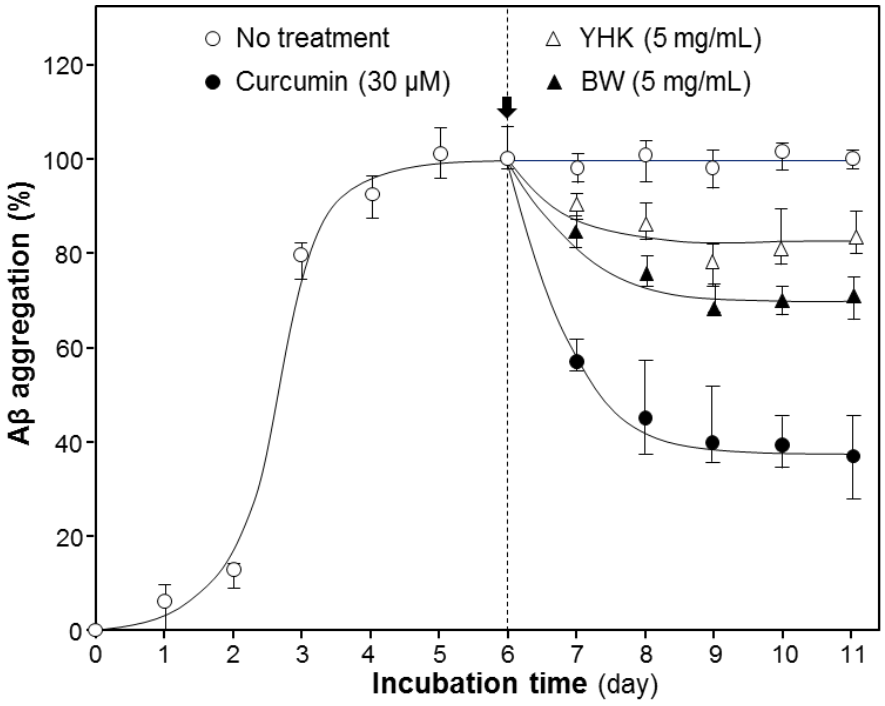

Fig.10 Time curve of YHK and Brainwell extracts on reversion of Aβ aggregation

4.7 Effect of YHK or Brainwell extracts on the growth of RAW264.7 cells.

Cell viability assay was performed in RAW264.7 cells to determine a safe concentration range (0-5 mg/mL) of each extract at which all extracts did not induce cell proliferation or death. Result showed that the cell viability was not significantly affected by YHK and Brainwell extracts at concentrations from 0-5 mg/mL (Fig. 11 & Fig. 12). Therefore, the concentrations, not more than 5 mg/mL, were selected for the following study.

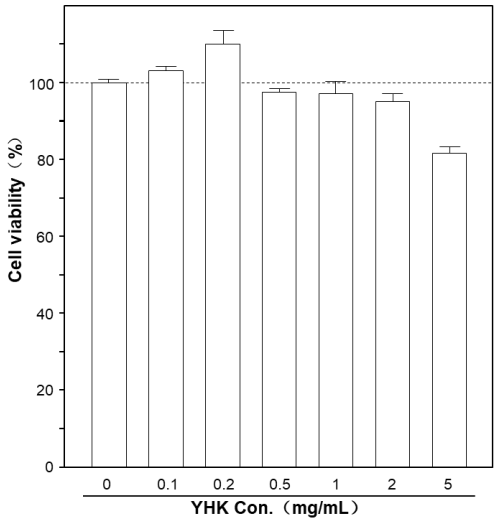

Fig.11 Effects of different concentrations of YHK on RAW264.7 cell viability

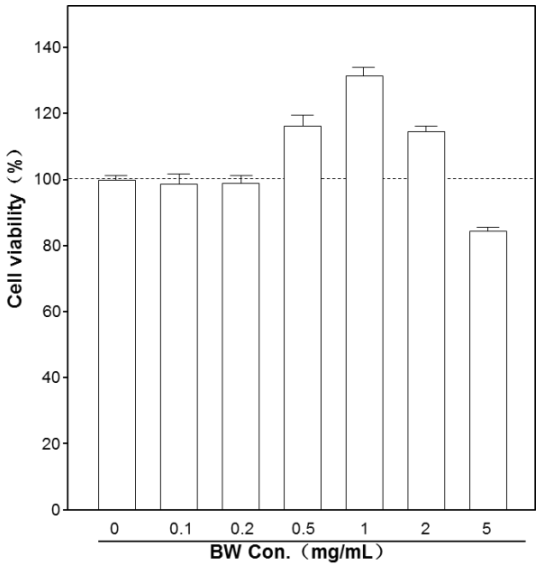

Fig.12 Effects of different concentrations of Brainwell on RAW264.7 cell viability

4.8 Establishment of LPS and ethanol-induced inflammatory cell models

LPS (100 ng/mL)-induced inflammation indicated by increasing of proinflammatory cytokines is firmly established. From the results, mRNA expression of IL-6 could not be detected in control group, while greatly induced by LPS-treatment. Pre-treatment of 10 mM ethanol could enhance LPS-induced mRNA expression of IL-6. However, pre-treatment of 10 μM 4-HNE could relieve LPS-induced mRNA expression of IL-6 (Fig. 13, left panel). Similar results were observed in mRNA expression of TNF-α (Fig. 13, right panel). From these results, it was clear that 10 mM ethanol could enhance LPS-induced inflammation in RAW264.7 cells.

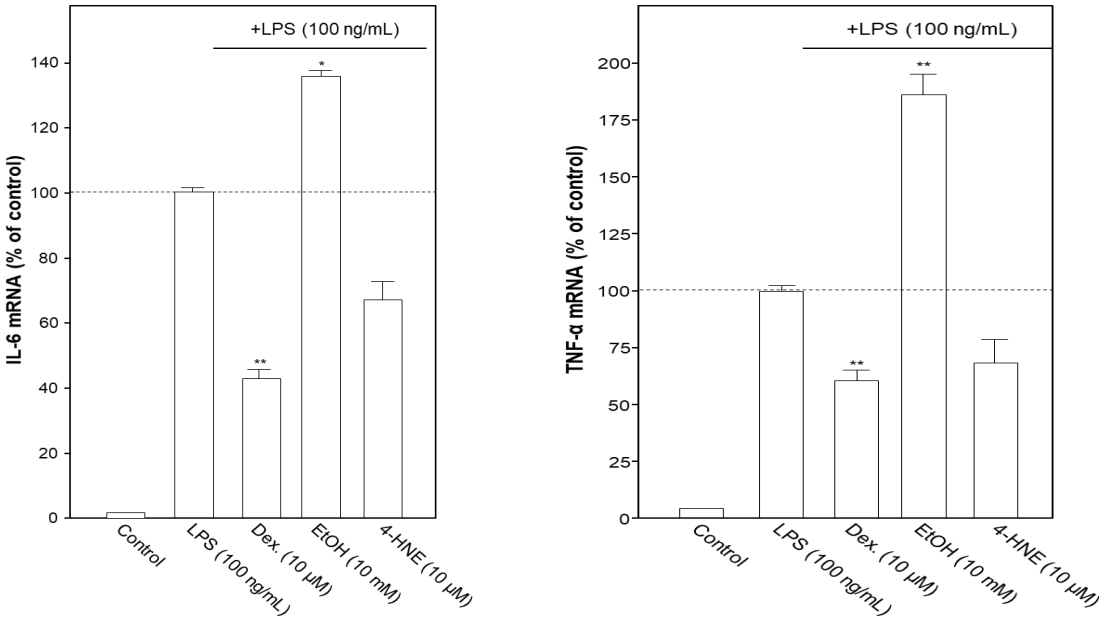

Fig. 13 Effects of ethanol and 4-HNE on LPS-induced mRNA expression of IL-6 and TNF-α

4.9 Effects of YHK and Brainwell extracts on LPS and ethanol-induced inflammatory responses

Application of YHK and Brainwell extracts at different concentrations (0.5 mg/mL, 1 mg/mL, and 2 mg/mL) on LPS and ethanol-induced RAW264.7 cells for 24 hours. Only YHK extracts at 2 mg/mL reduced LPS and ethanol-induced expression of IL-6 mRNA (Fig. 14). YHK or Brainwell extracts showed no significant reduction of LPS and ethanol-induced expression of TNF-α mRNA (Fig. 15).

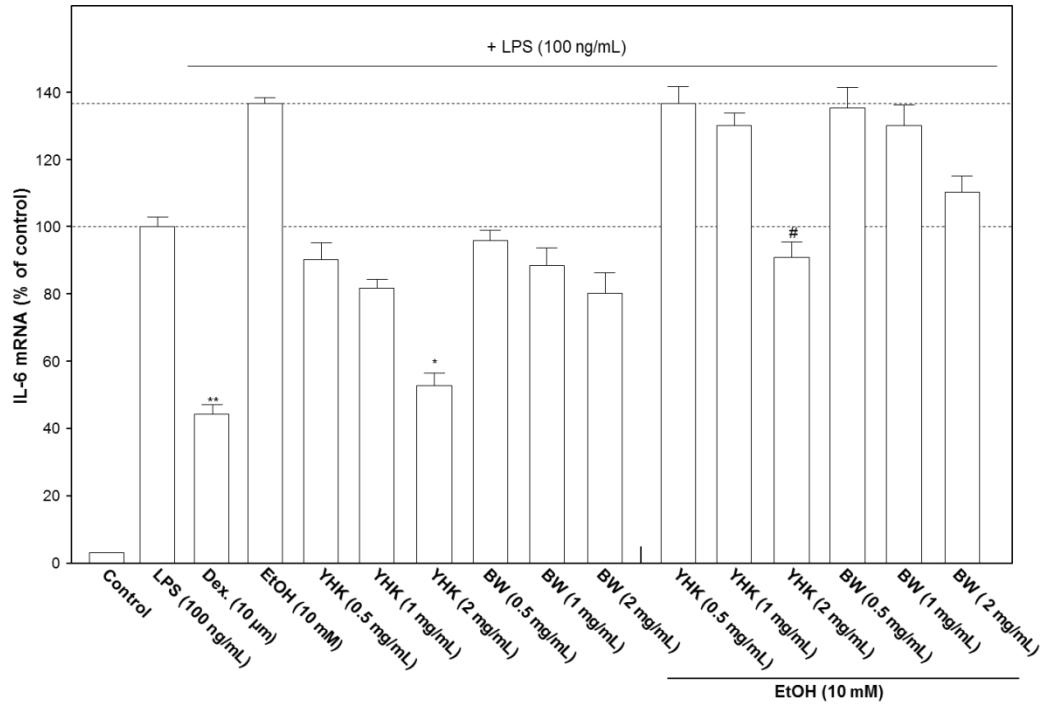

Fig.14 Effect YHK and Brainwell extracts on LPS and ethanol-induced IL-6 mRNA expression

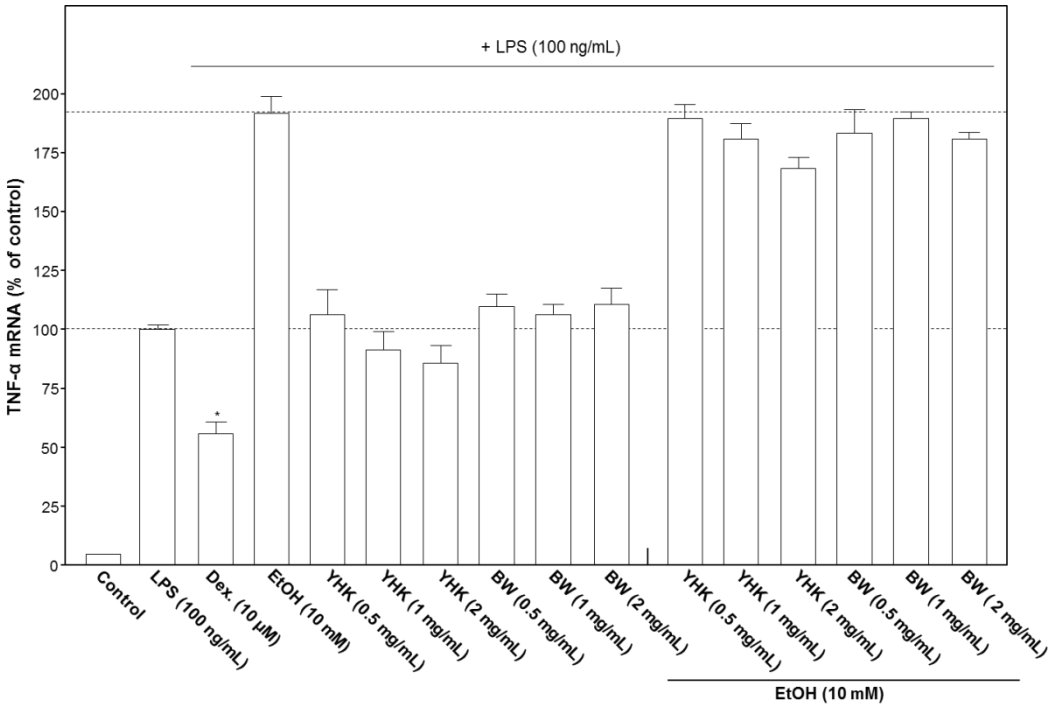

Fig.15 Effect YHK and Brainwell extracts on LPS and ethanol-induced TNF-α expression

4.10 Establishment of tBHP-induced oxidation cell models

In order to explore the potential effect of YHK and Brainwell on oxidation, tBHP (150 mM)-induced oxidation model is firmly established. Cultured PC12 cells were treated with tBHP and other chemicals. Results showed that the cell viability of PC12 cells was significantly affected by tBHP. Therefore, pre-treatment of Vitamin C (1 mM) and Ethanol (10mM) can reduce the cell death caused by oxidation, while pre-treatment of 4-HNE (10mM) enhanced the oxidation. Vitamin C can still act as a positive control in 4-HNE enhanced oxidation model (Fig. 16).

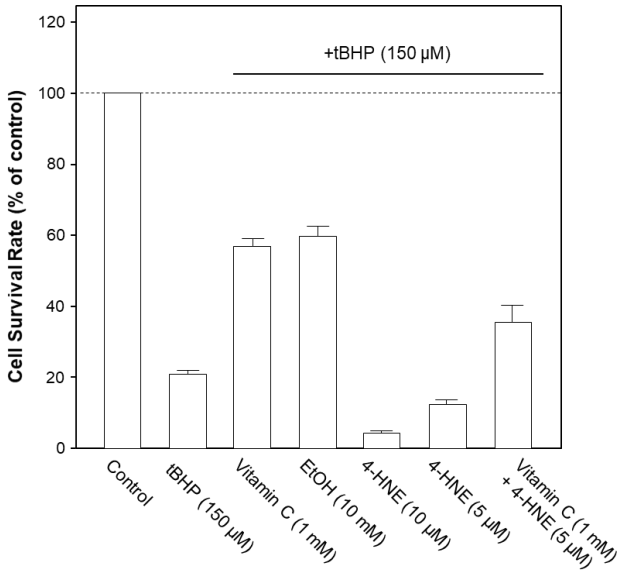

Fig.16 Effects of ethanol and 4-HNE on tBHP-induced oxidation of PC12 cells

4.11 Effects of YHK and Brainwell extracts on tBHP-induced oxidation in PC12 cells

PC12 cells were pre-treated with various drugs (YHK and Brainwell extracts) at different concentrations (0.1 mg/mL, 0.31 mg/mL, 1 mg/mL and 3 mg/mL). Then the oxidation model of PC12 cells was established by treating cell with tBHP (150 mM) for 3 h. Results showed that YHK extracts can dose-dependently prevent the oxidation induced by tBHP, and shows stronger effect than Brainwell extract. PC12 cells were co-treated by 4-HNE and various drugs (YHK and Brainwell extracts), and then were treated with tBHP for 3 hrs. From the results, YHK and Brainwell extracts can reverse the effect of 4-HNE which can enhance the oxidation of PC12 cells (Fig. 17).

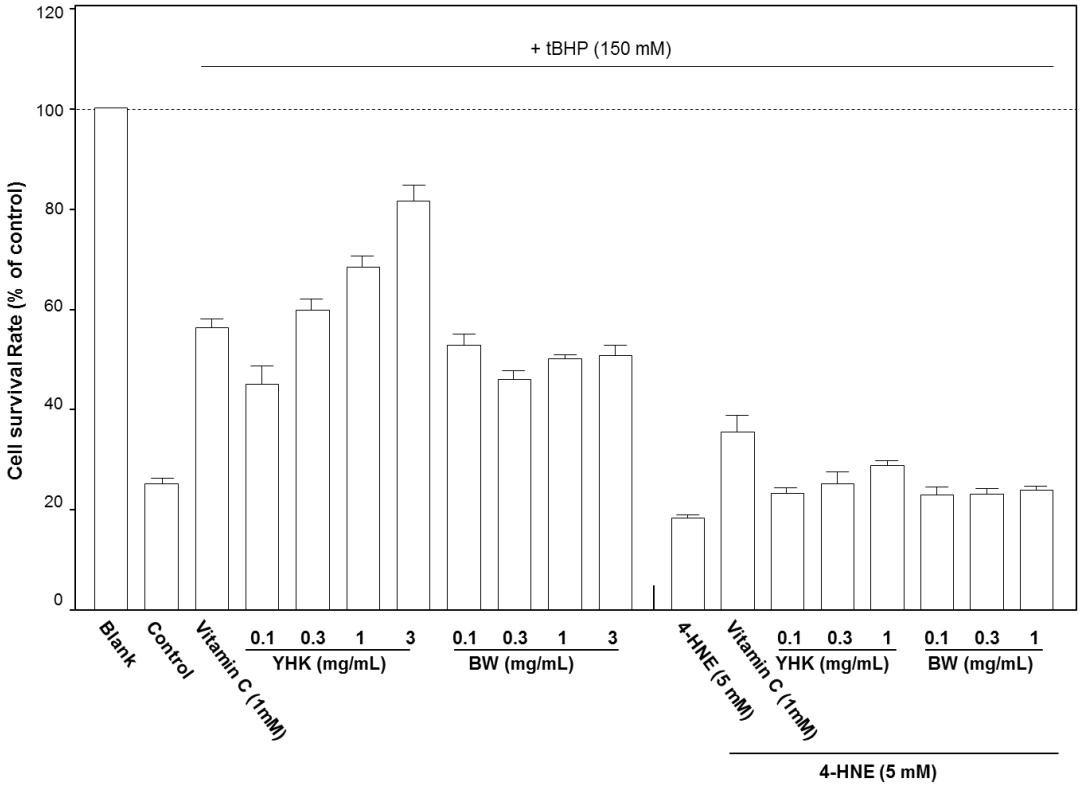

Fig.17 Effects of YHK and Brainwell on tBHP-induced oxidation of PC12 cells

5. Conclusion

(1) Application of YHK or Brainwell at high concentration (5 mg/mL) could inhibit the 4-HNE-induced Aβ aggregation.

(2) Application of YHK or Brainwell at high concentration (5 mg/mL) could inhibit Aβ aggregation-induced cell death and 4-HNE-enhanced cell death.

(3) Application of YHK or Brainwell at high concentration (5 mg/mL) could reverae Aβ aggregation.

(4) Application of YHK at high concentration (2 mg/mL) could reduce LPS and ethanol-induced expression of IL-6 mRNA. However, YHK or Brainwell extracts showed no significant reduction of LPS and ethanol-induced expression of TNF-α mRNA.

(5) Application of YHK or Brainwell at high concentration (3 mg/mL) could inhibit tBHP-induced oxidation and 4-HNE-enhanced oxidation of PC12 cells. The inhibition effect of YHK was in a dose-dependent manner.

(6) Application of YHK or Brainwell at high concentration (3 mg/mL) could inhibit tBHP-induced and 4-HNE-enhanced PC 12 cell death. The inhibition effect of YHK was in a dose-dependent manner.

6. Further work

(1) The anti-inflammatory effects of YHK and Brainwell extracts in primary cultured astrocytes could be investigated.

Reference:

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