J Chin Med 27(1): 2701002, 9 pages, 2016
DOI: 10.3966/241139642016062701002
E-ISSN: 24113964
http://www.nricm.edu.tw/jcm/

中醫藥雜誌
Journal of Chinese Medicine

Protective Effect of Methanol-Soluble Extract from *Terminalia chebula* Retzius against $A\beta_{25-35}$-Induced PC12 Cells Apoptosis through Modulation of PI3K/Akt-Dependent Repression of GSK-3 Activity

Yu-Chang Hou[1,9,#], Chia-Lin Chang[3,4,#], Chang-Ming Chern[6,7], Yea-Hwey Wang[5,6], Kuo-Tong Liou[10], Chien-Chih Chen[3], Che-San Lin[3], Yuh-Chiang Shen[2,5,8,#,*]

[1]*Department of Traditional Medicine, Taoyuan General Hospital, Ministry of Health and Welfare, Taoyuan, Taiwan*
[2]*National Research Institute of Chinese Medicine, Ministry of Health and Welfare, Taipei, Taiwan*
[3]*Research Institute of Biotechnology, College of Medicine and Nursing, Hung-Kuang University, Taichung, Taiwan*
[4]*Department of Nursing, College of Medicine and Nursing, Hung-Kuang University, Taichung, Taiwan*
[5]*National Taipei University of Nursing and Health Science, Taipei, Taiwan*
[6]*Stroke and Neurovascular Disease Center, Section of Cerebrovascular Disease and Taipei Municipal Gan-Dau Hospital, Department of Neurology, Taipei Veterans General Hospital and School of Medicine, Taipei, Taiwan*
[7]*National Yang-Ming University, Taipei, Taiwan*
[8]*Institute of Biomedical Sciences, College of Life Science, National Chung Hsing University, Taichung, Taiwan*
[9]*Department of Bioscience Technology, Chuan Yuan Christian University, Taoyuan, Taiwan*
[10]*Department of Chinese Martial Arts and Graduate Institute of Sport Coaching Science, Chinese Culture University, Taipei, Taiwan*

Amyloid beta protein (Aβ) is a key pathological protein that induces neuronal apoptosis and mediates degeneration in Alzheimer's disease (AD). There is no effective medication for AD treatment so far. To explore whether methanol-soluble extract from the fruit of *Terminalia chebula* Retzius (Tech-MeOH) is neuroprotective, $A\beta_{25-35}$-induced apoptosis of rat pheochromocytoma cells (PC12 cells) was used to examine the protective potential and mechanisms involved. The Tech-MeOH extract was effective in the inhibition of $A\beta_{25-35}$-induced PC12 cells apoptosis and an active pure compound named ellagic acid (Tech-ME-A) was isolated. Mechanism elucidation showed that $A\beta_{25-35}$ induced time-dependent activation of GSK-3 which peaked at 4hr after $A\beta_{25-35}$ induction. However, both Tech-MeOH and Tech-ME-A, as well as a GSK-3 inhibitor (AR-A014418) all significantly prevented GSK-3 activation, most possibly through modulation of phosphatidylinositol-3 kinase (PI3K)/Akt-dependent repression of GSK-3 activity, in turn, activated CREB-dependent upregulation of BcL2 and down-regulation of caspase 3. We conclude that methanol-soluble extract and ellagic acid isolated from *T. chebula* showed anti-apoptotic effects through modulation of PI3k/Akt-dependent inhibition of GSK-3 activity to reduce $A\beta_{25-35}$ toxicity.

Key words: Apoptosis, amyloid β-protein (Aβ), ellagic acid, glycogen synthase kinase 3 (GSK-3), phosphatidylinositol-3 kinase (PI3K)/Akt, *Terminalia chebula* Retzius

Received 22 September 2015, Accepted 30 October 2015, Available online 1 June 2016

***Correspondence**: Yuh-Chiang Shen, National Research Institute of Chinese Medicine, Ministry of Health and Welfare, No. 155-1, Li-Nung Street, Sec. 2, Peitou, Taipei 112, Taiwan, Tel: +886-2-28201999 ext. 9101, Fax: +886-2-28264276, E-mail: yuhcs@nricm.edu.tw

#These authors contributed equally in this work, respectively.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive decline of memory and cognition. Accumulation of amyloid beta protein (Aβ), activation of microglial cells, induction of neurofibrillary tangles, synaptic loss, and degeneration of cholinergic neurons are neuropathological hallmarks of AD [1,2]. Evidence has been established that the oxidative stress-mediated tissue damage plays a major role in neurological degeneration of AD [3]. Oxidative stress refers to the physiological condition at which the capacity of the endogenous antioxidant system fails to cope with the damaging effects of free radicals.

Recent investigations have shown that antioxidant therapy has proven to be successful in improving cognitive function and behavioral deficits in patients with mild to moderate AD and natural products are proposed to be the biggest source of antioxidants which can be beneficial for AD therapy [3]. The fruit of *Terminalia chebula* Retzius (Combretaceae) is a very popular traditional medicine that has been used for treatment of various diseases in Taiwan. Various pharmacological activities of chemicals from *T. chebula* are reported including antioxidant, antibacterial, antifungal, antiviral, antiprotozoal, anticarcinogenic, radioprotective, antimutagenic, chemo-preventive, hepatoprotective, cardioprotective, cytoprotective, antidiabetic, renoprotective, antiinflammatory, antiarthritic, adaptogenic, antianaphylactic, hypolipidemic, hypocholesterolemic, antiulcer, antispasmodic, anticaries, wound healing, purgative, antiallergic and immunomodulatory, etc [4].

Although *T. chebula* has many biological effects including antioxidant effects, there is no report related to the effects of the extracts from the *T. chebula* against Aβ-induced neuronal damage. Therefore, this present study is aimed to assess the neuroprotective activities of the methanol-soluble extract from *T. chebula* (Tech-MeOH) and one compound named ellagic acid (Tech-ME-A) isolated from *T. chebula* against the oxidative stress induced by $A\beta_{25-35}$, a well-accepted inducer for AD model, in the PC12 cells.

2. Materials and Methods

2.1. Chemicals and reagents

Methanol, hexane, ethyl acetate (Echo Chemical Corporation, LTD., Miaoli, Taiwan, R.O.C.) and chloroform (Uni-Onward Corporation, Taipei, Taiwan, R.O.C.) were purchased as ACS grade reagents. Deionized water was obtained from an Ultrapure Water System (Putity-UV, Suntex Instruments Corporation, LTD., Taipei, Taiwan, R.O.C.). Poly-L-lysine hydrobromide was obtained from Sigma-Aldrich Corporation, Shanghai, China. Dimethyl sulphoxide (DMSO) was purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated horse serum (HS), heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin and L-glutamine were purchased from HyClone (Thermo Scientific, USA). $A\beta_{25-35}$ was purchased from Kelowna International Scientific Inc. (Taipei, Taiwan, R.O.C.). The standard ellagic acid, 97%, was purchased from Alfa Aesar (MA, USA). The 100 mm and 60 mm cell culture dishes and 24-well cell culture plate were purchased from Greiner Bio-One (NC, USA).

2.2. Extraction and purification

The air-dried fruit of *T. chebula* was purchased from Xin Long Pharmaceutical Limited Company (Taichung, Taiwan, R.O.C.). *T. chebula* was pulverized into fine powder using a stainless steel blender (Waring Commercial, Torrington, CT, U.S.A.). Air-dried powder of *T. chebula* (300 g) was extracted with methanol (3 L×3) and agitated in an ultrasonic cleaner (model DC200H, Chemist Scientific Corporation, Taipei, Taiwan, R.O.C.) for 15 min at room temperature then filtered as described previously [9]. The dried methanol extract (Tech-MeOH) was a brown solid (142.9 g), and was stored at -20°C prior to neuroprotective activity analyses. The methanolic extract (30 g) was resuspended in deionized water (900 mL) and then partitioned in turn with hexane (900 mL×3), chloroform (900 mL×3) and ethyl acetate (900 mL×3). The ethyl acetate fraction (Tech-ME-A) was evaporated under reduced pressure to yield crude crystals. The crude crystals were repeatedly recrystallized from methanol to obtain pure product (80 mg).

2.3. General experimental procedures

Ultraviolet (UV) spectra were measured with a Hitachi U-1900 spectrophotometer (Lab Merchant Limited, London, United Kingdom). Infrared (IR) was recorded with KBr disks using a Nicolet Avatar 320 FT-IR spectrometer (Thermo Nicolet, Sparks, Nevada, U.S.A.). The

finnigan LCQ Advantage MAX Ion Trap mass spectrometer with ESI source (Thermo Electron Corporation, San Jose, CA, U.S.A.) was used for the mass spectrometric determination. Silica Gel 60 F254 plates (Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC). The chromatographic substances on TLC plates were visualized by UV-lamp (254 nm). A COSMOSIL 5C18-AR-II column (5 μm, 250 mm × 4.6 mm; Nacalai USA, Inc., San Diego, CA, U.S.A.) was used for the high-performance liquid chromatography (HPLC) analyses. HPLC spectra were performed on a Hitachi series apparatus equipped with an L-2400 UV spectrophotometric detector, an L-2130 pump and an L-2200 autosampler (SpectraLab Scientific Inc., Toronto, Ontario, Canada). Nuclear magnetic resonance (NMR) spectra were measured on a Varian Unity Plus 600 (600 MHz, Varian Inc., Palo Alto, CA, U.S.A.) spectrometer. The pure product was thoroughly characterized by UV, IR, mass, TLC, HPLC and NMR techniques.

2.4. Preparation of the $A\beta_{25-35}$ stock solution

The $A\beta_{25-35}$, the most toxic peptide fragment derived from amyloid precursor protein, was pre-aggregated prior to use in this study. Synthetic $A\beta_{25-35}$ was prepared at a concentration of 1 mM in deionized water, and aggregated at 37°C for 7 days. The stock solution was diluted to desired concentrations immediately before use and added to cell culture medium.

2.5. $A\beta_{25-35}$-induced cell apoptosis and quantification

PC12 cells (ATCC; USA) were plated on poly D-lysine-coated 100 mm cell culture dishes, and grown in DMEM, supplemented with 10% HS, 1% FBS, a mixture of 1% penicillin/streptomycin, and 1% L-glutamine at 37°C in a 95% humidified air-5% CO_2 chamber. Cells were subcultured for no more than ten passages. Cellular viability was determined using the trypan blue exclusion test. Only cell preparations with 95% or greater viability were used. PC12 cells were seeded in poly D-lysine-coated 60 mm cell culture dishes (1×10^6 cells/dish) with complete DMEM for 24 h. To study the $A\beta_{25-35}$ induced apoptosis on the PC12 cells, the PC12 cells were exposed to $A\beta_{25-35}$ (10 μM) for 24 h. The control cell was incubated without the addition of $A\beta_{25-35}$ solution. Quantification of apoptosis. Annexin V/propidium iodide (PI) assay kits (Invitrogen, CA, USA) were used to detect phosphatidylserine externalization, which is a hallmark of the early phase of apoptosis. In a typical procedure, PC12 cells (1×10^5 cells/well) were cultured in poly D-lysine-coated six-well plates. After pretreating with testing drug for 12 h, the cells were washed with PBS and treated with $A\beta_{25-35}$ solution (10 μM) for 24 h. The cells were harvested, then washed with cold PBS, and then incubated with 1× Annexin V working solution that contains PI (1 μg/mL final concentration) for 15 min in the dark at room temperature. After adding 400 μL 1× binding buffer, the cells were immediately analyzed using a FACSCalibur flow cytometer (BD Biosciences, CA, USA).

2.6. Western blotting

Equal amounts (50 μg) of protein from different treatment were subjected to 12% of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-transferred to a hydrophobic polyvinylidene difluoride membrane. After blocking, the membrane was incubated overnight at 4°C with an antibody against phospho-AKT (pAKT) (Santa Cruz Biotechnology), Y-pGSK-3 (Abcam, Cambridge, UK), S-pGSK-3 (Cell Signaling Technology, MA, USA), phospho-CREB (Millipore, Temecula, CA, USA), BcL2 (Cell Signaling Technology, MA, USA), cleaved caspase 3 (cCAP3, Cell Signaling Technology, MA, USA), or β-actin (Sigma-Aldrich). After incubation with a properly titrated second antibody, the immunoblot on the membrane was visible after development with an enhanced chemiluminescence (ECL) system and was quantified using an imaging program.

2.7. Statistical analysis

All results in the text and figures are presented as the mean±S.E.M. (standard error of the mean). Data were analyzed by one-way analysis of variance (ANOVA) followed by the post hoc Student-Newman-Keuls (S-N-K) t-test for multiple comparisons. Values of $P<0.05$ were considered significant.

3. Results

3.1. Structure elucidation of the compound

The UV spectrum of the Tech-ME-A showed absorption λmax 214 and 273 nm. The IR spectrum indicated carbonyl absorption at ν 1712 /cm and hydroxyl absorptions at ν 3473, 3555 /cm. The ESI-MS (negative mode) *m/z* is observed at 301.1 $[M-H]^-$. Its molecular formula was deduced to be $C_{14}H_6O_8$. The identity of the Tech-ME-A was confirmed by co-TLC with an

authentic standard ellagic acid. The Tech-ME-A and authentic standard were separated by TLC using methanol: water, 3:2 (v/v) as developing solvents. Compared to the authentic standard, the Tech-ME-A was found to produce the similar R_f value (R_f = 0.55) under UV light (254 nm). The Tech-ME-A and authentic standard were also analyzed by HPLC, and the mobile phase consisted of water-acetonitrile (100:0 gradient to 0:100, v/v for 0-30 min). Both samples were sonicated and filtered through 0.22 μm Millipore membrane filters. The injection volume was 2.0 μL. Both analyses were performed at room temperature. The flow rate was 1.0 mL/min and the UV detection was carried out at 254 nm. The retention time of the Tech-ME-A and authentic standard was 16.5 and 16.1 min, respectively. The ^{1}H-NMR (DMSO-d_6) spectrum of the Tech-ME-A showed two signals at δ 7.44 (s, 2H, ArH) and δ 10.66 (s, 4H, -OH). The ^{13}C-NMR (DMSO-d_6) spectrum of the Tech-ME-A exhibited seven signals at δ 107.48 (C-6), 110.15 (C-5), 112.33 (C-1), 136.35 (C-2), 139.72 (C-3), 148.12 (C-4), 159.14 (C-7). The above spectral data indicated that the Tech-ME-A was ellagic acid. Ellagic acid was obtained as a colorless amorphous powder. The yield is 0.27% (w/w; weight of ellagic acid/weight of methanol soluble extract of *T. chebula*). The structure of ellagic acid is shown in Fig. 1.

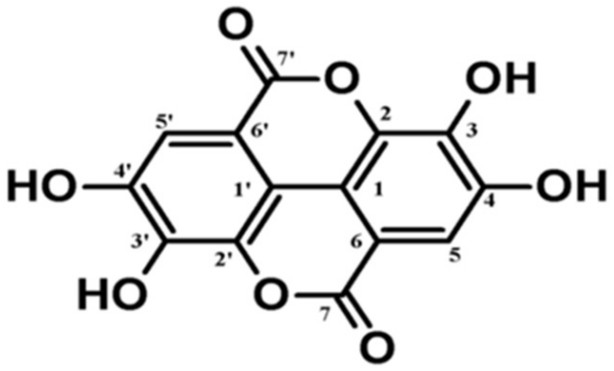

Fig. 1. The structure of ellagic acid.

3.2. Aβ$_{25-35}$-induced a dramatic apoptosis in PC12 cells and was prevented by Tech-MeOH, Tech-ME-A and a GSK-3 inhibiter

To study the neuroprotective effects of the Tech-MeOH and Tech-ME-A (ellagic acid), we used Aβ$_{25-35}$-induced PC12 cells apoptosis as an in vitro model to examine the neuroprotective effect by these samples. For concentration-dependency evaluation of the anti-apoptosis effects by Tech-MeOH (5~20 μg/mL) and Tech-ME-A (ellagic acid, 10~20 μM), we found that Tech-MeOH (5 μg/mL) and Tech-ME-A (ellagic acid, 20 μM) displayed the most effectiveness in this assay (Fig. 2). Fig. 3 shows that the Aβ$_{25-35}$ dramatically induced cell apoptosis in PC12 cells to around 32% (late apoptosis, Q1) and 8% (early apoptosis, Q4) (Fig. 3, upper panel). Cell viability decreased to around 40% when exposed to 10 μM Aβ$_{25-35}$ for 24 h (Fig. 2, lower panel, Living cell (%)). Pretreatment with Tech-MeOH (5 μg/mL) and Tech-ME-A (ellagic acid, 20 μM), as well as a GSK-3 inhibitor (Ar-A014418, 0.1 μM) and caspase inhibitor I (20 μM) all significantly prevent apoptosis both in early (Q4) and late phase (Q1). The surviving cell also dramatically enhanced to around 60% (Fig. 3, lower panel).

3.3. Aβ$_{25-35}$-induced apoptosis paralleled to activation of PI3K/Akt-dependent GSK-3 signaling and was suppressed by Tech-MeOH, Tech-ME-A and a GSK-3 inhibiter

To examine the possible underlying mechanism of action for the anti-apoptosis effect by Tech-MeOH (5 μg/mL) and Tech-ME-A (ellagic acid, 20 μM) in Aβ$_{25-35}$-induced cell death, Western blotting analysis was performed to examine the signaling and molecular targets mediated by these drugs. Aβ$_{25-35}$-induced a time-dependent increase of GSK-3 activity which was expressed as in the increased ratio of Y-pGSK-3 (activated form)/S-pGSK-3 (inactivated form), and this ratio peaked at 4h after Aβ$_{25-35}$ challenge (Fig. 4). Here we also found that challenge of PC12 cells for 4h by Aβ$_{25-35}$, GSK-3 activity was significantly enhanced that paralleled to the less activation of Akt signaling (pAkt) and down regulation of phosphor-CREB (pCREB) and BcL2, as well as upregulation of cleaved caspase 3 and cell apoptosis (Fig. 5). All of the above signaling changes by Aβ$_{25-35}$ challenge were significantly prevented by pretreatment of Tech-MeOH (5 μg/mL) and Tech-ME-A (ellagic acid, 20 μM), as well as a GSK-3 inhibitor, AR-A014418 (Fig. 5).

4. Discussion

Alzheimer's disease (AD) is a neurodegenerative disease described by extracellular Aβ deposits, intracellular neurofibrillary tangles and mitochondrial dysfunction. Mitochondrial dysfunction involves formation of reactive oxygen species (ROS) in mediating

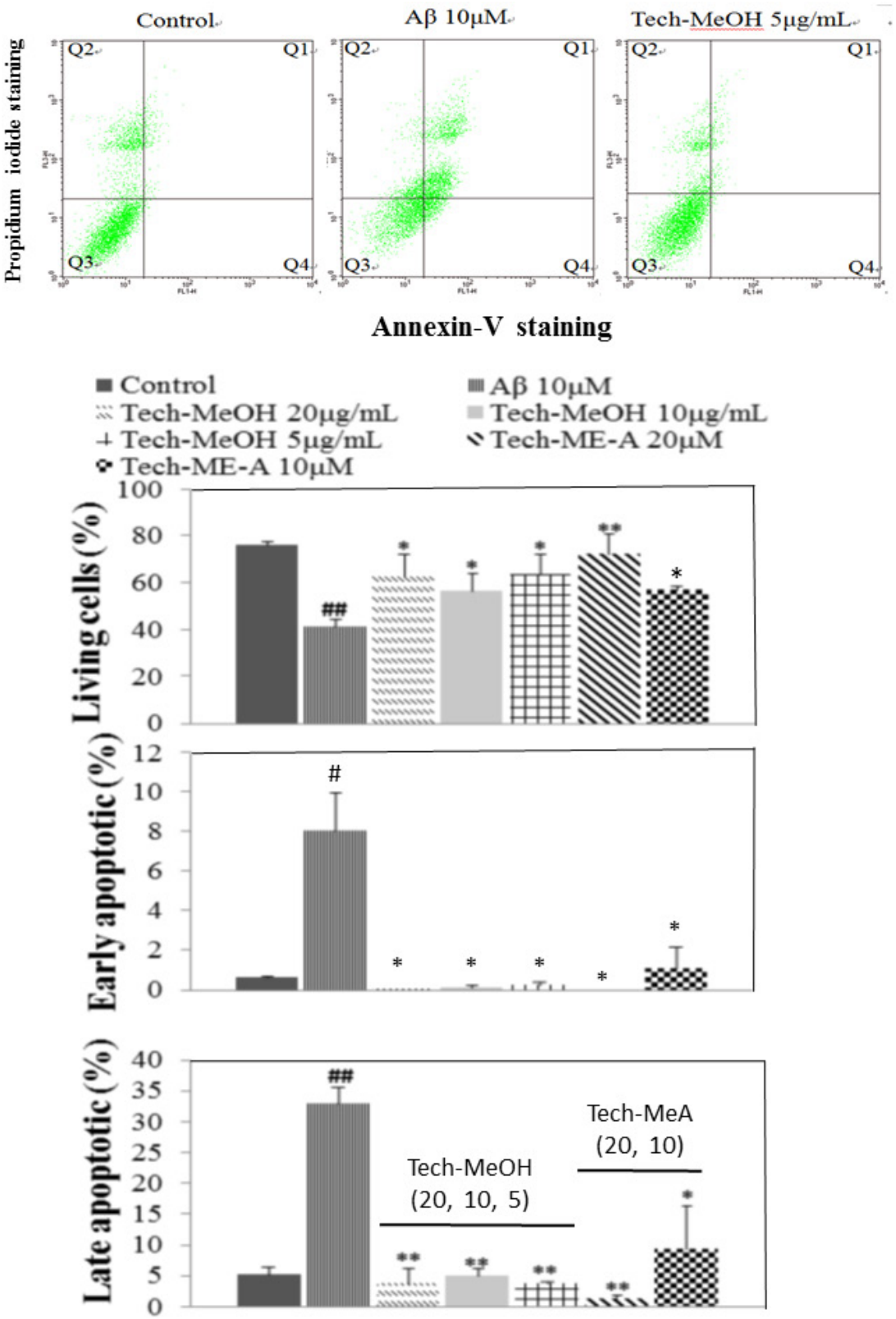

Fig. 2. Concentration-dependent effects of Tech-MeOH and Tech-ME-A on the changes of $A\beta_{25-35}$ induced apoptosis in PC12 cells. After pretreating with testing drug for 12 h, the cells were treated with $A\beta_{25-35}$ (10 μM; Aβ (10)) for 24 h. After harvested, cells were stained with annexin V (for apoptosis) and propidium iodide (for necrosis), and were immediately analyzed using a flow cytometer (FACSCalibur). Upper panel, a representative plot from flow cytometry. Lower panel, percentage (%) of cells in in different quarter (Q) indicating: Q1 (late apoptosis), Q2 (necrosis), Q3 (living cell) and Q4 (early apoptosis). Data are mean±SEM, N=5. #,*, P<0.05 or ##,**, P<0.01 as compared to control or Aβ (10), respectively by one-way ANOVA followed by S-N-K t-test.

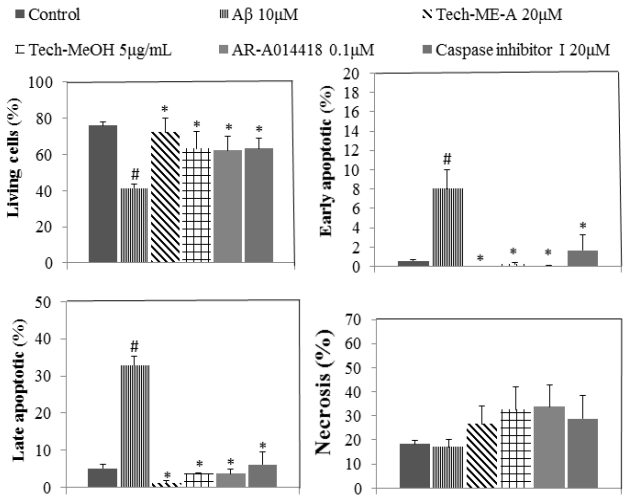

Fig. 3. Effects of Tech-MeOH, Tech-ME-A, AR-A014418 and caspase inhibitor I on the changes of $A\beta_{25-35}$ induced apoptosis in PC12 cells. After pretreating with testing drug for 12 h, the cells were treated with $A\beta_{25-35}$ (10 μM; Aβ (10)) for 24 h. After harvested, cells were stained with annexin V (for apoptosis) and propidium iodide (for necrosis), and were immediately analyzed using a flow cytometer (FACSCalibur). Data are mean±SEM, N=5. #,*, $P<0.05$ as compared to control or Aβ (10), respectively by one-way ANOVA followed by S-N-K t-test.

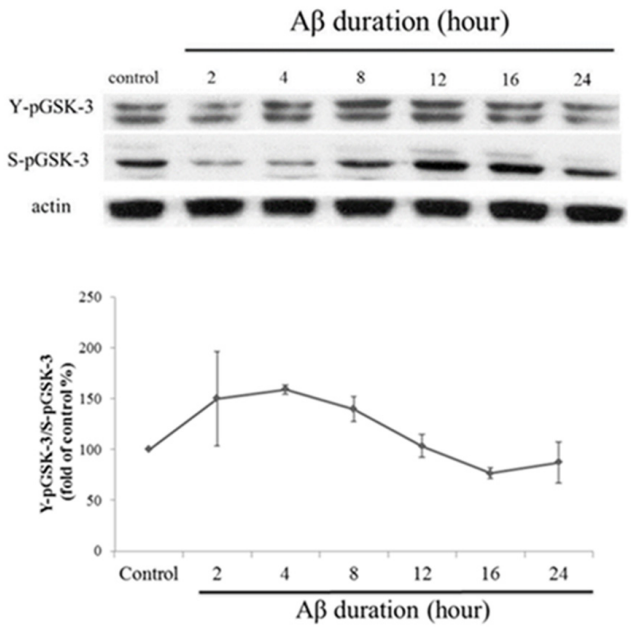

Fig. 4. Effect of $A\beta_{25-35}$ on activity of GSK-3 at different time intervals on PC12 cells. PC12 cells were treated with control (PBS), 10 μM of $A\beta_{25-35}$ for control (0h), 2 h, 4 h, 8 h, 12 h, 16 h, and 24 h. Western blotting was performed to examine the expression level of activated form (Y-pGSK-3) and inhibited form (S-pGSK-3). Data are intensity ratio of Y-pGSK-3/ S-pGSK-3 and was expressed as mean±SEM, N=3.

oxidative stress and enhanced apoptosis. Various bioenergetics (e.g., coenzyme Q10) and antioxidants from natural products (e.g., triterpenoids, *Ginkgo biloba*) have been tried or under different investigational phase against AD and other neurodegenerative disorders [5]. Among these pathological factors, formation of ROS plays an important neurotoxic pathway in mediating these neuropathological changes. During aging, oxidative stress is worse as a consequence of both an accelerated generation of ROS and a gradual decline in cellular anti-oxidant defending mechanisms [6]. ROS can easily initiate the lipid peroxidation of the membrane lipids, causing damage of the cell membrane of phospholipids, lipoprotein by propagating a chain reaction cycle [7]. Most living species have efficient defense systems to prevent themselves from oxidative stress induced by ROS [8]. Recent investigations have shown that the antioxidant properties of plants could be correlated with their oxidative stress defensing ability in the management of different human diseases and aging process etc [9].

Various pharmacological activities of chemicals from *T. chebula* are reported including antioxidant. Some compounds isolated from *T. chebula* are ellagic acid, chebulic acid, neochebulic acid, gallic acid, 2,4-chebulyl-beta-D-glucopyranose, chebulinic acid, hydroxy-benzoic acid derivatives, hydroxycinnamic acid derivatives, falvonol aglycones and chebulagic acid [4]. In our previous study, the methanol, 95% ethanol and water extracts of *T. chebula* exhibited potent antioxidant activities on luminol radicals, superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide (H_2O_2). Furthermore, the phytochemical composition analyses for total phenolic, triterpenoid and tannin content were also examined. The three extracts of *T. chebula* were rich of phenolic compounds compared with total triterpenoid and tannin content. The methanol extract had the greatest total triterpenoid content. The methanol and water extracts also exhibit neuroprotective activities against H_2O_2-induced

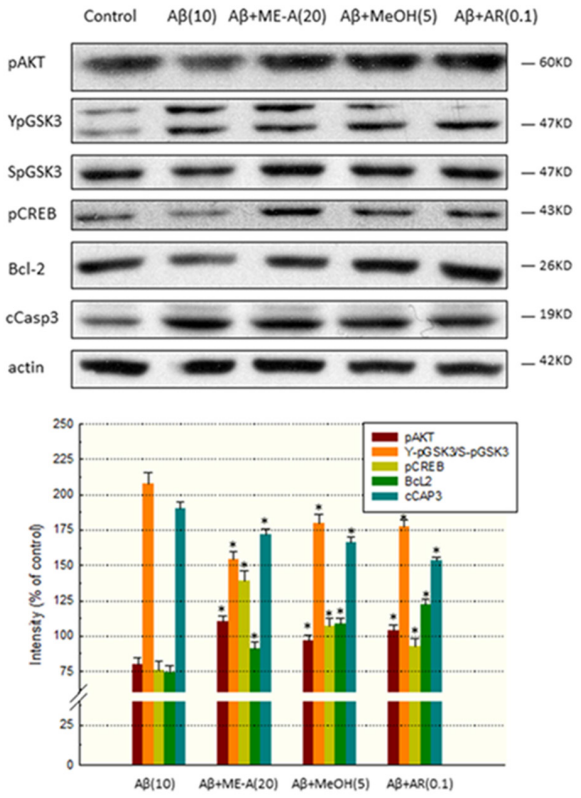

Fig. 5. Effects of Tech-MeOH, Tech-ME-A, and AR-A014418 on the changes of GSK-3 associated protein signals at 4h after $A\beta_{25-35}$ challenge on PC12 cells. PC12 cells were treated with control (PBS), 10 μM of $A\beta_{25-35}$ (Aβ(10)) for 4 h after pretreatment of Tech-MeOH (5 μg/ml, Aβ+MOH(5)), Tech-ME-A (20 μM; Aβ+MEA(20)), and AR-A014418 (0.1 μM; Aβ+AR(0.1)). Western blotting was performed to examine the expression levels of protein signals. Upper panel, a representative plot of protein bands from Western blotting including phospho-Akt (pAKT), Y-pGSK-3, S-pGSK-3, phospho-CREB (p-CREB), BcL2, and cleaved caspase 3 (cCAP). β-actin was included as a protein loading control for normalization. Lower panel, statistical results. Data are intensity ratio of treatment/ control in each column. For GSK-3 activity, it was further expressed as Y-pGSK-3/ S-pGSK-3. All data sets were expressed as mean±SEM, N=3. *, $P<0.05$ as compared to Aβ(10), respectively by one-way ANOVA followed by S-N-K t-test.

toxicity toward the rat pheochromocytoma cell line (PC12 cells) [10]. Based on our previous report regarding antioxidative and neuroprotective effects of *T. chebula* extracts [10], we proposed that extracts from *T. chebula* may have protective effects on neuronal cell death induced by $A\beta_{25-35}$. In this study, we clearly demonstrated that extract (Tech-MeOH) and compound (Tech-ME-A) isolated from *T. chebula* display effectiveness against $A\beta_{25-35}$-induced apoptosis in PC12 neuronal cells. Regarding that the potency of Tech-MeOH (5 μg/mL), containing only 0.27% of Tech-ME-A, displays almost equal potency to that of Tech-ME-A (20 μM, around 5 μg/mL), indicating that existing other active components within Tech-MeOH needs further elucidation.

To examine other action mechanisms other than the antioxidant effect of Tech-MeOH and Tech-ME-A in $A\beta_{25-35}$-induced cell apoptosis, this study focused on the PI3K/Akt/GSK-3 pathway. This is due to that activation of GSK-3 has been reported to inhibit cAMP response element-binding protein (CREB) signaling, a protein closely associate with Alzheimer's disease [11], and down regulate anti-apoptosis protein BcL2 expression, leading to caspase 3 activation and cell apoptosis [12]. Inhibition of GSK-3 activity by PI3K/Akt activation can reduce GSK-3 activity by phosphorylation of GSK-3 at serine (9) residue [11]. Our results showed that $A\beta_{25-35}$ induced a time-dependent increase of GSK-3 activity (as estimated by YpGSK-3/SpGSK-3) that peaked at 4h after $A\beta_{25-35}$ challenge and paralleled to the less activation of Akt signaling (pAkt) and down regulation of phosphor-CREB (pCREB) and BcL2, as well as upregulation of cleaved caspase 3 and cell apoptosis. All of the above signaling changes by $A\beta_{25-35}$ challenge were significantly prevented by pretreatment of Tech-MeOH and Tech-ME-A (ellagic acid), as well as a GSK-3 inhibitor, AR-A014418.

Similar observation has been revealed that Aβ-induced toxicity was aligned with decrement of the phosphorylated Akt (pAkt) which was prevented by insulin. The PI3 kinase inhibitor, LY294002, decreased pAkt and abolished the protective effect of insulin [13]. On the contrary, it has been found that inhibition of caspase-3 and the prevention of the caspase-mediated cleavage of Akt were able to completely prevent the Aβ-mediated inhibition of LTP [14]. Thus, it seems that Aβ activates caspase-3, which cleaves Akt to persistently activate GSK-3β and thereby trigger synaptosis which is termed CAG cascade [14]. In that case, what is not known is how Aβ activates casapse-3 to initiate the process, though an action via mitochondria seems likely. Moreover, recently, Aβ-induced PC12 cells apoptosis has been reported to mediate production of reactive oxygen species [5,15], and drug with anti-oxidative capacity was able to reduce Aβ-induced PC12 cells apoptosis [15]. With strong

anti-oxidative potential of *T. chebula* extracts [10], the protective effects of Tech-MeOH and ellagic acid on this model could be achieved by their antioxidant activities. Finally, we propose that both (1) modulation of PI3k/Akt/GSK-3 pathway as well as (2) inhibition of caspase-3 activity to modulate Akt/GSK3 activity through mitochondria protecting via anti-oxidative effect play important roles to signal the anti-apoptosis effects by extract (Tech-MeOH) and compound (Tech-ME-A) isolated from *T. chebula*.

4. Conclusion

Our data suggest that methanol-soluble extract and ellagic acid isolated from *T. chebula* showed significant anti-apoptotic effects in PC12 cells through inhibition of GSK-3 activity to reduce $A\beta_{25-35}$ toxicity by upregulation CREB signal and BcL2 expression.

Acknowledgements

This work was supported by projects from the Research Institute of Biotechnology, Hungkuang University, Taichung, Taiwan, Republic of China and the National Research Institute of Chinese Medicine, Ministry of Health and Welfare, Taipei City, Taiwan, Republic of China.

References

1. Kar S, Slowikowski SP, Westaway D, *et al*. Interactions between beta-amyloid and central cholinergic neurons: implications for Alzheimer's disease. *J. Psych. Neurosci.*, 29:427–441, 2004.
2. Yan Z, Feng J. Alzheimer's disease: interactions between cholinergic functions and beta-amyloid. *Curr. Alzheimer Res.*, 1:241–248, 2004.
3. Feng Y, Wang X. Antioxidant therapies for Alzheimer's disease. *Oxid. Med. Cell. Longev.*, ID 472932, 2012.
4. Bag A, Bhattacharyya SK, Chattopadhyay RR, *et al*. The development of *Terminalia chebula* Retz. (Combretaceae) in clinical research. *Asian Pac. J. Trop. Biomed.*, 3:244–252, 2013.
5. Kumar A, Singh A. A review on mitochondrial restorative mechanism of antioxidants in Alzheimer's disease and other neurological conditions. *Front. Pharmacol.*, 6:206, 2015.
6. Viña J, Lloret A, Giraldo E, *et al*. Antioxidant pathways in Alzheimer's disease: possibilities of intervention. *Curr. Pharm. Des.*, 17:3861–3864, 2011.
7. Lobo V, Patil A, Phatak A, *et al*. Free radicals, antioxidants and functional foods: Impact on human health. *Phcog. Rev.*, 4:118–126, 2010.
8. Rahman K. Studies on free radicals, antioxidants, and co-factors. *Clin. Interv. Aging*, 2:219–236, 2007.
9. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature*, 408:239–247, 2000.
10. Chang CL, Lin CS. Phytochemical composition, antioxidant activity and neuroprotective effect of *Terminalia chebula* Retzius extracts. *Evid. Based Complement. Alt.*, ID 125247, 2012.
11. Kim WY, Snider WD. Functions of GSK-3 signaling in development of the nervous system. *Front. Mol. Neurosci.*, 4:44, 2011.
12. Chuang DM, Wang Z, Chiu CT. GSK-3 as a target for lithium-induced neuroprotection against excitotoxicity in neuronal cultures and animal models of ischemic stroke. *Front. Mol. Neurosci.*, 4:15, 2011.
13. Ghasemi R, Moosavi M, Zarifkar A, *et al*. The interplay of Akt and ERK in Aβ toxicity and insulin-mediated protection in primary hippocampal cell culture. *J. Mol. Neurosci.*, Aug 13 [Epub ahead of print], 2015.
14. Jo J, Whitcomb DJ, Olsen KM, *et al*. $A\beta_{1-42}$ inhibition of LTP is mediated by a signaling pathway involving caspase-3, Akt1 and GSK-3β. *Nat. Neurosci.*, 5:545–547, 2011.
15. Zeng GF, Zong SH, Zhang ZY, *et al*. The role of 6-gingerol on inhibiting amyloid β protein-induced apoptosis in PC12 cells. *Rejuvenation Res.*, 5:413–421, 2015.

J Chin Med 27(1): 2701002, 9 pages, 2016
DOI: 10.3966/241139642016062701002
E-ISSN: 24113964
http://www.nricm.edu.tw/jcm/

中醫藥雜誌 Journal of Chinese Medicine

訶子甲醇萃取物修飾 PI3K/Akt 途徑抑制 GSK-3 活性降低 $A\beta_{25-35}$ 造成的 PC12 神經細胞凋亡

侯毓昌[1,9,#]、張嘉麟[3,4,#]、陳昌明[6,7]、王雅惠[5,6]、劉國同[10]、陳建志[3]、林哲三[3]、沈郁強[2,5,8,#,*]

[1] 衛生福利部桃園醫院中醫科，桃園，臺灣
[2] 衛生福利部國家中醫藥研究所，台北，臺灣
[3] 弘光科技大學生物科技系，台中，臺灣
[4] 弘光科技大學護理系，台中，臺灣
[5] 國立台北護理健康大學，台北，臺灣
[6] 臺北榮民總醫院神經醫學部及臺北市立關渡醫院，台北，臺灣
[7] 國立陽明大學腦科學研究所，台北，臺灣
[8] 國立中興大學生物醫學研究所，台中，臺灣
[9] 中原大學生物科技學系，台中，臺灣
[10] 中國文化大學技擊運動暨國術學系，台北，臺灣

β 澱粉樣蛋白 (Amyloid β-protein，Aβ) 是造成神經細胞凋亡引起神經退化導致海默氏症 (Alzheimer's disease，AD) 的主要病理因素，目前並無有效治療 AD 的藥物。本研究利用 $A\beta_{25-35}$ 造成神經細胞 PC12 凋亡的細胞模式來探討中藥訶子 (*Terminalia chebula* Retz) 的甲醇萃取物 (Tech-MeOH) 是否具有神經保護作用及可能作用機制。研究結果顯示 Tech-MeOH 非常有效抑制 $A\beta_{25-35}$ 造成神經細胞 PC12 凋亡，並從 Tech-MeOH 分離出純化合物 Tech-ME-A (證實為 ellagic acid)。機制探討發現 $A\beta_{25-35}$ 會隨著時間活化 glycogen synthase kinase 3 (GSK-3)，在 4 小時達到最高峰。而 Tech-MeOH、ellagic acid 及 AR-A014418 (一種 GSK-3 抑制劑) 都可以顯著抑制 GSK-3 的活性，並且逆轉 $A\beta_{25-35}$ 所誘發的 PC12 細胞凋亡；蛋白質訊息分子機制探討，發現它們最可能是藉由修飾 PI3K/Akt 依賴的 GSK-3 抑制作用而間接活化了 CREB 及其相關的抗凋亡蛋白 BcL-2 表現增加及抑制凋亡蛋白 caspase 3。我們結論認為從訶子的甲醇萃取物 (Tech-MeOH) 及進一步分離純化的 ellagic acid 都可藉由修飾 PI3K/Akt 途徑抑制 GSK-3 的活化及相關機制而降低 $A\beta_{25-35}$ 所造成之細胞凋亡作用。

關鍵字：凋亡、β 澱粉樣蛋白、肝醣合成酶激酶 -3 (GSK-3)、phosphatidylinositol-3 kinase (PI3K)/Akt、訶子

104 年 9 月 22 日受理
104 年 10 月 30 日接受刊載
105 年 6 月 1 日線上出版

*** 聯絡人**：沈郁強，衛生福利部國家中醫藥研究所，11221 台北市北投區立農街二段 155-1 號，電話：02-28201999 分機 9121，電子郵件信箱：yuhcs@nricm.edu.tw

#：作者貢獻度同等