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ORIGINAL ARTICLE

Phytoestrogen alpha-zearalanol attenuate endoplasmic reticulum stress to against cultured rat hippocampal neurons apoptotic death induced by amyloid beta25–35

Yilong Dong [1], Ruiqian Li [2], Aimei Jiang [3], Zhong Xu [3], Yanmei Wang [3]

1 School of Medicine, Yunnan University, Kunming, Yunnan, People's Republic of China
2 The Third Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, People's Republic of China
3 The First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, People's Republic of China

Correspondence to: Yanmei Wang, MD.
The First Affiliated Hospital of Kunming Medical University
295 Xichang Road, Kunming, Yunnan, 650031, People's Republic of China.
TEL: +86-871-65324888-2428; E-MAIL: wangym_kmmu@126.com

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Abstract

OBJECTIVE: Our previous studies demonstrated both phytoestrogen α-zearalanol (α-ZAL) and estrogen is effective decrease Alzheimer's disease (AD)-like apoptotic neuron death, but α-ZAL showed significantly less side-effect on breast and endometrial tissue compared to estrogen, it suggested that α-ZAL can be used as a potential substitute for estrogen. However, the molecular mechanism by which α-ZAL prevents neuron damage remains unclear. Growing evidence suggests that endoplasmic reticulum (ER) stress plays an important role in the process of cell apoptosis in AD; in addition, our published data indicated that α-ZAL possessed the potential ability to stabilize ER function. We therefore hypothesized that ER-stress mechanism maybe involved in the antiapoptotic effect of α-ZAL in this study.
METHODS: Primary rat hippocampal neurons have been cultured and subsequently followed exposed to β-peptide fragment 25–35($A\beta_{25-35}$) with or without α-ZAL pre-treatment, and then western blot and flow cytometry techniques has been used to evaluate the intracellular calcium balance, ER stress and apoptotic cell death.
RESULTS: The results showed that $A\beta_{25-35}$ treatment for 24h induced dramatic neuronal apoptosis, accompanied by an increase in calpain2 expression, a marker of intracellular calcium overload. On the other hand, ER stress sensitive hallmarks, glucose-regulated protein 78 (GRP78), double-stranded RNA-dependent protein kinase (PKR)-like ER-resident kinase (PERK) and C/EBP homologous protein-10 (CHOP10) expressions were up-regulated after $A\beta_{25-35}$ administration. Importantly, α-ZAL pre-treatment effectively attenuated above changes.
CONCLUSION: These results demonstrated that α-ZAL protects cells against AD-like apoptosis and the effects at least partially by attenuating severely ER stress.

Abbreviations

Aβ	- Amyloid β
AD	- Alzheimer's disease
ATF6	- Activating transcription factor 6 (ATF6)
CHOP	- C/EBP homologous protein-10
ER	- Endoplasmic reticulum
GRP78	- 78-kDa glucose-regulated protein
eIF2α	- Eukaryotic translation-initiation factor 2α
IRE1	- Inositol-requiring kinase 1
PBS	- Phosphate buffer saline
PERK	- Double-stranded RNA-dependent protein kinase (PKR)-like ER-resident kinase
PI	- Propidium iodide
ROS	- Reactive oxygen species
α-ZAL	- α-zearalanol

INTRODUCTION

Alzheimer's disease (AD) is the most common progressive neurodegenerative disorder in the aging population, which is pathologically characterized by extracellular amyloid-beta (Aβ) deposits, intracellular aggregates of hyperphosphorylated tau, and neurofibrillary tangles (Nisbet *et al.* 2015). Although the cause of AD is still unknown, postmenopausal depletion of endogenous estrogens is considered as a significant risk factor for onset of AD in women; therefore, estrogen has been used as an efficacious medicine to reduce the incidence of AD in postmenopausal women (Canderelli *et al.* 2007; Simpkins *et al.* 2009; Valen-Sendstad et al.2010). However, the fact that estrogen may predispose women to a much higher incidence of breast and endometrial cancers has undoubtedly impeded the clinical application of estrogen (Chlebowski et al.2010).

In our previous study, we have reported that α-zearalanol (α-ZAL), a plant-derived phytoestrogen, may effectively maintain serum estrogen concentration in ovariectomized animals but showed less adverse effects on endometrial tissue compared to estrogen (Dong et al.2006). Meanwhile, Deng and colleagues found that α-ZAL decreased the expression of c-myc, the most commonly amplified oncogenes in human breast cancer, but estrogen increased it in breast tissue, which means α-ZAL was safer than estrogen on breast (Deng et al.2010). Subsequently, our study confirmed that α-ZAL treatment protected cells against apoptosis induced by Aβ as it can be seen after estrogen treatment (Dong *et al.* 2011; Dong *et al.* 2015). Our previous results (Dong et al.2006; Dong *et al.* 2011; Dong *et al.* 2015) in conjunction with the other's findings (Deng et al.2010), suggest that a-ZAL may be used as a safe substitute of estrogen for AD prevention in postmenopausal women. Although the protective role of α-ZAL in AD has been supported, the molecular mechanism of how α-ZAL prevents cell death remains to be fully elucidated.

In the eukaryotic cell, endoplasmic reticulum (ER) is an important organelle responsible for protein synthesis, correction of protein folding, post-translation modification and transport. Perturbance of ER functions by accumulation of unfolded proteins, excessive amount of reactive oxygen species (ROS) or changes in intracellular calcium homeostasis triggering an evolutionarily conserved response, termed ER stress (Lindholm *et al.* 2006). In general, cells respond to ER stress by increasing transcription of ER chaperones, especially 78-kDa glucose-regulated protein (GRP78), and then activated multiple signaling such as double-stranded RNA-dependent protein kinase (PKR)-like ER-resident kinase (PERK) pathway, which facilitates proper protein folding or elimination of misfolded proteins. Moderate ER stress can relieve cellular dysfunction and enhance the chance for survival. However, when stress is severe and prolonged, ER stress itself can induce cell apoptotic death via transcriptional induction genes such as C/EBP homologous protein-10 (CHOP, also known as GADD153) (Tabas& Ron, 2011).

Increasing studies have shown ER stress to be implicated in AD. For instance, increased levels of GRP78 were observed in the hippocampus of AD brains (Hoozemans et al.2009), and PERK over activation has been suggested as a major neuronal degradation pathway in AD animal (O'Connor *et al.* 2008). Besides, neuronal apoptotic death can be attenuated when silencing CHOP gene in AD animal (Prasanthi *et al.* 2011), indicating that improvement of severe ER stress shall be a protective factor of AD. Meaningfully, our published data found that α-ZAL possessed the ability to regulate the Ca^{2+} homeostasis and inhibit the generation of ROS (Dong et al.2006; Dong *et al.* 2007).It means that α-ZAL removing the factors which may induce ER dysfunction and potentially attenuating severe ER stress. Therefore, we hypothesized that ER-stress mechanism maybe involved in the advantages of α-ZAL in the present study. To demonstrate the hypothesis, cell samples were collected from Aβ treated primary cultured rat hippocampal neurons with or without α-ZAL pretreated, the expression of parvalbumin and calretinin (the calcium-binding protein benefit to keep intracellular Ca^{2+} balance), calpain2 (a marker of intracellular calcium overload) has been measured, ER stress and apoptosis also has been evaluated to analyse the signaling process involved in neuroprotection of α-ZAL.

MATERIAL &METHODS

Materials

α-ZAL was a gift from Prof.Pingping Zuo at Perking Union Medical College. $A\beta_{25-35}$, DNAase1 and propidium iodide (PI) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Neurobasal medium, B27supplement and trypsin was obtained from Invitrogen Corporation (Carlsbad, CA, USA). The protease inhibitor mixture, BCA Protein Assay Kit and enhanced chemiluminescence substrate Kit was obtained from Pierce Biotechnology (Rockford, IL, USA). RIPA lysis buffer was purchased from Beyotime Biotechnology (Shang-

hai, China). Parvalbumin, calretinin, calpain2 and PERK antibody was purchased from Abcam (Abcam, England), GRP78 and CHOP antibody was purchased from Cell Signaling Technology (Boston, MA, USA), and GAPDH antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Annexin V-FITC apoptosis detection kit was purchased from BD Pharmingen (San Diego, CA, USA). All other chemicals used were of the highest grade commercially available.

Cell culture and treatment

Primary hippocampal neurons were prepared from the Sprague-Dawley rats at embryonic day 18 as was described previously with some alterations (Dong *et al.* 2007). Briefly, hippocampal tissues were incubated with trypsin (0.25%) and DNAase1 (50 μg/ml) at 37 °C for 10 min, followed by mechanical trituration with a fire-polished Pasteur pipette. The supernatant was transferred into a tube and centrifuged at 1 200 rpm for 3 min. The cells were resuspended in neurobasal medium containing 2% B27 supplement, 100 μg/ml streptomycin, and 100 U/ml penicillin, and were plated at a density of 5.0×10^5 cells/ml on poly-l-lysine-coated cell culture plates in a humidified 5% CO_2 atmosphere at 37 °C. The animal research protocols were reviewed and approved by the Animal Care Committee of the Yunnan University. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

All treatments were performed after 7 days of culture. Cells were incubated for 12h with 10^{-7} M α-ZAL, and then exposed to 10 μM $A\beta_{25-35}$ for another 24 h. α-ZAL was dissolved in DMSO and added to the medium by bath application with the final concentration of DMSO ≤0.1%. Control cells received an equal amount of DMSO, while model cells just receive $A\beta_{25-35}$ and without α-ZAL pretreatment. $A\beta_{25-35}$ peptide was dissolved in phosphate buffer saline (PBS), and then incubated for 72 h at 37 °C in order to induce aggregation. The aggregated $A\beta_{25-35}$ was then stored at −80 °C until use (Dong *et al.* 2011).

Flow cytometry assay using Annexin V/PI staining

Apoptotic cell death was quantified by flow cytometry using an AnnexinV-FITC kit, according to the manufacturer's protocol. Briefly, cells were harvested by trypsinization and pelleted by centrifugation. After wash twice with cold PBS, the pellets were re-suspended in 200 μl binding buffer, and then incubated with 5 μl Annexin V-FITC and 5 μl PI at room temperature in the dark. Samples were analyzed using FACScalibur flow cytometer (BD Biosciences, San Diego, CA, USA) using software supplied with the instrument. This allows discrimination of living cells (unstained with either fluorochrome) from early apoptotic cells (stained with Annexin V only) and late apoptotic cells (stained with both annexin V and PI). The samples were tested in six duplicates.

Western blot

Cells were dissociated by trypsin and collected by centrifuge. The cell pellet was washed three times with cold PBS, and then lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail. Cell lysates were centrifuged at 12 000 rpm at 4 °C for 10 min. The supernatants were used for SDS-PAGE after quantification the amount of total protein by using BCA Protein Assay kit. After separated, proteins were transferred on to PVDF membrane and blocked with 5% non-fat milk for 2 h at room temperature and then overnight incubation at 4 °C with primary antibodies: anti-parvalbumin, anti-calretinin, anti-calpain2, anti-PERK, anti-GRP78 (1:2 000), anti-CHOP, and anti-GAPDH (1:5 000). All the antibodies were diluted at 1:1000 except the special statement. After washing, the membranes were incubated in horseradish peroxidase (HRP)-conjugated secondary antibody (1:5 000) for 1h at room temperature. Immunoreactive bands were detected with the enhanced chemiluminescence. Band intensities were quantified using Image-Pro Express 6.0 software (Media Cybernetics Inc. Rockville, MD, USA).

Statistical analysis

Data were expressed as mean ± standard deviation (S.D.). Experimental results were analyzed using factorial analysis of variance (ANOVA) with appropriate post hoc test (SPSS version 13.0). Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

α-ZAL attenuated Ca^{2+}overload induced by $A\beta_{25-35}$

To investigate the relation between intracellular Ca^{2+} overload and α-ZAL, the expression of calpain2, a marker of Ca^{2+} overload was evaluated. Western data (Figure 1) showed that calpain2 protein was significantly increased in the cells treated with $A\beta_{25-35}$ alone ($p<0.01$, compare to control group), the treatment with α-ZAL before $A\beta_{25-35}$ significantly diminished the expression of calpain2 ($p<0.01$, compared to Aβ group). Meanwhile, parvalbumin and calretinin expression was significantly decreased in cells treated with $A\beta_{25-35}$ alone (both $p<0.01$, compare to control group), but the effect of $A\beta_{25-35}$ on protein expression has been attenuated by α-ZAL pretreatment ($p<0.01$, compared to Aβ group).

α-ZAL attenuated ER stress induced by $A\beta_{25-35}$

As shown in Fig.2, GRP78 protein showed a significant rise in cells treated with $A\beta_{25-35}$ alone ($p<0.01$, compare to control group), α-ZAL pretreatment partially but significantly attenuated up-regulation of GRP78 ($p<0.01$, compare to Aβ group). Furthermore, western blot analyses also showed a significantly increased PERK protein expression in cells received $A\beta_{25-35}$ alone ($p<0.01$, compare to control group), however, the expression of PERK induced by Aβ was signifi-

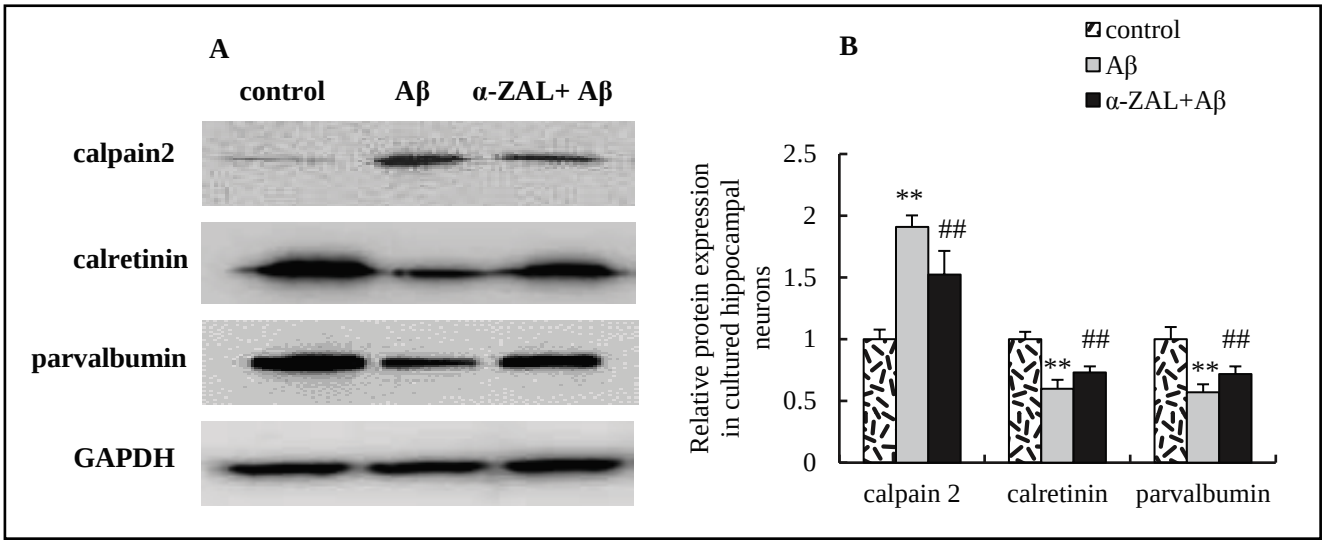

Fig.1 A. α-ZAL pretreatment alleviated intracellular Ca^{2+} overload and the parvalbumin and calretinin decline induced by $A\beta_{25-35}$. **B.** Quantitative analysis of protein levels by densitometry. The data from western blot were normalized by taking the value of control group as 1. **p<0.01 vs. control group; ##p<0.01 vs. Aβ group. Data are representative of six independent experiments.

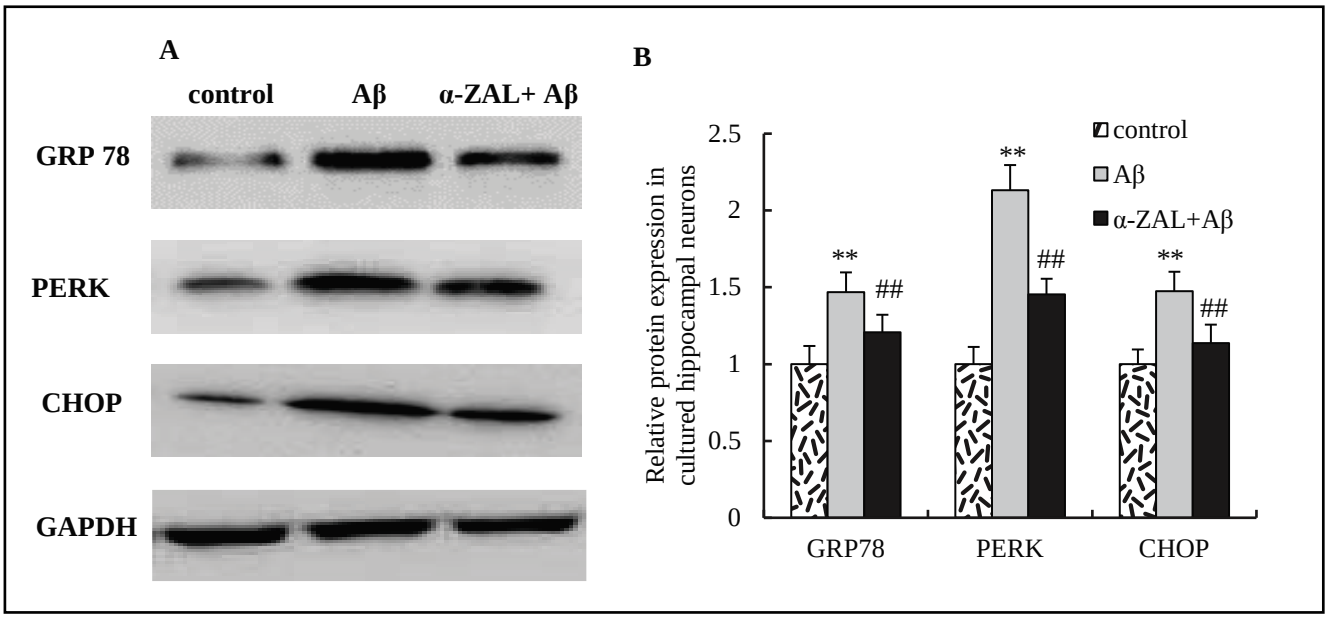

Fig. 2 A. α-ZAL pretreatment attenuated up-regulated of GRP78, PERK and CHOP induced by $A\beta_{25-35}$. **B.** Quantitative analysis of protein levels by densitometry. The data from western blot were normalized by taking the value of control group as 1. **p<0.01 vs. control group; ##p<0.01 vs. Aβ group. Data are representative of six independent experiments.

cantly attenuated while cells pretreatment with α-ZAL (p<0.01, compare to Aβ group).

α-ZAL attenuated the elevation of CHOP expression and apoptotic cell death

As suggested by western analysis (Figure 2), $A\beta_{25-35}$ alone significantly up-regulated CHOP expression (p<0.01, compare to control group), and the up-regulation of CHOP expression was attenuated by α-ZAL pretreatment (p<0.01, compare to Aβ group). Consistent with CHOP changes, flow cytometry assay demonstrated the apoptotic rate was sharply increased from 2.63% in control cells to 25.34% in cells received $A\beta_{25-35}$ alone, but pretreatment with α-ZAL significantly attenuated apoptosis induced by $A\beta_{25-35}$ which was shown by decrease of the apoptotic rate to 12.12% (Figure 3).

DISCUSSION

Apoptotic neuronal death induced by Aβ is one of the pathological features of AD, although most studies suggested a role of mitochondria signaling in apoptosis, increasing evidences supported that ER stress is involved in the neurotoxicity of Aβ (Thummayot *et al.*

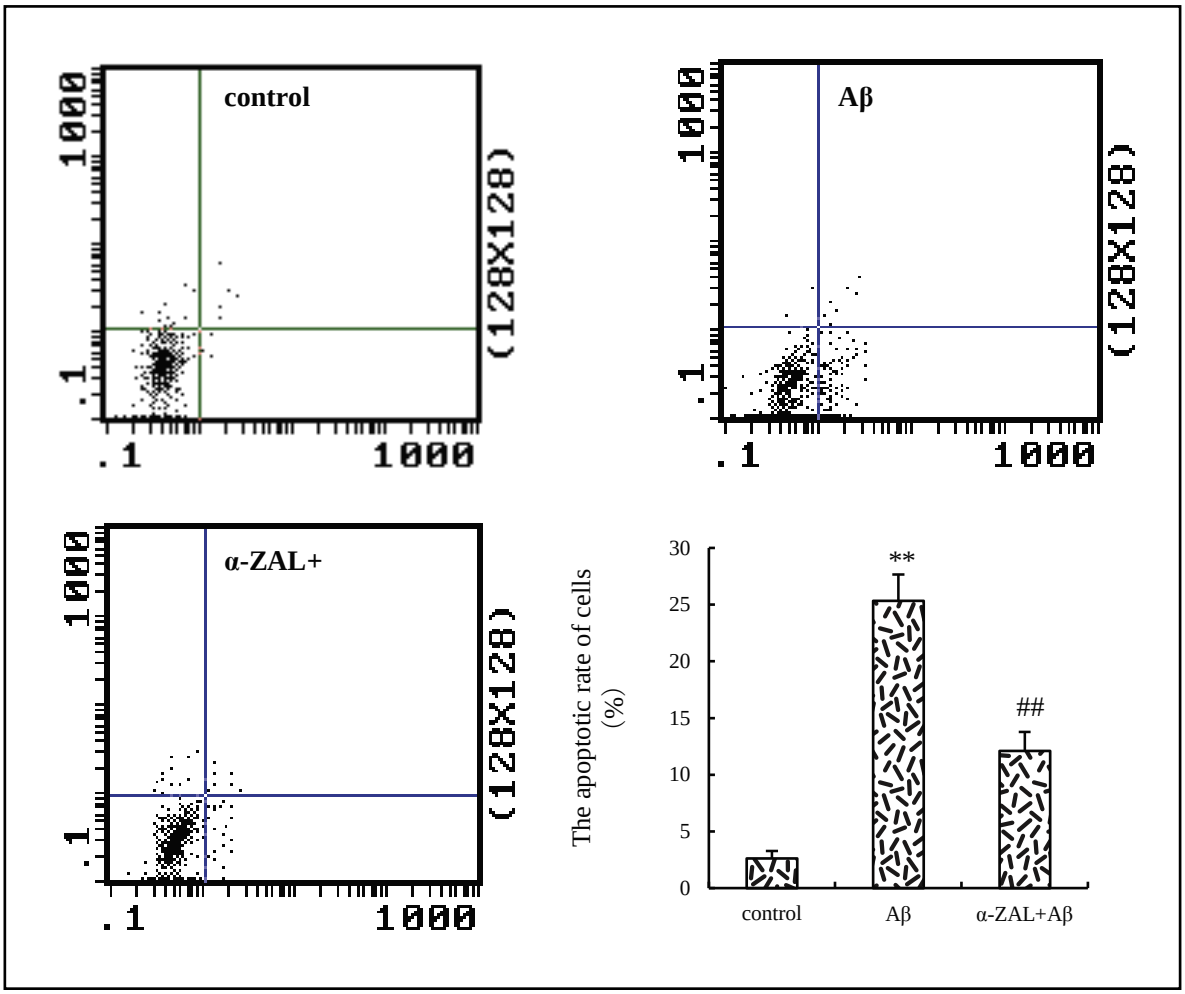

Fig. 3. Apoptotic rate was assessed by flow cytometry. α-ZAL attenuated hippocampal neurons apoptosis induced by $A\beta_{25-35}$. $^{**}p<0.01$ vs. control group; $\#\#p<0.01$ vs. Aβ group. Data are representative of six independent experiments.

2016; Zhou *et al.* 2016; Kong & Ba 2012). In the present study, we demonstrated that Aβ triggered intracellular calcium overload followed by ER stress, activated ER-related apoptosis signaling to induce apoptotic cell death. However, α-ZAL pretreatment alleviates calcium overload and ER stress induced by Aβ, thereby providing beneficial effects on anti-apoptosis.

Aβ is a soluble protein 40–42 amino acid residue in length which has been found to play a key role in AD. The predominant forms of Aβ in the human brain are Aβ (1–40) and Aβ (1–42), but Aβ (25–35) fragment, is the most toxic region and significantly contributes to the initiation of neurodegenerative changes in AD and is broadly used in research (Millucci *et al.* 2010).

Accumulating evidence shows that a rise of intracellular Ca^{2+} and its activated downstream signaling pathways is responsible for $A\beta_{25-35}$-induced neuronal death (Kim *et al.* 2015; Wu *et al.* 2015). We monitored the change of intracellular Ca^{2+}. Although the concentration of cytoplasm Ca^{2+} has not been evaluated directly in the present study, the increase in the expression of calpain2 has been found when cells received Aβ. Calpain2 is a calcium-dependent protein and its expression only can be up-regulated by millimolar calcium level in cytoplasm (Nguyen & Chen 2014). Under physiology condition, cytoplasm calcium concentration maintained at nanomolar level, when the cytoplasm Ca^{2+} increased sharply, the excess Ca^{2+} may trigger caplain2 expression. In the present study, we found that caplain2 expression increased following Aβ treatment, which suggested that there was a non-physiological Ca^{2+} accumulation in the cytoplasm. However, when cells in the presence of Aβ were pretreatment with α-ZAL, the low caplain2 were observed compared with no α-ZAL treatment, suggesting that α-ZAL significantly improved Ca^{2+} overload induced by Aβ. In addition, cells treated with Aβ showed a significantly decline in expression of parvalbumin and calretinin, the calcium-binding protein has been implicated in keeping Ca^{2+} homeostasis in cytoplasm. The down-regulation of parvalbumin and calretinin also has been found in AD animals (Popović *et al.* 2008), and this decrease may be a major contributing factor to the alternations in Ca^{2+} homeostasis. Our present findings indicated that

the decrease of parvalbumin and calretinin has been attenuated by α-ZAL. Thus, it maybe postulated that an initial increase in intracellular Ca^{2+} in neurons following $A\beta_{25-35}$ exposure, and α-ZAL may improve Ca^{2+} overload through up-regulate calcium-binding protein.

As a result of intracellular Ca^{2+} overload, ER stress can be activated. We then evaluated the expression of GRP78. GRP78 is a chaperon protein belonging to the HSP70 family and predominantly resides in the lumen of the ER. Under physiological conditions, GRP78 bind to PERK, activating transcription factor 6 (ATF6) and inositol-requiring kinase 1 (IRE1), which keeps them inactive. During ER stress, GRP78 releases these receptors leading to their activation (Bertolotti *et al.* 2000). Therefore, GRP78 has been considered as a gatekeeper of the ER response and the unregulated GRP78 is often studied as a biomarker of ER stress (Lee 2005). Similar to Zhou's findings (Zhou *et al.* 2016), the present study showed the up-regulation of GRP78 when cells treated with Aβ, the elevation of the sensitive ER stress hallmarks suggested that an ER stress occurred in these cells. Meanwhile, our research shows that α-ZAL pretreatment significantly reduced the expression of GRP78, which demonstrates that the excessive ER stress triggered by Aβ is attenuated by α-ZAL.

As stated early, moderate ER stress can relieve cellular dysfunction. However, if the ER stress is severe or prolonged, apoptotic cell death can be induced, which is mainly mediated by PERK signaling (Hetz 2012). PERK activation leads to the phosphorylation of eukaryotic translation-initiation factor 2α (eIF2α) and selectively induces ATF4, which both enhance the expression of CHOP and subsequent mediated apoptosis (Tabas & Ron, 2011). The increased expression of PERK and CHOP accompanied with high apoptotic rate has been observed in cells received Aβ in the present study. Pretreatment with α-ZAL was found to significantly decrease the expression of these proteins and alleviated the apoptotic cell death induced by Aβ. Therefore, this study suggests that α-ZAL offered a protective effect on apoptosis against Aβ assault and attenuated excessive ER stress play a role in these benefits.

Furthermore, it is need to point out, our previews study have compared the efficiency of different α-ZAL concentration on cell viability and found that low concentration (10^{-9} M) α-ZAL could not provide protective effect on cells, and there was no significant difference in protective of α-ZAL between medium and high concentration (10^{-7} and 10^{-6} M) (Dong *et al.* 2007), this is why we just chose one α-ZAL concentration in the present study. We also found that α-ZAL owns the ability to interact with estrogen receptor (Dong *et al.* 2015), so we can speculate that α-ZAL may regulate ER-related protein expression through estrogen receptor pathway, further research is necessary to determine this conjecture.

Although there were limits in this work, for example, the inter-action between ER and mitochondria in the protective of α-ZAL need to evaluate. Our present results supported that Aβ treated hippocampal neurons undergo apoptosis and α-ZAL exerts anti-apoptotic effects at least in part, attributable to its property neutralize calcium overload and subsequently excessive ER stress. Thus, the protective effects of α-ZAL against AD-associated neurotoxins may help to provide the pharmacological basis of its future clinical usage in the prevention of this neurodegenerative disease especially in postmenopausal women.

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Competing Interests: *The authors declare that they have no competing interests.*

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