ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2007, **39**(2): 123–130 **CN** 31-1940/Q

Protective Effects of Vitamin E against Oxidative Damage Induced by $A\beta_{1-40}Cu(II)$ Complexes

Xueling DAI[1], Yaxuan SUN[2], and Zhaofeng JIANG[2]*

[1] *College of Life Science, Capital Normal University, Beijing 100037, China;*
[2] *College of Applied Sciences and Humanities of Beijing Union University, Beijing 100083, China*

Abstract β-amyloid peptide (Aβ) is considered to be responsible for the formation of senile plaques, which is the hallmark of Alzheimer's disease (AD). Oxidative stress, manifested by protein oxidation and lipid peroxidation, among other alterations, is a characteristic of AD brain. A growing body of evidence has been presented in support of $A\beta_{1-40}$ forming an oligomeric complex that binds copper at a CuZn superoxide dismutase-like binding site. $A\beta_{1-40}Cu(II)$ complexes generate neurotoxic hydrogen peroxide (H_2O_2) from O_2 via Cu^{2+} reduction, though the precise reaction mechanism is unclear. The toxicity of $A\beta_{1-40}$ or the $A\beta_{1-40}Cu(II)$ complexes to cultured primary cortical neurons was partially attenuated when (+)-α-tocopherol (vitamin E) as free radical antioxidant was added at a concentration of 100 μM. The data derived from lactate dehydrogenase (LDH) release and the formation of H_2O_2 confirmed the results from the MTT assay. These findings indicate that copper binding to $A\beta_{1-40}$ can give rise to greater production of H_2O_2, which leads to a breakdown in the integrity of the plasma membrane and subsequent neuronal death. Groups treated with vitamin E exhibited much slighter damage, suggesting that vitamin E plays a key role in protecting neuronal cells from dysfunction or death.

Key words $A\beta_{1-40}Cu(II)$ complex; vitamin E; neuronal oxidative damage; H_2O_2 generation; lactate dehydrogenase (LDH) release

β-amyloid peptide (Aβ), a proteolytic product derived from the larger amyloid precursor protein (APP), is the major component of innumerable amyloid deposits in Alzheimer's disease (AD) [1–6]. Aβ peptides range in length from 39 to 43 residues [6,7]. Among these peptides, the 40-mer ($A\beta_{1-40}$) and the 42-mer ($A\beta_{1-42}$) are abundant in suffered brains, and these two peptides are the main constituents of the neuritic plaques in the parenchyma of AD brain [6–8]. It is previously reported that Aβ is strikingly precipitated by certain metals *in vitro*, in particular Cu^{2+} [8,9]. Studies in mice and humans show that copper level increases with normal aging in brain, thus copper regulation may have a causal relationship with aging [10]. As a redox active metal, copper plays important catalytic roles in many enzymes, such as CuZn superoxide dismutase [11–13]. Redox chemistry arising from transition metals such as copper is proposed to be the main chemical origin of the radicals and reactive oxygen species (ROS) implicated in the pathogenesis of AD [14,15], thus the level of copper must be strictly regulated to prevent aberrant ROS production resulting in cellular toxicity [16].

Recently, we reported that Cu^{2+} could bind to $A\beta_{1-40}$ in physiological conditions forming $A\beta_{1-40}Cu(II)$ complex [18], which possesses the property of catalyzing the O_2-dependent production of H_2O_2 [12,18], through the reduction of Cu^{2+} to Cu^{+} [16,17]. Moreover, a transition on the secondary structure of the peptide has been observed in the presence of copper [18,19], facilitating the oligomerization of Aβ then deposits prevalent in AD.

This interaction of Aβ with copper mediates higher cytotoxicity compared with Aβ or copper alone [17,20].

Received: November 14, 2006 Accepted: December 20, 2006
This work was supported by the grants from the Technology Development Program of the Beijing Education Committee and the Key Program of the Beijing Natural Science Foundation (No. KZ200311417015)
*Corresponding author: Tel, 86-10-64900138; Fax, 86-10-64900143; E-mail, zhaofeng@buu.com.cn

DOI: 10.1111/j.1745-7270.2007.00261.x

Under this model, the aggregated peptide in concert with bound copper ions, initiates free radical processes [21] resulting in protein oxidation, lipid peroxidation, ROS formation, and cellular dysfunction leading to subsequent neuron death [16].

The polyunsaturated fatty acids of membrane lipids are the main targets for peroxide radicals, and brain is particularly vulnerable to lipid peroxidation for its high lipid content and remarkably high proportion of polyunsaturated fatty acids [22]. Vitamin E as a lipophilic free radical scavenger that protects cells against oxidative stress [23] can interact with redox chain reaction [24] that damages cells, but its protective effects on primary cultured neurons *in vitro* against neurotoxicity mediated by $A\beta_{1-40}$-copper ion interaction has not yet been reported to our knowledge.

Here we report that, in the presence of vitamin E, which is a generic term for a group of naturally occurring tocopherol derivatives with biologic activity similar to that of α-tocopherol [22], neurotoxicity mediated by $A\beta_{1-40}$ or $A\beta_{1-40}$Cu(II) complexes is markedly abrogated. Further, our results suggest that when vitamin E is present together with $A\beta_{1-40}$ or $A\beta_{1-40}$Cu(II) complexes, the production of H_2O_2 is partially eliminated, and the leakage of lactate dehydrogenase (LDH) originated from the breakdown in the integrity of the plasma membrane [25–27] dramatically decreases. On the basis of these data, we conclude that the oxidative stress hypothesis is well suited to explain the toxic effect of $A\beta_{1-40}$Cu(II) complexes in neuronal cultures, and this fatal cytotoxicity could be rescued by vitamin E, which may provide a therapeutic interdiction in AD.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), newborn calf serum (NCS), horse serum (HS), penicillin-streptomycin, Neurobasal™-A medium, and B-27 supplement minus AO were obtained from Invitrogen (Carlsbad, USA). Dimethyl sulfoxide (DMSO) was purchased from Amresco (Solon, USA). Poly-*L*-lysine, trypan blue, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (+)-α-tocopherol (vitamin E), and $A\beta_{1-40}$ were purchased from Sigma-Aldrich (St. Louis, USA). The purity of the peptide is determined by HPLC [28], and it has achieved 90% at least. Hydrogen peroxide assay kit was purchased from Beyotime Institute of Biotechnology (Haimen, China). Lactate dehydrogenase assay kit was obtained from Jiancheng Bioengineering Institute (Nanjing, China). Other chemical reagents including absolute ethyl alcohol, $CuCl_2 \cdot 2H_2O$, $NaHCO_3$, NaCl, and NaOH were obtained from Beijing Chemical Industry (Beijing, China), unless otherwise mentioned. Prior to use all buffers and stock solutions of metal ions were filtered through a 0.22-μm filter (Millipore, Cork, Ireland) to remove any particulate matter.

Peptide and Cu·$A\beta_{1-40}$ complex preparation

Working solutions of peptide were prepared by taking a known amount of $A\beta_{1-40}$ and dissolving it in HFIP. The peptide was incubated at 25 ºC for 1 h to remove any preformed structure [29]. HFIP was removed by evaporation. Known amounts of peptide were aliquoted out, dissolved in deionized water to a concentration of 1 mg/ml. After being incubated for 5 d at 37 ºC, the stock solution was diluted in Neurobasal™-A medium to a final concentration of 50 μM, aliquoted and stored at –20 ºC [18]. The aliquots were diluted to final working concentrations before being added to culture medium. As for Cu·$A\beta_{1-40}$ complexes, $CuCl_2$ was added to Aβ sample and diluted to the required concentration from a 50 μM stock. Samples were used immediately after being mixed or incubated at 37 ºC for 15 min at least.

Preparation of vitamin E solution

For its extreme insolubility in water, vitamin E was dissolved in the mixture of absolute ethyl alcohol and dimethyl sulfoxide [30] at a ratio of 1:1 (*V*/*V*), and the stock solution was then aliquoted and stored at –20 ºC. When vitamin E was required, the aliquots were diluted to the final working concentration desired in culture medium. The working solution was allowed to take effect in the medium for no longer than 2 d, because alcohol and dimethyl sulfoxide may exert toxic influence upon neuronal cells.

Primary neuronal culture

Cortical neuronal cultures were obtained according to an existing protocol [18,29] with modification. Briefly, cortices of new born Sprague-Dawley rats were removed, dissected free of meninges and blood vessels, cut into 1–2 mm^3 tissues, and dissociated in 0.125% (*W*/*V*) trypsin, shaking every 5 min. Fifteen minutes later, 5% serum was immediately added to terminate the dissociation. The dissociated cells were triturated using a filter-plugged fine pipette tip, pelleted, and resuspended in plating medium

(DMEM with 10% NCS and 10% HS plus 1% penicillin-streptomycin). Viable cells were counted manually using a 1-mm^2 grid (10×objective) stained with 0.4% trypan blue. Afterwards, dissected cells were plated into poly-*L*-lysine-coated 48-well culture plates or 25-cm^2 cell culture flasks (Corning, Corning, USA) at a density of 125,000 cells/cm^2 in plating medium. All cultures were maintained in an incubator set at 37 ºC with 5% CO_2. After 2 h the plating medium was replaced with fresh Neurobasal™-A medium plus B27 supplements, 100 U/ml penicillin, 100 μg/ml of streptomycin, and 0.5 mM *L*-glutamine. This method resulted in cultures highly enriched for neurons (>95% purity) with minimal astrocyte and microglial content as determined by immunostaining of culture preparations using specific marker antibodies.

The neuronal cells were allowed to mature for 7 d in culture before treatment using freshly prepared serum-free Neurobasal™-A medium plus B27 supplements minus antioxidants [18]. Samples were freshly prepared at molar ratio of peptide versus Cu^{2+} of 0:1, 1:1 and 2:1, respectively, then coincubated at 20 ºC for 15 min, finally the mixtures were added to neurons for 5 d. For treatment of neuronal cultures, samples were diluted to the final concentration of 5 μM in serum-free Neurobasal™-A medium plus B27 supplements. Groups treated with vitamin E were cultured in the same conditions. Cell viability and other properties were then assayed.

Cell viability assay

Cell survival was monitored by phase contrast microscopy, and cell viability was quantitated using MTT assay as described previously [27,31,32]. Briefly, cells were washed two times with 250 μl of D-Hanks buffer, then placed in Neurobasal™-A medium plus B27 lacking antioxidants (250 μl), and 25 μl of MTT was added to each well in a 48-well plate and incubated for 4 h at 37 ºC with 5% CO_2. After removal of the medium, blue formazan formed was solubilized in DMSO and absorbance (570 nm) was determined using a spectrometer (Bio-Tek, Winooski, USA), and background readings of MTT incubated in cell-free medium were subtracted. The data were normalized and calculated as a percentage of untreated control values before analysis.

Hydrogen peroxide assay

The reactions were carried out using Hydrogen peroxide assay kit. Firstly, the sample and schizolysis solution supplied by the kit were mixed at a ratio of 1:1, then the supernatants were gathered by centrifuging at 1100 *g* for 10 min for the following tests. All the operations were carried out on ice. Finally, the test tubes containing 50 μl of supernatants and 100 μl of test solutions were placed at room temperature for 20 min and measured instantly with a spectrometer at a wavelength of 560 nm. The concentration of H_2O_2 released was calculated according to standard concentration curve originated from standard solutions upon the identical experiments.

Lactate dehydrogenase assay

LDH is a stable cytoplasmic enzyme presented in all cells including neurons [25,27]. It is rapidly released into the cell culture supernatant when the cell plasma membrane is damaged. Therefore, the LDH level in the culture medium is a dependable biochemical index for neuronal plasma membrane damage. In this study, LDH release from cytosol of damaged neurons into the medium following $A\beta_{1-40}$ or $A\beta_{1-40}Cu(II)$ complexes exposure was detected using Lactate dehydrogenase assay kit, which determined the LDH activity in the medium to enzymatically convert the lactate and NAD^+ to pyruvate and NADH. The dinitrophenylhydrazine salt produced in the enzymatic reaction was then reduced to red formazan in the presence of pyruvate, thus allowing a colorimetric detection for neuronal membrane integrity.

Statistical analysis

We diluted and measured each sample at least three times performed in triplicate and used the averaged value for analysis. Error bars represent SE of the mean of each group analyzed. Data were evaluated statistically using SPSS 11.0 software (Chicago, USA). Comparisons were analyzed using one-way analysis of variance (ANOVA) followed by *post-hoc* Student-Newman-Keuls methods and/or Student's *t* test. Minimum significance level was set at $P<0.01$.

Results

Morphology on primary neuronal culture

Two hours after first plating, the plating medium was replaced with fresh Neurobasal™-A medium plus B27 supplements that designed for the long-term viability of neurons in the central nervous system, and resulted in cultures highly enriched for neurons. The neuronal cells were allowed to mature for 7 d before treatment. Whereas untreated group exhibited typical morphology of neurons: abundant cell body, rich cytoplasm and big cell nucleus, etc. [**Fig. 1(A)**]. The treatment of Aβ-copper complexes

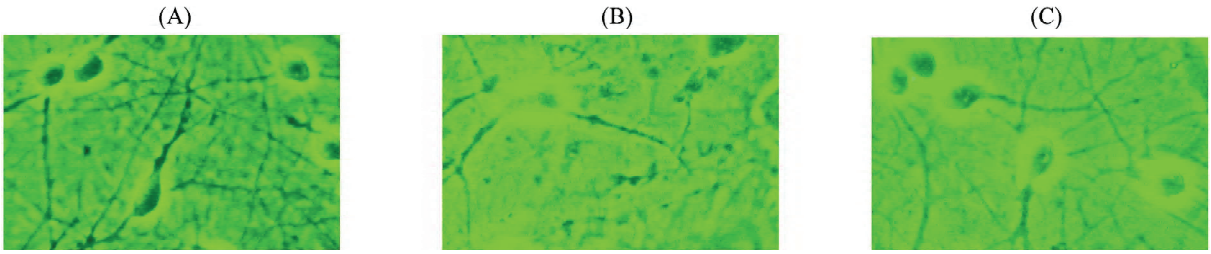

Fig. 1 Neurotoxicity of AβCu complexes and partial abolition by vitamin E

Light microscopic photomicrographs show the morphology of cortical neurons in cultures. Primary cortical neurons were grown at low density (1.25×10^5 cells/cm^2) for 7 d, and the morphology of control group (A), exposed to 5 μM $A\beta_{1-40}Cu$(II) (B) for 5 d, or incubated with 5 μM $A\beta_{1-40}Cu$(II) plus 100 μM vitamin E (C) for 5 d was recorded by photomicrographs. Magnification, 400×.

for 5 d exhibited greater neurotoxicity on neurons compared with copper or Aβ alone (data not shown). These changes included neuron loss, decreasing in light halo, distortions of cell bodies, and a growing trend for the neural network to decrease or even extinguish [**Fig. 1 (B)**]. Groups cultured with Aβ-copper complexes plus vitamin E performed much slighter damage than groups treated with Aβ-copper complexes, where much more distinct neural network and holonomic cell body could be observed [**Fig. 1(C)**].

Effect of vitamin E determined by MTT assay

It was shown in **Fig. 2** that the cell viability of groups exposed to either $A\beta_{1-40}$ or $A\beta_{1-40}Cu$(II) complexes determined by MTT assay was markedly decreased compared to untreated group, suggesting their severe cytotoxicity on neuronal cells. With respect to cytotoxicity comparison between $A\beta_{1-40}$ and $A\beta_{1-40}Cu$(II) complexes, group with addition of copper led to significantly greater toxicity than those treated with $A\beta_{1-40}$ alone using statistical comparison. Additional treatment of vitamin E markedly blunted the decrease of viability in groups exposed to either $A\beta_{1-40}$ or $A\beta_{1-40}Cu$(II) complexes, but hardly any difference could be detected between normal control groups and those exclusively treated with vitamin E. These data indicated that vitamin E could partly prevent neuronal cells from death mediated by $A\beta_{1-40}$- or $A\beta_{1-40}$-copper ion complex-induced cytotoxicity.

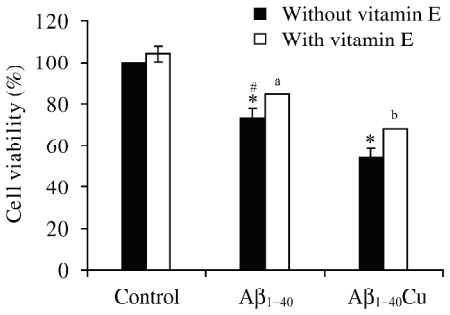

Fig. 2 Cell death caused by $A\beta_{1-40}$, $A\beta_{1-40}Cu$ complexes, and protective effects of vitamin E against toxicity

Primary neuronal cells were grown at low density (1.25×10^5 cells/cm^2) for 7 d, then neurons were exposed to 5 μM $A\beta_{1-40}$ or 5 μM $A\beta_{1-40}Cu$ complexes for 5 d in serum free medium, with addition of 100 μM vitamin E or not. The viability of these cells following different treatments was determined by measuring the inhibition of MTT reduction. Multiple comparisons on group exposed to 5 μM $A\beta_{1-40}$, 5 μM $A\beta_{1-40}Cu$ complexes versus control ($^*P<0.01$) were done using one-way ANOVA followed by *post-hoc* Student-Newman-Keuls method. Comparison of group exposed to 5 μM $A\beta_{1-40}$ versus 5 μM $A\beta_{1-40}Cu$ complexes ($^{\#}P<0.01$) or group treated with vitamin E versus without vitamin E ($^aP<0.05$, $^bP<0.01$) was done using Student's *t* test. Data are shown as mean±SE. Each experiment was carried out in triplicate.

Hydrogen peroxide generation

The ability of Aβ peptides incubated with Cu^{2+} to generate H_2O_2 has been reported previously [11,18]. Here we tested the ability of control group, group treated with copper, exposure to $A\beta_{1-40}$, $A\beta_{1-40}Cu$(II) or $(A\beta_{1-40})_2Cu$ (II) respectively to generate H_2O_2. The results (data not shown) confirmed our preceding studies [18], suggesting the strongest redox activity of $A\beta_{1-40}Cu$(II) among all the treatments. The amount of H_2O_2 release induced by $A\beta_{1-40}$ or $A\beta_{1-40}Cu$(II) complexes was significantly greater than those untreated (**Fig. 3**), and the capability of $A\beta_{1-40}$ to generate H_2O_2 was strengthened in the presence of Cu^{2+}. Further, we explored the protective effects of vitamin E against H_2O_2 production induced by control group, and those exposed to $A\beta_{1-40}$ or $A\beta_{1-40}Cu$(II) complexes. According to comparison between parallel groups in which vitamin E made an exclusive distinction, group treated with vitamin E significantly lowered the production of

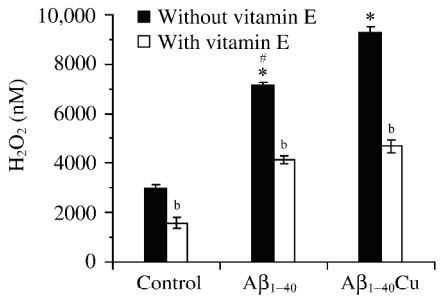

Fig. 3 Production of H_2O_2 induced by $A\beta_{1-40}$, $A\beta_{1-40}Cu$ complexes, or parallel treated with vitamin E against toxicity

Primary cortical neurons were grown at low density (1.25×10^5 cells/cm^2) for 7 d in serum-free medium. On the 8th day, these cells were exposed to 5 μM $A\beta_{1-40}$ or 5 μM $A\beta_{1-40}Cu$ for 5 d in the presence of 100 μM vitamin E or not. Production of H_2O_2 was compared on group exposed to 5 μM $A\beta_{1-40}$, 5 μM $A\beta_{1-40}Cu$ complexes versus control (*P<0.01) using one-way ANOVA, while comparison of group exposed to 5 μM $A\beta_{1-40}$ versus 5 μM $A\beta_{1-40}Cu$ complexes ($^{\#}P$<0.01) or group treated with vitamin E versus without vitamin E (^{b}P<0.01) was done using Student's t test. Each experiment was carried out in triplicate. Data are shown as mean±SE.

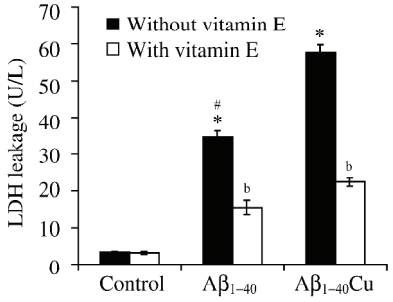

Fig. 4 Activity of LDH released by $A\beta_{1-40}$, $A\beta_{1-40}Cu$ complexes, or under the same dose treated with vitamin E against cytotoxicity

Primary cortical neurons were allowed to mature for 7 d in serum-free medium. Then, 5 μM $A\beta_{1-40}$ or 5 μM $A\beta_{1-40}Cu$ was added to the medium in the presence of 100 μM vitamin E or not for 5 d. Statistical comparisons between group treated with $A\beta_{1-40}$ versus group with $A\beta_{1-40}Cu$ complexes ($^{\#}P$<0.01), or between group treated with vitamin E versus without vitamin E (^{b}P<0.01) were done using Student's t test. Activity of LDH released was compared on group exposed to 5 μM $A\beta_{1-40}$, 5 μM $A\beta_{1-40}Cu$ complexes versus control group (*P<0.01) using one-way ANOVA followed by *post-hoc* Student-Newman-Keuls methods. Each experiment was carried out in triplicate. Data are shown as mean±SE.

H_2O_2 compared with those without vitamin E in all the groups tested.

Leakage of LDH

Increasing activity of LDH released from cells into culture medium also reflects the damage of neurons [25, 27]. LDH activity in the supernatant of control groups, group exposed to $A\beta_{1-40}$ or $A\beta_{1-40}Cu(II)$ complexes was assessed, and both groups treated with $A\beta_{1-40}$ in the presence of copper or $A\beta_{1-40}$ alone had markedly increasing release of LDH versus normal control groups (**Fig. 4**). Groups treated with $A\beta_{1-40}Cu(II)$ complexes gave rise to more LDH release compared with those with $A\beta_{1-40}$. Furthermore, our data implicated that both groups exposed to $A\beta_{1-40}$ or $A\beta_{1-40}Cu(II)$ complexes in the presence of vitamin E had significantly lowered the leakage of LDH from cells into medium compared to parallel groups without vitamin E, but the comparison between control groups and that plus vitamin E had not achieved statistically significant difference (P>0.01).

Discussion

Mounting evidence demonstrates that aberrant metal homeostasis is observed in AD patients [33], with enhancing the formation of ROS and toxic Aβ oligomers and facilitating the formation of amyloid deposits in AD brain, which may contribute to AD pathogenesis [9,34]. Furthermore, copper released from synaptic activity has been shown to induce parenchymal and cerebrovascular amyloid in transgenic mice [35].

The levels of copper in the brain rise markedly with aging [10,33], and it is proposed that the consequent abnormal decoration of Aβ with Cu^{2+} [36] leads to two principle abnormal reactions: redox activity leading to H_2O_2 production [11,18] and aggregation [9,34,37]. The role of toxicity of Aβ in AD is strengthened by *in vitro* findings showing that Aβ reduces Cu^{2+} to Cu^{+} in a catalytic manner that converts O_2 to H_2O_2 [10,11,17].

Amyloid-associated pathophysiology has been reported to be involved in oxidative stress [1,4], and the ROS induced by $A\beta_{1-40}Cu(II)$ complexes can directly undermine the integrity of neuronal cell membrane [26], resulting in the release of substantive bioactive substances into the extracellular space, including enzymes such as LDH and glutathione peroxidase [27].

In the present study, the primary cultured cortical neurons were exposed to low concentrations of $A\beta_{1-40}Cu(II)$ complexes or $A\beta_{1-40}$ at 5 μM, yet morphologic damage appeared via light microscopic photomicrograph compared to control groups [**Fig. 1(A)**]. When compared in the same dose, group treated with $A\beta_{1-40}Cu(II)$ complexes [**Fig. 1 (B)**] exhibited much greater destruction than that with $A\beta_{1-40}$ or copper alone (data not shown). In the parallel

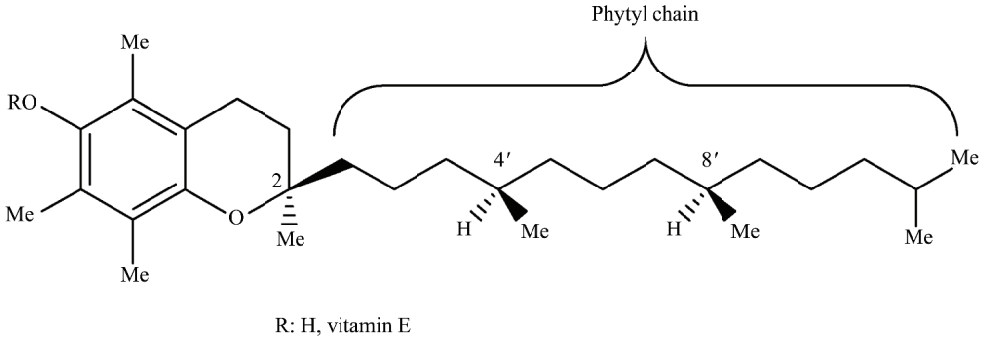

Fig. 5 Chemical structure of vitamin E (α-tocopherol, α-TOH)

Vitamin E as a natural antioxidant plays a vital role in the maintenance of tissue homeostasis and cellular defence against oxidative stress.

treated group plus vitamin E, however, cellular damage induced by Aβ-copper complexes was partially abrogated [**Fig. 1(C)**]. All the phenomena observed were in good correspondence to the decrease in viability as assessed by MTT assay (**Fig. 2**).

We further explored the formation of H_2O_2 in groups treated with copper, $A\beta_{1-40}$, $A\beta_{1-40}Cu(II)$ or $(A\beta_{1-40})_2Cu(II)$ respectively. Groups treated with $A\beta_{1-40}Cu(II)$ gave rise to maximum H_2O_2 production among all the groups involved (data not shown), which provided evidence for our previous study [18] hypothesizing copper bound to Aβ in a molar ratio of 1:1. Because groups treated with copper or $(A\beta_{1-40})_2Cu(II)$ released less amount of H_2O_2 than that with $A\beta_{1-40}$ or $A\beta_{1-40}Cu(II)$ complexes respectively, we chose groups that exposed to $A\beta_{1-40}$ or $A\beta_{1-40}Cu(II)$ complexes from the alternatives with addition of vitamin E. As shown in **Fig. 3**, results indicated that groups incubated with vitamin E obviously decreased the formation of H_2O_2 compared to those parallel groups with $A\beta_{1-40}$ or $A\beta_{1-40}Cu(II)$ complexes.

With respect to the destruction in the integrity of neuronal cell membrane induced by $A\beta_{1-40}$ or $A\beta_{1-40}Cu(II)$ complexes-mediated cytotoxicity, LDH activity in the extracellular fluid of cells was detected [25,26]. According to the data (**Fig. 4**), leakage of LDH in groups exposed to $A\beta Cu^{2+}$ was greater than that observed in cells exposed to Aβ, whereas groups treated with vitamin E in paralleled groups exhibited much milder cytotoxicity to neuronal cells, suggesting vitamin E may exert all its influence on preventing cell membrane from breakdown.

The activity of LDH released into extracellular fluid and the formation of H_2O_2 confirmed the results of the MTT assay. These results favor the view that oxidative damage is involved in the cytotoxicity induced by Aβ-copper ion complexes [8,13], while vitamin E can partially reverse the effects. Tissue damage and cell death caused by lipid peroxidation can be prevented by vitamin E [38], but possible molecular mechanisms underlying vitamin E-mediated resistance of neurons to oxidative stress are not fully understood. There is a phenolic hydroxyl group in aromatic ring of vitamin E (**Fig. 5**) that provides a proton to detoxify lipid peroxidation [24] by trapping the peroxyl radicals [39], which is proposed to be the prerequisite of a neuroprotective effect against oxidative challenges [40].

We assume that the blockage of the intracellular production of hydrogen peroxide in the presence of vitamin E is causally related to its neuroprotective effect. This antioxidant is believed to be capable of suppressing lipid peroxidation, which is further confirmed by the fact that a decrease in the activity of LDH released from cells into culture medium (**Fig. 4**). The abolition of lipid peroxidation and free radicals results in the elevation of cell viability [39]. In conclusion, we here present evidence that $A\beta_{1-40}Cu(II)$ complexes cause oxidative stress via the initiation of intracellular peroxide accumulation followed by the leakage of LDH released in primary cortical neuron cultures. At molecular cellular level, $A\beta_{1-40}Cu(II)$ complexes induce the cytotoxic peroxide and ultimately cause neuronal cell death that can be prevented by vitamin E to a less degree [33]. With respect to the mechanism involved, we propose that vitamin E may have a dual activity by blocking the immediate rise in peroxides and the subsequent lipid peroxidation [38], and by suppressing the $A\beta_{1-40}Cu(II)$ complexes-induced activation of oxidative stress.

Comparatively high copper level in AD brain forming Aβ-copper complexes may trigger oxidative stress in neuronal cells [8,10,41], resulting in long-lasting potentially irreversible damage [5]. As discussed above, the oxidative stress hypothesis is well suited to explain the toxic effect of Aβ-copper ion complexes in neuronal

cultures [38], and the consequential untoward injury could potentially be prevented by antioxidants such as vitamin E. However, our data indicate that the neuronal damage induced by Aβ-copper could not be completely abrogated by addition of vitamin E, suggesting other potential therapeutic strategies such as clioquinol [42] may be involved in the treatment of AβCu(II)-mediated neurotoxicity, and these are deserved further investigation.

References

1 Hodges JR. Alzheimer's centennial legacy: Origins, landmarks and the current status of knowledge concerning cognitive aspects. Brain 2006, 129: 2811–2822

2 Selkoe DJ. Translating cell biology into therapeutic advances in Alzheimer's disease. Nature 1999, 399(Suppl): A23–A31

3 Goedert M, Spillantini MG. A century of Alzheimer's disease. Science 2006, 314: 777–781

4 Huang X, Moir RD, Tanzi RE, Bush AI, Rogers JT. Redox-active metals, oxidative stress, and Alzheimer's disease pathology. Ann NY Acad Sci 2004, 1012: 153–163

5 Roberson ED, Mucke L. 100 years and counting: Prospects for defeating Alzheimer's disease. Science 2006, 314: 781–784

6 Selkoe DJ. Alzheimer's disease: Genes, proteins, and therapy. Physiol Rev 2001, 81: 741–766

7 Kim W, Hecht MH. Sequence determinants of enhanced amyloidogenicity of Alzheimer Aβ42 peptide relative to Aβ40. J Biol Chem 2005, 280: 35069–35076

8 Syme CD, Nadal RC, Rigby SE, Viles JH. Copper binding to the amyloid-β (Aβ) peptide associated with Alzheimer's disease. J Biol Chem 2004, 279: 18169–18177

9 Atwood CS, Moir RD, Huang X, Scarpa RC, Bacarra NM, Romano DM, Hartshorn MA *et al*. Dramatic aggregation of Alzheimer Aβ by Cu(II) is induced by conditions representing physiological acidosis. J Biol Chem 1998, 273: 12817–12826

10 Maynard CJ, Cappai R, Volitakis I, Cherny RA, White AR, Beyreuther K, Masters CL *et al*. Overexpression of Alzheimer's disease amyloid-β opposes the age-dependent elevations of brain copper and iron. J Biol Chem 2002, 277: 44670–44676

11 Opazo C, Huang X, Cherny RA, Moir RD, Roher AE, White AR, Cappai R *et al*. Metalloenzyme-like activity of Alzheimer's disease β-amyloid. Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H_2O_2. J Biol Chem 2002, 277: 40302–40308

12 Curtain CC, Ali F, Volitakis I, Cherny RA, Norton RS, Beyreuther K, Barrow CJ *et al*. Alzheimer's disease amyloid-β binds copper and zinc to generate an allosterically ordered membrane-penetrating structure containing superoxide dismutase-like subunits. J Biol Chem 2001, 276: 20466–20473

13 White AR, Multhaup G, Maher F, Bellingham S, Camakaris J, Zheng H, Bush AI *et al*. The Alzheimer's disease amyloid precursor protein modulates copper-induced toxicity and oxidative stress in primary neuronal cultures. J Neurosci 1999, 19: 9170–9179

14 Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR. Copper, iron and zinc in Alzheimer's disease senile plaques. J Neurol Sci 1998, 158: 47–52

15 Bush AI. The metallobiology of Alzheimer's disease. Trends Neurosci 2003, 26: 207–214

16 Smith MA, Rottkamp CA, Nunomura A, Raina AK, Perry G. Oxidative stress in Alzheimer's disease. Biochim Biophys Acta 2000, 1502: 139–144

17 Huang X, Cuajungco MP, Atwood CS, Hartshorn MA, Tyndall JD, Hanson GR, Stokes KC *et al*. Cu(II) potentiation of Alzheimer Aβ neurotoxicity: Correlation with cell-free hydrogen peroxide production and metal reduction. J Biol Chem 1999, 274: 37111–37116

18 Dai XL, Sun YX, Jiang ZF. Cu(II) potentiation of Alzheimer Aβ1–40 cytotoxicity and transition on its secondary structure. Acta Biochim Biophys Sin 2006, 38: 765–772

19 Yoshiike Y, Tanemura K, Murayama O, Akagi T, Murayama M, Sato S, Sun X *et al*. New insights on how metals disrupt amyloid β-aggregation and their effects on amyloid-β cytotoxicity. J Biol Chem 2001, 276: 32293–32299

20 White AR, Multhaup G, Galatis D, McKinstry WJ, Parker MW, Pipkorn R, Beyreuther K *et al*. Contrasting, species-dependent modulation of copper-mediated neurotoxicity by the Alzheimer's disease amyloid precursor protein. J Neurosci 2002, 22: 365–376

21 Butterfield DA, Castegna A, Lauderback CM，Drake J. Evidence that amyloid β-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. Neurobiol Aging 2002, 23: 655–664

22 Grundman M. Vitamin E and Alzheimer disease: The basis for additional clinical trials. Am J Clin Nutr 2000, 7: 630S–636S

23 Futakawa N, Kondoh M, Ueda S, Higashimoto M, Takiguchi M, Suzuki S, Sato M. Involvement of oxidative stress in the synthesis of metallothionein induced by mitochondrial inhibitors. Biol Pharm Bull 2006, 29: 2016–2020

24 Jin L, Beswick RA, Yamamoto T, Palmer T, Taylor TA, Pollock JS, Pollock DM *et al*. Increased reactive oxygen species contributes to kidney injury in mineralocorticoid hypertensive rats. Physiol Pharmacol 2006, 57: 343–357

25 Zhao L, Brinton RD. Select estrogens within the complex formulation of conjugated equine estrogens (Premarin) are protective against neurodegenerative insults: Implications for a composition of estrogen therapy to promote neuronal function and prevent Alzheimer's disease. BMC Neurosci 2006, 7: 24

26 Li L, Zhou QX, Shi JS. Protective effects of icariin on neurons injured by cerebral ischemia/reperfusion. Chinese Med J 2005,118: 1637–1643

27 Saad B, Dakwar S, Said O, Abu-Hijleh G, Al Battah F, Kmeel A, Aziazeh H. Evaluation of medicinal plant hepatotoxicity in co-cultures of hepatocytes and monocytes. Evid Based Complement Alternat Med 2006, 3: 93–98

28 Kicinski HG, Adamek S, Kettrup A. Trace enrichment and HPLC analysis of polycyclic aromatic hydrocarbons in environmental samples, using solid phase extraction in connection with UV/VIS diode-array and fluorescence detection. Chromatographia 1998, 28: 203–208

29 Smith DP, Smith DG, Curtain CC, Boas JF, Pilbrow JR, Ciccotosto GD, Lau TL *et al*. Copper mediated amyloid-β toxicity is associated with an intermolecular histidine bridge. J Biol Chem 2006, 281: 15145–15154

30 Liu YH, Huang GW, Chang H. Antioxidation of soybean isoflavone in vascular endothelial cells with oxidative damage. Zhongguo Lin Chuang Kang Fu 2006, 10: 170–172

31 White AR, Huang X, Jobling MF, et al. Homocysteine potentiates copper- and amyloid beta peptide-mediated toxicity in primary neuronal cultures: Possible risk factors in the Alzheimer's-type neurodegenerative pathways. J Neurochem 2001, 76:1509–1520

32 Stoddart MJ, Furlong PI, Simpson A, Davies CM, Richards RG. A comparison of non-redioactive methods for assessing viability *in ex vivo* cultured cancellous bone: Technical note. Eur Cell Mater 2006, 12: 16–25

33 Finefrock AE, Bush AI, Doraiswamy PM. Current status of metals as

therapeutic targets in Alzheimer's disease. J Am Geriatr Soc 2003, 51: 1143–1148

34 Cuajungco MP, Faget KY, Huang XD, Tanzi RE, Bush AI. Metal chelation as a potential therapy for Alzheimer's disease. Ann NY Acad Sci 2000, 920: 292–304

35 Maynard CJ, Bush AI, Masters CL, Cappai R, Li QX. Metals and amyloid-β in Alzheimer's disease. Int J Exp Pathol 2005, 86: 147–159

36 Crouch PJ, Barnham KJ, Duce JA, Blake RE, Masters CL, Trounce IA. Copper-dependent inhibition of cytochrome c oxidase by Aβ(1–42) requires reduced methionine at residue 35 of the Aβ peptide. J Neurochem 2006, 99: 226–236

37 Atwood CS, Scarpa RC, Huang X, Moir RD, Jones WD, Fairlie DP, Tanzi RE *et al*. Characterization of copper interactions with Alzheimer amyloid β peptides: Identification of an attomolar-affinity copper binding site on amyloid β1–42. J Neurochem 2000, 75: 1219–1233

38 Munoz FJ, Opazo C, Gil-Gomez G, Tapia G, Fernandez V, Valverde MA, Inestrosa NC. Vitamin E not 17 estradiol protects against vascular toxicity induced by amyloid wild type and the dutch amyloid variant. J Neurosci 2002, 22: 3081–3089

39 Naiki H, Hasegawa K, Yamaguchi I, Nakamura H, Gejyo F, Nakakuki K. Apolipoprotein E and antioxidants have different mechanisms of inhibiting Alzheimer's beta-amyloid fibril formation *in vitro*. Biochemistry 1998, 37: 17882–17889

40 Behl C, Skutella T, Lezoualc'h F, Post A, Widmann M, Newton CJ, Holsboer F. Neuroprotection against oxidative stress by estrogens: Structure-activity relationship. Mol Pharmacol 1997, 51: 535–541

41 Miranda S, Opazo C, Larrondo LF, Munoz FJ, Ruiz F, Leighton F, Inestrosa NC. The role of oxidative stress in the toxicity induced by amyloid β-peptide in Alzheimer's disease. Prog Neurobiol 2000, 62: 633–648

42 White AR, Du T, Laughton KM, Volitakis I, Sharples RA, Xilinas ME, Hoke DE *et al*. Degradation of the Alzheimer disease amyloid-peptide by metal-dependent up-regulation of metalloprotease activity. J Biol Chem 2006, 281: 17670–17680

Edited by
Liqin ZHAO