ORIGINAL ARTICLE

Induction of DNA Damage and Cell Death by Beta Amyloid Peptide and Its Modification by Tocotrienol Rich Fraction (TRF)

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ABSTRAK

Penyakit Alzheimer berkait dengan peningkatan kematian sel neuron dan gangguan fungsi kognitif. Penyakit ini dicirikan oleh pembentukan plak di otak. Pengumpulan peptida beta amiloid (Aβ) dipercayai merupakan penanda awal kepada patofisiologi penyakit Alzheimer. Walau bagaimanapun, mekanisma toksisiti Aβ masih tidak diketahui tetapi mungkin melibatkan peningkatan tekanan oksidatif. Oleh itu, kajian ini dijalankan untuk menentukan kesan toksik Aβ terhadap DNA - biomolekul penting yang dioksidakan oleh radikal bebas, dan apoptosis sel serta modulasinya oleh fraksi kaya tokotrienol (TRF), sejenis antioksidan. Sel titian neuroblastoma diperlakukan sama ada dengan 10μM peptida Aß, 5μg/ml TRF diikuti dengan 10μM peptida Aß atau 10μM peptida Aß peptide diikuti dengan 5μg/ml TRF. Sel yang tidak dperlakukan dengan Aß atau TRF dijadikan sebagai kawalan. Kerosakan DNA ditentukan dengan menggunakan asai komet, viabiliti sel ditentukan menggunakan asai 3-(4,5-dimetiltiazol-2-il)-5-(3-karboksimetoksifenil)2-(4-sulfofenil)-2H-tetrazolium (MTS) dan pewarnaan propidium iodida dan calcein-AM dijalankan untuk menentukan bilangan sel yang hidup dan sel yang mengalami apoptosis. Keputusan menunjukkan bahawa peptida Aß meningkatkan kerosakan DNA secara signifikan berbanding kawalan ($p<0.05$) dan meningkatkan kematian sel. Walau bagaimanapun, perlakuan dengan TRF mengurangkan kerosakan DNA secara signifikan, meningkatkan bilangan sel hidup dan mengurangkan jumlah sel yang mengalami apoptosis berbanding kumpulan yang diaruh oleh peptida Aß sahaja ($p<0.05$). Oleh itu, kajian ini menujukkan peptida Aß menyebabkan kerosakan DNA dan kematian sel melalui apoptosis kemungkinan disebabkan oleh aruhan kerosakan oksidatif pada DNA. Ini disokong oleh fakta di mana TRF, suatu antioksidan, berupaya melindungi kerosakan DNA dan apoptosis.

Kata kunci: Kerosakan DNA, peptida beta amiloid, TRF, neurodegenerasi, apoptosis, penyakit Alzheimer

ABSTRACT

Alzheimer's disease (AD) is associated with increase neuron cell death and decline in cognitive function. This disease is characterized by plaque formation in the brain. It is

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believed that accumulation of beta amyloid peptide (Aβ) is an early sequence in the pathophysiology of AD. However, the mechanism of Aβ toxicity is unknown but may involve increase oxidative stress. This study was thus undertaken to determine the toxic effect of Aβ on DNA - an important biomolecule which is oxidized by free radicals, and cell apoptosis and its modulation by tocotrienol rich fraction (TRF). Neuroblastoma SH-SY5Y cell lines were treated either with 10μM Aß peptide; 5μg/ml TRF followed by 10μM Aß peptide or 10μM Aß peptide followed by 5μg/ml TRF. Untreated cells served as control. DNA damage was evaluated by the alkaline comet assay, cell viability by the 3-(4,5-dimetiltiazol-2-il)-5-(3-karboksimetoksifenil)2-(4-sulfofenil)-2H-tetrazolium (MTS) and the propidium iodide & calcein-AM staining to determine the number of viable and apoptotic cells. Results showed that Aß peptide induced a significantly higher DNA damage compared to control ($p<0.05$) and higher number of cell death. However treatment with TRF resulted in significantly less DNA damage, higher cell survival and decreased number of apoptotic cells as compared to Aß peptide treated cells ($p<0.05$,). Thus this study showed that Aß peptide causes DNA damage and ultimately cell death via apoptosis probably by inducing oxidative DNA damage. This is further supported by the fact that TRF is able to prevent the DNA damage and apoptosis.

Key words: DNA damage, beta amyloid peptide, TRF, neurodegeneration, apoptosis, Alzheimers disease

INTRODUCTION

Alzheimers disease (AD) is characterized by regional neurodegeneration, synaptic loss and the presence of senile plaques (SantaCruz et al. 2005). The senile plaques consist of abnormal interneuronal growth, dead cells and beta amyloid. There has been increasing evidence which suggests that the buildup and aggregation of beta amyloid peptide (Aβ) in the brain plays a primary role in the pathogenesis of AD (Piccini et al. 2005).

Although Aβ has been demonstrated to be neurotoxic in cell culture (Butterfield et al. 2004), the mechanism by which it exerts its toxicity is still unclear. Several mechanisms have been suggested including increased sensitivity to excitotoxins, alterations to calcium homeostasis (Celsi et al. 2006), activation of receptor that precipitates cell destruction and activation of inflammatory pathways (Zou et al. 2006).

Recently, the involvement of oxidative stress in the pathogenesis of AD has been suggested (Wang et al. 2008). The AD brain was reported to have increased lipid peroxidation, increased carbonyl modification of proteins and increased oxidation of mitochondrial DNA (Arimura & Kaibuchi 2005).

Increase oxidative stress occurs when the balance between free radical generation and antioxidant capacities shifts toward free radical generation leading to oxidative damage to lipids, protein, RNA and DNA. This is not surprising as the brain is more susceptible to oxidative damage compared to other organs or tissues, due to its high rate of oxygen consumption, high polyunsaturated lipid content, and relative paucity of antioxidant enzymes (Puttfarcken 1996).

Understanding how Aβ acts and its probable association to oxidative stress is important for the formulation of treatment and preventive steps against neurodegeneration. Therefore, in this study,

the involvement of oxidative damage in the mechanism of Aβ-toxicity resulting in neuronal cell death was determined by measuring oxidative DNA damage. The involvement of oxidative damage was further elucidated by determining the effect of TRF.

MATERIALS AND METHODS

SH-SY5Y neuroblastoma cell culture

Human neuroblastoma SH-SY5Y cells were gifts from Dr. Coral Sanfeliu of the Institut d'Investigacions Biomèdiques de Barcelona, Spain. Cells were grown under the following condition: 50% of EMEM with Earles Salt pH 7.2 (Flowlab, Australia) and 50% Ham's F-12 pH 7.2 (Sigma, USA) supplemented with 200mM Non-essential amino acid (NEAA) (Flowlab, Australia), 10mg/ml gentamicin (PAA, Austria) and 10% Foetal Bovine Serum (FBS), heat inactivated (PAA, Austria). Cells were subcultured after wash with phosphate buffered saline (PBS) and trypsin. Cells were then seeded at 1.5 x 10^4 cells per ml on 96-well plates. Cultures were maintained in 5% CO_2 / 95% air at 37^{O}C. Experiments were carried out on the 7th-9th day.

Preparation of Aggregated Aβ

The stock solution of $A\beta_{1-42}$ (Calbiochem, German) was dissolved at 1mg/ml in 50Mm Tris-HCL, pH ≥ 9.0. The peptide was diluted to 20μM with calcium, magnesium-free PBS and stored at -20^{o}C. The stock solution was then diluted to a final concentration of 10μM followed by incubation at 37^{o}C for 24 hours before use.

Experimental Design

Cells were divided into 4 groups: untreated cells served as control, treated with 10μM Aß peptide for 24 hours, 5μg/ml TRF for 24 hours followed by 10μM Aß peptide for 24 hours (pre treatment) or 10μM Aß peptide for 24 hours followed by 5μg/ml TRF for 24 hours (post treatment). All cultures were maintained in 5% CO_2 / 95% air at 37^{o}C. TRF was purchased from Golden Hope Biorganic Sdn. Bhd. (Malaysia). DNA damage, cell viability and apoptosis were determined by using MTS assay, comet assay and Propidium Iodide & Calcein-AM staining respectively.

Comet assay

DNA damage was determined by using the Comet assay according to the method described by Singh et al. (1988), with minor modifications. 105μl of normal melting agarose (NMA) was added to frosted microscope slides (Curtin Matherson, USA), covered with coverslips and kept for 10 minutes to solidify. Coverslips were removed and 20μl cells with 80μl low melting point agarose (LMA) were added to the slides (Sigma, USA). The slides were then immersed in a jar containing cold lysing solution (2.5 M NaCl, 100 mM ethylene-diaminetetra-acetic acid, 10 mM Tris at pH 10, 1% Triton X-100, 1% sodium N-lauroyl sarcocinate, 10% dimethylsulfoxide) for at least 1 hour. After lysis, the slides were placed in electrophoresis box filled with electrophoresis buffer (0.3 M NaOH and 1 mM ethylene-diaminetetra-acetic acid). The cells were incubated for 20 minutes to allow for unwinding of DNA and expression of alkali-labile sites. All of these steps were conducted under dim light to prevent additional DNA damage. After electrophoresis, neutralization buffer (0.4M Tris, pH 7.5) was added to neutralize the alkali, and the slides were left at room temperature for 5 minutes. This step was repeated twice. Ethidium bromide (Sigma, USA) was added to each slide, covered with a coverslip, kept in a humidified box and finally analyzed using

a fluorescence microscope (AxioCam MRC, Carl Zeiss, Germany).

MTS assay

The MTS assay purchased from (Promega, USA), employs MTS and the electron coupling agent phenazine methosulphate (PMS). MTS is converted into a medium soluble formazan product by dehydrogenase enzymes found in metabolically active cells. 20 µl of MTS reagents was pipetted into each well containing the samples in 100µl of culture medium. The plates were then incubated for 2 hours in 5% CO_2/95% air at 37^{o}C. The optical density (OD) of the wells was determined using a microplate reader at 490-nm wavelength (Versa max, Japan).

Propidium Iodide & Calcein-AM staining

Staining to detect cell death is based on the principle that live cells have intracellular esterase that converts non-fluorescent cell-permeable calcein-AM to the intensely fluorescent calcein which is retained within the cells. Viable cell membranes are impermeable to propidium iodide. Dead cells however, allow propidium iodide to enter and bind to nucleic acid. Thus live cells stained green with calcein-AM while dead and apoptotic cells stained red with propidium iodide (Sigma, USA). 30µg/ml calcein-AM and 7.5 µg/ml propidium iodide were added to the cell cultures in chamber slides (Nunc, Denmark) and incubated for 30 minutes. Thereafter, cultures were washed with PBS, fixed with fresh 4% paraformaldehyde and then incubated for 30 minutes at 37^{O}C. Slides were washed and coverslips mounted for microscopic examination (AxioCam MRC, Carl Zeiss, Germany).

RESULTS

DNA Damage Studies

Figure 1 shows DNA damage in the treated and untreated cells as determined by comet assay. Cells were grouped according to the length of comet tail (%). Cells without DNA fragmentation were classified as no DNA damage, cells with DNA fragmentation were classified

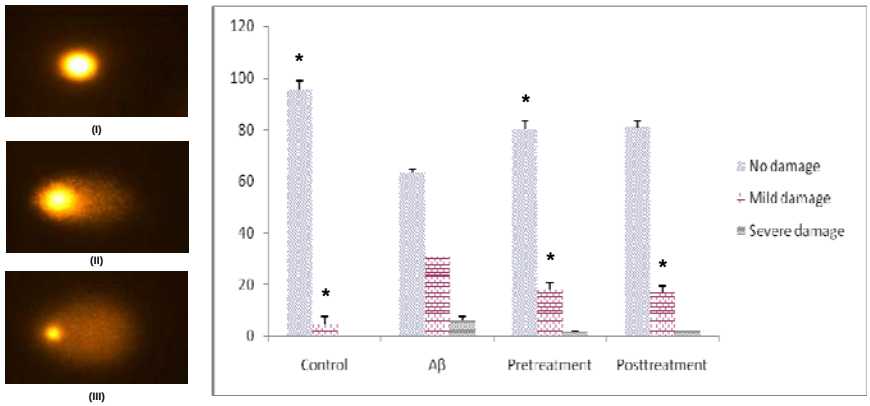

Figure 1 : DNA damage in untreated (control) and cells treated with either 10µM Aβ peptide, Aβ then 5 µg/ml TRF or TRF then Aβ peptide. DNA was stained with ethidium bromide and was visible as comet tail under fluorescence microscope. Length of comet tail is associated with severity of DNA damage. Thus cell was classified as having no damage (I), mild damage (II) and severe damage (III). Results showed that neuron cells exposed to 10µM Aß peptide showed a significantly ($p<0.05$) higher percentage of DNA damage compared to control. Treatment with TRF significantly lowers the DNA damage induced by 10µM Aß. *Denotes $p<0.05$ compared to cells exposed to Aβ peptide only. Data is presented as means $\pm$ SD. n=9.

as having mild damage and cells with major fragmentation as severely damaged. Results showed that incubation with 10μM Aß peptide significantly increased the DNA damage ($p<0.05$). However, cells treated with TRF before (pre treatment) or after (post treatment) incubation with Aβ was observed to have a significantly reduced number of DNA damage. There was no significant difference observed between the number and severity of DNA damage in pre and post TRF treated groups.

Determination of Aβ Peptide toxicity

The concentration of Aβ peptide which can induce 50% cell death (IC_{50}) was determined by incubating cells with increasing concentration of Aβ peptide. Figure 2 shows that this concentration was 10μM and hence 10μM Aβ peptide was the concentration used in subsequent experiments.

Neuroprotection Studies

Figure 3 shows that 10μM Aβ significantly increased neuronal death. Pre and post treatment with 5μg/ml TRF significantly increased the number of viable cells compared to the cells treated with Aβ only and thus demonstrated that TRF was able to prevent cell death induced by Aß peptide.

Figure 4 shows the results of fluorescence staining using calcein AM and propidium iodide. Control live neuron cells were stained green (a) whereas cells exposed to 10μM Aß peptide were stained red with (i) cell shrinkage and (ii) condensed chromatins, characteristics of apoptotic cells (b). Cells pretreated with 5μg/ml TRF before exposure to Aß underwent early apoptosis but still retained membrane integrity (c). TRF post treated cells showed increased number of viable cells compared to cells exposed to Aβ only demonstrating that post treatment of cells with TRF prevented cell death induced by Aß (d).

DISCUSSION

Recent evidence suggests that Aβ peptide may contribute to the progressive neuronal loss and/or may be directly neurotoxic both in vitro and in vivo (Pike et al. 1993). This is consistent with the present finding where Aβ peptide was

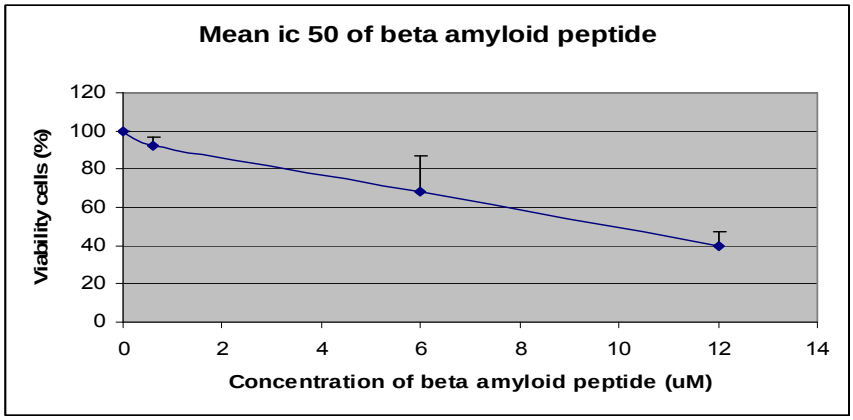

Figure 2 : Viability of neuron cells exposed to Aß peptide was determined using the MTS assay. Incubation with varying concentrations of Aß peptide for 24 hours significantly ($p<0.05$) decreased the number of viable neuron cells. Concentration of Aß peptide which reduced 50% of neuron cells (IC_{50}) was found to be 10μM Aß.

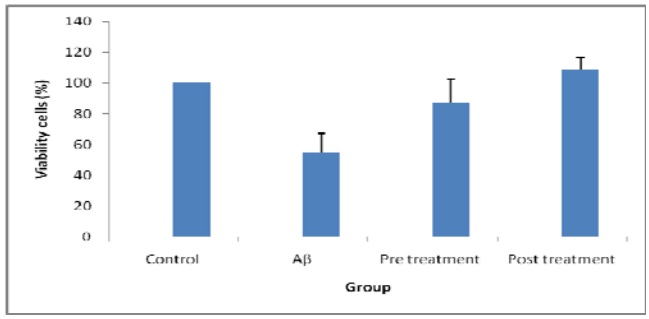

Figure 3 : Neuroprotection of TRF against Aß peptide induced cell death using MTS assay. Neuron cells pretreated and posttreated with 5µg/ml TRF for 24 hours showed a significant increase in percentage of viable cells compared to cells exposed to Aβ peptide. TRF was able to protect against cell death at a concentration of 5µg/ml. *Denotes $p<0.05$ compared to the Aß treated group. Data is presented as means ± SD. n=9.

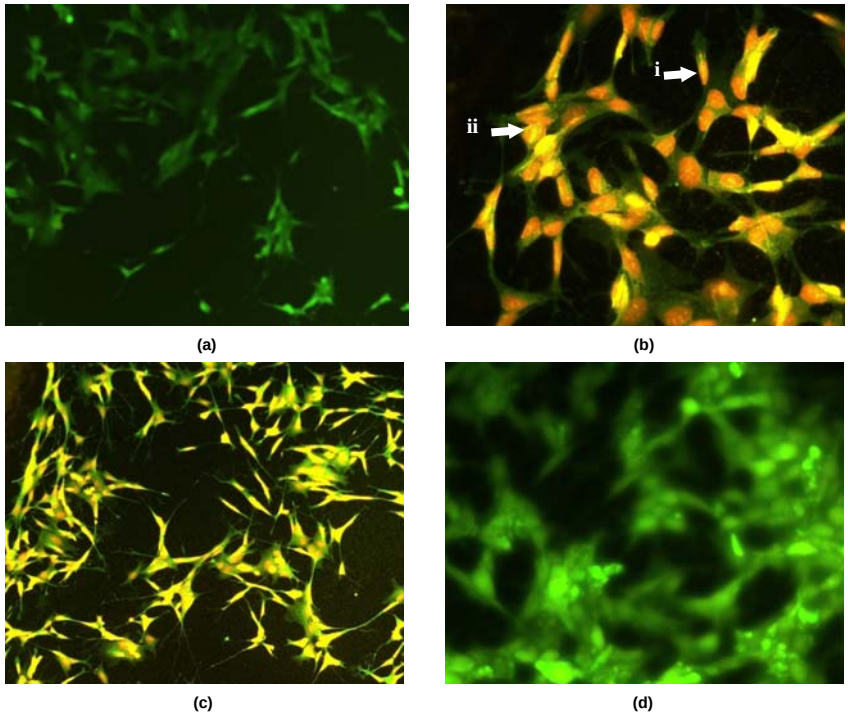

Figure 4: Neuroprotective effect of TRF against Aß peptide induced cell death using the Calcein AM and propidium iodide staining. Live cells were stained green, dead cells were stained red. Micrographs are labeled as a) control : live cells, b) exposed to 10µM Aß peptide : cells underwent apoptosis with the characteristic (i) cell shrinkage and (ii) condensed chromatins, c) 5µg/ml TRF followed by 10µM Aß peptide : underwent early apoptosis but still retained membrane integrity d) 10µM Aß peptide followed by 5µg/ml TRF respectively : number of viable (green) cells increased and apoptotic (red) cells decreased.

observed to significantly decrease the number of viable cells (Figure 2). The induction of cell death may be elicited via apoptosis (Behl et al. 1994). The present finding (Figure 4b) showed that the number of apoptotic cells was increased while the number of viable cells decreased when exposed to Aβ peptide.

Aβ peptide may exert its cytotoxic effects by activating the apoptotic pathway via free radical damage and/or induction of the signaling molecules (Pereira et al. 1999). Free radicals are constantly generated *in vivo* and have been reported to be significant contributors to the development of chronic degenerative disorders such as Alzheimers disease (Ames et al. 1993). Although multiple antioxidant repair systems exist to help control this process, reactive species still cause damage to biomolecules, cells and tissues.

DNA damage is probably the most significant biological target because this may ultimately lead to altered gene expression, disruption of cellular repair mechanism and distorted cellular function (Heaton et al. 2002). Thus DNA damage was measured in this study after cells were incubated with Aβ peptide with and without treatment with TRF.

Comet assay was used to measure DNA damage and visual scoring method was applied to evaluate the amount of DNA damage (Collins 2004). The present results showed that DNA damage was significantly increased in cells incubated with Aβ peptide (Figure 1). This may be attributed to the observation that Aβ peptide could generate ROS which contribute to the DNA damage.

Treatment with TRF, either before (pre) or after (post-treatment) incubation with Aβ peptide confirmed the neuroprotective effect of TRF. The data from this study showed that TRF prevented cell death, minimized apoptosis and reduced the DNA damage induced by Aβ peptide. Many studies have shown that vitamin E and its isomers exert neuroprotective effects against ROS-induced cell death in vitro (Sen et al. 2006; Mazlan et al. 2006). A recent report also demonstrated that AD patients with moderately severe impairment respond favorably to α-tocopherol by slowing the progression of the disease (Sano et al. 1997). An epidemiological study involving 633 persons over 65 years old suggests that the use of high dose vitamin E supplements may lower the risk of AD (Morris et al. 1998). The neuroprotective effect of TRF may be exerted via its antioxidant effects or by acting at the gene level, affecting the expressions of the signaling molecules of the apoptotic pathway.

The results thus show the relationship between the oxidative DNA damage, apoptosis and cell death induced by Aβ peptide which is prevented by TRF. In conclusion, Aβ peptide cause DNA damage and ultimately cell death via apoptosis probably by inducing oxidative DNA damage. This is further supported by the fact that TRF, an antioxidant is able to prevent the DNA damage, apoptosis and cell death.

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REFERENCES

Ames, B.N., Shigenaga, M.K., Hagen, T.M. 1993. Oxidants, antioxidants and the degenerative diseases of aging. *Proc Natl Acad of Sci USA* **90**: 7915–7922.

Annahazi, A., Mracsko, E., Sule, Z., Karge, E., Penke, B., Bari, F., Farkas, E. Pre-treatment and post- treatment with α- tocopherol attenuates hippocampal neuronal damage in experimental cerebral hypoperfusion. *Eur J Pharm* **84:** 726-738.

Arimura, N., Kaibuchi, K. 2005. Key regulators in neuronal polarity. *Neuron* **48**: 881–884.

Behl, C., Davis, J.B., Lesley, R., Schubert, D. 1994. Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell* **77**: 817– 827.

Butterfield, D.A., Boyd-Kimball, D. 2004. Amyloid β-Peptide(1-42) contributes to the oxidative stress and neurodegeneration found in Alzheimer disease brain. *Brain Pathol* **14**: 426-432.

Celsi, F., Svedberg, M., Unger, C., Cotman, C.W., Carri, M.T., Ottersen, O.P., Nordberg, A., Torp, R. 2007. Beta-amyloid causes downregulation of calcineurin in neurons through induction of oxidative stress. *Neurobiol Dis* **26**: 342-352.

Collins, A.R. 2004. The comet assay for DNA damage and repair: principles, application and limitations. *Mol Biotechnol* **26**:249–261.

Heaton, P.R., Reed, C.F., Mann, S.J., Ransley, R., Stevenson, J., Charlton, C.J., Smith, B.H., Harper, E.J., Rawlings, J.M. 2002. *J Nutr* **132**:1720–1724.

Mazlan, M., Then, S.M., Mat Top, G., Wan Ngah, W.Z. 2006. Comparative effects of gamma tocopherol and tocotrienol against hydrogen peroxide induce apoptosis on primary cultured astrocytes. *J Neurol Sci*. **243**:5-12.

Morris, M.C., Beckett, L.A., Scherr, P.A., Hebert, L.E., Bennet, D.A., Field, T.S., Evans, D. 1998. Vitamin E and vitamin C supplement use and risk of incident Alzheimer disease. *Alzheimers Dis Assoc Disord* **12**: 121-126.

Pereira, C., Santos, M.S., Oliveira, C. 1999. Involvement of oxidative stress on the impairment of energy metabolism induced by a beta peptides on PC12 cells: protection by antioxidants. *Neurobiol Dis* **6**: 209-219.

Piccini, A., Russo, C., Gliozzi, A., Relini, A., Borghi, R., Giliberto, L., Armirotti, A., D'Arrigo, C., Bachi, A., Cattaneo, A., Canale, C., Torrassa, S., Saido, T.C., Markesbery, W., Gamberti, P., Tabaton, M. 2005. Beta amyloid is different in normal aging and in Alzheimer disease. *J Biol Chem* **280**: 34186 - 34192

Pike, C.J., Burdick, D., Walencewicz, A.J., Glabe, C.G., Cotman, C.W. 1993. Neurodegeneration induced by beta amyloid peptides in vitro: The role of peptide assembly state. *J Neurosci* **13**: 1676-1687.

Puttfarcken, P.S., Manelli, A.M., Neilly, J., Frail, D.E. 1996. Inhibition of Age-Induced β-Amyloid Neurotoxicity in Rat Hippocampal Cells. *J Exp Neurol* **138**: 73-81.

Sano, M., Ernesto, C., Thomas, R.G., Klauber, M.R., Schafer, K., Grundman, M., Woodbury, P., Growdon, J., Cotman, C.W., Pfeiffer, E., Schneider, L.S., Thal, L.J. 1997. A controlled trial of selegiline, α-tocopherol, or both as treatment for Alzheimer.s disease. *NewEngl J Med* **336**: 1216-1223.

SantaCruz, K., Lewis, J., Spires, T., Paulson, J., Kollinek, L., Ingelsson, M., Guimaraes, A., DeTure, M., Ramsden, M., McGowan, E., Forster, C., Yue, M., Orne, J., Janus, C., Mariash, A., Kuskowski, M., Hyman, B., Hutton, M., Ashe, K.H. 2005. Tau Suppression in a Neurodegenerative Mouse Model Improves Memory Function. *Science* **309**: 476-481.

Sen, C.K., Khanna, S., Roy, S. 2006. Tocotrienol: vitamin E beyond tocopherols. *Life Sci* **78 :** 2008-2098.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Biol* **175**: 184 –91.

Wang, X., Su, B., Siedlak, S.L., Moreira, P.I., Fujioka, H., Wang, Y., Casadesus, G., Zhu, X. 2008. Beta amyloid overproduction causes abnormal mitochondrial dynamics via differential modulation of mitochondrial fission/fusion proteins. *Proc Natl Acad Sci USA* **105**: 19318-19323

Zou, L.B., Mouri, A., Iwata, N., Saido, T.C., Wang, D., Wang, M.W., Mizoguchi, H., Noda, Y., Nabeshima, T. 2006. Inhibition of neprilysin by infusion of thiorphan into the hippocampus causes an accumulation of amyloid and impairment of learning and memory. *J Pharm Exp Ther* **317**: 334-340.