Sains Malaysiana 39(1)(2010): 145–156

Comparison of the Effects of α-Tocopherol and γ-Tocotrienol against Oxidative Stress in Two Different Neuronal Cultures

(Perbandingan Kesan α-Tokoferol dan γ-Tocotrienol Terhadap Tekanan Oksidatif Pada Dua Kultur Neuron Yang Berlainan)

THEN SUE-MIAN*, WAN ZURINAH WAN NGAH, GAPOR MAT TOP & MUSALMAH MAZLAN

ABSTRACT

Tocopherols and tocotrienols have been shown in previous studies to protect neurons from oxidative injuries, especially from hydrogen peroxide (H_2O_2) and buthionine sulfoximine (BSO) induced oxidative stress. In this study, we compared two vitamin E isomers, γ-tocotrienol (GTT) and α-tocopherol (ATF) in their neuroprotective effects against H_2O_2-induced apoptosis in primary rat cortical neurons and human neuroblastoma cell line SH-SY5Y. Cytotoxicity screening of H_2O_2, GTT and ATF was done to determine the IC_{50} levels. To screen for neuroprotective effects, cortical neurons and SH-SY5Y cell cultures were pre-incubated with GTT or ATF, respectively at different concentrations for 1 hour before concurrent treatment of H_2O_2 at IC_{50}. Results of these treatments were compared to cells treated with H_2O_2 only and control cells. Cytotoxicology screening showed that IC_{50} of H_2O_2 for cortical neuron is at 50 µM while SH-SY5Y have higher IC_{50} of 100 µM. GTT is cytotoxic to cortical neurons at ≥50 µM and SH-SY5Y at ≥100 µM while ATF did not show any toxicity within the range of concentration tested (1-750 µM). Results from neuroprotection screening showed that GTT and ATF were able to protect both cortical neurons and SH-SY5Y from H_2O_2-induced oxidative stress at concetration of ≤10 µM. Cellular uptake of GTT is higher in both cortical neurons and SH-SY5Y as compared to ATF when both cortical neuron and SH-SY5Y were incubated with 10 µM GTT or ATF, respectively for 24 hour. Although primary rat cortical neurons and human neuroblastoma SH-SY5Y were different culture system, the effects of GTT and ATF are similar in both H_2O_2 –induced culture which strongly suggest that both GTT and ATF act as free radical scavenger to exert their neuroprotective effects.

Keywords: Apoptosis; neurons; oxidative stress; tocopherol; tocotrienol

ABSTRAK

Kajian terdahulu menunjukkan bahawa kedua-dua tokoferol and tokotrienol mempunyai potensi untuk melindungi neurons daripada tekanan oksidatif, khasnya daripada apoptosis yang diaruh hidrogen peroksida (H_2O_2) dan buthionine sulfoximine (BSO). Dalam kajian ini, dua vitamin E isomer, iaitu γ-tokotrienol (GTT) dan α-tokoferol (ATF) dibandingkan dalam kesan neuroproteksi terhadap apoptosis yang diaruh oleh H_2O_2 pada sel neuron korteks tikus dan titian sel neuroblastoma manusia SH-SY5Y. Penyaringan sitotoksik terhadap H_2O_2, GTT dan ATF dilakukan untuk menentukan aras IC_{50}. Untuk menyaring kesan neuroproteksi, kultur sel neuron korteks dan SH-SY5Y diberi pra-eraman GTT atau ATF masing-masing pada kepekatan berlainan selama 1 jam sebelum diperlakukan bersama H_2O_2 pada kepakatan IC_{50}. Keputusan ini dibanding dengan bacaan daripada sel yang diperlakukan H_2O_2 sahaja dan kawalan. Penyaringan sitotoksikologi menunjukkan bahawa IC_{50} H_2O_2 untuk neuron korteks adalah 50 µM manakala SH-SY5Y mempunyai IC_{50} H_2O_2 yang lebih tinggi, iaitu 100 µM. GTT adalah sitotoksik kepada neuron korteks pada ≥50 µM dan SH-SY5Y pada ≥100 µM manakala ATF tidak menunjukkan ketoksikan pada jurang kepekatan yang diuji (1-750 µM). Keputusan daripada penyaringan neuroproteksi menunjukkan bahawa kedua-dua GTT dan ATF dapat melindungi neuron korteks dan SH-SY5Y daripada stress oksidatif yang diaruh oleh H_2O_2 pada kepekatan ≤10 µM. Pengambilan GTT oleh sel adalah lebih tinggi oleh kedua-dua neuron korteks dan SH-SY5Y berbanding dengan pengambilan ATF apabila kedua-dua sel neuron korteks dan SH-SY5Y masing-masing dieram dengan 10 µM GTT atau ATF selama 24 jam. Walaupun kultur neuron korteks tikus dan titian sel neuroblastoma manusia SH-SY5Y adalah sistem kultur sel yang berbeza, kesan GTT dan ATF yang serupa terhadap kedua-dua kultur yang diaruh H_2O_2 mencadangkan bahawa GTT dan ATF bertindak sebagai peraih radikal bebas untuk memberikan kesan neuroproteksinya.

Kata kunci: Apoptosis; neurons; tekanan oksidatif; tokoferol; tokotrienol

INTRODUCTION

According to Floyd and Hensley (2002), the brain has a very high potential for oxidative damage as the brain is highly aerobic, processing about 20% of O_2 consumed of which 1-2% of the O_2 consumed is converted to reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive

chlorine species (Clarke & Sokoloff 1999; Cui et al. 2004). ROS (i.e. H_2O_2, $HO^{\bullet}$, $O^{\bullet}$) and RNS ($NO^{\bullet}$, $ONOO^{-}$, NO_2) are the causes of oxidative stress in the central nervous system (CNS) as they are produced in large amounts in pathologic conditions of many neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotropic lateral sclerosis (ALS) (Emerit et al. 2004; Klein & Ackerman 2003; Mariani et al. 2005). In addition to its high oxygen consumption, the brain also have high level of unsaturated lipids, Fe^{2+} and ascorbic acid, which increases its vulnerability to lipid peroxidation (Floyd & Hensley 2002).

H_2O_2 and $OH^{\bullet}$ are highly deleterious to neurons by initiating inreversible injuries to neurons via lipid peroxidation, oxidative alteration of proteins and DNA damage as well as neuronal apoptosis (Fang et al. 2002; Yuan & Yankner 2000). H_2O_2 is generated by the action of several enzymes such as superoxide dismutase (SOD), amino acid oxidase and mono amine oxidase (Cadenas & Davies 2000). H_2O_2 by itself is only slightly toxic, however it is a substrate for the Fe^{2+}-catalyzed generation of the highly reactive hydroxyl radicals ($OH^{\bullet}$) by Fenton reactions which rapidly react with DNA, membrane lipids and proteins (Goldsteins et al. 1993; Vervaart & Knight 1996). H_2O_2 molecules could cross cell membranes easily, where either it is detoxified by neighbouring astrocytes or it causes cellular damage to the DNA in susceptible surrounding cells, which subsequently leads to apoptosis (Higuchi 2003; Hockenbery et al. 1993).

Antioxidants, especially vitamin E has been touted as potential therapy for neurodegenerative diseases with underlying conditions of oxidative stress such as Alzheimer's disease and Down's syndrome but results has been inconclusive. Epidemiological studies suggested that intake of antioxidant nutrient such as vitamin E and vitamin C is able to reduce the risk of developing Alzheimer's disease (Morris et al. 1998; Sano et al. 1997) and Parkinson's disease (Hellenbrand et al. 1996). Clinical studies has been controversial, some studies showed that dietary antioxidant, e.g. vitamin E and C confer protection against neurodegenerative diseases (Golbe et al. 1998) and is related to lower risk of Alzheimer's disease (Engelhart et al. 2002); whereas other studies have shown that dietary intake of antioxidant vitamin E and C has no effect against amyotrophic lateral sclerosis (ALS) (Galbussera et al. 2006; Graf et al. 2005). Some *in vivo* studies have supported the suggestion that dietary antioxidant provide defence against disease. Joseph et al. (1999) has shown that supplementation of diets with vegetables and fruits that is rich in antioxidants has shown positive effects against age related neuronal degeneration in old rats. Neurodegeneration in Down syndrome is prevented by treatment with antioxidants such as vitamin E (Busciglio & Yankner 1995). There were also studies done on α-tocopherol and tocotrienols as neuroprotective agents in oxidative stress induced apoptotic neuronal death (Osakada et al. 2003, Sen et al. 2000). Eventhough nutritional antioxidant studies has not been conclusive, it remains as a potential preventive tool against premature neurodegeneration such as Alzheimer's disease (Behl & Moosmann 2002). The latest Cochrane report reanalyzed all of the published reports on vitamin E treatment for Alzheimers and mild cognitive impairment and found that overall, there is insufficient evidence to confirm efficacy of vitamin E treatment for Alzheimer's disease. There is a need for more randomized double-blind placebo-controlled trials to confirm or contradict the little available evidence (Isaac et al. 2008).

All tocopherol and tocotrienol derivatives come under one generic description that is vitamin E. Both tocopherols and tocotrienols have isomers, designated as α-, β-, γ-, δ-, which differ by the number and position of the methyl groups on the chromanol ring (Wang & Quinn 1999). Tocopherols have a phytyl chain while tocotrienols have similar chain but with three double bonds at position 3', 7' and 11' (Wang & Quinn 2000). Tocopherol and tocotrienol have different biological activities towards free radicals (Yoshida et al. 2003) and may play significant role as signaling molecule in cellular activities such as gene expression (Roy et al. 2002). In rat striatal neuron cultures, α-tocopherol has been demonstrated to protect against oxidative damage (Osakada et al. 2003). α-Tocotrienol and γ-tocopherol was also found to be able to prevent cerebral ischemia-induced brain damage in mice (Mishima et al. 2003). α-Tocotrienol was shown to be able inhibit glutamate-induced apoptosis in HT4 neuronal cells (Sen et al. 2000).

Previous studies with γ-tocotrienol showed that the neuroprotective effect of γ-tocotrienol against oxidative stress induced by H_2O_2 in astrocyte culture (Mazlan et al. 2006) and cerebellar granule neurons (Then et al. 2009). We extended our investigation to further examine the protective effects of α-tocopherol (ATF) and γ-tocotrienol (GTT) in cortical neurons of rat and human neuroblastoma SH-SY5Y exposed to H_2O_2.

Materials & Methods

Materials

ATF and GTT extracted from palm oil, were supplied by the Malaysia Palm Oil Board (MPOB). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) and trypsin from Flowlab (Australia), poly-L-lysine, gentamicin, insulin, hydrogen peroxide, Calcein-AM, propidium iodide, paraformaldehyde from Sigma (USA), p-aminobenzoic acid from ICN (USA), 96-well plates, chamber slide from Nunc (Denmark), CellTiter 96®AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS) from Promega (USA), ssDNA Apoptosis ELISA kit from Chemicon (USA), 70 μM cell strainers and other tissue culture materials were brought from BD Falcon (USA).

PREPARATION OF PRIMARY NEURON CULTURES FROM RAT

This study was approved by the Universiti Kebangsaan Malaysia (UKM) animal ethics committee (UKMAEC) with the approval number for the project is BIOK/MUSALMAH/12-JUNE/097.

Primary cultures of cortical neurons were prepared as follows: The forebrain were removed aseptically from *in utero* 15th day (E15) fetuses of Wistar rats under a stereoscopic microsope. The tissue was placed in cold Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate buffer saline (CMF-DPBS, pH 7.4) containing 3 g/L BSA, 1 g/L glucose, and 50 µg/mL gentamicin (Sebastia et al. 2004). The meninges were removed from the brain tissue and the tissues were placed on sterile Teflon board and were diced with a clean sterile scapel in a perpendicular manner before being transferred to 50 ml collection tube. It was then briefly centrifuged, and the pellet was resuspended in 12 mL of warm 0.025% trypsin and 0.04% DNAse solution. The suspension was incubated with the solution above in shaking water bath for 10 minutes, then 1 ml of fetal calf serum (FCS) was added to inhibit trypsin activity and 1 ml of DNAse 1 mg/ml to lessen DNA clumping of cells. The suspension was then centrifuge at 800 rpm for 5 minute. After removing the supernatant, the pellet was resuspended and slowly triturated with fire-polished glass pipette in Dulbecco's modified Eagle's medium (DMEM, pH 7.4) containing 19 mM $NaHCO_3$, 26.2 mM KCl, 7 µM p-aminobenzoic acid, 100 mU/l insulin, 50 µg/ml gentamicin and 10% FCS. The cells were plated at a density of 1.5×10^6 cells per ml on 96-well plates, Petri dishes and 8-wells chamber slides pretreated overnight with poly-L-lysine. Cultures were maintained in 5% CO_2/95% air at 37°C. Experiments were carried out on the 3rd – 7th day. Immunocytochemical analysis determined that 90% of cells were neurons (data not shown).

CELL CULTURE OF SH-SY5Y

Human neuroblastoma cell line SH-SY5Y, were cultured in DMEM /Ham's F-12, pH7.4 at ratio of 1:1 containing 2 mM L-glutamine, 50 µg/ml gentamicin, 100 µg/ml non-essential acid amino, 10% FCS and maintained in 5% CO_2/95% air incubator at 37°C. Cells were differentiated by addition of 10 µM retinoic acid on the 2nd day, media were changed and fresh retinoic acid was added on the 4th day. Experimental test were undertaken on 6th – 8th day of the culture.

CYTOTOXICITY OF H_2O_2, α-TOCOPHEROL AND γ-TOCOTRIENOL

Cortical neuron and SH-SY5Y culture were incubated with varying concentrations of H_2O_2 (1-500 µM), ATF and GTT at the range of 1-750 µM for 24 hour at 37°C. Cytotoxicity of H_2O_2, ATF and GTT was assessed by determining cell viability using MTS and LDH release assays, rate of apoptosis by using ssDNA Apoptosis ELISA (Chemicon) and by morphological studies of fluorescence staining (propidium iodide and Calcein-AM).

NEUROPROTECTIVE EFFECTS OF α-TOCOPHEROL AND γ-TOCOTRIENOL

To study the neuroprotective effects of ATF and GTT, IC_{50} of H_2O_2 for cortical neurons and SH-SY5Y culture were first pre-determined. Varying concentration of ATF and GTT (0.5-100 µM) were incubated with the cells above for 1 hour at 37°C. H_2O_2 was added after that and the cells were further for 24 hour at 37°C before assessing cell viability and apoptosis as stated earlier.

MTS ASSAY

Cell viability was assessed with CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, USA) which uses 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulfophenyl)-2H-tetrazolium (MTS) and the electron coupling agent phenazine methosulphate (PMS). MTS compound is reduced by dehydrogenase enzymes found in metabolically active cells into a formazan product that is soluble in the medium. The amount of coloured formazan product is proportional to the number of viable cells. Briefly, 50 µl of media in each well were transferred to another 96-well plate for LDH assays. MTS solution was premixed with media at the ratio 2:3 before adding 50 µl of the solution to each well of the remaining culture supernatant and incubated in humidified incubator at 37°C in 5% CO_2 for 2-4 hour. The quantity of formazan product present was determined by measuring the absorbance at 490 nm with a microtiter plate reader (VersaMax, Molecular Devices, USA).

LACTATE DEHYDROGENASE (LDH) ASSAY

Lactate dehydrogenase (LDH) release assay was performed to measure the amount of LDH release into culture medium. In this assay, LDH released from cells into culture supernatant is determined by an enzymatic method that uses the LDH-catalyzed reaction of pyruvate to lactate by oxidation of NADH + H^+ to NAD^+ by measuring the amount of NADH disappearing from the incubation buffer (Koh & Choi 1987). Briefly, LDH standard was prepared by serial dilution of Triton-X treated cells (100% maximal LDH activity) to 50%, 25% and 12.5% maximal LDH activity. 50 µl of the transferred culture supernatant from the original plate was diluted with 50 µl PBS. 100 µl of freshly prepared β-NADH solution (4.58 mg pyruvate 5.32 mg NADH and 7.49 mg $NaHCO_3$ for 10 ml potassium phosphate buffer 0.05 M, pH 7.4) was added to each well of culture supernatant, mixed well and incubated for 1 min before the absorbance was read at 340 nm, 25°C (VersaMax, Molecular Devices, USA).

SINGLE-STRANDED DNA APOPTOSIS ELISA

The ELISA kit used is called ssDNA Apoptosis ELISA (Chemicon, USA) which is based on the selective denaturation of DNA in apoptotic cells by formamide and detection of denatured DNA with monoclonal antibody to single stranded DNA (ssDNA) (Frankfurt & Krishan

2001). Formamide denatures DNA in apoptotic cells but not in necrotic cell or in cell with DNA breaks in the absence of apoptosis. Briefly, treated cells were fixed with 80% methanol in PBS, then treated with formamide. DNA in apoptotic cells were then denatured by heating to 75°C for 10 minute, which was immediately followed by cooling to 4°C for 5 minute. Positive controls provided by the kit were pre-fixed in a separate 96-well plate with 100 µl of positive controls added to the each wells and dried overnight according to protocol. The fixed positive control wells were rinsed with PBS before use and can be stored dry, covered until ready to use. The fixed positive controls were then concurrently processed together with the experimental plates according to subsequent steps of protocol. The absorbance reading of the positive control should be between 1.5 and 2.8 which indicates good assay sensitivity and will be denoted as 100% apoptosis. The apoptosis rate of all sample readings were calculated in relative to the positive control readings. For negative controls, 100 Units/ml SI nuclease was added and incubated at 37°C for 30 minute. To block non-specific binding sites, 3% nonfat milk was added and incubated at 37°C for 1 hour before incubating with antibody mixture for 30 minute. ABTS solution was added after washing. The absorbance was then read at 405 nm in microplate reader (VersaMax, Molecular Devices, USA).

CELL DEATH STAINING

Cell death staining make use of the fact that live cells have intracellular esterase that convert non-fluorescent cell-permeable calcein-AM to the intensely fluoresecent 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 (Rosa et al. 1997). Thus live cells stained with calcein-AM while dead and apoptotic cells stained with propidium iodide to give green and red fluorescence respectively. 30 µg/ml calcein-AM and 7.5 µg/ml propidium iodide were added to the cultures in chamber slides and incubated for 30 minute. Thereafter, cultures were washed with PBS, fixed with fresh 2% paraformaldehyde and then coverslips mounted for microscopic examination.

DETERMINATION OF VITAMIN E UPTAKE BY HPLC

Uptake of ATF and GTT were analysed using reverse-phase high performance liquid chromatography (HPLC) Fluorescent EM 330 nm, EX294 detector (Shimadzu, Japan). Treated (with 10 µM of either ATF or GTT) and untreated neurons were harvested and counted before adding 50 mg/dl BHT in 95% ethanol to stop auto-oxidation. After incubation for 10 min at room temperature, ice-cold 95% ethanol was added to precipitate the protein. To extract vitamin E, cells were sonicated for 40 secs before addition of ice cold HPLC grade hexane. Cells were then centrifuged at 3000 X g, 5 minute before collecting the hexane layer of supernatant. Vitamin E extracts were then vacuum dried and stored at –70°C before analysis with HPLC. To prepare HPLC samples, 100 µl hexane was added before further dilution with hexane for HPLC analysis. Peaks of samples were compared with tocotrienol rich fraction (TRF) standard and concentrations of ATF and GTT uptake in cells were calculated in µM per million cells (Mazlan et al. 2006).

STATISTICAL ANALYSIS

Each experiment of cultures in microplates was carried out in triplicate with at least three independent cultures. Experiments of cultures in petri dishes and chamber slides was carried out in three independent cultures. Data were reported as the mean ± SD from at least 3 independent experiments. Comparisons between groups were made using Student's t-test and two-way ANOVA. A p value less than 0.05 was considered statistically significant.

RESULTS

CYTOTOXICITY SCREENING

Cytotoxic effects of H_2O_2 in the cortical neurons and human neuroblastoma cell line, SH-SY5Y were investigated with Figure 1 showing H_2O_2 cytotoxicity screening of cortical neurons and human neuroblastoma SH-SY5Y. MTS assay results (Figure 1a) showed that rat cortical neurons were more susceptible to H_2O_2 toxicity where the numbers of viable cells decreased by 40-50% when incubated with 50 µM H_2O_2. The human cell line SH-SY5Y has higher tolerance for H_2O_2 toxicity whereby the number of viable cells decreased by 50% when incubated with 100 µM H_2O_2 (Figure 1b). IC_{50} of H_2O_2 for cortical neurons is 50 µM where as IC_{50} of H_2O_2 for SH-SY5Y is 100 µM. Thus in the subsequent neuroprotection studies, cells were exposed to 50 µM H_2O_2 for cortical neuron culture and 100 µM H_2O_2 for SH-SY5Y. LDH release assay showed that the maximum LDH release from cells into the media was in the 40-60% range as the assay did not take into account of LDH that remained in the cells. At the IC_{50} concentration, the percentage of LDH release is almost half of the maximum LDH release. For cortical neurons, at 50 µM H_2O_2 the LDH release is 18% and as for SH-SY5Y, at 100 µM H_2O_2 the LDH release is 14%.

GTT was found to be cytotoxic to primary rat cortical neuron culture (Figure 2a) at concentrations higher than 50 µM and at concentrations higher than 100 µM for human neuroblastoma SH-SY5Y (Figure 2b) from the MTS assay. In the LDH release assay, GTT was toxic to cortical neurons at ≥200 µM while toxicity to SH-SY5Y was at 100 µM. ATF was non-toxic to both cortical neurons and SH-SY5Y up to concentration of 750 µM that was tested. The precentage of viable cells drop drastically at concentration above 100 µM for GTT whereby only around 20% viable cells remain. In subsequent testing for neuroprotection, MTS assay were used for cell viability evaluation unless otherwise indicated.

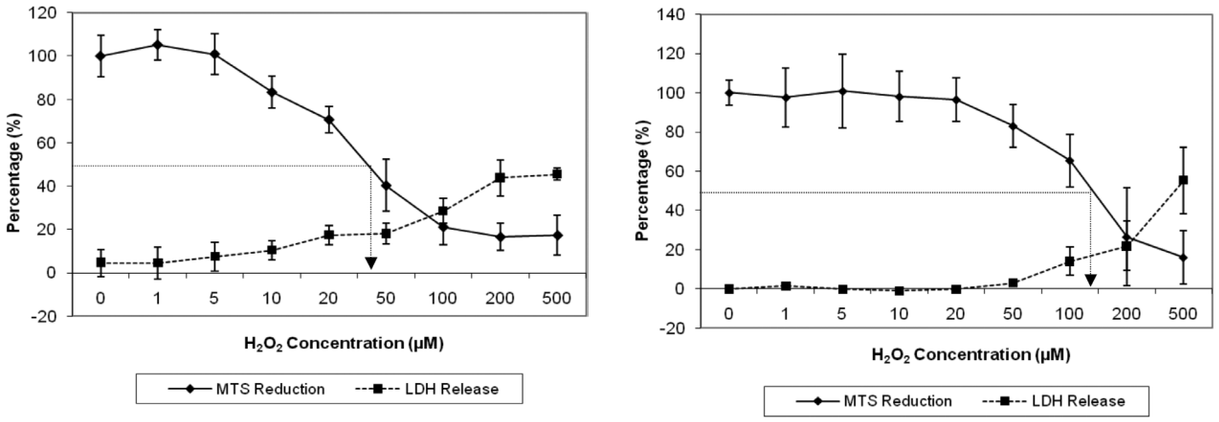


FIGURE 1. Effects of H_2O_2 cytotoxicity towards viability of (a) cortical and (b) human neuroblastoma SH-SY5Y as assessed by MTS and LDH release assay. The cultures were exposed to different concentration of H_2O_2 for 24 hrs at 37°C. For cortical neurons, no significant difference of cell viability between control and H_2O_2-exposed cultures when the concentration of H_2O_2 was less than 5 μM. Cell viability decreased 50% when exposed to 50 μM. At more than 100 μM H_2O_2 almost all cell were dead. The IC_{50} of H_2O_2 for cortical neurons was found to be 50 μM. SH-SY5Y is more resistant to H_2O_2 whereby H_2O_2 cytotoxicity is at concentration more than 100 μM. Data is presented as the means ± SD (n=9)

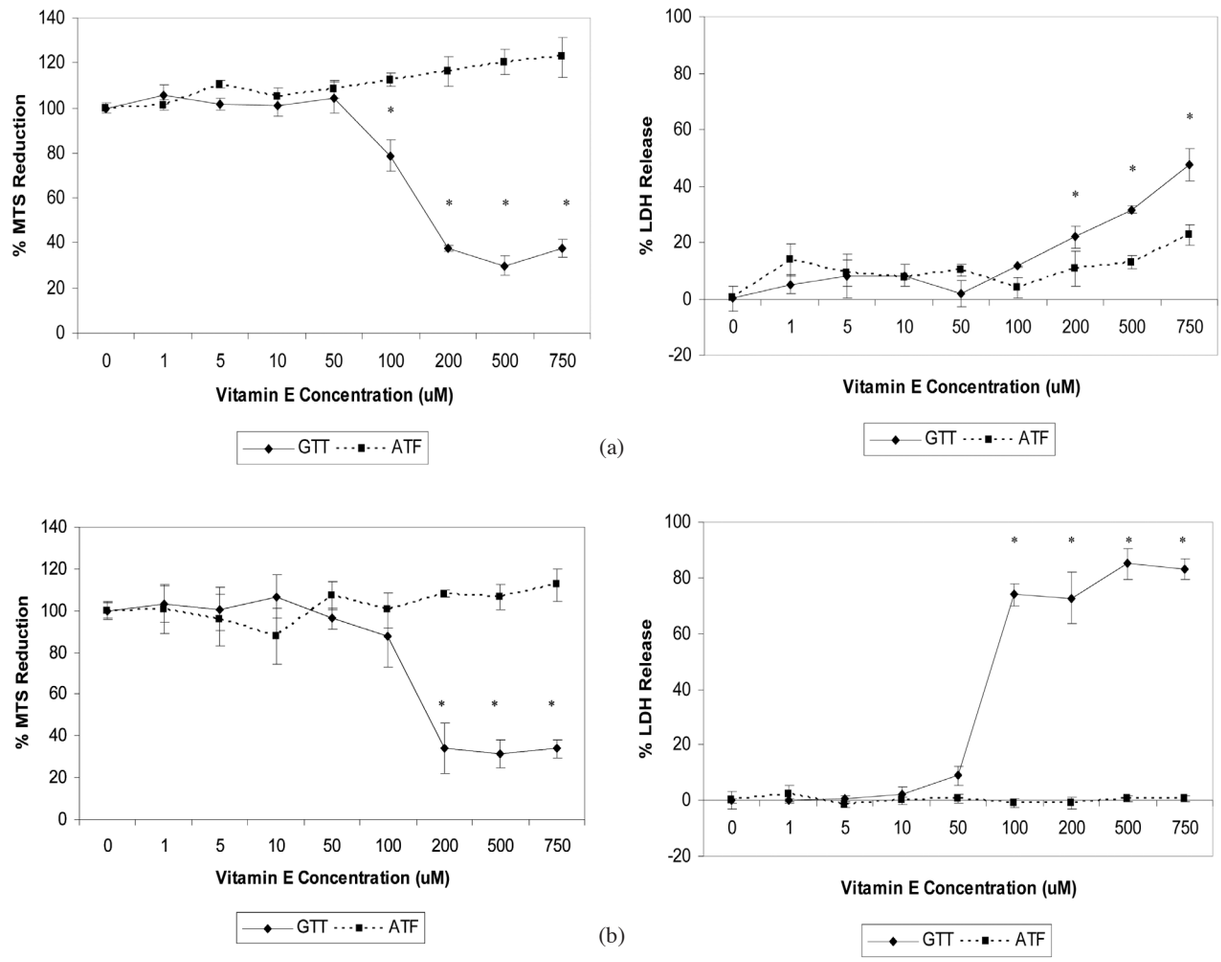
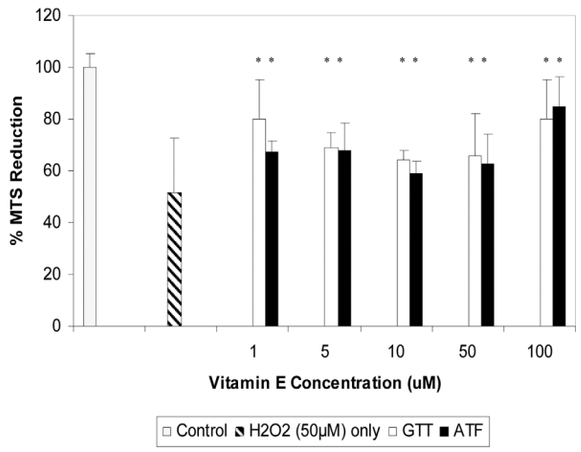


FIGURE 2(a). Cytotoxic effects of α-tocopherol (ATF) and γ-tocotrienol (GTT) on (a) cortical neuron culture and (b) human neuroblastoma SH-SY5Y as assessed by MTS assay and LDH assay. ATF and GTT were incubated overnight with FCS at 37°C and working concentrations were prepared fresh before the test. Neurons were incubated with different concentration of ATF and GTT for 24 hrs at 37°C. At the range tested (1-750 μM), ATF showed no cytotoxic effects towards neurons from MTS assay and LDH assay. GTT was shown to be toxic to cortical neurons at ≥50 μM while GTT cytotoxicity for SH-SY5Y was at ≥100 μM from MTS assay. Data is presented as mean ± SD, n=9

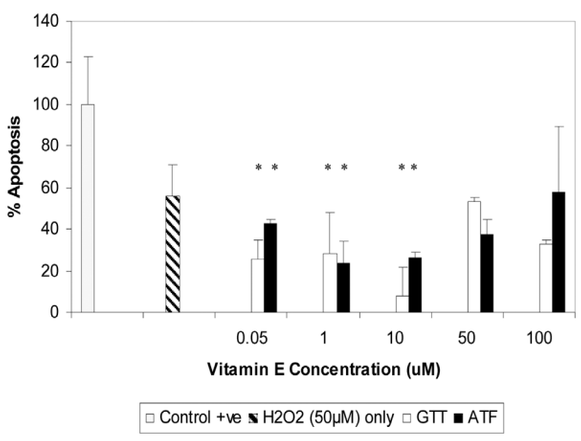
NEUROPROTECTION SCREENING

Figure 3a showed that GTT was able to confer neuroprotection to cortical neurons from H_2O_2-induced cell death as assessed by MTS assay. Similar results were obtained for SH-SY5Y whereby MTS assay showed cell viability increased with pre-incubation of GTT up to 50 μM and up to 100 μM for ATF (Figure 4a). GTT was as effective as ATF at protecting neurons with higher viability of cells when pre-incubated with ATF or GTT at concentration ≤100 μM as assessed by MTS assay.

The neuroprotective effects of GTT is further substantiated by the results of the ssDNA Apoptosis ELISA (Figure 3b and Figure 4b) which showed that GTT protected cortical neurons from H_2O_2-induced apoptosis with the effective concentration 0.05-10 μM and SH-SY5Y at concentration of 1-10 μM. Pre-incubation of ≤10 μM GTT and ATF decreased the rate of apoptosis in cortical neurons that were exposed to oxidative stress (Figure 3b). Results also showed that both ATF and GTT confer protection to SH-SY5Y cells at concentration ≤10 μM (Figure 4b). The fluorescent staining

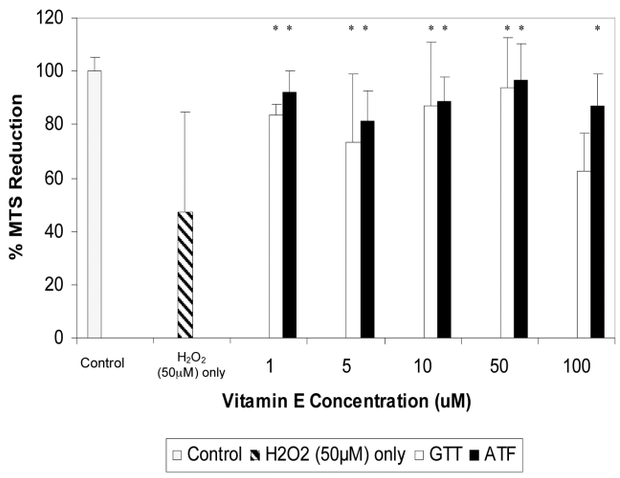


(a)

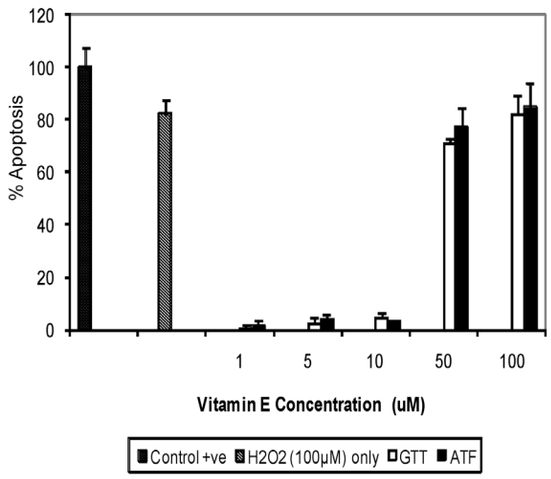


(b)

FIGURE 3. Protective effects of α-tocopherol (ATF) and γ-tocotrienol (GTT) against H_2O_2-induced cell loss in cortical neuron cultures whereby cell viability was determined using MTS assay and ELISA kit for single-stranded DNA (ssDNA) from Chemicon, USA. Neurons were pretreated with different concentration of ATF and GTT for 1h before exposure to 100 μM H_2O_2 for 24 hour at 37°C. Both ATF and GTT were able to increase cell viability at concentration range of 1-100 μM while decrease apoptosis rate in H_2O_2-treated cells at concentration range of 1-10 μM. * denotes $p<0.05$ compared to control. Data is presented as mean ± SD, n=9.



(a)



(b)

FIGURE 4. Protective effects of α-tocopherol (ATF) and γ-tocotrienol (GTT) against H_2O_2-induced cell loss in human neuroblastoma SH-SY5Y cultures whereby cell viability was determined using MTS assay and ELISA kit for ssDNA. Neurons were pretreated with different concentration of ATF and GTT for 1h before exposure to 100μM H_2O_2 for 24 h at 37°C. ATF were able to increase cell viability at concentrations of 1-100 μM while GTT at 1-50 μM. Both GTT and ATF decreased apoptosis rate in H_2O_2-treated cells at concentration range of 1-10 μM. * denotes $p<0.05$ compared to control. Data is presented as mean ± SD, n=9

of PI and Calcien-AM (Figure 5 & 6) also showed H_2O_2 triggers apoptosis in neurons which was attenuate by pre-incubation with 10 µM GTT or ATF. Morfological studies of cortical neurons (Figure 5) showed that cells incubated with H_2O_2 (Figure 5b) undergo apoptosis characterized by shrinkage of cells, permeability of membrane which allowed the fluorescent dye PI to enter cells and bind to the nucleus DNA, condensation of nucleus and formation of apoptotic bodies. Both GTT (Figure 5c) and ATF (Figure 5d) protected cortical neurons when pre-incubated at concentration of 10 µM and the cells were viable did not undergo apoptosis with integrity of membrane cells maintained and there is no shrinkage of cells. Fluorescence staining of SH-SY5Y (Figure 6) showed similar results with the cortical neurons, with H_2O_2 (Figure 6b) caused cells to undergo apoptosis where as pre-incubation with GTT (Figure 6c) and ATF (Figure 6d) increased the numbers of viable cells stained with Calcein-AM.

UPTAKE OF A-TOCOPHEROL AND Γ-TOCOTRIENOL INTO CELLS

HPLC analysis of hexane extract of neurons pre-incubated with ATF and GTT revealed that there was a significant difference of uptake of the ATF and GTT in cortical neurons (Figure 7a) and SH-SY5Y cell line (Figure 7b). In both cortical neurons and SH-SY5Y, uptake of GTT is higher than uptake of ATF. Uptake of GTT was higher in SH-SY5Y compared with cortical neurons; while uptake of ATF was higher in cortical than in SH-SY5Y.

DISCUSSION

Our study found that high concentration of GTT (≥100 µM) was toxic to cortical neurons and SH-SY5Y. This was similar to the finding reported earlier in which GTT was found to be toxic to astrocytes at concentration ≥ 100 µM (Mazlan et al. 2006). Studies done by Nesaretnam et

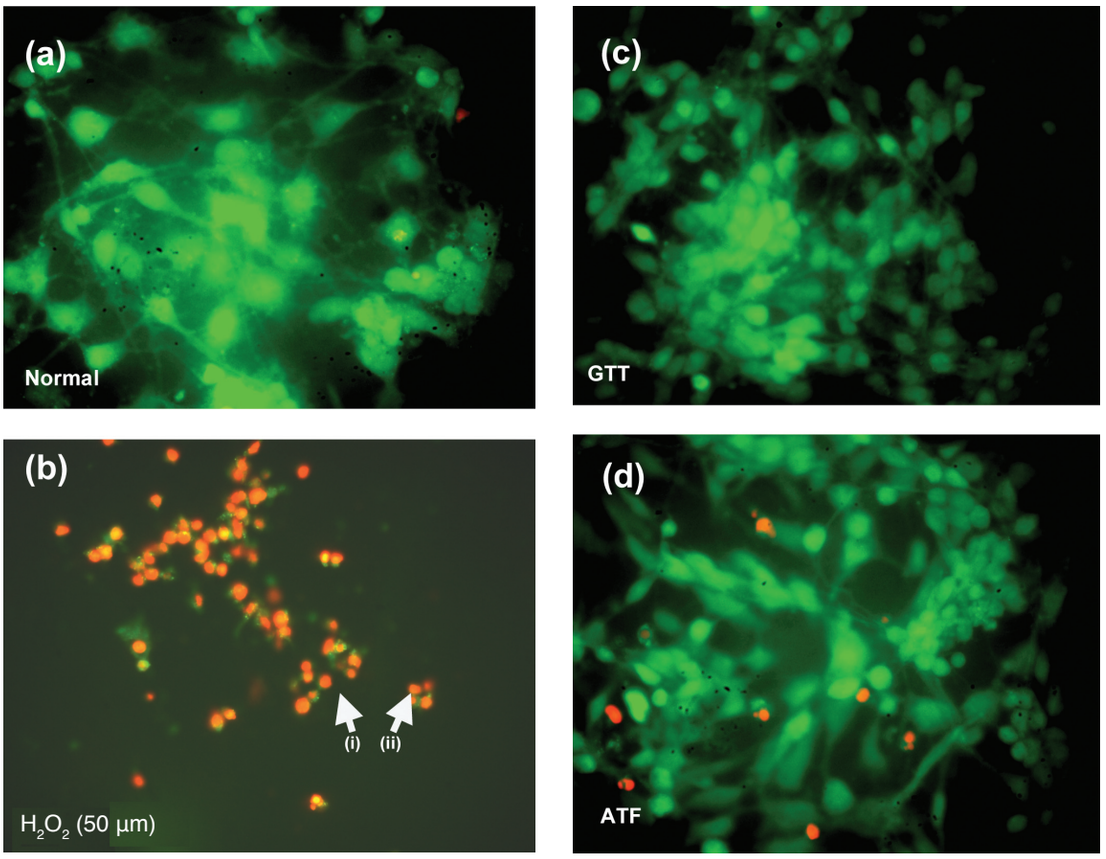


FIGURE 5. Fluorescence cell death staining of cortical neurons exposed to different treatments: (a) control, (b) 50 µM H_2O_2 treatment, (c) pre-incubation of GTT (10 µM) 1h followed by H_2O_2 treatment, (d) pre-incubation of ATF (10 µM) 1h followed by H_2O_2 treatment. (Micrographs are shown at X40 magnification). Live cells were stained with 30 µM Calcein-AM (green) and dead cells were stained with propidium iodide, PI (red). Neurons undergo early apoptosis as membrane permeability increase and cells' nucleus were stained with PI. Nucleus also showed (i) condensation and (ii) formation of apoptotic bodies when exposed to 50 µM H_2O_2. When neurons were pre-incubated with either GTT (c) or ATF (d), cells remain viable and membrane integrity retained as compared to H_2O_2-treated cells

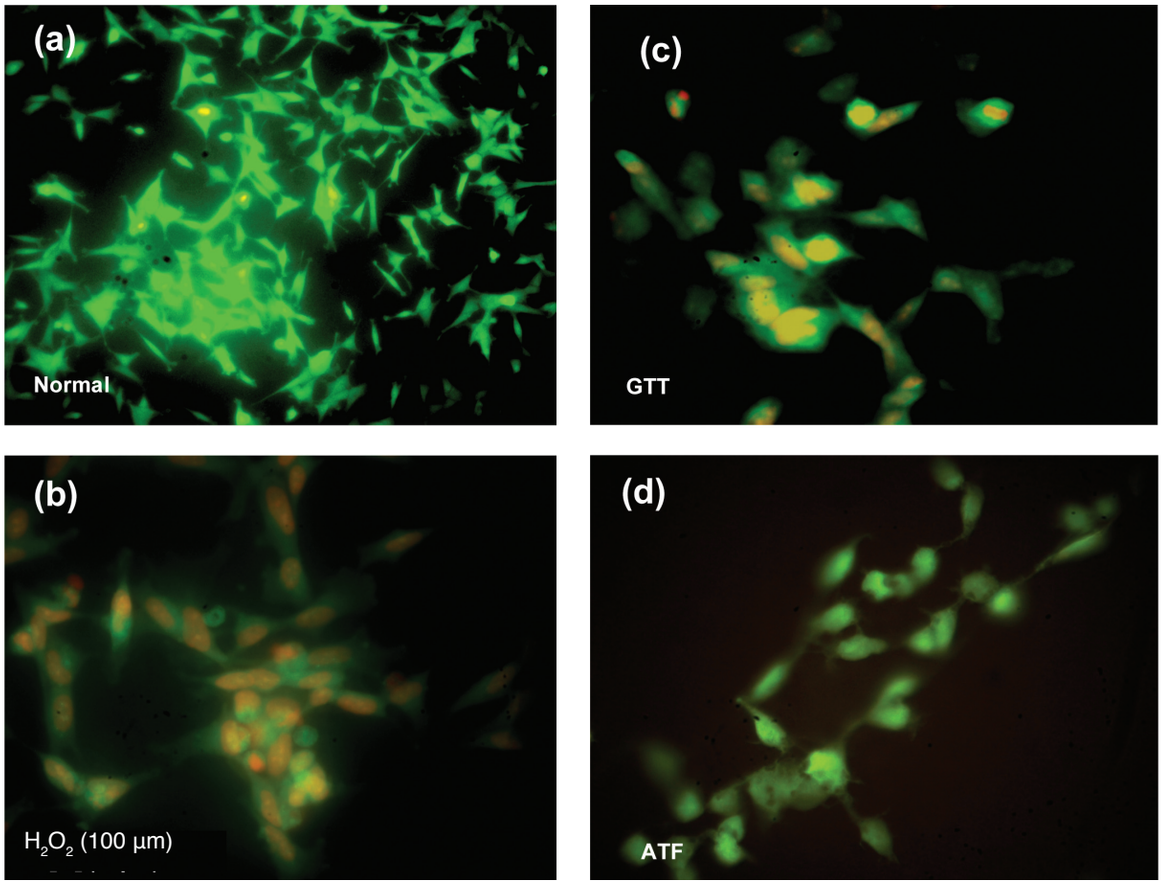
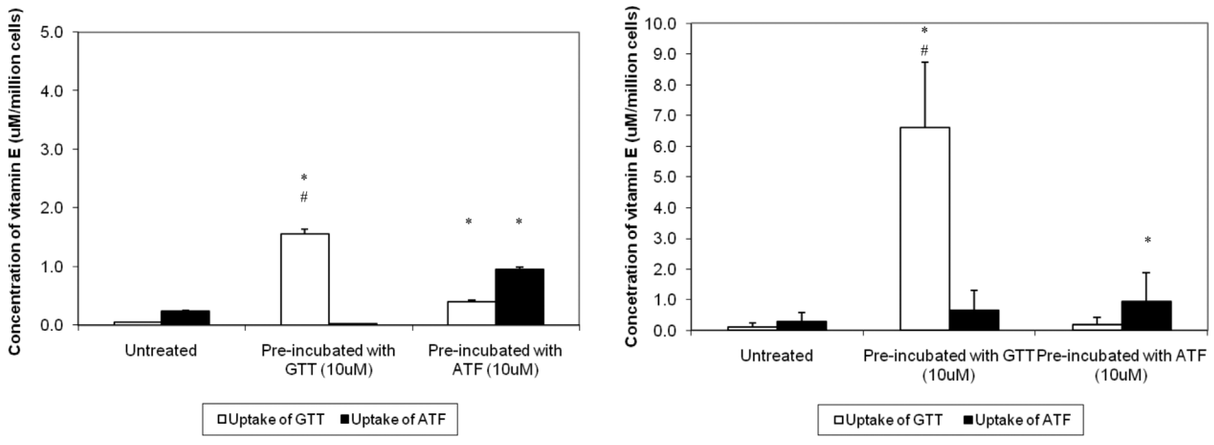


FIGURE 6. Fluorescence staining of human neuroblastoma SH-SY5Y exposed to different treatments: (a) control, (b) 50 µM H_2O_2 treatment, (c) pre-incubation of GTT (10 µM) 1h followed by H_2O_2 treatment, (d) pre-incubation of ATF (10µM) 1h followed by H_2O_2 treatment. (Micrographs are shown at X40 magnification). Live cells were stained with 30 µM Calcein-AM (green) and dead cells were stained with propidium iodide, PI (red). Similar to cortical neurons, SH-SY5Y showed nucleus condensation and formation of apoptotic bodies when exposed to 100 µM H_2O_2. When neurons were pre-incubated with either GTT (c) or ATF (d), cells remain viable and membrane integrity retained as compared to H_2O_2-treated cells



(a) (b)

FIGURE 7. Uptake of α-tocopherol (ATF) and γ-tocotrienol (GTT) in (a) cortical neurons and (b) human neuroblastoma SH-SY5Y. Neuron cultures were incubated with 10 µM of either ATF or GTT for 24 hrs at 37°C. Cells were harvested and prepared for HPLC as described in text. Cellular uptake of GTT and ATF in cells treated respectively with GTT and ATF is significantly higher than control. Uptake of GTT is significantly higher than ATF uptake in both cortical neurons and SH-SY5Y while uptake of GTT in SH-SY5Y is higher than uptake of GTT in cortical neurons. Data is presented as mean ± SD, n=3

al. (1995) has shown that GTT act as an anti-cancer agent at high dose (≥180 μg/ml) by inhibiting proliferation and inducing apoptosis of breast cancer cells. Recent study showed that tocotrienols, including GTT displayed more potent apoptotic activities in neoplastic mammary epithelial cells (Slyvester & Shah 2005). GTT was also showed to induce apoptosis to liver cancer cells in rats (Makpol et al. 1997) and recently Sakai et al. (2004) showed that GTT induced apoptosis in rat hepatoma cells. Study by Conte et al. (2004) showed that GTT at high concentration has anti-proliferative effects against prostate cancer cell and high dose of GTT (400 mg/kg) was also showed to sensitize prostate cancer tumor to radiation-induced lipid peroxidation which cause the tumor to destruct and reduced in size (Kumar et al. 2006). A latest study by Xu et al. (2009) showed that GTT at 60 μM has strong inhibition and apoptosis towards human colon carcinoma cell line. Our previous study has also shown that in the absence of H_2O_2, GTT has stronger pro-apoptotic tendency than ATF by while in the presence of H_2O_2, both GTT and ATF exert neuroprotection by modulating the apoptosis signaling pathway (Then et al. 2009). Therefore, in this study, GTT may function as apoptosis-inducing agent at concentration ≥100 μM in cortical neurons and SH-SY5Y but at low concentration was not harmful to neuron cells. Both GTT and ATF may act as antioxidant to protect cells from oxidative assault.

ATF was not toxic to cortical neurons and SH-SY5Y within the concentration range tested. ATF has been shown to confer neuroprotection to neurons exposed to oxidative stress in *in vitro* studies (Osakada et al. 2003) and in *in vivo* studies (Rebrin et al. 2005). Zhang et al. (2004) demonstrated that ATF confer neuroprotection against cerebral ischemia and neuronal death. Our findings are consistent with the report of Osakada and colleagues (2004) which demonstrated the neuroprotection of α-, γ- and δ-tocotrienol in striatal neurons exposed to various pro-oxidants, including H_2O_2. Their findings suggested that GTT mainly protects striatal neurons via it's antioxidant properties and this is in agreement with our results in primary cortical neurons and in SH-SY5Y cell lines. Sen et al. (2000) showed that α-tocotrienol at nanomolar (nM) was able to confer neuroprotection against glutamate-induced oxidative stress in HT4 neuronal cell line.

ATF uptake in the biological system has been widely established as explain here: vitamin E is taken up in the proximal part of the intestine, emulsified together with the fat-soluble components of food and form lipid droplets which leads to spontaneous formation of mixed micelles and absorbed into membrane cells by passive diffusion (Mardones & Rigotti 2004). Together with other hydrophobic molecules such as triglycerides, phospholipids, cholesterol and alipoproteins, tocopherols are reassembled to chylomicrons which are stored as secretory granule and excreted by exocytosis to the thoracic lymph compartment and finally reach the blood stream (Brigelius-Flohe & Traber 1999). Remnant chylomicron-associated vitamin is delivered to the liver where the ATF form is selectively incorporated into very-low-density lipoprotein (VLDL) by hepatic α-tocopherol transfer protein (αTTP) (Kaempf-Rotzoll et al. 2003). How vitamin E is then taken into cells from plasma is through multiple pathway, which is 1) facilitated and by lipid transfer proteins and lipase, 2) receptor-mediated lipoprotein endosytosis, 3) selective lipid uptake via α-tocopherol transporter (αTT) (Azzi & Stocker 2000). It was suggested that there are no specific mechanisms of ATF cellular uptake but this substance take advantage of known lipid uptake pathways (Mardones & Rigotti 2004). Enzymes such as lipoprotein lipase (LPL) helps mediate transfer of ATF from chylomicron to cells by binding to the cell surface (Traber & Arai 1999), as well as delivery of ATF across the blood-brain barrier (BBB) into the central nervous system (Nakamura et al. 1998). As for other isomers of tocopherols and tocotrienols, it is excreted because of their low affinity for α-TTP. The excess amount of α-tocopherol are metabolized to α-CEHC and γ-tocotrienol are metabolized to γ-CEHC which are both excreted into urine in humans and rats (Ikeda et al. 2003). The discrimination of vitamin E isoforms by α-TTP however does not completely explain the tissue-specific distribution of tocotrienol, especially in the adipose tissue and skin as reported by Ikeda et al. (2001).

In this study, the different neuronal cells has different absoption efficacy for ATF and GTT and this is in agreement with studies done by Roy et al. (2002a) that ATF and GTT content is different in various organ which showed differencial cellular uptake in various organ. In our previous findings of vitamin E uptake in astrocytes (Mazlan et al. 2006), ATF and GTT uptake into astrocytes was not significantly different as compared with the uptake in neurons. Data showed here that uptake of GTT is higher compared to α-tocopherol in rat neurons and SH-SY5Y cultures. Sen et al. (2000) also demonstrated that uptake of α-tocotrienol is much higher than α-tocopherol in HT4 neuronal cell line. A recent study also showed that α-tocotrienol is localized at the same place as α-tocopherol which is near the membrane surface and tocotrienol has higher membrane permeability than tocopherol which strongly suggested that transport of γT3 across the cell membrane of cortical neurons and neuroblastoma SH-SY5Y cell line is more efficient than transport of αT (Yoshida et al. 2007). We suggest that there is a different mechanism of uptake between astrocytes and neurons which may contribute to the better antioxidant protection of GTT in neurons at lower concentration. *In vivo s*tudies by Khanna et al. (2005) suggested that the presence of ATF inhibits the cellular absoption of tocotrienol. Mice feed on a tocotrienol-rich diet has a higher absorption of α-tocotrienol compared to mice feed on diet contained both tocopherol and tocotrienol even until the 5th generation. A study done by Ikeda et al. (2003) showed that the presence of α-tocopherol decrease the concentration of α-tocotrienol but not GTT in tissues of rat. Both groups suggested that uptake mechanism of GTT is not dependent

on α-TTP mechanism (Khanna et al. 2005; Ikeda et al. 2003). However there is still no clear cellular mechanism for tocotrienol uptake.

Conclusion

GTT triggers cell death at high concentration (>100 μM) in neuronal cell cultures but exerts neuroprotective effects at low concentration (<50 μM) against H_2O_2-induced apoptosis in cortical neurons and human neuroblastoma cell line SH-SY5Y. These results demonstrated that GTT at low dose is a consistent neuroprotectant in different neuronal cultures that is efficiently uptake into neuronal cells in primary rat neuronal culture and human neuroblastoma cell line. At high dose, GTT is cytotoxic to neuronal cells while ATF is non-toxic to neuronal cells. Uptake of GTT is far more efficient through the membrane cells of neuron, thus this may explain why GTT is toxic at high concentration but is neuroprotective at low dose. GTT and ATF may act as potent antioxidant to protect neuronal cells from H_2O_2-induced oxidative stress.

Acknowledgements

This study was funded by the Ministry of Science, Technology and Innovation, Malaysia under the Intensified Research Prioritised Area (IRPA) grant 06-02-02-0022/ PR0008/09-07. We thank Dr. Coral Sanfeliu from the Department of Pharmacology & Toxicology, Institute d'Investigacions Biomediques de Barcelona (IIBB), Spain for her invaluable assistant in setting of the culture system in our laboratory and the human neuroblastoma cell line SH-SY5Y was a gift from her.

References

Azzi, A. & Stocker, A. 2000. Vitamin E: non-antioxidant roles. *Prog. Lipid Research* 39: 231-255.

Behl, C. & Moosmann, B. 2002. Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach. *Free Radic. Biol. Med.* 33(2): 182-191.

Brigelius-Flohe R. & Traber M.G. Vitamin E: function and metabolism. *FASEB J.* 13(10): 1145-1155.

Busciglio, J. & Yankner, B.A. 1995. Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons *in vitro*. *Nature* 378: 776-779.

Cadenas, E. & Davies, K.J.A. 2000. Mitochondrial free radical generation, oxidative stress and aging. *Free Radic. Biol. Med.* 29: 222-230.

Clarke, D.D. & Sokoloff, L. 1999. Circulation and energy metabolism of the brain. In *Basic Neurochemistry: Molecular, Cellular and Medical Aspects,* edited by Siegel, G.J., Agranoff, B.W., Albers, R.W., Fisher, S.K. & Uhler, M.D. hlm. 637-669. Philadelphia: Lippincott-Raven.

Conte, C., Floridi, A., Aisa, C., Piroddi, M., Floridi, A. & Galli, F. 2004. Gamma-tocotrienol metabolism and antiproliferative effects on prostate cancer cells. *Ann. N. Y. Acad. Sci.* 1031: 391-4.

Cui, K., Luo, X.L., Xu, K.Y., & Ven Murthy, M.R. 2004. Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress nutraceutical antioxidants. *Prog. Neuropharma. Biol. Psych.* 28: 771-799

Emerit, J., Edeas, M. & Bricaire, F. 2004. Neurodegenerative diseses and oxidative stress. *Biomed. Pharmacother* 58: 39-46

Engelhart, M.J., Geerlings, M.I., Ruitenberg, A., van Swieten, J.C., Hofman, A., Wittenman, J.C.M., & Breteler, M.M.B. 2002 Dietary intake of antioxidant and risk of Alzheimer's disease. *JAMA* 287: 3223-3229

Fang, Y.Z., Yang, S., & Wu, G. 2002. Free radicals, antioxidant and nutrition. *Nutr.* 18: 872-9.

Floyd, R.A. & Hensley, K. 2002. Oxidative stress in brain aging: implications for therapeutics of neurodegenerative diseases. *Neurobiol. Aging* 23: 795-807.

Frankfurt, O.S. & Krishan, A. 2001. Enzyme-linked immunosorbent assay (ELISA) for the specific detection of apoptotic cells and its application to rapid drug screening. *J. Immunol. Methods*. 253(1-2): 133-144.

Golbe, L.I., Farrell, T.M. & Davis, P.H. 1998. Case-control study of early life dietary factors in Parkinson's disease. *Arch. Neurol*. 45: 1350-1353

Goldstein, S., Meyerstein, D. & Czapski, G. 1993. The Fenton reagents. *Free Radic. Biol. Med.* 15(4): 435-445.

Graf, M., Ecker, D., Horowski, R., Kramer, B., Riederer, P., Gerlach, M., Hager, C., Ludolph, A.C., Becker, G., Osterhage, J., Jost, W.H., Schrank, B., Stein, C., Kostopulos, P., Lubik, S., Wekwerth, K., Dengler, R., Troeger, M., Wuerz, A., Hoge, A., Schrader, C., Schimke, N., Krampfl, K., Petri, S., Zierz, S., Eger, K., Neudecker, S., Traufeller, K., Sievert, M., Neundorfer, B. & Hecht, M. 2005. High-dose vitamin E therapy in amyotrophic lateral sclerosis as add-on therapy to riluzole: a result of placebo controlled double blind study. *J. Neural. Transm.* 112(5): 649-60.

Galbussera, A., Tremolizzo, L., Brighina, L., Testa, D., Lovati, R., Ferrarese, C., Cavaletti, G. & Filippini, G. 2006. Vitamin E intake and the quality of life in amyotrophic lateral sclerosis patients: a follow-up case series study. *Neurol. Sci.* 27(3): 190-3.

Hellenbrand, W., Boeing, H., Robra, B.P., Seidler, A., Vieregge, P., Nishan, P. Joerg, J., Oertel, W.H., Schneider, E., & Ulm, G. 1996. Diet and Parkinson's disease II: a possible role for the past intake of specific nutrients. *Neurol*. 47: 644-650.

Higuchi, Y. 2003. Chromosomal DNA fragmentation in apoptosis and necrosis induced by oxidative stress. *Biochem. Pharmacol*. 66: 1527-1535.

Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Millian, C.L. & Korsmeyer, S.J. 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 75: 241-251.

Ikeda, S., Toyoshima, K. & Yamashita, K. 2001. Dietary sesame seeds elevate alpha- and gamma-tocotrienol concentrations in skin and adipose tissue of rats fed the tocotrienol-rich fraction extracted from palm oil. *J. Nutr.* 131: 2892-2897.

Ikeda, S., Tohyama, T., Yoshimura, H., Hamamura, K., Abe, K. & Yamashita, K. 2003. Dietary α-tocopherol decreases α-tocotrienol but not γ-tocotrienol concentration in rats. *J Nutr*. 133(2): 428-434.

Isaac, M.G.E.K.N., Quinn, R. & Tabet, N. 2008. Vitamin E for Alzheimer's disease and mild cognitive impairment (Review). *Cochrane Database Syst Rev.* 16(3): CD002854.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Bielinski, D., Martin, A., McEven, J.J. & Bickford, P.C. 1999. Reversal of age-related declines in neuronal signal transduction, cognitive and motor behavioral deficits with bluberry,

spinach or strawberry dietary supplementation. *J. Neurosci*. 19: 8114-8121.

Kaempf-Rotzoll, D.E., Traber, M.G. & Arai, H. 2003. Vitamin E and transfer proteins. *Curr. Opin. Lipidol*. 14(3): 249-54.

Khanna, S., Patel, V., Rink, C., Roy, S., Sen, C. K. 2005. Delivery of orally supplemented α-tocotrienol to vital organs of rats and tocopherol-transport protein deficient mice. *Free Radic. Biol. Med*. 39: 1310-1319.

Klein, J.A. & Ackerman, S.L. 2003. Oxidative stress, cell cycle and neurodegeneration. *J. Clin. Invest*. 111: 785-793.

Koh, J.Y. & Choi, D.W. 1987. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci. Methods* 20(1): 83-90.

Kumar, K.S., Raghavan, M., Hieber, K., Ege, C., Mog, S., Parra, N., Hildabrand, A., Singh V., Srinivasan, V., Toles, R., Karikari, P., Petrovics, G., Seed, T., Srivastava, S. & Papas, A. 2006. Prefencial radiation sensitization of prostate cancer in nude mice by nutraceutical antioxidant γ-tocotrienol *Life Sci*. 78: 2099-2104.

Makpol, S., Shamaan, N.A., Jarien, Z., Top, A.G., Khalid, B.A. & Wan Ngah, W.Z. 1997. Different starting times of alpha-tocopherol and gamma-tocotrienol supplementation and tumor marker enzyme activities in rat chemically induced with cancer. *Gen. Pharmacol*. 28(4): 589-92.

Mardones, P. & Rigotti, A. 2004. Cellular mechanism of vitamin E uptake: relevance in α-tocopherol metabolism and potential implications for disease. *J. Nutr. Biochem*. 15: 252-260.

Mariani, E., Polidori, M.C., Cherubini, A. & Mecocci, P. 2005. Oxidative stress in brain aging, neurodegenerative and vascular disease: An overview. *J. Chromatogr. B* 827: 65-75

Mishima, K., Tanaka, T., Pu, F., Egashira, N., Iwasaki, K., Hidaka, R., Matsunaga, K., Takata, J/, Karube, Y. & Fujiwara, M. 2003. Vitamin E isoforms α-tocotrienol and γ-tocopherol prevent cerebral infarction in mice. *Neurosci Lett*. 337: 56-60.

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

Mazlan, M., Sue Mian, T., Mat Top, G. & Zurinah Wan Ngah, W. 2006. Comparative effects of α-tocopherol and γ-tocotrienol against hydrogen peroxide induced apoptosis on primary-cultured astrocytes. *J. Neurol. Science*. 243: 5-12.

Nakamura, T., Reicher, H. & Sattler, W. 1998. Comparison of RRR-alpha and all-rac-alpha-tocopherol uptake by permanent rat skeletal muscle myoblasts (L6 cells): effects of exogenous lipoprotein lipase *Lipids*. 33(10): 1001-8.

Nesaretnam, K. Guthrie, N., Chambers, A.F. & Carroll, K.K. 1995. Effect of tocotrienols on the growth of a human breast cacer cell line in culture. *Lipids* 30: 1171-1177.

Osakada, F., Hashino, A., Kume, T., Katsuki, H., Kaneko, S. & Akaike, A. 2003. Neuroprotective effects of α-tocoferol on oxidative stress in rat straital cultures. *Eur. J. Pharmacol*. 465: 15-22

Osakada, F., Hashino, A., Kume, T, Katsuki, H., Kaneko, S. & Akaike, A. 2004. α-tocotrienol provides the most potent neuroprotection among vitamin E analogs on cultured striatal neurons. *Neuropharmacol*. 47: 904-915.

Rebrin, I., Zicker, S., Wedekind, K.J., Paetau-Robinson, I., Packer, L. & Sohal, R. S. 2005. Effect of antioxidant-enriched diet on glutathione redox status in tissue homogenates and mitochondria of the senencense-accelerated mouse. *Free Rad. Biol. Med*. 39: 549-557.

Rosa, R., Sanfeliu, C., Suñol, C., Pomés, A., Rodríguez-Farré, E., Schousboe, A., Frandsen, A. 1997. The mechanism for hexachlorocyclohexane-induced cytotoxicity and changes in intracellular Ca^{2+} homeostasis in cultured cerebellar granule neurons is different for the gamma- and delta-isomers. *Toxicol. Appl. Pharmacol*. 142(1): 31-39.

Roy, S., Venojarvi, M., Khanna, S. & Sen, C.K. 2002a. Simultaneous detection of tocopherols and tocotrienols in biological samples using HPLC-coulometric electrode array. *Meth. Enzymol*. 352: 326-332.

Roy, S., Lado, B.H., Khanna, S. & Sen, C.K. 2002b. Vitamin E sensitive genes in the developing rat fetal brain: a high-density oligonucleotide microarray analysis. *FEBS Lett*. 530: 17-23

Sebastia, J., Cristòfol, R., Martin, M., Rodriguez-Farrē, E. & Sanfeliu, C. 2004. Evaluation of fluorescent dyes for measuring intracellular gluthathione content in primary cultures of human neurons and neuroblastoma SH-SY5Y. *Cytometry Part A* 51A: 16-25.

Sakai, M., Okabe, M., Yamasaki, M., Tachibana, H. & Yamada, K. 2004. Induction of apoptosis by tocotrienol in rat hepatoma dRLh-84 cells. *Anticancer Res*. 24(3a): 1683-1688.

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, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N. Eng. J. Med*. 336(17): 1216-22.

Sen, C., K., Khanna, S., Roy, S. & Packer, L. 2000. Molecular basis of vitamin E: tocotrienol potently inhibits glutamate-induced pp60c-src kinase activation and death of HT4 neuronal cells. *J. Biol. Chem*. 275: 13049-13055.

Sylvester, P.W. & Shah, S. 2005. Intracellular mechanisms mediating tocotrienol-induced apoptosis in neoplastic mammary epithelial cells. *Asia Pac. J. Clin. Nutr.* 14(4): 366-73.

Then, S.M., Mazlan, M., Top, G.M. & Wan Ngah, W.Z. 2009. Is vitamin E toxic to neurons? *Cell. Mol. Neurobiol*. In Press.

Traber, M.G., Arai, H. 1999. Molecular mechanisms of vitamin E transport. *Ann. Rev. Nutr.* 19: 343-355.

Vervaart, P. & Knight, K.R. 1996. Oxidative stress and the cell. *Clin. Biochem. Rev.* 17: 3-16.

Wang, X. & Quinn, P.J. 1999. Vitamin E and its function in membranes. *Prog. Lipid Res*. 38(4): 309-336.

Wang, X. & Quinn, P.J. 2000. The location and function of vitamin E in membranes (review). *Mol. Membr. Biol*. 17(3): 143-56.

Xin, Y., Fong, Y.T., Wolf, G., Wolf, D. & Cao, W. 2001. Protective effects of XY99-5038 on hydrogen peroxide induced cell death in cultured retinal neurons. *Life Sci*. 69: 289-299.

Xu, W.L., Liu, J.R., Liu, H. K., Qi, G.Y., Sun, X.R., Sun, W.G. & Chen, B. Q. 2009. Inhibition of proliferation and induction of apoptosis by gamma-tocotrienol in human colon carcinoma HT-29 cells. *Nutr.* 25(5): 555-566.

Yoshida, Y., Niki, E. & Noguchi, N. 2003 Comparative study on the action of tocopherols and tocotrienols as antioxidants: chemical and physical effects. *Chem. Phys. Lipids* 123: 63-75.

Yoshida, Y., Saito, Y., Jones, L. & Shigeri, Y. 2007. Chemical preactivities and physical effects in comparison between tocopherols andtocotrienols: physiological significance and prospects as antioxidants. *J Biosci. Bioeng*. 104: 439-445.

Yuan, J. & Yankner, B.A. 2000 Apoptosis in the nervous system *Nature* 407: 802-809.

Zhang, B., Tanaka, J., Yang, L., Yang, L., Sakanaka, M., Hata, R., Maeda, N. & Mitsuda, N. 2004. Protective effect of vitamin E against focal brain ischemia and neuronal death through induction of target genes of hypoxia-inducible factor-1. *Neuronsci*. 126: 433-440.

Then Sue-Mian*
UKM Medical Molecular Biology Institute (UMBI)
Universiti Kebangsaan Malaysia Medical Centre (UKMMC)
53000 Kuala Lumpur
Malaysia

Wan Zurinah Wan Ngah & Musalmah Mazlan
Department of Biochemistry
Medical Faculty
Universiti Kebangsaan Malaysia
50300 Kuala Lumpur
Malaysia

Gapor Mat Top
Malaysian Palm Oil Board
Bangi, 43000 Selangor D.E.
Malaysia

*Corresponding author; email: suemian@ppukm.ukm.my

Received: 7 January 2009
Accepted: 7 June 2009