
**UNIVERSITI PUTRA MALAYSIA**

# *NEUROPROTECTIVE EFFECT OF TOCOTRIENOL-RICH FRACTION AND α-TOCOPHEROL OF VITAMIN E AGAINST GLUTAMATE TOXICITY IN NEURONAL CELLS AND ASTROCYTES*

**THILAGA RATI SELVARAJU**

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**By**

**THILAGA RATI SELVARAJU**



**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy**

**June 2015**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy.

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**THILAGA RATI SELVARAJU**

**June 2015**

**Chairman : Huzwah Binti Khaza'ai, PhD**
**Faculty : Medicine and Health Sciences**

Vitamin E is a fat-soluble antioxidant consisting of tocopherol and tocotrienol. The tocotrienol-rich fraction (TRF) palm oil extract comprises 25% α-tocopherol (α-TCP) and 75% tocotrienols. TRF has been shown to possess potent antioxidant, anti-inflammatory, anti-cancer, neuroprotective, and cholesterol-lowering activities. Glutamate is the main excitatory amino acid neurotransmitter in mammalian central nervous systems; it can be excitotoxic and has been suggested to play a key role in neurodegenerative disorders such as Parkinson's and Alzheimer's disease. In this study, the effects of vitamin E when supplemented before (pre-treatment) and after (post-treatment) glutamate insult were elucidated in neuronal and astrocyte cell lines. The neuroprotective effect of TRF and α-TCP were investigated. Glutamate-mediated cytotoxicity was diminished by pre- and post-treatment of TRF and α-TCP. Vitamin E acted as a potent antioxidant agent in recovering mitochondrial injury from elevated oxidative stress, and cells exhibited better survival following glutamate toxicity. Quantitative morphological studies were also conducted via an apoptosis detection kit using flow cytometric analysis. Pre- and post-treatment with TRF and α-TCP led to better survival and lower cell death rates following glutamate neurotoxicity. The flow cytometry morphological findings were validated by scanning electron microscopy analysis. Cell cycle analysis was also performed using an RNAse-propidium iodide assay. The presence of glutamate in the nerve cells caused DNA or protein damage and chromatin destruction; manipulating these nerve cells to re-enter the cell cycle promoted repair of the damage. Supplementation of TRF and α-TCP enhanced the DNA repair process, with higher numbers of nerve cells accumulating in the S and G2/M phases, indicating active replication and repair of the DNA damage that had occurred during the previous cell cycle. In both TRF and α-TCP pre- and post-treatment groups, glutamate-injured cells exhibited significant reductions in concentrations of the lipid peroxidation biomarker malondialdehyde. Ferric reducing antioxidant power (FRAP) assay was employed to determine the total antioxidant power in the cells. There was significantly increased antioxidant capacity in the cells treated with TRF or α-TCP as compared to

glutamate-treated cells, which indicated good neuroprotection. Exposure to glutamate also reduced the concentrations of glutathione, superoxide dismutase, and catalase, important natural antioxidants synthesized in neurons and astrocytes. Both pre- and post-treatment of vitamin E markedly increased antioxidant activity. Subsequently, the expression of traumatic brain injury markers for neuron-specific enolase (NSE) and S100 calcium-binding protein B (S100 β) were elucidated using real-time PCR. The results revealed the downregulation of *NSE* and *S100B* upon glutamate challenge in neuronal cells and astrocytes following treatment with different concentrations of TRF and α-TCP, a sign of the recovery process. Human apoptosis quantitative PCR array analysis determined that post-treatment with 200 ng/mL TRF and α-TCP upregulated the anti-apoptotic genes and downregulated the pro-apoptotic genes in both the neuronal and astrocyte cell lines. In conclusion, TRF and α-TCP have protective and recovery properties against glutamate toxicity in neuronal cells and astrocytes. From the average value calculation, TRF preceded α-TCP in neuroprotection against glutamate insult in both astrocytes and neuronal cells. Hence, the present study can serve as a platform for further studies on the effects of TRF and α-TCP, as they could be developed into potential treatment agents for neurodegenerative diseases.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah.

**KESAN PERLINDUNGAN SARAF OLEH FRAKSI KAYA TOKOTRIENOL DAN α-TOKOFEROL DARIPADA VITAMIN E TERHADAP KETOKSIKAN GLUTAMAT DALAM SEL NEURON DAN ASTROSIT**

Oleh

**THILAGA RATI SELVARAJU**

**Jun 2015**

**Pengerusi : Huzwah Khaza'ai, PhD**
**Fakulti : Perubatan dan Sains Kesihatan**

Vitamin E ialah sejenis antioksidan larut lemak yang terdiri daripada tokoferol dan tokotrienol. Fraksi kaya tokotrienol (TRF) ialah satu ekstrak minyak kelapa sawit yang terdiri daripada 25% α-tokoferol dan 75% tokotrienol. TRF juga telah dibuktikan mempunyai ciri-ciri yang berkesan, misalnya, sebagai antioksidan, neuroproteksi, anti-radang, anti-kanser, dan menurunkan paras kolesterol. Keracunan glutamat merupakan penyebab utama neurodegenerasi sistem saraf. Glutamat merupakan salah satu asid amino utama yang menjadi perangsang dalam sistem saraf pusat mamalia, di mana kepekatan yang tinggi boleh mengakibatkan eksisitoksisiti dan ia memainkan peranan penting dalam penyakit neurodegenerasi seperti penyakit Parkinson dan Alzheimer. Dalam kajian ini, kesan vitamin E telah diuji apabila ditambah sebelum (pra-rawatan) dan selepas (pasca-rawatan) keracunan glutamat dalam sel neuron dan astrosit. Kesan neuroproteksi TRF dan α-tokoferol (α-TCP) telah diuji dalam kajian ini. Kesitotoksikan glutamat telah berkurangan melalui pra- dan pasca-rawatan TRF dan α-TCP. Vitamin E bertindak sebagai agen antioksidan yang kuat dalam pemulihan kecederaan mitokondria akibat tekanan oksidatif tinggi. TRF dan α-TCP juga mempamerkan kemandirian yang lebih baik apabila dikenakan ketoksikan glutamat. Kajian morfologi berbentuk kuantitatif juga telah dijalankan dengan menggunakan analisis sitometri aliran. Pra- dan pasca-rawatan dengan pelbagai kepekatan TRF dan α-TCP dapat meningkatkan kadar hidup sel di samping mengurangkan kadar kematian sel berikutan ketoksikan glutamat dalam sel. Data morfologi yang diperolehi melalui analisa sitometri aliran telah disahkan melalui analisis mikroskop imbasan elektron. Analisis kitaran sel juga telah dilaksanakan melalui ujian RNase-propidium iodide. Kehadiran glutamat dalam sel-sel saraf boleh menyebabkan kerosakan DNA atau protein dan kemusnahan kromatin; memanipulasikan sel-sel saraf untuk memasuki semula kitaran sel untuk membaiki kerosakan. Rawatan TRF dan α-TCP berjaya meningkatkan proses pemulihan DNA di fasa S dan G2/M di mana sel-sel saraf telah aktif mereplikasi DNA mereka dan membaiki kerosakan DNA yang berlaku semasa fasa terdahulu dalam kitaran sel. Sel-sel yang mengalami kecederaan glutamat menunjukkan pengurangan kepekatan malondialdehid, penanda biooksidasi lipid berikutan kedua-dua pra- dan pasca-

rawatan TRF dan α-TCP masing-masing. Ujian penurunan ferik kuasa antioksidan (FRAP) telah digunakan untuk menentukan jumlah kuasa antioksidan dalam sel-sel. Nilai kapasiti antioksidan kedua-dua jenis vitamin E memaparkan neuroproteksi yang sangat baik berbanding dengan sel-sel yang dikenakan kecederaan glutamat. Pendedahan kepada glutamat juga didapati menyebabkan pengurangan kepekatan glutation (GSH), superoksida dismutas (SOD), dan katalas (CAT), yang dikenali sebagai antioksidan semulajadi yang penting yang disintesis dalam sel neuron dan astrosit. Kedua-dua pra- dan pasca-rawatan vitamin E menyebabkan peningkatan yang ketara dalam aktiviti antioksidan. Expressi penanda kecederaan otak, iaitu enolase khusus neuron (NSE) dan S100 protein pengikat kalsium B (S100 β), juga telah dikaji menggunakan kaedah PCR masa nyata. Keputusannya, rawatan TRF dan α-TCP mengurangkan ekspressi gen *NSE* dan *S100B* sebagai proses tanda pemulihan apabila cabaran glutamat dikenakan atas sel-sel neuron dan astrosit. Analisis apoptosis manusia melalui PCR kuantitatif (qPCR) dengan rawatan dos 200 ng/mL TRF dan α-TCP mendapati bahawa TRF dan α-TCP mendorong peningkatan ekspressi gen anti-apoptotik dan menyebabkan pengurangan ekspressi gen pro-apoptotik dalam kedua-dua jenis sel. Kesimpulannya, TRF dan α-TCP melindungi dan memulihkan sel neuron dan astrosit yang telah menghadapi ketoksikan glutamat. Berdasarkan pengiraan nilai purata, kesan TRF mendahului α-TCP dari segi neuroproteksi dalam kedua-dua jenis sel yang diuji. Kajian ini boleh digunakan sebagai dasar untuk mendalami kebaikan TRF dan α-TCP kerana ia boleh dibangunkan sebagai ejen rawatan yang berpotensi untuk merawat penyakit neurodegeneratif.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

**Huzwah Khaza'ai, PhD**
Senior lecturer,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia.
(Chairman)

**Sharmili Vidyadaran, PhD**
Associate Professor,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia.
(Member)

**Abdah MD Akim, PhD**
Senior Lecturer,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia.
(Member)



_____
**BUJANG KIM HUAT, PhD**
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Name of Chairman of Supervisory Committee:
**Dr. Huzwah Khaza'ai**

Signature: _____
Name of Member of Supervisory Committee:
**Assoc. Prof. Dr. Sharmili Vidyadaran**

Signature: _____
Name of Member of Supervisory Committee:
**Dr. Abdah MD Akim**

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| | |
|---|---|
| % | Percentage |
| ± | More or less than |
| °C | Celsius |
| µM | Micromolar |
| 12-LOX | 12-Lipoxygenase |
| 15-LOX | 15-Lipoxygenase |
| 5-LOX | 5-Lipoxygenase |
| AD | Alzheimer's disease |
| AIF | Apoptosis-inducing factor |
| *AKT1* | V-Akt murine thymoma viral oncogene homolog 1 |
| ALS | Amyotrophic lateral sclerosis |
| AMPA | α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate |
| ANOVA | Analysis of variance |
| *AP15* | Apoptosis inhibitor 5 |
| *APAF1* | Apoptotic peptidase activating factor 1 |
| apoB | Apolipoprotein B |
| APP | Amyloid precursor protein |
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| AV | Annexin V |
| *AVEN* | Apoptosis, caspase activation inhibitor |
| Aβ | Beta amyloid |
| *BAD* | BCL2-associated agonist of cell death |
| *BAG1* | BCL2-associated athanogene |
| *BAG3* | BCL2-associated athanogene 3 |
| *BAG4* | BCL2-associated athanogene 4 |
| *BAK1* | BCL2-antagonist/Killer 1 |
| *BAX* | BCL2-associated X protein |
| BBB | Blood-brain barrier |
| *BBC3* | BCL2-binding component 3 |
| *BCL10* | B-cell CLL/Lymphoma 10 |
| *BCL2* | B-cell CLL/Lymphoma 2 |
| *BCL2A1* | BCL2-related protein A1 |
| *BCL2L1* | BCL2-like 1 |
| *BCL2L10* | BCL2-like 10 (apoptosis facilitator) |
| *BCL2L11* | BCL2-like 11 (apoptosis facilitator) |
| *BCL2L14* | BCL2-like 14 (apoptosis facilitator) |
| *BCL2L2* | BCL2-like 2 |
| *BCL6* | B-cell CLL/Lymphoma 6 |
| *BFAR* | Bifunctional apoptosis regulator |
| *BID* | BH3 interacting domain death agonist |
| *BIK* | BCL2-interacting killer (apoptosis-inducing) |
| *BIRC2* | Baculoviral IAP repeat-containing 2 |
| *BIRC3* | Baculoviral IAP repeat-containing 3 |
| *BIRC5* | Baculoviral IAP repeat-containing 5 |
| *BNIP1* | BCL2/adenovirus E1B 19 kDa interacting protein 1 |
| *BNIP2* | BCL2/adenovirus E1B 19 kDa interacting protein 2 |
| *BNIP3* | BCL2/adenovirus E1B 19 kDa interacting protein 3 |

| | |
|---|---|
| *BNIP3L* | BCL2/adenovirus E1B 19 kDa interacting protein 3-like |
| *BOK* | BCL2-related ovarian killer |
| BSA | Bovine serum albumin |
| BSO | L-buthionine-S,R-sulfoximine |
| $Ca^{2+}$ | Calcium ion |
| CAG | Cytosine, adenine, guanine |
| CAPS9 | Caspase-9, apoptosis-related cysteine peptidase |
| *CARD10* | Caspase recruitment domain family, member 10 |
| *CASP2* | Caspase-2, apoptosis-related cysteine peptidase |
| *CASP3* | Caspase-3, apoptosis-related cysteine peptidase |
| *CASP6* | Caspase-6, cysteine aspartic acid-specific protease 6 |
| *CASP7* | Caspase-7, apoptosis-related cysteine peptidase |
| *CASP8* | Caspase-8, apoptosis-related cysteine peptidase |
| *CASP9* | Caspase-9, cysteine aspartic acid-specific protease 9 |
| Caspase | Cysteine aspartic acid-specific protease |
| CAT | Catalase |
| *CD40* | CD40 molecule, TNF receptor superfamily member 5 |
| CDK | Cyclin-dependent kinase |
| cDNA | Complementary DNA |
| *CLU* | Clusterin |
| CNS | Central nervous system |
| $CO_2$ | Carbon dioxide |
| CPR | Cardiopulmonary resuscitation |
| Cq | Quantification cycle |
| CSF | Cerebrospinal fluid |
| Cu/ZnSOD | Copper- and zinc-containing SOD |
| *DAD1* | Defender against cell death 1 |
| *DAP* | Death-associated protein |
| *DAPK1* | Death-associated protein kinase 1 |
| *DAPK2* | Death-associated protein kinase 2 |
| dATP | Deoxyadenosine triphosphate |
| DBTRG-05MG | Glioblastoma |
| *DDIT3* | DNA damage-inducible transcript 3 |
| DEPC | Diethylpyrocarbonate |
| *DFFA* | DNA fragmentation factor, 45 kDa, alpha polypeptide |
| *DIABLO* | Diablo, IAP-binding mitochondrial protein |
| DMBA | 7,12-Dimethylbenz(α)anthracene |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| *DPF2* | D4, zinc and double PHD fingers family 2 |
| DS | Down's syndrome |
| DTNB | 5,5'-Dithiobis-2-nitrobenzoic acid |
| E | Efficiency |
| *E2F1* | E2F transcription factor 1 |
| *E2F2* | E2F Transcription factor 2 |
| EAA | Excitatory amino acid |
| EAAC1 | Excitatory amino acid carrier 1 |
| EAAT | Excitatory amino acid transporter |
| EAAT5 | Excitatory amino acid transporter 5 |
| EDTA | Ethylenediaminetetraacetic acid |

| | |
|---|---|
| EDX | Energy dispersive X-ray |
| ELISA | Enzyme-linked immunosorbent assay |
| *FADD* | Fas (TNFRSF6)-associated via death domain |
| *FAS* | Fas cell surface death receptor |
| *FASLG* | Fas ligand (TNF superfamily, member 6) |
| Fe (II)-TPTZ | Ferrous tripyridyltriazine |
| Fe (III)-TPTZ | Ferric tripyridyltriazine |
| $FeSO_4 \cdot 7H_2O$ | Ferrous sulphate heptahydrate |
| *FGFR3* | Fibroblast growth factor receptor 3 |
| FITC | Fluorescein isothiocyanate |
| *FOXO3* | Forkhead box O3 |
| FRAP | Ferric reducing antioxidant power |
| g | Gram |
| G1 | Gap 1 |
| G2 | Gap 2 |
| GABA | Gamma-aminobutyric acid |
| *GADD45G* | Growth arrest and DNA damage-inducible, gamma |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GFAP | Glial fibrillary acidic protein |
| GLAST | Glutamate-aspartate transporter |
| GLT | Glutamate transporter |
| GluRs | Glutamate receptors |
| GP | GSH peroxidase |
| GPR | Global pattern recognition |
| GPx | Glutathione peroxidase |
| *GPx1* | Glutathione peroxidase 1 |
| GPx4 | Glutathione peroxidase 4 |
| GR | GSH reductase |
| GRx | Glutathione reductase |
| GSH | Glutathione |
| *GSK3B* | Glycogen synthase kinase 3 beta |
| $H_2O$ | Water |
| $H_2O_2$ | Hydrogen peroxide |
| HCl | Hydrochloric acid |
| HD | Huntington's disease |
| HDL | High-density lipoprotein |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| *HIF1A* | Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) |
| *HIPK2* | Homeodomain-interacting protein kinase 2 |
| *HRK* | Harakiri, BCL2-interacting protein (contains only BH3 domain) |
| *HSPA1A* | Heat shock 70 kDa protein 1A |
| *HTRA2* | HtrA serine peptidase 2 |
| IAPs | Inhibitor of apoptosis proteins |
| *IGF1* | Insulin-like growth factor 1 (somatomedin C) |
| iGluR | Ionotropic glutamate receptor |
| IL-1β | Interleukin-1β |
| IL3 | Interleukin-3 |
| KA | Kainite |
| LCPUFA | Long chain polyunsaturated fatty acid |

| | |
|---|---|
| LDH | Lactate dehydrogenase |
| LDL | Low-density lipoprotein |
| LR | Lower right |
| *LTA* | Lymphotoxin alpha |
| *LTBR* | Lymphotoxin beta receptor (TNFR superfamily, member 3) |
| MBP | Myelin basic protein |
| *MCL1* | Myeloid cell leukemia sequence 1 (BCL2-related) |
| MDA | Malondialdehyde |
| MEM | Minimum essential media |
| mg | Milligram |
| mGluR | Metabotropic glutamate receptors |
| mL | Milliliter |
| mM | Millimolar |
| MMP | Mitochondrial membrane potential |
| MnSOD | Manganese-containing SOD |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide |
| $Na^+$ | Sodium ion |
| NAC | Non-Aβ component |
| NADH | β-Nicotinamide adenine dinucleotide |
| *NAIP* | NLR family, apoptosis inhibitory protein |
| NaOH | Sodium hydroxide |
| NBT | Nitroblue tetrazolium |
| *NFKB1* | Nuclear factor of kappa light polypeptide gene enhancer in B-cell 1 |
| NFTs | Neurofibrillary tangles |
| ng | Nanogram |
| nm | Nanometer |
| nM | Nanomolar |
| NMDA | *N*-methyl-D-aspartate |
| nmol/mg | Nanomole per milligram |
| $NO^-$ | Nitric oxide |
| NSE | Neuron-specific enolase (gene: *NSE*) |
| NTC | Non-template control |
| $O_2^-$ | Superoxide |
| $OH^-$ | Hydroxyl radical |
| $ONOO^-$ | Peroxynitrite |
| P13K-AKT | Phosphatidylinositol-3-kinase and protein kinase B |
| *PAWR* | PRKC, apoptosis, WT1, regulator |
| PBS | Phosphate-buffered saline |
| PD | Parkinson's disease |
| *PERP* | PERP, TP53 apoptosis effector |
| pH | Potential of hydrogen |
| PI | Propidium iodide |
| *PMAIP1* | Phorbol-12-myristate-13-acetate-induced protein 1 |
| PNS | Peripheral nervous system |
| *PRDX2* | Peroxiredoxin 2 |
| PS | Phosphatidylserine |
| *PTEN* | Phosphatase and tensin homolog |
| PTP | Permeability transition pore |
| PUFA | Polyunsaturated fatty acids |

| | |
|---|---|
| Q1 | Quadrant 1 |
| Q2 | Quadrant 2 |
| Q3 | Quadrant 3 |
| Q4 | Quadrant 4 |
| qPCR | Quantitative polymerase chain reaction |
| *RAD21* | RAD21 homolog (*Schizosaccharomyces pombe*) |
| Rho 123 | Rhodamine 123 |
| *RIPK1* | Receptor-interacting serine/threonine-protein kinase 1 |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| rpm | Revolutions per minute |
| RPMI | Roswell Park Memorial Institute |
| rRNA | Ribosomal RNA |
| RT-PCR | Real-time polymerase chain reaction |
| S | Synthesis |
| S100 β | S100 calcium-binding protein B (gene: *S100B*) |
| SEM | Standard error of the mean |
| SHRSP | Stroke-prone spontaneously hypertensive rats |
| *SIRT1* | Sirtuin 1 |
| SK-N-SH | Neuroblastoma |
| SOD | Superoxide dismutase |
| *SOD1* | Superoxide dismutase 1 |
| SPSS | Statistical Package for the Social Sciences |
| *STAT5A* | Signal transducer and activator of transcription 5A |
| *STAT5B* | Signal transducer and activator of transcription 5B |
| SWNCT | single-walled carbon nanotubes |
| system $x_c^-$ | Cystine/glutamate antiporter |
| TAE | Tris-acetate-EDTA |
| TBA | Thiobarbituric acid |
| TBARS | Thiobarbituric acid-reactive substances |
| TBE | Tris-borate-EDTA |
| TBI | Traumatic brain injury |
| TCA | Trichloroacetic acid |
| TCA cycle | Tricarboxylic acid cycle |
| TEP | 1,1,3,3-Tetraethoxypropane |
| *TGFB1* | Transforming growth factor, beta 1 |
| TNB | 5-thionitrobenzoic acid |
| *TNF* | Tumor necrosis factor |
| *TNFAIP3* | Tumor necrosis factor, alpha-induced protein 3 |
| TNFR | Tumor necrosis factor family receptors |
| *TNFRSF10A* | Tumor necrosis factor receptor superfamily, member 10A |
| *TNFRSF11B* | Tumor necrosis factor receptor superfamily, member 11B |
| *TNFRSF1A* | Tumor necrosis factor receptor superfamily, member 1A |
| *TNFRSF1B* | Tumor necrosis factor receptor superfamily, member 1B |
| *TP53* | Tumor protein P53 |
| *TP53INP1* | Tumor protein P53 inducible nuclear protein 1 |
| TPTZ | 2,4,6-Tri(2-pyridyl)-1,3,5-triazine |
| *TRADD* | TNFRSF1A-associated via death domain |
| *TRAF2* | TNF receptor-associated factor 2 |
| *TRAF4* | TNF receptor-associated factor 4 |

| | |
|---|---|
| TRF | Tocotrienol-rich fraction |
| UK | United Kingdom |
| *UNC5B* | Netrin receptor Unc-5 homolog B (*Caenorhabditis elegans*) |
| UR | Upper right |
| USA | United States of America |
| UV | Ultraviolet |
| V | Volt |
| v/v | Volume per volume |
| VEGF | Vascular endothelial growth factor |
| *VEGFA* | Vascular endothelial growth factor A |
| VLDL | Very low-density lipoprotein |
| *XIAP* | X-linked inhibitor of apoptosis |
| α | Alpha |
| α-TCP | α-Tocopherol |
| α-TTP | α-TCP transfer protein |
| β | Beta |
| γ | Gamma |
| δ | Delta |
| μg | Microgram |
| μl | Microliter |
| μM | Micromolar |
| τ | Tau |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of the study

Vitamin E is the general name for lipid-soluble tocopherol and tocotrienol. Tocopherol and tocotrienol contain structural features of a common chromanol with a C-2 side chain (Osakada et al., 2004). Tocotrienol and tocopherol differ in terms of the unsaturated phytyl tail in the former and the saturated side chain in the latter. Tocopherols and tocotrienols are differentiated into compounds that differ according to methyl substitution number and position on the chromanol ring (Shichiri, Takanezawa, Uchida, Tamai, & Arai, 2007). The natural vitamin E family consists of eight chemically distinct isomers: alpha (α), beta (β), gamma (γ), and delta (δ)-tocotrienol, and alpha (α), beta (β), gamma (γ), and delta (δ)-tocopherol (Baxter et al., 2012).

Large quantities of tocotrienols (up to 800 mg/kg) are found in crude palm oil obtained from oil palm fruits, and mostly consist of α-, β-, γ-, and δ-tocotrienols (Sen, Khanna, & Roy, 2006). Tocotrienols are also found in oil derived from barley, rice bran, rye, and wheat germ. Refined palm oil contains approximately 350-440 ppm vitamin E, which comprises tocopherol (30%) and tocotrienol (70%) (Sambanthamurthi, Sundram, & Tan, 2000). Currently, there is an increasing demand for analysis of the dietary and physiological elements of vitamin E from palm oil, specifically that in the tocotrienol-rich fraction (TRF). TRF is an extract that consists of 25% α-tocopherol (α-TCP) and 75% tocotrienol. TRF has potent antioxidant, anti-cancer, anti-inflammation, and cholesterol-lowering properties (Choi, Kim, Lee, & Choi, 2008; Minhajuddin, Beg, & Iqbal, 2005; Wu, Liu, & Ng, 2008). Thus, interest in studying the neuroprotective effects of tocotrienol and tocopherol has increased over time.

Glutamate plays a major role as a neurotransmitter in the central nervous system (CNS). Elevated levels of extracellular glutamate, however, are neurotoxic to nerve cells (Coyle & Puttfarcken, 1993; Lau & Tymianski, 2010). Glutamate is considered a predominant excitatory amino acid neurotransmitter that can be found in the mammalian CNS; it can be toxic and it has been suggested that it plays a crucial role in the development of neurodegenerative diseases (Zou & Crews, 2005). Glutamate-mediated injury is a main contributor to pathological cell death in the nervous system (Saito et al., 2007).

There are two types of glutamate-induced toxicity: receptor-initiated excitotoxicity and non-receptor-mediated oxidative toxicity (Saito et al., 2007). Receptor-mediated glutamate excitotoxicity involves excessive stimulation of the glutamate receptors (GluRs), which subsequently leads to excessive calcium ion ($Ca^{2+}$) influx and

activates a cell death cascade involving the accumulation of mitochondrially generated reactive oxygen species (ROS). Studies have shown that excessive extracellular glutamate causes nerve cell death via stimulation of the *N*-methyl-D-aspartate (NMDA) receptors in cases of stroke or trauma (Lipton, 2004; Rothman & Olney, 1986). On the other hand, non-receptor-mediated oxidative toxicity involves the breakdown of the cystine/glutamate antiporter (system $x_c^-$) mechanism, which leads to the depletion of glutathione (GSH) and subsequently causes oxidative stress and cell death. System $x_c^-$ couples the import of cystine to the export of glutamate. Elevated concentrations of extracellular glutamate inhibit the uptake of cystine, which is essential for synthesis of the intracellular antioxidant GSH. GSH plays a key role in the disposal of peroxides by brain cells and in protection against ROS (Dringen, 2000; Murphy, Miyamoto, Sastre, Schnaar, & Coyle, 1989). Depletion of GSH renders the cell vulnerable to oxidative stress and ultimately leads to cell death. GSH reduction renders the cell unable to eliminate ROS, which are constantly formed in the mitochondria as well as during some enzyme reactions, and eventually results in cell death via oxidative injury (Lewerenz, Klein, & Methner, 2006). The stimulation of oxidative destruction via glutamate has been established as a primary cytotoxic mechanism in C6 glial cells (Han et al., 1997; Kato, Negishi, Mawatari, & Kuo, 1992), PC-12 neuronal cells (Froissard, Monrocq, & Duval, 1997; Pereira & Oliveira, 1997), immature cortical neuronal cells (Murphy et al., 1989) and oligodendroglial cells (Oka, Belliveau, Rosenberg, & Volpe, 1993).

The nervous system is the network of nerve cells that transmits nerve impulses throughout the body (Figure 1.1). It is rich in both unsaturated fats and iron, rendering it particularly susceptible to oxidative stress and damage (Aliev et al., 2008). Oxidative stress involves interference of the redox equilibrium between the production and removal of highly ROS and reactive nitrogen species (RNS) (Aksenova, Aksenov, Mactutus, & Booze, 2005). Oxidative stress further damages cell lipids, proteins, and DNA (Higuchi, 2004). It also plays a role modulating critical cellular functions, such as apoptosis program stimulation, ion transportation, and $Ca^{2+}$ mobilization, which leads to cell death (Emerit, Edeas, & Bricaire, 2004; Scandalios, 2002). Numerous studies have attempted to prevent neuronal cell death caused by oxidative stress by administering free radical-scavenging antioxidants, such as vitamin E. A previous study showed that vitamin E, known as a chain-breaking antioxidant, increased the survivability of neuronal cells with glutamate injury (Sen, Khanna, Roy, & Packer, 2000).

We aimed to elucidate the protective role of vitamin E against glutamate toxicity and how recovery by vitamin E, in terms of viability, antioxidant activity, traumatic brain injury (TBI) marker expression, and apoptotic gene regulation, is possible. In concert with improving neuronal cell and astrocyte survival against glutamate-induced toxicity, we expected to observe advanced neuroprotection. We also expected that tocotrienols and tocopherols would have prophylactic and preventive properties against neurodegeneration and eventually propose that they are alternative nutrition-based therapeutics.

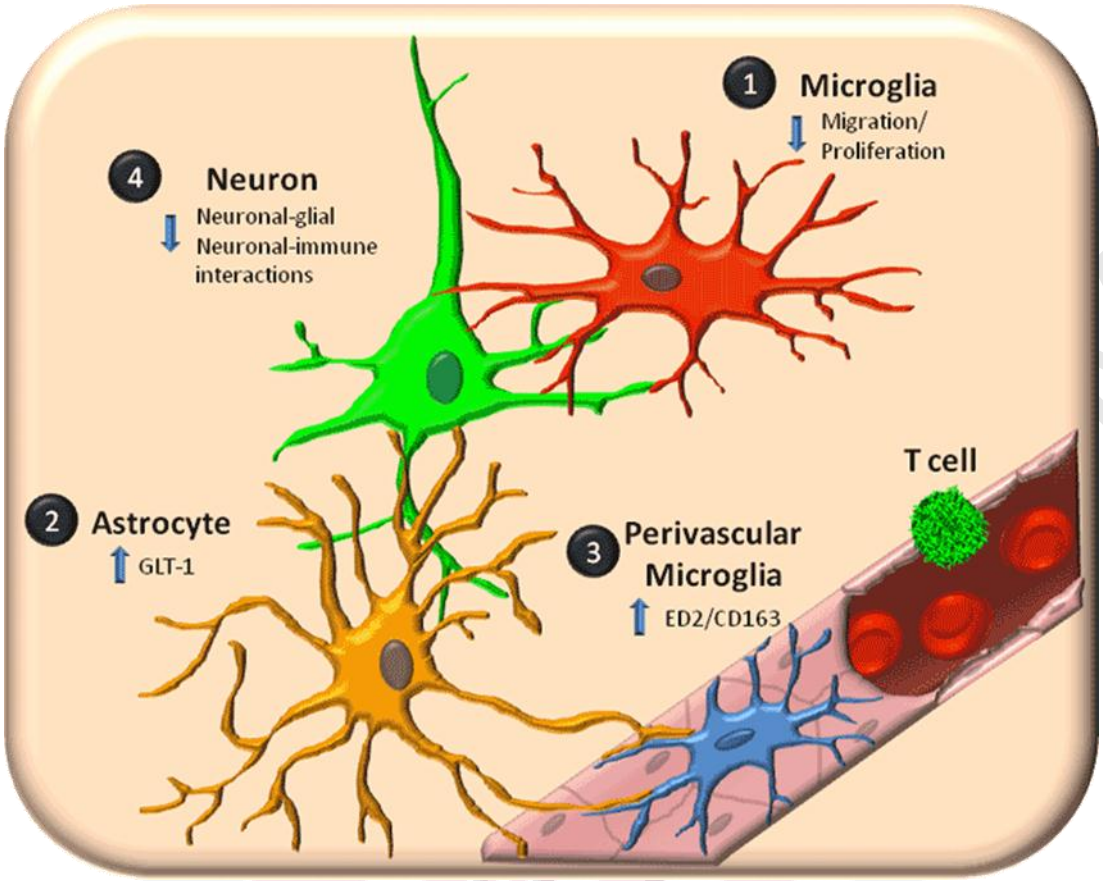


**Figure 1.1: Neuron-glia interaction**. Source: (Kato, Watabe, & Kanba, 2013)

## 1.2 Problem statement

Neurodegenerative diseases are considered the next great public health challenge and are one of the most prevalent health concerns worldwide. The limitations of available drugs and the lack of potent therapeutic agents against neurotoxicity-induced neurological disorders have spurred rapid development of further neuroprotection-related research. Increased concentrations of glutamate in the CNS may lead to oxidative stress, which contributes to nerve cell death and subsequently leads to neurodegenerative disorders. Protection against oxidative stress can be conferred by free radical scavenger compounds such as vitamin E in the form of tocopherol as well as tocotrienol. Therefore, this study was designed to evaluate the potency of both forms of vitamin E in protecting neuronal cells and astrocytes against glutamate-initiated toxicity. Our elucidation of the possible underlying mechanisms focused on several molecular targets, including antioxidant enzymes, TBI markers, and the expression of anti- and pro-apoptotic genes.

## 1.3 Research objectives

### 1.3.1 General objective

To elucidate the potential of TRF and α-TCP as neuroprotective agents in glutamate induced injury in neuronal cells and astrocytes.

### 1.3.2 Specific objectives

- To elucidate the role of TRF and α-TCP in prophylactic and recovery processes against glutamate-injured neuronal cells and astrocytes in terms of cell viability.

- To investigate the antioxidant activity of TRF and α-TCP on glutamate-challenged nerve cells.

- To measure the expression of neuron-specific enolase (*NSE*) and S100 calcium-binding protein B (*S100B*) as TBI markers to assess functional damage due to glutamate toxicity and the recovery processes following supplementation of TRF and α-TCP.

- To define the effect of TRF and α-TCP on anti- and pro-apoptotic genes and their relationship in the apoptosis pathway in glutamate-injured neuronal cells and astrocytes.

## 1.4 Hypothesis

TRF confers better neuroprotection than α-TCP against glutamate-induced toxicity in neuronal cells and astrocytes.

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