The comparative effects between tocotrienol-rich fraction (TRF) and α-tocopherol on glutamate toxicity in neuron-astrocyte mono- and co-culture systems

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Abstract

Background: Vitamin E, which can be categorized into tocotrienols and tocopherols, is known to protect cells from glutamate neurotoxicity. Studies have shown that tocotrienol-rich fraction (TRF) protecting the brain against oxidative damage more efficient than α-tocopherol. The role of astrocyte in promoting neuronal survival and recovery after glutamate neurotoxicity is also increasingly appreciated.
Aims: To elucidate the effects of TRF and α-tocopherol and the synergism between astrocyte and neuron against glutamate neurotoxicity.
Methods: Astrocyte and neuron were subjected to glutamate injury followed by TRF and α-tocopherol treatments (100 – 300 ng/ml). Effects of TRF and α-tocopherol on nerve cell viability and glutathione contents against glutamate toxicity were examined. The synergism between astrocyte and neuron was elucidated through co-culture model. Statistical analysis was performed using one way ANOVA.
Results: Both TRF and α-tocopherol improved approximately 10% of glutamate-injured astrocyte and neuronal cell viability. In co-culture model, TRF and α-tocopherol provided nearly complete protection from glutamate toxicity. Besides, TRF and α-tocopherol treatments significantly restored at least 20% of glutathione contents in glutamate-injured neurons. In the presence of astrocyte, 300 ng/ml TRF and α-tocopherol completely restored glutathione contents in glutamate-injured neuron.
Conclusions: TRF and α-tocopherol had shown promising neuroprotective effects in astrocyte and neuron from glutamate toxicity. Great scavenging effect of both TRF and α-tocopherol against glutamate toxicity was observed in neuron. Similar protective effects between TRF and α-tocopherol were observed. Co-culture model demonstrated the synergistic properties between neuron and astrocyte. Supplementation of TRF and α-tocopherol in co-culture further improved the recovery process.
Keywords: Tocotrienol-rich fraction; α-tocopherol; glutamate neurotoxicity; neuron-astrocyte co-culture

1. Introduction

Elevated concentration of glutamate is the major contributor to neuronal cell death under pathological conditions
such as ischemic insults and traumatic brain damage which could be related to the etiology of most of the neurodegenerative
diseases\textsuperscript{1}. Glutamate toxicity in nerve cells exists in two forms which are receptor-initiated excitotoxicity\textsuperscript{2} and non-receptor
mediated oxidative glutamate toxicity\textsuperscript{3}. Receptor-initiated excitotoxicity involves over-stimulation of glutamate receptor
(GluRs) which leads to excessive Ca\textsuperscript{2+} influx and activates a cell death cascade\textsuperscript{4}. On the hand, non-receptor mediated
oxidative glutamate toxicity involves the breakdown of the cystine/glutamate antiporter (\(x\textsubscript{-}c\)) mechanism, which leads to the
depletion of glutathione (GSH) and subsequently causes oxidative stress and cell death\textsuperscript{3}.

Nervous system, which is rich in both unsaturated fats and iron, makes it particularly susceptible to oxidative stress
and damage\textsuperscript{5}. Oxidative stress plays a significant role in the modulation of critical cellular functions, such as apoptosis
program activation, ion transport and calcium mobilization\textsuperscript{6}, which often leading to cell death\textsuperscript{7}. However, cell death caused
by oxidative stress can be prevented by administration of free radical scavenging antioxidants, such as vitamin E. Vitamin
E, which is a fat-soluble antioxidant, prevents lipid peroxidation in biological membranes. It exists in eight forms, namely
\(\alpha\)-, \(\beta\)-, \(\gamma\)- and \(\delta\)-tocopherols and \(\alpha\)-, \(\beta\)-, \(\gamma\)- and \(\delta\)-tocopherols\textsuperscript{8}. Alpha-tocotrienol has been found to be 40 to 60 times more
potent in preventing lipid peroxidation as compared to \(\alpha\)-tocopherol\textsuperscript{9}. On the other hand, many studies demonstrated that
tocotrienol-rich fraction (TRF) possesses powerful antioxidant\textsuperscript{10}, anti-inflammation\textsuperscript{11} and cholesterol-lowering
properties\textsuperscript{12}. Tocotrienols differ from tocopherols by possessing an unsaturated isoprenoid side chain instead of a saturated
phytlyl tail. The unsaturated side chain of tocotrienol is claimed to allow better penetration and distribution into saturated
lipid layers such as brain and liver\textsuperscript{13}. In previous studies, \(\alpha\)-tocopherol was shown to protect cells from glutamate-induced
cell death in micromolar concentration. However, in recent studies, \(\alpha\)-tocotrienol had shown better protection from
 glutamate-induced cell death in nanomolar concentration by inhibiting glutamate-induced early activation of c-Src kinase\textsuperscript{14}.
This finding showed that \(\alpha\)-tocotrienol has potent signal transduction regulatory properties which was independent of its
antioxidant properties. Thus, the interest to study the neuroprotection of tocotrienols from glutamate toxicity, either by
antioxidant activity or by antioxidant-independent activity has increased with time.

Nervous system consists of glial and neuron. Astrocyte, subtype of glial cell, is known to protect neuron from
oxidative stress through transcriptional up-regulation of glutathione synthesis and removal of extracellular glutamate\textsuperscript{15}.
Astrocytes death after ischemia or reperfusion may strongly affect neuronal survival due to the absence of trophic and
metabolic support to neurons and astrocytic glutamate uptake\textsuperscript{16}. Thus, the role of astrocytes in promoting neuronal survival
and recovery after a cerebral insult is becoming increasingly appreciated.

1.1 Objectives

To elucidate the role of TRF and \(\alpha\)-tocopherol in recovery processes by supplementation to glutamate-injured
astrocyte, neuron as well as neuron in co-culture system.

To examine the synergism between astrocyte and neuron against glutamate toxicity with the supplementation of
TRF and \(\alpha\)-tocopherol.

2. Materials and Methods

Minimum essential media (MEM), RPMI 1640 media and phosphate buffer saline were purchased from Invitrogen
(USA) while fetal bovine serum, trypsin-EDTA and penicillin/streptomycin were purchased from PAA (Austria). Sodium
pyruvate, sodium bicarbonate, L-glutamic acid monosodium salt hydrate and dimethyl sulphoxide (DMSO) were supplied
by Sigma (USA). Tocotrienol-rich fraction (TRF) was purchased from Sime Darby while \(\alpha\)-tocopherol was supplied by
ICN Biochemicals (USA). The 3-[4, 5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) powder was
purchased from PhytoTechnology Laboratories (USA). ApoGSH\textsuperscript{TM} colorimetric glutathione detection kit was obtained
from Biovision (USA). Six well culture inserts (transparent polyethylene terephthalate 0.4 \(\mu\)m pores membrane) were
purchased from Becton, Dickinson and Company (USA).

2.1 Cell culture

Human neurons (SK-N-SH) and human astrocytes (DBTRF-05MG) were obtained from American Type Culture
Collection (ATCC, USA). Neurons and astrocytes were cultured in MEM and RPMI 1640, respectively. Both cell lines were
supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin) throughout
the experiments. Cultures were maintained at 37\degree C in 5% CO\textsubscript{2} and 95\% air in a humidified incubator. In order to mimic the
Spatial interaction between neurons and astrocytes, neurons were co-cultured with astrocytes in co-culture membrane insert model prior to treatment.

2.2 TRF, α-tocopherol and glutamate preparation

TRF and α-tocopherol were freshly prepared in absolute ethanol with concentration of 100, 200 and 300 µg/ml (10^3 × working concentration) prior to treatment. Glutamate solutions with various concentrations were prepared in phosphate buffer saline (PBS).

2.3 TRF and α-tocopherol treatment

2.3.1 Mono-culture

Neurons and astrocytes were individually seeded in 6 well plates at cell density of 1 × 10^6 cells per well (total medium per well = 2 ml) followed by 24 hours incubation in humidified incubator at 37°C, 5% CO_2. After 24 hours incubation, neurons and astrocytes were exposed to 60 mM and 180 mM glutamate, respectively for 30 minutes prior to TRF and α-tocopherol treatments. After 30 minutes of glutamate treatment, cells were treated with 100, 200 and 300 ng/ml of TRF and α-tocopherol in the respective wells. Positive control (glutamate-injured cells) was treated with equal volume of absolute ethanol (0.1%, v/v) to replace TRF or α-tocopherol. Meanwhile, negative control (without any treatment) was added with equal volume of absolute ethanol (0.1%, v/v) while glutamate was replaced with PBS. Plate was then incubated in humidified incubator at 37°C, 5% CO_2 for another 24 hours and ready for cell viability assay and glutathione assay.

2.3.2 Co-culture

Astrocytes were seeded in 6 well culture inserts which were placed in an empty 6 well plate. Meanwhile, neurons were seeded in 6 well plate. Both cells were seeded with cell density of 1 × 10^6 cells per well. Both cell lines were then incubated in humidified incubator at 37°C, 5% CO_2 for 2 hours to allow cell attachment. After that, inserts containing astrocytes were transferred to the 6 well plate containing neurons. The cultures were left to adapt for 1 day prior to treatment. On the second day, TRF and α-tocopherol treatments were carried out as described earlier in section 2.3.1.

2.4 Determination of cell viability

MTT cell viability assay was carried out after 24 hours of TRF and α-tocopherol treatments. Culture medium in 6 well plate was removed followed by addition of 2 ml fresh medium. In co-culture model, inserts containing astrocytes were removed before removal of culture medium and addition of fresh medium. Then, 500 µl MTT (2 mg/ml) was added to each well followed by 4 hours incubation at 37°C with 5% CO_2. A total volume of 1 ml DMSO was then added to each well to dissolve the formazan formed. Plate was gently agitated for 5 minutes before being transferred to a sterile 96 well plate for optical density measurement. Negative controls were measured as 100% cell viability.

2.5 Glutathione assay

Cells were treated with TRF or α-tocopherol against glutamate toxicity in 6 well plate as described earlier in section 2.3.1 and section 2.3.2. After 24 hours of TRF and α-tocopherol treatments, plate was then ready for glutathione assay. The cell lines were washed twice in PBS followed by cell detachment with trypsin. Cells were then collected through centrifugation at 700 × g for 5 minutes at 4°C. The remaining procedure was performed according to the manufacturer’s instructions. Lastly, total glutathione was measured at wavelength of 450 nm with microplate reader (BioTex-ELx800, USA). Glutathione content in negative control samples was calculated as 100%.

2.7 Statistical analysis

Data were reported as mean ± SEM of 3 independent experiments. Comparisons between groups were made by using one way analysis of variance (ANOVA) post hoc analysis (SPSS 17.0). A p-value less than 0.05 was considered as statistically significant.

3. Results

3.1 Effects of TRF and α-tocopherol on glutamate treatment

The half maximal inhibitory concentration (IC_{50}) of glutamate for neurons and astrocytes were 80 mM and 230
mM, respectively based on the finding from dose response study (data not shown). In order to cause cell injury, \( \text{IC}_{20} \) of glutamate was used throughout this study. 60 mM glutamate was used to cause injury on neuron while 180 mM glutamate was used to cause injury on astrocyte.

3.2 Cell viability

![Graph A](image1.png)

**Figure 1.** Effects of TRF and α-tocopherol on neurons, (A) astrocytes, (B) neurons in co-culture model, (C) against glutamate toxicity in term of cell viability. Data are presented as mean ± SEM of 3 independent experiments (n = 3 in each experiment). \*\( p<0.05 \), compared with control group; \( \Delta p<0.05 \), TRF and α-tocopherol-treated group compared with glutamate-treated group; \#\( p<0.05 \), TRF-treated group compared with α-tocopherol-treated group.

At concentrations of 100 to 300 ng/ml, both TRF and α-tocopherol increased neuronal cell viability against glutamate toxicity in a dose-response manner (Figure 1A). Both TRF and α-tocopherol treatments at concentration of 300 ng/ml recovered approximately 10% of the neuronal cell viability upon glutamate treatment. On the other hand, both TRF and α-tocopherol treatments at concentration of 100 and 200 ng/ml recovered more than 10% of astrocyte cell viability from glutamate toxicity (Figure 1B). In contrast with neuron, only minor increment of astrocyte cell viability was observed in TRF treatment with concentration of 300 ng/ml. In the presence of astrocyte (co-culture), TRF and α-tocopherol further improved neuronal cell viability to nearly 100% cell viability from glutamate toxicity (Figure 1C). In terms of cell viability, TRF and α-tocopherol showed similar protecting effect in both cell lines against glutamate challenge.
3.3 Determination of cellular glutathione (GSH) level

**Figure 2.** Glutathione production (%) of glutamate-injured neurons, (A) astrocyte, (B) neurons in co-culture model, (C) upon TRF and α-tocopherol treatments. Data are presented as mean ± SEM of 3 independent experiments (n = 3 in each experiment). *p<0.05, compared with control group; Δp<0.05, TRF and α-tocopherol-treated group compared with glutamate-treated group; #p<0.05, TRF-treated group compared with α-tocopherol-treated group.

GSH concentration of both cell lines decreased at least 30% after 24 hours of glutamate challenge (Figure 2A and 2B). At concentrations of 100 to 300 ng/ml, TRF and α-tocopherol significantly restored glutathione content of neuron after glutamate challenge (Figure 2A). At concentration of 300 ng/ml, TRF completely restored glutathione content of glutamate-injured neuron. Similar finding was observed with α-tocopherol treatment at concentration of 200 ng/ml. However, α-tocopherol treatment at concentration of 300 ng/ml did not exert better effect in restoring glutathione content as compared to 200 ng/ml. In astrocyte, both TRF and α-tocopherol did not restore glutathione content upon glutamate challenge (Figure 2B). In co-culture model, TRF and α-tocopherol at concentration of 200 and 300 ng/ml further improved the glutathione content in neuron (Figure 2C). At concentration of 300 ng/ml, both TRF and α-tocopherol completely restored glutathione content in neuron in the presence of astrocyte.
4. Discussion

The model of glutamate-induced cell death had been widely used for the identification of agents that provide neuroprotective effect. In the present study, glutamate induced toxicity on neuron and astrocyte in a dose-dependent and time-dependent manner (data not shown). This study showed that 80 mM of glutamate was required to cause 50% of neuronal death which was similar to the study on SH-SY5Y neuronal cell, a subclone of SK-N-SH neuronal cells, against glutamate challenge for 24 hours. On the other hand, a much higher concentration of glutamate was required to cause toxicity in astrocyte as compared to neuronal cell. This demonstrated that astrocytes were more resistant to glutamate-induced toxicity when compared with neurons, which can be explained by the distribution of glutamate transporters in neurons and astrocytes. Excessive extracellular glutamate re-uptake is needed from synaptic cleft into neurons or astrocytes through glutamate transporter to prevent neurotoxicity. Previous study showed that astrocytic glutamate transporters (EAAT1 and EAAT2) are responsible for more than 80% of glutamate uptake in the brain through glutamate transporter to prevent neurotoxicity.

5. Conclusion

Neurons were more vulnerable to glutamate toxicity as compared to astrocyte. Both TRF and α-tocopherol at concentration of 100 to 300 ng/ml inhibited astrocyte and neuronal cell death against glutamate toxicity. Great scavenging effect of both TRF and α-tocopherol against glutamate toxicity was observed in neuron. Similar effect between TRF and α-tocopherol in recovering neurons and astrocytes from glutamate toxicity was observed. Co-culturing models have demonstrated that neuronal survival is dependent on astrocytes survival. In the presence of astrocytes, neurons were more resistant to glutamate toxicity.
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