RESEARCH ARTICLE

# Tocotrienol supplementations ameliorate oxidative stress in the thalamus of rats exposed to low-to-moderate doses of ethanol

Pitchaiah Dasari, Kaushik Raghavendra, Prasunpriya Nayak*


## ABSTRACT

*Background*: Functional deterioration of thalamic structures has been reported on many occasions of ethanol-induced neurodegeneration. The neuroprotective role of tocotrienol (T3) is well established along with its antioxidant property. The interaction of oral T3 supplementation modalities with the thalamic oxidative stress parameters was studied in low-to-moderate doses of ethanol (Et) exposures. *Materials and Methods*: Four phases of experiments were carried out with nil (Et-0) and three doses of Et exposures (Et-I, Et-II and Et-III) for 4 weeks. In each phase, 4 groups of Wistar rats were maintained with sham supplementation (NT3), prior supplementation (PT3), simultaneous supplementation (ST3) and total supplementation (TT3) with T3 for 6 weeks. Thalamic levels of reduced glutathione (GSH), oxidized glutathione (GSSH) and lipid peroxidation (LPO) were estimated. *Results*: Two-way ANOVA demonstrated that the thalamic GSH and LPO were significantly influenced by the modalities of T3 supplementation. However, low-to-moderate doses of Et exposures contributed significantly to alterations of only the LPO level of the thalamus. *Conclusion*: Out of the tested modalities, the best protection in terms of oxidative stress was observed when prior and simultaneous supplementation modalities were combined (TT3).


**Keywords:** Tocotrienol, Thalamus, Ethanol, Reduced glutathione, Oxidized glutathione, Lipid peroxidation.

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## Introduction

One of the main causes of morbidity and death is alcohol abuse.[1] Regardless of dosage, alcohol consumption is linked to a consistently elevated risk of stroke and hypertension.[2] On the other hand, research has also shown that moderate to low alcohol use has benefits in lowering the risk of cardiovascular illnesses[3,4] by lowering the activity of a stress-related brain network.[5] Although the results are conflicting, low to moderate alcohol intake has been linked, in addition to its effects on physical health, to the development of conditions like dementia and cognitive impairment that are closely linked to cardiovascular disorders.[1,6] While some studies have found no, very little, or even negative effects related to alcohol intake, others have demonstrated advantages to cognitive function associated with low to moderate alcohol consumption.[1,7,8]

Tocotrienols (T3) are the unsaturated isomers of tocopherols having isoprenoid sidechains.[9] Based on the presence of methyl group in different positions of the chromanol ring, there are four isomers of T3, namely α-T3, β-T3, γ-T3, and δ-T3.[10] Even though it is rare in nature, the experimentally observed superior antioxidative capacity has been assigned to its better distributions within the lipid layers of cell membranes.[9] It can protect the lipid-rich body from the damaging effects of oxidative stress, including the brain. Remarkably, T3 also provides neuroprotection, which might be supplementary to the defense against oxidative stress[11] and that to even at nanomolar concentrations.[12,13] On the other hand, excess and long-term supplementation of T3 may cause a build-up of itself and potentially may have pro-oxidant and even neurotoxic effects in vitro.[12,14]

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Thalamic dysfunction is linked to alcohol abuse.[15] The thalamic area is reported to be susceptible to neurodegenerative diseases[16] and oxidative stress.[17] While, the thalamus could maintain the level of lipid peroxidation during normal aging, enhanced lipid peroxidation was reported in response to degenerative threats like aluminum exposure.[18] Oxidative stress can suggestively damage the specific nuclei in the thalamus leading to failure in suppression of distracting information and attention deficit in individuals.[19] The importance of the thalamic region in oxidative stress and neurodegeneration was also demonstrated in a rat model of thiamine deficiency.[20] As the frequency of low-to-moderate alcohol consumption is very high globally,[2] evaluation of cognitive function is worthy and as the thalamus is an important area involved in alcohol-related neurobehavioral deterioration,[21,22] study with thalamus is worthy.

In this context, the thalamic levels of reduced glutathione and lipid peroxidation were measured in rats exposed to low-to-moderate doses of ethanol and supplemented with tocotrienol through different modalities.

## Materials And Methods

### Materials

Oryza tocotrienol©-90 was kindly donated by the Oryza Oil and Fat Chemical Co. Ltd, Japan. Other chemicals were of analytical grade and procured from reputed companies.

### Animal Maintenance and Treatment

The experimental protocol was approved by the Institutional Animal Ethics Committee. Male albino Wistar rats weighing 120-140 g were obtained from NCLAS, National Institute of Nutrition, Hyderabad, maintained and treated in the Central Animal House of NRI Medical College and General Hospital and the procedures were performed according to the guidelines of the Committee for Control and Supervision of Experiments on Animals (Lt. No. 43/Chairman-IAEC, NRI Medical College and GH, Chinakakani; Dated 16-06-2014). Figure 1 depicts the experimentation protocol used for the current study. All the experiments were carried out in four phases – Et-0, Et-I, Et-II and Et-III with 0, 0.2, 0.4 and 0.6 g Et exposure/kg body weight for four weeks, respectively. In each phase, after one week of acclimatization, rats were randomly divided (with the help of Random Allocation Software Version 1.0, May 2004) into four groups [NT3, PT3, ST3 and TT3] containing 6 animals each. In the NT3 group, animals received only sham supplementation. Animals of the PT3 group received T3 supplementation for 2 weeks prior to 4 weeks of Et exposure. Animals with T3 supplementation for 4 weeks during the Et exposure are assigned to the ST3 group. In the TT3 group, animals were supplemented with T3 for 6 weeks while exposed to Et for the last 4 weeks. Based on earlier results of varied doses and durations of T3 supplementation, 10 mg T3/day/rat for 4 weeks was used for the current study. Both Et exposures and T3 supplementations were carried out through oral feeding. Feeding of Et and distilled water was done in the morning session, while, feeding with T3 supplementation and sham feeding were done in the evening session daily for the whole 6 weeks.

### Isolation of Thalamus

Overnight fasted rats were sacrificed by cervical dislocation. The whole brain was removed and washed with ice-cold saline. Under the dissection microscope, the thalamic area was immediately separated, weighed and preserved in the ice chamber for biochemical processing.

### Biochemical Parameter

The thalamic areas were homogenized in ice-cold 0.1M phosphate buffer (pH 7.4), cold centrifuged at 1000 rpm for 5 minutes and the supernatants were used for the determination of biochemical parameters.[23] For the estimation of thalamic-reduced glutathione (GSH), equal volumes of homogenate were mixed with 4% (w/v) sulfosalicylic acid was immediately mixed. After shaking well, the mixtures were centrifuged at 3000 rpm for 10 min. For the estimation of thalamic oxidized glutathione (GSSG), the homogenates were treated with 2-vinyl pyridine and then incubated with glutathione reductase and NADPH. After the incubation, the reaction was stopped with sulfosalicylic acid and centrifuged. Supernatants are mixed with 5,5'-dithiobis 2-nitrobenzoic acid (0.1 mmol, in 0.01M phosphate buffer, pH 8.0). After 2 minutes, absorbances were recorded at 412 nm. The level of GSH was quantified with the help of a standard curve prepared with the same procedure as originally mentioned.[24]

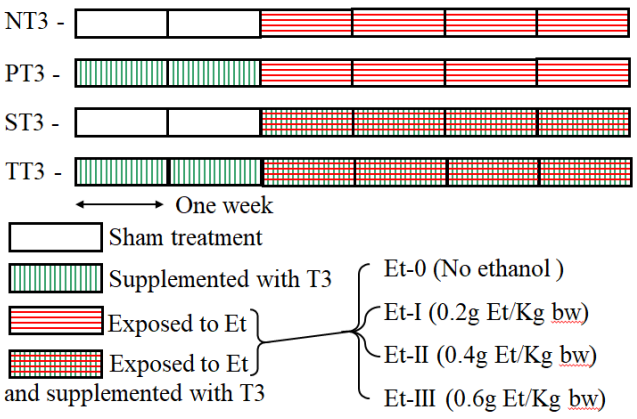


**Figure 1:** Protocol of experimentation with low-to-moderate doses of ethanol (Et) exposures and different modalities of tocotrienol (T3) supplementation. NT3 = Without T3 supplementation, PT3 = 2 weeks of T3 supplementation prior to Et exposure, ST3 = 4 weeks of T3 supplementation along with Et exposure, TT3 = 6 weeks of T3 supplementation starting from 2 weeks prior to Et exposure. Et-0 = Without Et exposure, Et-I, Et-II and Et-III = Et exposure at a dose of 0.2, 0.4 and 0.6 g/ kg body weight, respectively.

The level of lipid peroxidation was estimated by measuring the level of malonaldehyde or malondialdehyde (MDA) in samples with the help of thiobarbituric acid (TBA) as described elsewhere.[25] To 500µL of homogenate sample, 1 ml of 10% TCA was added followed by 2 ml of 0.67% TBA. The mixtures were heated in a water bath at 80°C (instead of 100°C to minimize the interference of some carbohydrates) for 15 min. After cooling and centrifugation at 3000 rpm for 10 min, the absorbances of the supernatants were read at 535 nm. A reagent blank was prepared following the same procedure using water instead of a sample. The extent of lipid peroxidation was expressed as nmol MDA formed in the processed sample using the molar extinction coefficient for MDA of 1.56 ×10 M-1 cm.[26]

### Statistical Analyses

Box and whisker plots have been used to present the data graphically showing the median value (bold horizontal line), interquartile range (boxes on either side of the line) along with range (dotted lines) and outliers (small circles), if any. After checking the normalcy of collected data, influences of the Et exposure and T3 supplementation were evaluated by two-way ANOVA. The differences between the groups were analyzed by Tukey's post-hoc test accepting the probability

of 5% or less as significant using PAST statistical software (ver. 3.12; Copyright: Ø. Hammer 1999-2016).[27]

## Results

The present study evaluated the impacts of low-to-moderate doses of Et exposure on GSH, GSSG, their ratio and LPO of thalamic areas in rat brains. As already reported, these ethanol exposures and varied modalities of T3 supplementation affected the changes in body weight in dose- and supplementation-specific ways.[11]

For the thalamic GSH content, the influence of T3 supplementation was statistically significant without any significant interaction with the low-to-moderate doses of Et exposures. Among the supplementation modalities, thalamic GSH contents of TT3 groups were significantly higher than the others during all four phases of the study (Figure 2A). Interestingly, both ST3 and TT3 modalities demonstrated significant differences from the other modalities of T3 supplementation. However, responses of ST3 and TT3 modalities were in opposite directions. In concurrence with this, comparable slopes of gradual increment in thalamic GSH contents were observed in all the Et exposure phases (Figure 2C). On the other hand, the pattern of changes in thalamic GSH content with a gradual rise in Et exposure level was specific to supplementation modality (Figure 2B).

On the other hand, no significant alteration in the level of GSSG has been noted in either of the T3 supplementation modalities as well as in any of the Et exposure doses (Figure 3). Notably, the differences between the modalities

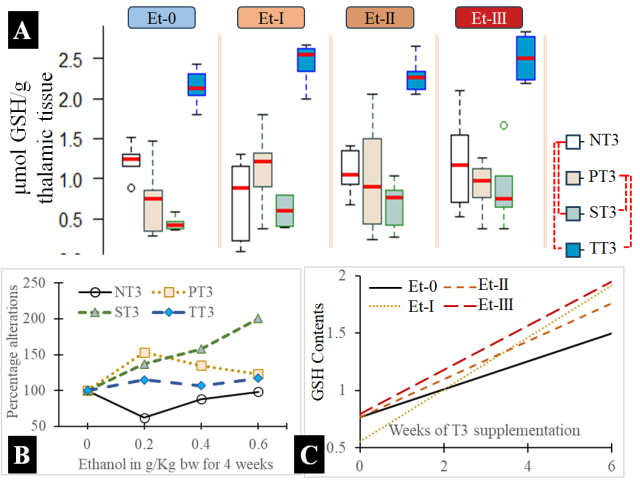


**Figure 2:** (A) Box and whisker plot of thalamic reduced glutathione (GSH) contents; Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Square brackets (red dotted lines) between the experimental study phases and supplemental modalities indicate significant differences between them ($p < 0.05$, Tukey's post-hoc test). (B) Line diagram showing percentage alterations of group means of thalamic GSH values plotted against doses of Et exposure. (C) Trend lines for cumulative GSH data (µmol GSH/g thalamic tissue) of each phase of the experiment are drawn against the duration of T3 supplementation.

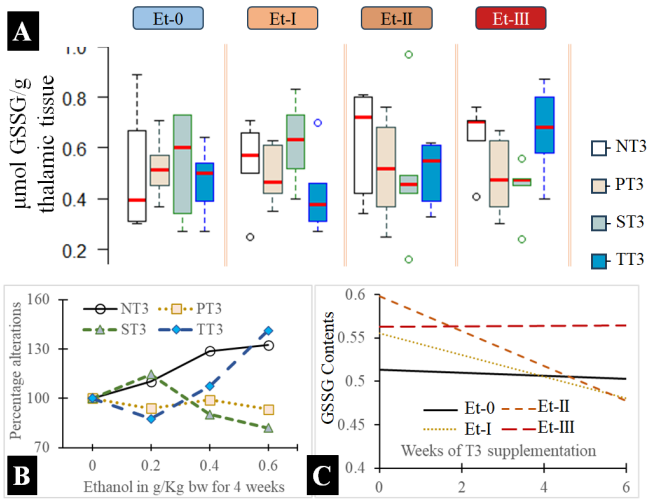


**Figure 3:** (A) Box and whisker plot of thalamic oxidized glutathione (GSSG) contents; Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Square brackets (red dotted lines) between the experimental study phases and supplemental modalities indicate significant differences between them ($p < 0.05$, Tukey's post-hoc test). (B) Line diagram showing percentage alterations of group means of thalamic GSSG values plotted against doses of Et exposure. (C) Trend lines for cumulative GSSG data (µmol GSSG/g thalamic tissue) of each phase of the experiment are drawn against the duration of T3 supplementation.

of T3 supplementation were getting larger with the increase in doses of Et exposures; however, NT3 and PT3 supplementations only demonstrated uniformity in changes (Figure 3B). On the other hand, the trends in GSSG contents in the thalamic area of ET-0 and ET-III groups were maintained throughout the six weeks of experimentations (Figure 3C).

Figure 4 depicts the alterations in the GSH/GSSG ratio in the thalamic tissues of studied experimental animals. Irrespective of the changes noted in Figures 2 and 3, the ratio gradually improved along the duration of the study protocol in all the Et exposure groups (Figure 4C). Without supplementation group, demonstrated nearly 50% reductions in all the doses of Et exposures, while maximal improvement in the ratio was noted in the TT3 group (Figure 4B). Though the influence of ethanol on the thalamic GSH/GSSG ratio was not significant, the influences of T3 and its interactions with Et were statistically significant. Accordingly, the TT3 supplementation group was significantly different from all the other T3 supplement groups (Figure 4A).

Influences of T3 supplementation and Et exposure were statistically significant in causing alterations in LPO of the thalamic areas of rats. Accordingly, thalamic LPO in the Et-0 phase was significantly different from the Et-II and Et-III, and Et-I was significantly different from Et-III. Considering all the phases of Et exposures, among the used modalities, ST3 and TT3 were significantly distinct from NT3 (Figure 5A). Interestingly, the PT3 modality was the highest sufferer when faced highest dose of Et exposure (Figure 5B). With

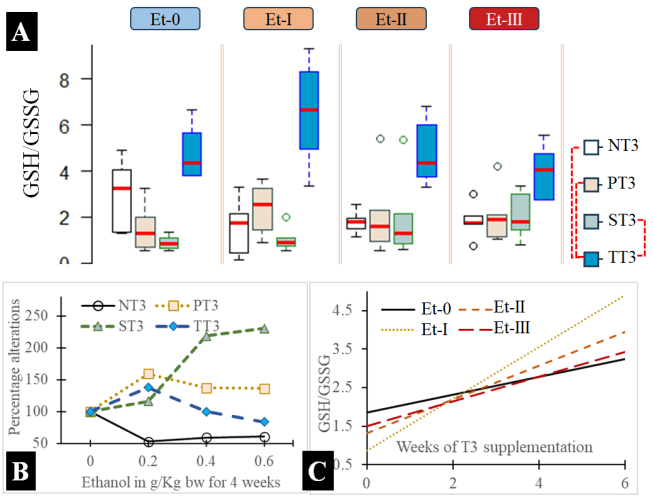


**Figure 4:** (A) Box and whisker plot of the ratio of thalamic reduced and oxidized glutathione levels (GSH/GSSG); Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Square brackets (red dotted lines) between the experimental study phases and supplemental modalities indicate significant differences between them ($p < 0.05$, Tukey's post-hoc test). (B) Line diagram showing percentage alterations of group means of thalamic GSH/GSSG values plotted against doses of Et exposure. (C) Trend lines for cumulative GSH/GSSH data of each phase of the experiment are drawn against the duration of T3 supplementation.

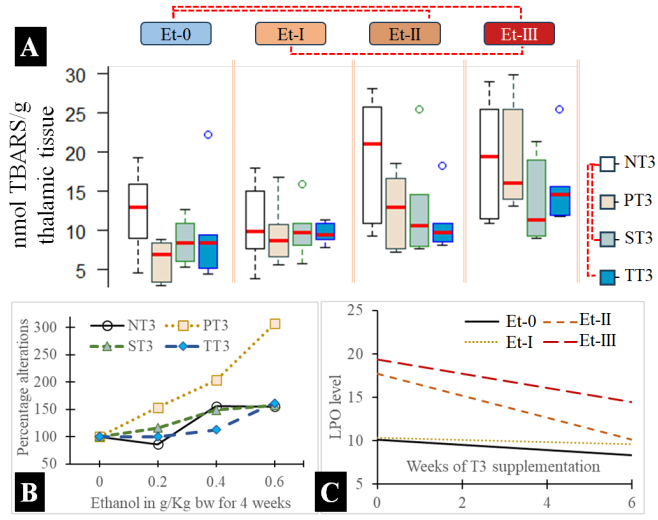


**Figure 5:** (A) Box and whisker plot of thalamic lipid peroxidation (LPO) levels; Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Square brackets (red dotted lines) between the experimental study phases and supplemental modalities indicate significant differences between them ($p < 0.05$, Tukey's post-hoc test). (B) Line diagram showing percentage alterations of group means of cerebellar LPO values plotted against doses of Et exposure. (C) Trend lines for cumulative LPO data (nmol TBARS/g thalamic tissue) of each phase of the experiment are drawn against the duration of T3 supplementation.

weeks of T3 supplementations, a decline in LPO levels were noticed in higher doses of Et exposure groups (Figure 5C); however, only nominal changes in LPO were noted in Et-0 and Et-I groups that had lower thalamic LPO level even without supplementation.

## Discussion

Consumption of alcohol is on the rise in India and some other Asian countries and the trend is likely to endure beyond 2030[28] Rises in disposable income[28] and social acceptance towards alcoholic beverages[11] refute the WHO mandate of a 10% reduction in harmful uses of alcohol by 2025. Therefore, this work assessed the effectiveness of different modalities of T3 supplementation against the thalamic oxidative stresses caused by low-to-moderate dosages of Et exposures. With low-to-moderate doses of ethanol intoxication, as in the case of social drinking, the blood level of alcohol reaches a range of 5-20 mM.[29] This amount of blood alcohol can reduce anxiety and may produce mild sedation. However, when the blood alcohol exceeds this level, it generally leads to wakeless sedation, cognitive disablement, uncoordinated movements and loss of memory.[29] With ethanol exposure, brain glucose metabolism is limited and it has to depend on the acetate for energy.[30] Restricted expression of monocarboxylic acid transporter in the thalamus, the availability of acetate is likely to be insufficient to the thalamus and eventually, it would face greater threats of degenerative changes.[30] The thalamus is involved in the sleep-wake cycle and cognitive functions,[31] these degenerative changes in the thalamus would likely cause more severe problems within a short time. Alcohol abuse is linked to malfunctioning of the thalamus[15] and the high possibility is that these changes start with oxidative stress.[3]

Appreciable quantitative measures of oxidative stress in tissue are the levels of LPO and GSH within that. A rise in LPO indicates an increase in oxidative stress level, whereas a fall in GSH contents also indicates the same. Even in the presence of a mechanism of replenishing the GSH, commonly GSH content is reduced in the tissue facing the oxidative stress. The level of GSSG and ratio GSH/GSSG indicate the strength of the replenishment machinery of the tissue. The current study demonstrated an increase in LPO thalamus area with the exposure to gradual higher doses of Et in the without supplementation groups of experimental animals (Figure 5B); whereas, all the tested modalities of T3 supplementation restricted that rise in thalamic LPO level (Figure 5B). Similarly, falls in GSH/GSSG ratio were noted in the case of without T3 supplementation group, which was compensated by almost all the modalities of T3 supplementation (Figure 4B). On the other hand, thalamic GSH content was maintained even with exposure to a moderate dose of Et exposure for 4 weeks even without T3 supplementation (Figure 2B) and along with an increase in thalamic GSSG content (Figure 3B). Alterations in GSH content in the thalamic area of experimental animals with low-to-moderate doses of Et exposure were reported

earlier (16,17). Additionally, the highest baseline GSH concentration and potentially higher GSH recycle rate in the thalamus of F344 rats have also been reported.[32]

Both Et exposure and T3 supplementation influenced the level of thalamic LPO in the present investigation. The changes in thalamic LPO levels in ST3 and TT3 supplementation modalities were comparable to those in the NT3 supplementation group that had no T3 supplementation. Thus, a considerable role of inherent cellular antioxidative mechanisms could be suggested for the observed resistance against the consequences of Et exposures in the thalamus. However, the mechanism was limited and able to withstand only the threats of low doses of Et exposure. With moderate doses of Et exposures, there was a significant elevation of the thalamic LPO levels (Figure 5A). In addition, cytochrome P450, an enzyme involved in ethanol catabolism is found to be inducible at the thalamus.[33] This report also supports the inherent Et handling capability of the thalamus. On the other hand, a very high level of increase in thalamic LPO in the PT3 group (Figure 5B) suggests that the pre-exposure supplementation modality possibly weakens the inherent oxidative stress handling capacity. By this, considering data presented for GSH, GSSG and their ratio in the thalamic area, upregulation of GSH machinery in the thalamus can be suggested in response to used grades of low-to-moderate Et exposure.

Therefore, the current investigation suggests that the thalamic area, a brain area of cognitive and behavioural importance, can withstand some degree of low-dose Et insult. However, with a moderate dose of Et exposure, the thalamic area is susceptible to oxidative stress. Supplementation with T3 is supportive of the inherent capability of the thalamus to withstand this oxidative stress. Nevertheless, the modality of T3 supplementation should be chosen carefully so that the inherent capability of the brain area is not disturbed.

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## PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.