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Original Article

Evaluation of Varied Modalities of Tocotrienol Supplementations to Counter the Cerebellar Oxidative Stress Caused by Low-to-moderate Doses of Ethanol in Rats

Pitchaiah Dasari, Prasunpriya Nayak*

ABSTRACT

Background and Aim: Self-intoxication with Ethanol (Et) is a common problem worldwide. Being a psychoactive drug, neurotoxic effects of Et are well known. Cerebellum is highly vulnerable to Et exposure. Tocotrienols (T3) are relatively rare components of vitamin E and have the potential to prevent the oxidative stress and act as neuroprotective. Varied modalities of T3 supplementations were evaluated to identify the possibilities of countering cerebellar oxidative stress caused by low-to-moderate doses of Et exposure. **Methods:** Four phase 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. Cerebellar levels of reduced Glutathione (GSH), Lipid Peroxidation (LPO) and activities of catalase, Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) and Glutathione Reductase (GR) were estimated and Superoxide and Peroxide Handling Capacities (SPHCs) were calculated. **Results:** All the tested cerebellar oxidative stress parameters and handling capacities were significantly influenced by the modalities of T3 supplementation. However, low-to-moderate doses of Et exposures contributed significantly in alterations of LPO level, GPx activity, GR activity and glutathione-dependent SPHC of cerebellum. **Conclusion:** Critical evaluation of studied parameters suggest insufficient overall performance of ST3 type of supplementation, whereas, TT3 type of supplementation was the best among the modalities of T3 supplementation. However, excess T3 supplementation may not be beneficial in some cases of oxidative stress parameters and SPHC of cerebellum.

Key words: Tocotrienol, Cerebellum, Ethanol, Oxidative stress, Superoxide and peroxide handling capacity (SPHC).

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INTRODUCTION

As estimated, India will possibly consume 6.83 billion liters of alcoholic beverages in the year 2020[1] which may leap into 16.8 billion liters by the year 2022.[2] With the rise in social-acceptance of alcohol intake, even in adolescents, the onset age of self-intoxication is coming down.[3] On the other hand, adolescents face relatively less motor impairments and this indulges them towards frequent self-intoxication and dependence on alcohol.[4] This, in turn, raises the overall years of alcohol consumption and changes the social binge drinkers into chronic drinkers. Not only India, being the most used psychoactive drug, alcohol abuse is a grave health problem worldwide and remains in top five causes of death in many countries.[5]

All the organs of our body, including brain, face detrimental effects of ethanol (Et) exposure. Cognitive impairment and regional brain damages were observed even in alcoholics without any overt sign of neurological problems.[5] Along with other brain regions, cerebellum faces neuronal loss in chronic Et exposure and this may have a relationship with loss of cognitive and motor functions.[4,6,7] In fact, compared to cerebral cortex, Et-induced apoptosis was higher in cerebellum.[8] Cerebellum, *per se*, is highly vulnerable to chronic Et exposure; nevertheless, the extent of damage depends on the profile of the subject as well as level of consumption.[9] To elucidate the region-specific response of brain to Et exposure, Dizon *et al.* reviewed articles showing toxic impact of Et in different brain areas.[10] They identified cerebellum as the most resistant organ during development, while same as most sensitive organ during adult life.[10] Alcoholic cerebellar degeneration is a neuropsychiatric disorder associated cerebellar injury because of chronic ethanolic insult to cerebellum. Cerebellar degenerations are frequently found in alcoholics, whether clinically appreciated or not.[11]

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The neuropathological alterations are attributed to structural or functional degeneration of Purkinje cells with special emphasis to thiamine deficiency.[11] There are reports supporting the Purkinje cell loss[12] and opposing that.[13] Similarly, reports of cerebellar atrophy without thiamine deficiency are also available.[14,15] Oxidative stress is a crucial component of alcohol-induced neurodegenerations.[16] Irrespective of age, oxidative stress can be a vital contributing mechanism for the Et-induced neurodegeneration in cerebellum.[17]

Tocotrienol (T3) improves systemic oxidative status and provides neuroprotection. However, these two properties are believed not to have a causal relationship. Even in nanomolar concentration,[18] T3 can counter oxidative stress and promotes cellular repair procedure.[19] With prolonged use, accumulation of T3 is possible and T3 may act as pro-oxidant and even neurotoxic *in vitro*.[19,20] Hence, the current study evaluates the efficacy of varied modalities of T3 supplementation against the cerebellar oxidative stress because of low-to-moderated doses of Et exposure and appraises the superoxide and peroxide handling capacities (SPHC) of cerebellum in such conditions.

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 the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India). 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 NT3 group, animals received only sham supplementation. Animals of 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 as ST3 group. In TT3 group, animals were supplemented with T3 for 6 weeks while exposed to Et for 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 whole 6 weeks.

Isolation of Cerebellum

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

Biochemical Parameter

The cerebella were homogenized in ice-cold 0.1M phosphate buffer (pH 7.4), cold centrifuged in 1000 rpm for 5 min and the supernatant used for the determination of biochemical parameters. All the biochemical parameters were estimated and calculated following the methods as described elsewhere.[21]

Statistical Analyses of Data

The weekly change in body weight was calculated as percentage alteration of mean weekly body weights of individual rats and are depicted as line diagram of the mean of six observations ± standard error of the mean. 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) or outliers (small circles), if any. 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).[22]

RESULTS

The detrimental effects of Et exposure are well known. Impacts of low-to-moderate doses of Et exposure significantly influenced the change in absolute body weight of adult rats during the 6 weeks of treatment protocol and the body weight changes in Et-III phase was statistically significant in comparison to Et-0 and Et-I phase (Figure 1B). Similarly, different modalities of T3 supplementation also influenced the changes in absolute body weights and all the T3 supplemented groups were significantly different from the sham-supplemented group (Figure 1B). However, of all the groups of all the phases, difference between the ST3 and NT3 groups of Et-0 phase only was statistically significant. Weekly changes in body weight were significantly influenced by the oral T3 supplementation modalities in all the phases of experimentation with nil and low-to-moderate doses of Et exposures (Figure 2). Predictably, the time of Et exposure along with initiation and duration of T3 supplementation effected the growth of the experimental rats with statistical significances. Moreover, the interactions between the T3 supplementation and modalities of supplementations were also contributed significantly in the observed change in growth rate. Except in ST3 of Et-II phase, all the tested T3 supplementation modalities of all the Et phases demonstrated significant differences in weekly body weight gains when compared to the sham supplementation group (Figure 2). Similarly,

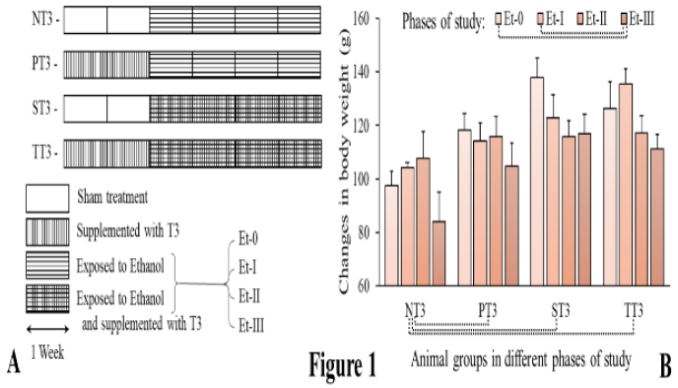

Figure 1

Figure 1: (A) Protocol of experimentation with different doses of Et exposure and different modalities of 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 bw, respectively. (B) Changes in absolute body weight during the 6 weeks of experimentation protocol. Dotted lines indicate significant differences ($p < 0.05$) between the groups or phase as per two-way ANOVA.

except Et-I phase, percentage changes in body weight during the 2nd week were significantly different from that of the week 0, in all the phases (Figure 2).

Current modalities of T3 supplementation significantly influenced the cerebellar GSH contents (Figure 3A). Interactions of T3 supplementation with low-to-moderate doses of Et exposures were also contributed significantly in the alterations of cerebellar GSH levels, though, only insignificant influences of Et exposure were noted for the same (Figure 3A). Indicating the importance of specific modality of T3 supplementation, cerebellar GSH contents of TT3 groups of rats were significantly different from the other modes of supplementation groups as well as no supplementation group. The mean cerebellar GSH contents of TT3 group were 58%, 97%, 48% and 77% higher in comparison to that of NT3 group of Et-0, Et-I, Et-II and Et-III phases, respectively; while, the PT3 (Et-I and Et-III) and ST3 (Et-0) groups demonstrated occasional improvement in cerebellar GSH contents only. On the other hand, the cerebellar GSH content of all the doses of Et exposure with T3 supplementation modality groups were maintained more or less similar to that of without Et exposure, except the ST3 supplementation groups (Figure 3B). Statistically, cerebellar GSH content of ST3 group of Et-0 phase was significantly higher than that of NT3 and PT3 groups of same phase; while it was higher than the ST3 groups of other Et phases. Cerebellar GSH contents of TT3 groups were higher in comparison to respective ST3 groups during Et-I, Et-II and Et-III phases; however, in Et-III phase the difference was without statistical significance. Nonetheless, all the Et phases demonstrated gradual rise in cerebellar GSH contents with increase in duration of T3 supplementation, irrespective of modality and the slope of increment in the Et-0 phase was distinctively higher than the other Et phases (Figure 3C).

Exposure to low-to-moderate doses of Et, employed modalities of T3 supplementations as well as their interactions were contributed significantly in alterations of levels of cerebellar LPO (Figure 4A). Statistical analyses identified Et-0 phase as significantly different from other Et phases, while ST3 group was significantly different from other T3 supplementation groups (Figure 4A). The levels of cerebellar LPO were significantly higher in ST3 groups of Et-I, Et-II and Et-III phases when compared with that of Et-0 phase. On the other hand, cerebellar LPO of PT3 and TT3 groups were significantly lower than that of ST3 group during Et-II phase of experimentation. In terms of percentage alterations of mean cerebellar LPO, NT3 group demonstrated almost gradual increase in it with the rise in doses of Et exposure. Similar dose-dependent response was not seen in any T3 supplementation groups (Figure 4B).

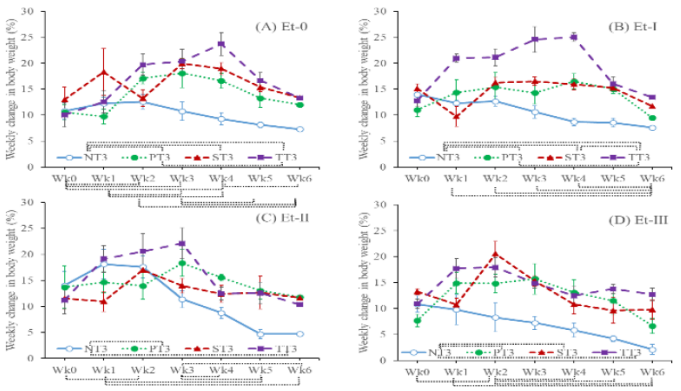

Figure 2: Weekly changes in body weight percentage compared to the initial body weight during different phases of experimentation. Dotted lines indicate significant differences ($p < 0.05$) between the groups or weeks as per two-way ANOVA.

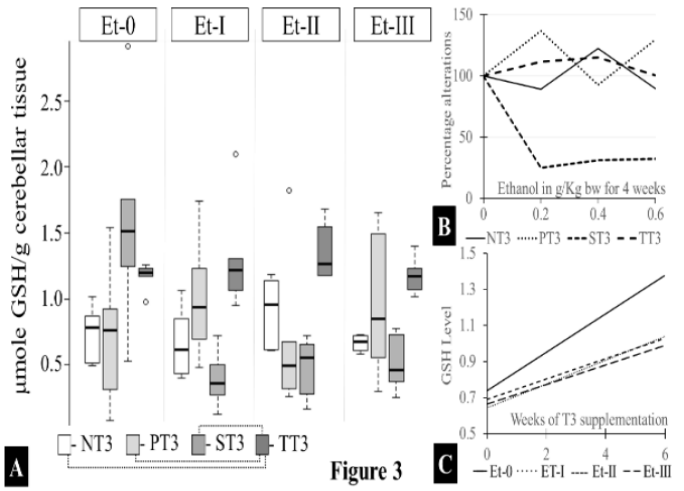

Figure 3: (A) Box and whisker plot of cerebellar reduced glutathione (GSH) levels; Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Dotted lines between the groups or phases indicate significant ($p < 0.05$) difference between them as per two-way ANOVA. (B) Line diagram showing percentage alterations of group means of cerebellar GSH values plotted against doses of Et exposure. (C) Trend-lines for cumulative data of each phase of experiment are drawn against duration of T3 supplementation.

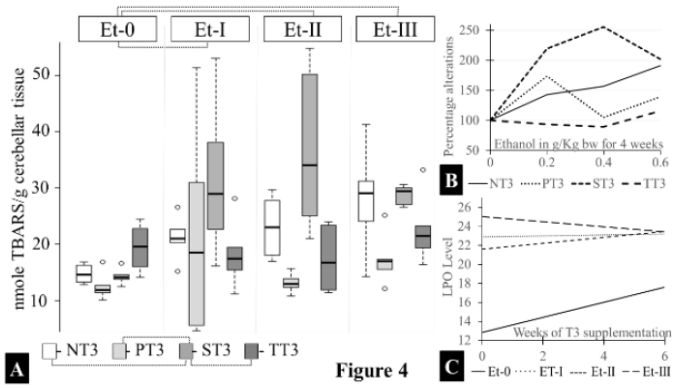

Figure 4: (A) Box and whisker plot of cerebellar lipid peroxidation (LPO) levels; Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Dotted lines between the groups or phases indicate significant ($p < 0.05$) difference between them as per two-way ANOVA. (B) Line diagram showing percentage alterations of group means of cerebellar LPO values plotted against doses of Et exposure. (C) Trend-lines for cumulative data of each phase of experiment are drawn against duration of T3 supplementation.

Trend-line for the collective data for all the T3 supplementation group and sham supplementation group depicted a gradual rise in cerebellar LPO as the duration of T3 supplementation increased during Et-0 phase (Figure 4C). Interestingly, slopes for cerebellar LPO trend-lines during other Et phases were close to 0 and comparable to each other, however, they crossed the vertical axis at substantially higher levels compared to Et-0 trend-line (Figure 4C).

Neither low-to-moderate doses of Et exposure nor their interactions with T3 supplementation modalities influenced the cerebellar catalase activities in the present study. Accordingly, none of the study groups differed significantly in between the Et exposure phases of experimentation.

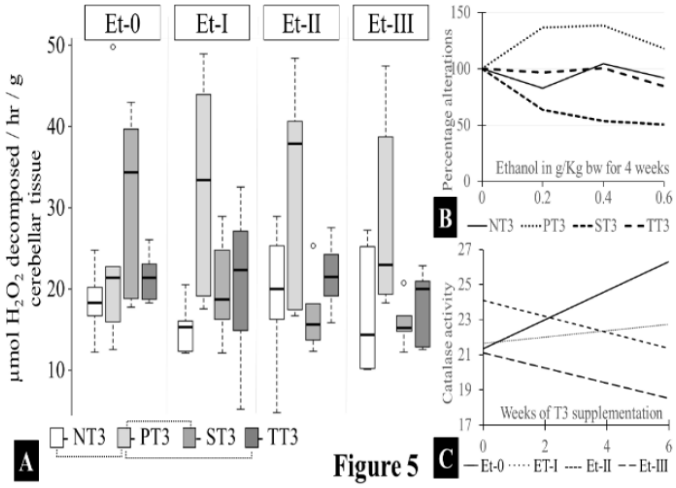

Figure 5: (A) Box and whisker plot of cerebellar catalase activities; Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Dotted lines between the groups or phases indicate significant ($p < 0.05$) difference between them as per two-way ANOVA. (B) Line diagram showing percentage alterations of group means of cerebellar catalase activities plotted against doses of Et exposure. (C) Trend-lines for cumulative data of each phase of experiment are drawn against duration of T3 supplementation.

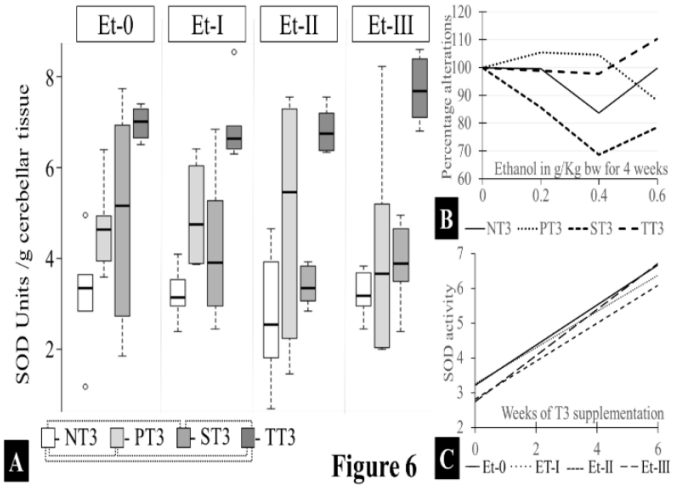

Figure 6: (A) Box and whisker plot of cerebellar superoxide dismutase (SOD) activities; Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Dotted lines between the groups or phases indicate significant ($p < 0.05$) difference between them as per two-way ANOVA. (B) Line diagram showing percentage alterations of group means of cerebellar SOD activities plotted against doses of Et exposure. (C) Trend-lines for cumulative data of each phase of experiment are drawn against duration of T3 supplementation.

Only the cerebellar catalase activity of PT3 group was raised significantly in comparison to that of NT3 and ST3 groups within the Et-I phase of study. On the other hand, T3 supplementation contributed significantly in the alterations of cerebellar catalase activities, which was significantly different in PT3 group in comparison to any of the other T3 supplementation groups (Figure 5A). During Et-I, Et-II and Et-III phases of study, mean cerebellar catalase activities were higher in PT3 group, lower in ST3 group while similar in NT3 and TT3 groups in comparison to that of Et-0 (Figure 5B). The trend-lines for Et phase of study indicated variable responses of cerebellar catalase activities in response to gradual increase in duration of T3 supplementation. During Et-0 phase of study, longer the duration of T3 supplementation, higher was the cerebellar activities. While, cerebellar catalase activities of Et-II and Et-III phases were gradually reduced with increase in duration of T3 supplementation (Figure 5C).

Cerebellar SOD activities were significantly influenced by the T3 supplementation modalities but not by the low-to-moderate doses of Et exposure. Accordingly, cerebellar SOD activities of T3 supplementation groups – PT3, ST3 and TT3 were significantly different from sham supplementation group, NT3 (Figure 6A). In addition, TT3 group was significantly different from the PT3 and ST3 groups, in terms of cerebellar SOD activities (Figure 6A). Statistically significant differences in cerebellar SOD activities between NT3 and TT3 groups were noticed in all the Et phases of experimentation. Similarly, the cerebellar SOD activities of TT3 groups were higher in comparison to respective ST3 groups also in Et-I, Et-II and Et-III phases of study. Additionally, the difference between PT3 and TT3 groups for cerebellar SOD activity was also statistically significant. When percentage alterations of mean cerebellar activities of Et-exposed groups were compared with Et-unexposed only ST3 group demonstrated substantial alterations (Figure 6B). Notably, alterations at highest used dose of Et exposure were different from the alterations at lower doses of Et exposures for all the T3 supplementation modalities (Figure 6B). On the other hand, all the phases of experimentation documented a very similar type of alterations in trend-lines of cerebellar SOD activities in response to increased duration T3 supplementation. Irrespective of doses of Et exposure or T3 supplementation modalities, approximating positive slope for trend-lines of cerebellar SOD activities were noted (Figure 6C).

Changes in cerebellar GPx activities during Et-II and Et-III phases of study were significantly different from that of the Et-0 phase of study (Figure 7A). Statistically significant differences were also observed between each other of NT3, ST3 and TT3 groups in terms of cerebellar GPx activities (Figure 7B). Hence, the cerebellar GPx activities were significantly influenced by the low-to-moderate doses of Et exposure as well as by the modalities of T3 supplementations. The interaction between them also contributed significantly in the alterations of cerebellar GPx activities during the present study. Post-hoc analyses found statistically significant rise in cerebellar GPx activities in ST3 group of Et-III phase study compared to ST3 groups of Et-0 and Et-I phases of study. Both ST3 and TT3 groups demonstrated significantly higher cerebellar GPx activities compared to both NT3 and PT3 groups during Et-III phase of study. Percentage alterations in mean cerebellar GPx activities of NT3 group indicated more or less unaltered operations of GPx in all the levels of Et exposure (Figure 7B). In PT3 group, the GPx activity was slightly decreased only in highest dose of Et exposure (Figure 7B). Both ST3 and TT3 groups demonstrated rise in GPx activities in all the Et doses, however, the increases were higher in the ST3 group (Figure 7B). The trend-lines for cumulative cerebellar GPx activity data of different Et phase demonstrated increasing trends during Et-0 and Et-III phases and decreasing trends during Et-I and Et-II phases of study (Figure 7C).

Statistically significant contributions of Et exposures and T3 supplementation modalities were observed in case of cerebellar GR activities. However, their interactions did not influence the cerebellar GR activities significantly. Among the Et phases, only Et-I phase was significantly different from that Et-0 phase in terms of cerebellar GR activities. When the T3 supplementation modalities were considered, cerebellar GR activities of NT3 group were significantly different from that of ST3, PT3 and TT3 groups (Figure 8A). During comparison between the T3 supplementation groups, only Et-I phase of study demonstrated differ-

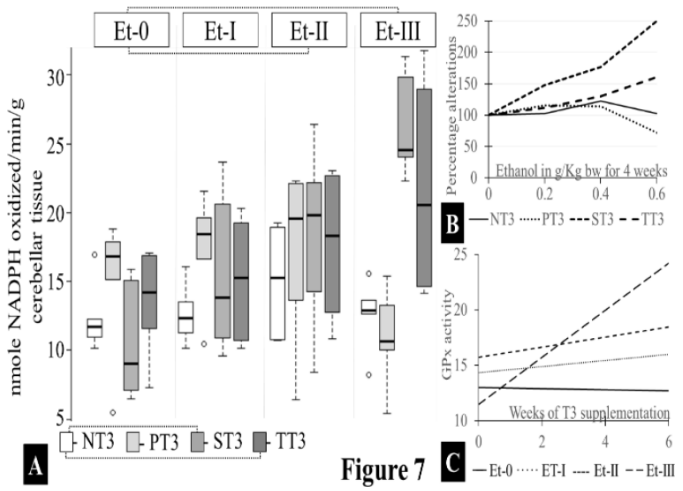

Figure 7: (A) Box and whisker plot of cerebellar glutathione peroxidase (GPx) activities; Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Dotted lines between the groups or phases indicate significant ($p < 0.05$) difference between them as per two-way ANOVA. (B) Line diagram showing percentage alterations of group means of cerebellar GPx activities plotted against doses of Et exposure. (C) Trend-lines for cumulative data of each phase of experiment are drawn against duration of T3 supplementation.

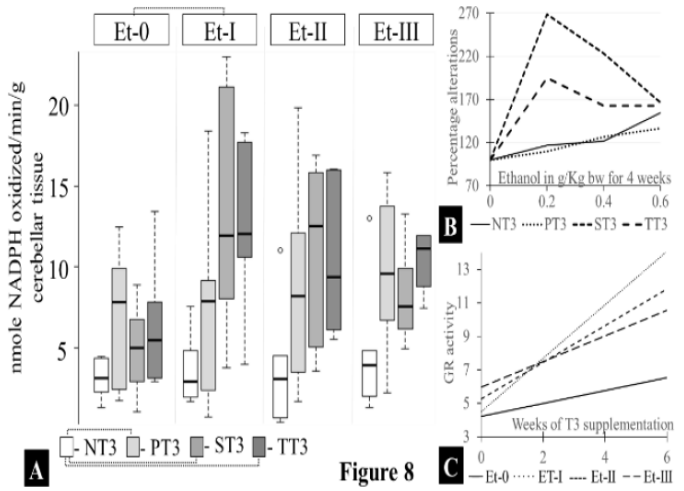

Figure 8: (A) Box and whisker plot of cerebellar glutathione reductase (GR) activities; Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Dotted lines between the groups or phases indicate significant ($p < 0.05$) difference between them as per two-way ANOVA. (B) Line diagram showing percentage alterations of group means of cerebellar GR activities plotted against doses of Et exposure. (C) Trend-lines for cumulative data of each phase of experiment are drawn against duration of T3 supplementation.

ences between NT3 - ST3 groups and NT3 –TT3 groups as statistically significant. Mean cerebellar GR activities of all the T3 supplementation groups were raised with the increase in doses of Et exposure; however, ST3 and TT3 groups demonstrated declines in increments of cerebellar GR activities with the moderately higher doses of Et exposure (Figure 8B). Trends in cumulative data distribution suggested increments in cerebellar GR activities with the increase in duration of T3 supplementation in all the Et phases of experimentations. The slope for the Et-0 trend-line was the lowest while that gradually decreased as Et-I > Et-II > Et-III (Figure 8C).

Statistical analyses of the current data demonstrated a significant influence of modalities of T3 supplementation on the cerebellar GI-SPHC. The doses of Et exposure or their interactions with T3 supplementation could not influence the cerebellar GI-SPHC significantly. Consequently, post-hoc analyses found the differences in cerebellar GI-SPHC in between NT3-TT3 and PT3-TT3 groups were statistically significant (Figure 9A). Mean cerebellar GI-SPHC in response to different doses of Et exposures were found to be comparable for NT3 and TT3 groups with relatively wider variations in NT3 group. Raised cerebellar GI-SPHC was noted during all the doses of Et exposures in case of PT3 group, while, lowered cerebellar GI-SPHC was noted during all the doses of Et exposures in case of ST3 group (Figure 9B). Trend-lines for the collective data of each Et phase demonstrated gradual decreases in cerebellar GI-SPHC with the increase in duration of T3 supplementation (Figure 9C). While the decreasing tendencies for each Et phase were comparable, it was highest during Et-II phase of experiments (Figure 9C).

Both doses of Et exposures and modalities of T3 supplementation contributed significantly in the observed alterations in cerebellar GD-SPHC; however, without significant interaction between themselves. Phase-wise comparison found the difference between cerebellar GD-SPHC of Et-0 and Et-II phases of study to be statistically significant (Figure 10A). Similarly, the NT3 and ST3 groups were statistically different from the TT3 groups in terms of their cerebellar GD-SPHC (Figure 10A). The differences between GD-SPHC of NT3 and TT3 groups as well as that of between ST3 and TT3 groups were statistically significant. Variations

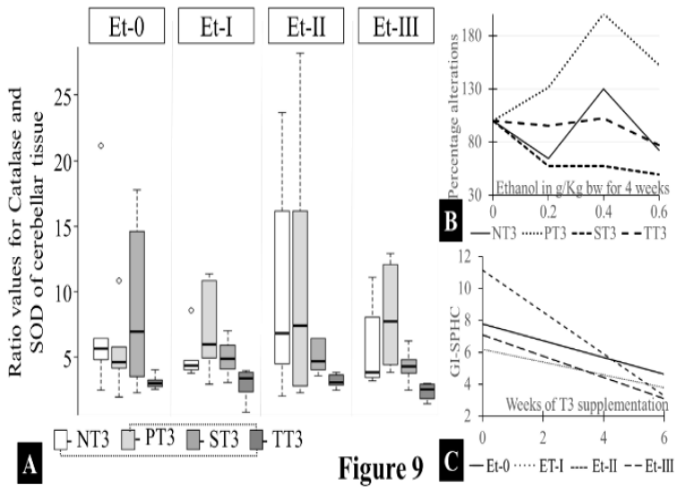

Figure 9: (A) Box and whisker plot of cerebellar glutathione-independent superoxide and peroxide handling capacity (GISPHC); Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Dotted lines between the groups or phases indicate significant ($p < 0.05$) difference between them as per two-way ANOVA. (B) Line diagram showing percentage alterations of group means of cerebellar GISPHC plotted against doses of Et exposure. (C) Trend-lines for cumulative data of each phase of experiment are drawn against duration of T3 supplementation.

in mean cerebellar GD-SPHC during phases of experimentation with gradual increase in Et doses were comparable for all the T3 supplementation modalities, except ST3, where cerebellar GD-SPHC was gradually increased with increasing doses of Et exposures (Figure 10B). On the other hand, with gradual increase in T3 supplementation duration, the trend-lines for cerebellar GD-SPHC demonstrated negative slopes of NT3, PT3 and ST3 (Figure 10C).

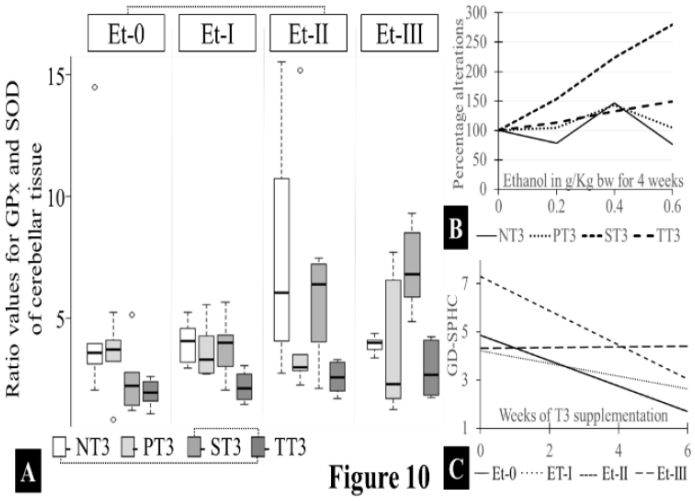

Figure 10: (A) Box and whisker plot of cerebellar glutathione-dependent superoxide and peroxide handling capacity (GDSPHC); Et-0, Et-I, Et-II and Et-III indicate the phases of experimentation and NT3, PT3, ST3 and TT3 are the T3 supplementation groups (as described in Animal maintenance and treatment section of Materials and Methods). Dotted lines between the groups or phases indicate significant ($p < 0.05$) difference between them as per two-way ANOVA. (B) Line diagram showing percentage alterations of group means of cerebellar GDSPHC plotted against doses of Et exposure. (C) Trend-lines for cumulative data of each phase of experiment are drawn against duration of T3 supplementation.

DISCUSSION

The cerebellum is one of the common targets of Et-induced neurodegeneration, though, the mechanism is not established yet.[11] Cerebellum plays important roles in planning and coordination of motor activities. It is also involved in fine adjustment of movements and motor memory. Maintenance of balance and skilled movements are likely to be compromised with damages in cerebellar functions. In the current study, different modalities of T3 supplementation were compared in terms of the oxidative stress faced by cerebellum and changes in its handling capacities of oxidative stress in rats when they were challenged with low-to-moderate doses of Et exposures.

When the overall changes in body weights were compared, all the groups of rats demonstrated only insignificant changes, except the positive effect of T3 in the Et-0 phase. This was in accordance with our earlier observation where the same dose of T3 showed significant changes in body weight.[23] The percentage changes in body weight, in the current study, were also influenced significantly by the Et exposures in all the groups except PT3. Therefore, even in the absence of overall change in absolute body weight, the currently used low-to-moderate doses of Et exposure was sufficient to contribute significantly in the slowing of body weight gain. Han *et al*. also recorded specific changes in body weight gain with 2g/kg bw/day Et exposure for 7 days.[24] On the other hand, Abulaiti *et al*. noticed only insignificant changes in body weight with 5% Et exposure of 8 weeks in comparison to pair-fed control rats.[11] Therefore, the change in body weight was likely to be due to change in food or calorie intake. No significant change in food intake was observed in either of the groups in the present study (data not shown) in corroboration with earlier report.[16] In addition, significant effects of T3 supplementation modalities on the weekly changes in body weight percentage was observed. Decreased volume of cerebellar vermis white matter was reported in alcoholics.[7] In corroboration to that, change in structure and size of the cerebellum were noticed in an experimental study with Et exposure of 6.5 g/Kg bw/day for 55 days to adolescent female rats; however, without any alteration in cerebellar mass.[4] No change in absolute cerebellar mass or cerebello-somatic index (absolute cerebellum weight / body weight) was also observed in the current study (data not shown). Even in chronic exposure, Et was ineffective to alter the brain weight while body weights were significantly less compared to control animals.[25]

Comparison of Et-induced neuronal damage in different brain regions were reviewed in Nayak *et al*.[26] which indicated the possibility of neurodegenerative impact on cerebellum even in moderate doses of Et exposure and demonstrated the significant alterations in cerebellar oxidative stress parameters with high dose of Et exposure. In the current study, only insignificant influences of Et exposure were observed in cerebellar GSH content, SOD activity and catalase activity. However, level of LPO, activities of GPx and GR and both handling capacities were significantly influenced by the current low-to-moderate doses of Et exposure for 4 weeks. Many direct or indirect pathways could be involved in the production of oxidative stress in brain.[27,28] Oxidant imbalance associated with Et exposure was observed in cerebellum[16,29] and the damages were noticed in Purkinje cells[14,30] as well as granule neurons[14,3] in response to Et exposure in both experimental[32] and clinical setup.[15,33] In addition to oxidative stress, cerebellar excytotoxicity, inflammation as well as apoptosis of cerebellar neurons were reported in response to Et exposure.[4] In this context, supplementation with T3 would have been an ideal curative measure as antioxidant, anti-inflammatory and neuroprotective roles of it are well known.[34] While, T3 was found to be useful against oxytosis, the neuroprotective efficacy was observed only when administered before predictable cerebral ischemia.[35] Therefore, the supplementation with T3 was provided in 3 different modalities – (a) for 2 weeks prior to the Et exposure, (b) along with the Et exposure for 4 weeks and (c) starting from 2 weeks prior while continued for 4 weeks along with the Et exposure.

A quantitative measure of oxidative stress is the level of LPO and GSH. Rise in LPO indicates increased oxidative stress level. Moreover, the severity of LPO can also be assessed by measuring the level of GSH.[36] In a study with 10%, 20% and 35% of Et exposure for 20 days, statistically significant decreases in cerebellar GSH were found in all the groups; however, there was no dose dependency.[37] Facing the oxidative stress, generally GSH content decreases. Even after citing references for Et-induced decreases, Smith *et al*. themselves found increase in cerebellar GSH content with prenatal exposure to Et and suggested the role of unique compensatory mechanisms.[36] Therefore, the regional level of GSH depends on the status of oxidative stress, antioxidant status, activities of enzymes involved in turnover of GSH and some local factors. In the present study, low-to-moderate doses of Et did not influence the cerebellar GSH content; while their interactions with the presence and modalities of T3 supplementation essentially influenced the same. Maintenance of systemic, organ or regional GSH level by vitamin E, TRF or T3 in presence of oxidative stressor had been reported times and again.[23,32,37-42] Increases in cerebellar GSH level were noticed in the ST3 group (statistically significant) and TT3 group (statistically significant) in comparison to NT3 and PT3 groups of Et-0 phase of current study. However, in presence of Et exposures, TT3 group abled to maintain the level of cerebellar GSH while ST3 group failed to do so. This observation indicated the importance of supplementation timing in maintenance of cerebellar GSH level or oxidant status. It is worthy to mention here that Smith *et al*. noticed increased level of cerebellar GSH and LPO in neonates and highlighted the mutual interplays between components involved in intracellular and extracellular oxidant status within cerebellum.[36]

Significant increases in local GSH levels between the T3 supplementation groups within Et exposure phases were associated with significant enhancements in cerebellar LPO levels. Similar reciprocal relationships between cerebellar GSH and LPO levels in response to varied doses of

Et exposure were reported earlier.[26,37] Increased production of hydroxyl radical and levels of lipid peroxidation were observed in Et-exposed cerebellar granule cell *in vitro*,[31] indicating direct contribution of Et exposure on the oxidative stress. Interestingly, almost a dose-dependent increase in cerebellar LPO levels was observed in NT3 group of the current study, however, without no substantial alteration in cerebellar GSH level. Therefore, the cerebellar peroxide formation should not be assessed as only direct effects of Et exposure on the oxidative status of cerebellum. Citing references, de Freitas *et al*.[16] suggested that Et could also indirectly enhance the oxidative stress in cerebellum by increasing the iron load for it, which in turn convert the superoxide and H_2O_2 into more potent oxidant like hydroxyl radical. Thus, cerebellar neurons are highly susceptible to LPO caused by Et exposure and accordingly, all the doses of Et exposures in the current study were significantly different from the Et-0 phase of study. On the other hand, Et-0 animals indicated an increasing trend of cerebellar LPO levels with the lengthening of duration of T3 supplementation. The cause of such change in cerebellar LPO level was not clear; however, with TRF supplementation, increased level of hepatic LPO was also observed in nude mice xenografted with SW620 cells.[43]

Two distinct but closely related functions of catalase was imperative in the current context. Together with SOD, it forms a part of the superoxide and peroxide handling capacity (SPHC) that are produced because of Et-induced oxidative stress. At the same time, involvement of catalase in conversion of Et into acetaldehyde.[44] Acetaldehyde could be produced from Et by – (a) catalase mediated pathway at the peroxisomes, (b) cytochrome P4502E1 mediated pathway at the microsomes and (c) alcohol dehydrogenase at the cytosol.[28,45] Catalase needs H_2O_2 for this activity whereas H_2O_2 itself is a substrate for catalase.[28,46] Conversion of H_2O_2 into water as well as Et into acetaldehyde are the functions of peroxisomal catalase and nearly half of the brain Et load is metabolized by it.[6] Additionally, involvement of catalase in production of H_2O_2 during Et metabolism in combination with xanthine oxidase and NADPH oxidase was also reported.[47] Therefore, the catalesmic metabolism plays pivotal role in Et oxidation in brain[48] and H_2O_2 could be a central regulating factor for catalase mediated Et metabolism. Presence of catalase was found in both neuronal and glial cells, however, the neuronal content was much greater.[48] While comparing the oxidative stress effect of Et on brain regions, Han *et al*. found most severe changes to occur in cerebellum.[24] Intraperitoneal administration of Et (2g/kg bw/day) for 7 days caused increase in LPO and decreases in catalase and SOD activities.[24] However, no significant influences of low-to-moderate doses of Et exposure on the cerebellar catalase and SOD activities was observed in the current study. Even, the interactions with modalities of T3 supplementation were also statistically insignificant for both these cerebellar enzymes. Accordingly, unaltered activities of cerebellar catalase were observed in both sham supplemented (NT3) and total supplemented (TT3) groups across the Et phases of the study. However, when the supplementation was not uniform during the study period, as in case of PT3 and ST3 groups, the cerebellar catalase activities behaved separately across the Et phase of the study. On the other hand, differential responses of cerebellar catalase activities towards low-to-moderate doses of Et exposure were also observed. In absence of Et insult, there was a tendency to enhance the cerebellar catalase activity with the increase in duration of T3 supplementation, while no change in the same with lowest dose of Et exposure. Similarly, dose-dependent increase in liver catalase activity of nude male mice harvested with SW620 cells.[43] Of note, this dose-dependent response of liver catalase was not observed in female mice with same treatment.[43] Contrary to these, with moderate doses of Et exposure (Et-II and Et-III), cerebellar catalase activities inclined to decrease with longer duration of T3 supplementation. Supplementation with T3 or TRF increased the activities of catalase in erythrocytes of transgenic AD mice,[49] in asthmatic lungs but not in normal lungs.[50] Nonetheless, the dietary T3 supplementation could not affect the expression of catalase in aged mice.[51] Similarly, in a study with 3 months and 8 months of TRF supplementation, no alteration in brain catalase was observed.[20] Considering the crucial role of catalase in Et metabolism and generation of oxidative stress and with the report of highest level of catalase mRNA expression cerebellum of rat,[44] detailed study of catalase with different levels of Et exposure and antioxidant supplementation is required.

Keeping the SOD expression unaltered, pretreatment with T3 increased the gastric mucosal SOD activity in response to stress.[52] In the same line, T3 treatment improved the SOD activity of house dust mite challenged lung along with increments in expression of all the three isoforms – CuZn SOD, Mn SOD and extracellular SOD; however, without any enhancement in SOD activity of unchallenged lungs.[50] On the contrary, only insignificant increase in SOD2 was observed in T3 supplemented aged mice even with increased T3 bioavailability by γ-cyclodextrin.[51] Collagen-induced loss of serum SOD activity was also prevented by T3.[40] An increasing trend of cerebellar SOD activity with longer duration of T3 supplementation was observed in all phases of the current study. These contrasting responses of catalase and SOD of cerebellum might have detrimental effects on handling the oxidative stress in cerebellum. Higher, cerebellar SOD activity surely benefitted cerebellum in removing the superoxide radicals but in expense of production of more H_2O_2. While, compromised cerebellar catalase activity might have failed to neutralize it and allowed to create scope for more oxidative stress. Both 3 months and 8 months of TRF supplementation increased the brain SOD activity of aged rats without much difference between the increments done by these supplementation schedules.[20] They also noticed differential response of catalase and SOD activities in response to TRF supplementation. In the present study, cerebellar SOD activity of TT3 group was significantly raised in comparison to the NT3 group in all the Et phases. In addition, other modalities of T3 supplementation also demonstrated significant differences with NT3 and TT3. In contrast, with higher doses of Et exposure, the cerebellar SOD activities were found to be somewhat decreased in all the Et phases. Like that of cerebellar catalase, SOD activities were also not significantly influenced by the Et exposures. Observation that the cerebellar SOD activity of TT3 group was significantly different from NT3 group in Et-0 phase, from NT3 and ST3 groups in Et-I and Et-II phases and from NT3, PT3 and ST3 groups in Et-III phase indicated importance of the dose of Et exposure in cerebellar SOD responses towards different modalities of T3 supplementation.

Only insignificant alterations in catalase, SOD and GPx activities of cerebrum and hippocampus of aged rats were observed in response to dietary T3 supplementation for 3 weeks.[53] However, aged rats fed with TRF for 8 months demonstrated significant increase in brain GPx activity, while the same study of 3 months duration failed to note any increment in brain GPx activity.[20] Therefore, duration of the T3 supplementation could be crucial for influencing the GPx activity. Corroborating this notion, an inclination towards increasing cerebellar GPx activity with the increase in duration of T3 supplementation was observed in the Et-0 phase of the current study. A similar trend in cerebellar GPx activity was observed in the Et-III phase of the study while opposite trends for the same were observed in Et-I and Et-II phases of study. However, alterations in cerebellar GPx activities during both Et-II and Et-III phases of the study were significantly different from that of Et-0 phase in the present experimentation. In studies with GPx of erythrocytes,[49] lungs,[50] liver,[24,54] T3 supplementation was ineffective in alteration of GPx activity. Besides, T3 supplementation with enhanced bioavailability did not find any alteration in brain GPx expression.[51] Different modalities

of T3 supplementation also did not cause any alterations in cerebellar GPx activity of rats without Et exposure. However, interaction between low-to-moderate doses of Et exposures and different modalities of T3 supplementation contributed significantly in the observed alterations of cerebellar GPx activity. Therefore, cerebellar GPx appeared to be highly sensitive to oxidative stress produced by Et exposure as well as mitigation of that by T3 in modality-specific way.

Alterations in the enzyme activities involved in combating oxidative stress could be possible by T3 supplementation. Administration of T3 in the form of tocomin (10 mg /day) protected the catalase, SOD, GPx along with GR in liver and kidneys from stress induced decrements in hamster.[55] However, the impact of T3 supplementation on those renal and hepatic enzymes of unstressed animals were not mentioned. When *in silico* interactions of these enzyme proteins with T3 were compared, GR demonstrated highest binding energy and lowest inhibition constant.[55] Therefore, the interaction of GR with T3 was better than that of catalase, SOD and GPx. Nevertheless, no effect of T3 supplementation was noticed on liver GR even after 9 months of supplementation.[54] On the other hand, 8 weeks of Et exposure at a dose of 3 g/kg body weight did not produce any significant alteration in brain GR activity.[56] However, Et exposure (2 g/Kg bw) for 2 months significantly reduced the GR activities in myocardial tissue of young and old rats.[57] In the current experimentation, both low-to-moderate doses of Et exposure and modalities of T3 supplementation contributed significantly in the alteration of cerebellar GR. Except the Et-0 phase, in all the other phases T3 supplementation showed some degree of elevation in cerebellar GR activities. Accordingly, with longer duration of T3 supplementation, the cerebellar GR activities tended to gain in activity. This was further corroborated with the trend-lines of cerebellar GSH, which showed matching tendencies with that of cerebellar GR. Isolated slopes of Et-0 trend-lines for both parameters indicated the supplementation with T3 was effective only when there was some extent of oxidative challenge in the cerebellum, even though the responses were not much dependent on the magnitude of challenges.

Both glutathione independent (GI) and glutathione dependent (GD) SPHCs of cerebellum in the current experimentations showed very similar trend-lines of decreasing performances with increased duration of T3 supplementation. In addition, both of those were influenced by the varied modalities of T3 supplementation only. Interestingly, none of the T3 supplementation groups showed statistically significant difference with either of the intra-phase or inter-phase study groups. However, Et-II phase of the study was distinctively separate amongst the other Et phase of study in terms of both GISPHC and GDSPHC.

CONCLUSION

Different modalities of oral T3 supplementation were compared for their effectiveness to prevent the cerebellar oxidative stress in rats exposed to low-to-moderate doses of Et. Most of the studied parameters demonstrated alterations towards prevention of cerebellar oxidative stress; nevertheless, there were differences amongst the doses of Et exposure. However, declining tendencies of SPHCs in some groups were cause of concern. These alterations indicate that oral T3 supplementations provide benefits against onidative stress, but at the cost of inherent cellular capacities. In addition, the effectiveness of oral T3 supplementation was better when that was maintained throughout the period of Et insult.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

T3: Tocotrienol; **Et:** Ethanol; **NT3:** Sham supplementation; **PT3:** Prior supplementation; **ST3:** Simultaneous supplementation; **TT3:** Total supplementation; **GSH:** Reduced glutathione; **LPO:** Lipid peroxidation; **SOD:** Superoxide dismutase; **GPx:** Glutathione peroxidase; **GR:** Glutathione reductase; **SPHC:**Superoxide and peroxide handling capacity; **GI:** Glutathione- independent; **GD:** Glutathione-dependent; **TRF:** Tocotrienol-rich fraction.

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