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Effect of Tocotrienol on medium dose ethanol-induced alternations in serum superoxide and peroxide handling capacities (SPHC) in rat

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Tocotrienols are members of vitamin E family present in low concentrations and possess high antioxidant activity. Consumption of ethanol is a common problem and induces oxidative stress. In this study, we evaluated the effect of tocotrienol against ethanol-induced oxidative stress. Male albino Wistar rats were divided into two sets; one set of rats were exposed with low to moderate doses of ethanol for 4 weeks, while another set was exposed to tocotrienol orally (10 mg/day) in addition to the 'low to moderate doses of ethanol for 4 weeks'. Oxidative stress parameters, like levels of reduced glutathione and lipid peroxidation, activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase were determined in serum before the initiation of treatment protocol and at the end of 2nd and 4th week of treatment. Serum levels of superoxide and peroxide handling capacities were also calculated in those three time points. Tocotrienol-treated rats showed statistically significant enhancement in reduced glutathione level, glutathione peroxidase and glutathione reductase activities. Glutathione-dependent superoxide and peroxide handling capacity of those rats were found to be higher. The current study suggests that the tocotrienol-induced protection against the oxidative stress is most likely mediated by glutathione-based system.

Keywords: AUDs, Glutathione peroxidase, Glutathione reductase, Lipid peroxidation, Oxidative stress, Reduced glutathione

Alcohol use disorders (AUDs) is a serious health concern in India with widened treatment gap for the afflicted abusers[1,2]. Interestingly, with a decreased trend in alcohol use in European Region, global consumption level of alcohol was mostly stable except for last 4-5 years of increased consumption[1]. In India, there is a rise in alcohol consumption which could be due to various factors, such as (i) increasing trend of alcohol use among younger generation; (ii) wide acceptability of alcohol in the upper-middle income groups; (iii) easy availability of home-made alcohol, particularly to the lower income group; and also (iv) increased income level[3].

Ethanol is metabolized into acetaldehyde and further into acetate by alcohol dehydrogenase and aldehyde oxidase or xanthine oxidase enzymes, respectively. In the process, it gives rise to reactive oxygen species (ROS) via cytochrome $P_{450}2E1$[4]. Ethanol-induced organ damage is indicated by altered liver[5] and kidney[6] parameters along with decreased total antioxidant capacity in a time dependent fashion[5]. In addition, reduction in antioxidant levels and compromised activities of antioxidative enzymes in hemolysate are also reported[5]. Decreased ascorbic acid and increased nitrite levels in serum have also been reported in chronic ethanol consumption[4]. Though several approaches have been put forward to deter the ethanol-induced formation of ROS and their effects on cellular antioxidant defense system[7], search for a novel and effective one is still on.

Tocotrienols are relatively rare components of vitamin E. They share structural (chromanol ring and farnesyl isoprenoid chain joined at C-2; Fig. 1) and functional (antioxidant activity) similarities with tocopherols. However, they have advantages of superior antioxidant and atoxic attributes. In addition, double bonds in side chain (3′, 7′ and 11′; Fig. 1) render them efficient penetration in liver[8,9] and allow

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Abbreviations: AUDs, alcohol use disorders; Et, ethnaol; GPx, Glutathione peroxidase; GR Glutathione reductase; GD-SPHC Glutathione-dependent superoxide and peroxide Handling Capacity; GI-SPHC Glutathione-independent superoxide and peroxide handling capacity; KW, Kruskal-Wallis test; MW, Mann-Whitney pairwise comparisons; GSH, reduced glutathione; SPHC, superoxide and peroxide handling capacity; SOD superoxide dismutase; TBARSThiobarbituric acid reactive substances; T3, tocotrienol; $T3_+/T3_0$, animals with/without tocotrienol supplementation

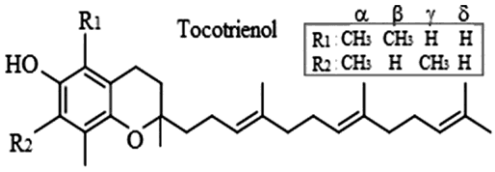

Fig. 1—Structure of tocotrienol.

them to exert its influence at the cellular level to contribute significantly in the antioxidant capacity. Both α and γ isomers of tocotrienol (T3) are effective in suppressing the lipid peroxidation by quenching and scavenging the free radicals in addition to augmentation of enzymatic antioxidant system[10]. However, being a desmethyl isomer, γ-tocotrienol is suggested to have higher bioavailability compared to the α-tocotrienol[11].

In this context, the current study evaluated the effect of Oryza tocotrienol©-90 oral supplementation against oxidative stress induced by low to moderate doses of ethanol exposures.

Materials and Methods

Materials

Oryza tocotrienol©-90 (Minimum tocotrienol content – 62.9%) was donated by the Oryza Oil & Fat Chemical Co. Ltd, Japan. All other reagents were of analytical grade and procured from Sigma, SRL, SDS, Merck, HiMedia.

Animal maintenance and treatment

The experimental protocol was approved by the Institutional Animal Ethics Committee. Two sets of male albino Wistar rats [$T3_0$: 20 animals without tocotrienol (T3) supplementation, and $T3_+$: 20 animals with T3 supplementation] weighing 120-140 g were obtained, maintained and treated in the Central Animal House of NRI Medical College & General Hospital, and the procedures were performed according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India)[12]. After one week of acclimatization, rats were randomly divided (with the help of Random Allocation Software Version 1.0, May 2004) into Et-0, Et-I, Et-II and Et-III groups (containing 5 animals in each group), and exposed to ethanol (at doses of 0.0, 0.2, 0.4 and 0.6 g/Kg body wt., respectively) daily through oral gavage for 4 wk. In another set of experiments, all these groups were exposed to Oryza-tocotrienol©-90 supplementation (10 mg/day) for 4 wk in addition to ethanol exposures. Both, ethanol and Oryza-tocotrienol©-90 treatments, were carried out through oral feeding. Ethanol or distilled water was given in the morning session while Oryza-tocotrienol©-90 was given in the evening session daily.

The doses of ethanol exposures were selected considering the ethanol content of accepted 'moderate' drinking[13] and previous publications where these doses had been proven to induce prooxidant status in rat brain[12,14]. On the other hand, the dose for Oryza-tocotrienol©-90 was selected on the basis of results obtained from preceding dose-dependent study in rat.

Serum collection and Biochemical assays

Nearly one mL of blood was collected through retro-orbital puncture in three occasions: (i) prior to the initiation of exposures (0^{th} wk); (ii) at the end of 2^{nd} wk of exposures; and (iii) at the end of 4^{th} wk of exposures. Collected serum samples were used to determine reduced glutathione (GSH) content, lipid peroxidation, activities of catalase, superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) as reported earlier[15]. Ratio of activities of CAT/SOD and GPx/SOD are used to calculate GSH-independent Superoxide and Peroxide Handling Capacity (SPHC) and GSH-dependent SPHC, respectively, as detailed earlier[16,17].

Statistical analyses

The collected values of each parameter from individual animal on 2^{nd} and 4^{th} week were processed as percentage alteration of parameter value of same rat and group data presented in Box and whiskers plot indicating Median, lower and upper quartile, range and outliers (if any) using R statistical package[18]. Variance and difference between the groups were analyzed by Kruskal-Wallis (KW) test and Mann-Whitney pairwise comparisons (MW), respectively, accepting the probability of 5% or less as significant using PAST statistical software[19].

Results

Values of serum parameters of 0^{th} wk (Table 1) were used as baseline for evaluation of impact of varied doses of ethanol exposure and tocotrienol treatment. At the end of 2^{nd} and 4^{th} wk of treatments, serum GSH levels of $T3_0$ animals, but not $T3_+$ animals, were significantly decreased in Et-I, II and III groups compared to that of Et-0 group. Significant differences between $T3_+$ and $T3_0$ set of animals of Et-I, II and III groups in terms of serum GSH level is evidenced by significant KW value for both 2^{nd} and 4^{th} wk data, as well as MW tests (Fig. 2A).

Table 1—Pretreatment values of serum oxidative stress parameters in the used sets of rats.

Serum Parameters (Units)	Mean±SEM (n = 40)
Reduced Glutathione (μg/ml serum)	37.93±5.59
Lipid peroxidation (μmol TBARS/ml serum)	119.47±17.50
Superoxide dismutase (Units/ml serum)	32.67±1.37
Catalase (μmol H_2O_2 decomposed/h/ml serum)	13.62±1.55
Glutathione peroxidase (nmole NADPH oxidized/min/ml serum)	5.87±0.73
Glutathione reductase (nmole NADPH oxidized/min/ml serum)	1.36±0.20
Glutathione-independent superoxide and peroxide handling capacity	0.43±0.05
Glutathione-dependent superoxide and peroxide handling capacity	0.16±0.02

Significant KW values for both 2nd and 4th wk data along with MW tests indicate acceptable difference in serum TBARS levels of $T3_0$ and $T3_+$ animals at the end of 2nd and 4th wk (Fig. 2B). Throughout the study, $T3_+$ animals demonstrated lower serum TBARS level compared to the baseline level, while $T3_0$ animals recorded greater or equal to that during the similar period of treatment in all the ethanol exposure groups.

Hardly any change was noticed in the SOD activities of $T3_0$ animals of Et-I, II and III groups compared to that of Et-0 group of same set in either of the fortnightly data presented. However, ethanol treated groups showed reduced SOD activity up to 20% compared to 0th wk activity (Fig. 2C). On the other hand, similar comparison in $T3_+$ set indicates 20-40% increases at the end of 4th wk but without any

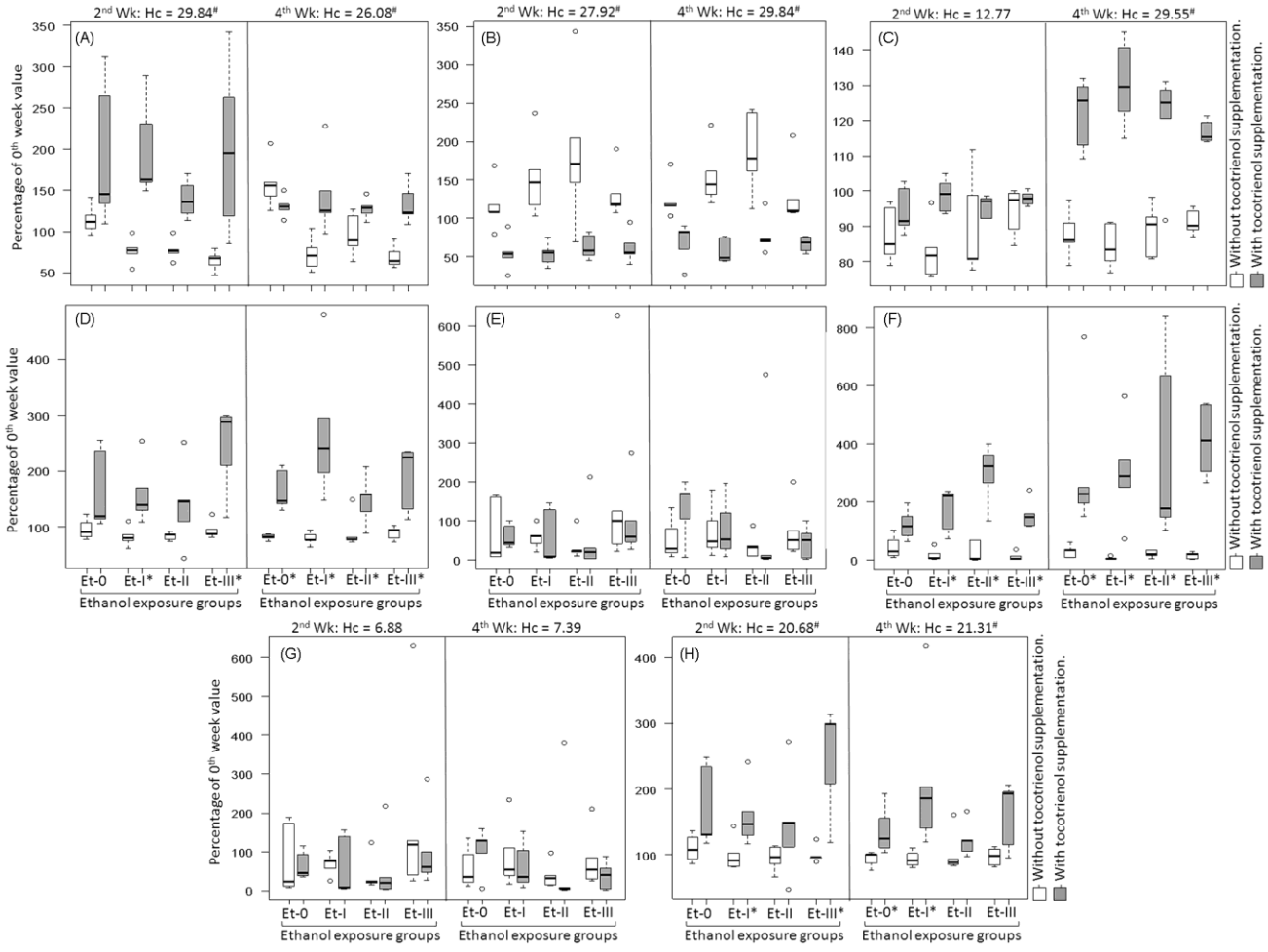

Fig. 2—Percentage alterations of (A) serum reduced glutathione level; (B) lipid peroxidation level; (C) superoxide dismutase activity; (D) glutathione peroxidase activity; (E) catalase activity; (F) glutathione reductase activity; (G) glutathione-independent superoxide and peroxide handling capacity (GI-SPHC); and (H) glutathione-dependent superoxide and peroxide handling capacity (GD-SPHC) after 2nd and 4th week of ethanol exposures compared to the level of pre exposure. [*indicates significant differences between the animals with tocotrienol supplementation and animals without tocotrienol supplementation of same ethanol exposure group (Et). Hc indicates tie corrected Chi-square value of Kruskal-Wallis test and # indicates $P < 0.05$]

appreciable change at the end of 2nd wk. This duration dependent variation in the T3 treatment was evidenced by higher KW value in 4th wk data ($p = 0.0001$) compared to that of 2nd wk data ($p = 0.08$). While the differences between $T3_0$ and $T3_+$ sets in all the Et groups were obvious at the end of 4th wk, only Et-I group showed such difference at the end of 2nd wk with $p = 0.04$ (Fig. 2C).

Similarly, time dependent effects T3 treatment is observed in the alteration of serum GPx activities in the present study (Fig. 2D). At the end of 2nd wk, appreciable differences in alterations of serum GPx activities was noticed in the $T3_+$ set of animals of all the Et groups, however, MW test identified $T3_+$ *vs*. $T3_0$ differences was significant only in Et-I and Et-III groups (Fig. 2D). On the other hand, changes in all the Et groups of $T3_+$ set were statistically significant against changes in respective Et group of $T3_0$ set of animals at the end of treatment (Fig. 2D). Corroborating the same, KW value for the data of 2nd wk ($P = 0.001$) was slightly lower than that of 4th wk data ($P = 0.0002$).

Activities of serum catalase are found to remain unaltered throughout the study (Fig. 2E) for both $T3_0$ and $T3_+$ sets of animals in all the ethanol exposure groups. Levels of serum GR activities in all the $T3_0$ animals are almost equal to the base line activities even at the end of 4th week (Fig. 2F). In case of $T3_+$ set of animals, serum GR activities at the end of 2nd and 4th weeks were found raised in all the Et groups when compared with their 0th wk value, and enhanced in 4th wk appreciably higher than that of 2nd wk (Fig. 2F). While difference between $T3_0$ and $T3_+$ were significant in Et-I, II and III groups even in 2nd wk and continued till 4th wk, the difference in Et-0 group was significant only at the end of 4th wk (Fig. 2F).

Changes in SPHCs for $T3_0$ and $T3_+$ animals of Et groups are presented in Fig. 2 G and H. Glutathione-independent (GI) SPHC, at both time periods, showed only insignificant changes compared to the pre-treatment levels (Fig. 2G). Significant KW values were recorded for both 2nd and 4th wks' data of glutathione-dependent (GD) SPHC (Fig. 2H). MW tests revealed that the augmentations of GD-SPHC in $T3_+$ set of animals were significant compared to $T3_0$ set of animals in Et-I group for both 2nd and 4th wk data. Augmentations in Et-III group at the end of 2nd wk and in Et-0 group at the end of 4th wk were also statistically significant (Fig.2H).

Discussion

Oxidative stress originating from 'heavy' or chronic ethanol abuse has been well established. However, very few studies are available with 'light' to 'moderate' doses of ethanol abuse[12,16,20]. The current study demonstrates amelioration of ethanol-induced decrements in serum GSH levels by supplementation with T3. Similar rise in blood GSH level in rats was noted with treatment of γ-T3 at a dose of 5 mg/kg body wt for 21 days[21]. Notably, the serum GSH levels of three groups of rats, in the current study, exposed to different doses of ethanol also did not differ significantly. Therefore, the used doses of ethanol exposure aggresses the overall antioxidant defense system equally as indicated by the decreased GSH level. On the other hand, the serum GSH values of two different time points indicate that duration of current ethanol exposure is also ineffective, like that of variable doses. Similarly, T3-supplemented groups of rats also demonstrated significant decline in serum TBARS level compared to their respective counterparts without T3 supplementation, irrespective of duration of treatment and dose of ethanol exposure. The observed decrease in lipid peroxidation is in agreement with the published reports on T3-induced reduction in serum lipid peroxidation[22,23] as well as tissue specific TBARS levels[24,25] in experimental animals. Recently, 8 wk intervention study with 200 mg T3/day in type-2 diabetic patients demonstrated significantly decreased serum malonaldehyde level along with significant increase in the total antioxidant capacity of serum[26]. Decreased serum GSH levels in Et-I, II and III groups compared to that of Et-0 group indicates that the oxidative stress is induced in $T3_0$ animals because of ethanol exposure, nevertheless, it is not influencing the level of lipid peroxidation significantly. Changes in the serum levels of GSH and TBARS by the end of 2nd wk of supplementation, suggests T3-mediated antioxidant dominance. The reduction in serum lipid peroxidation products indicates the damage of the cell membrane and hence, the cellular rampant by the free radicals could be kept under control with the use of T3 supplementation.

SOD is instrumental in converting highly noxious superoxide radicals ($O_2^{\bullet-}$) into H_2O_2, a relatively less potent oxidant. However, it is further liquidated into water by catalase and GPx. Most of the T3 studies found raised SOD activities either in studied tissues[10] or in serum[10,21]. However, SOD activities remain

predominantly unaltered at the end of 2nd wk in the current study. Elevated SOD activities in $T3_+$ animals at the end of 4th wk but not at the end of 2nd wk of treatment (except Et-I group), indicates that it requires time to reach a specific level of T3 as well as influence the transcription and translation of SOD, as revealed by earlier studies[21,24,27]. This observation of SOD activities indicates time-dependent effect of current treatment protocol, which is not apparent in the alterations of GSH or TBARS. Likewise, all the Et groups demonstrated increased serum GPx activity at the end of 4th wk of treatment, while similar increment was found only in Et-I and Et-III groups at the end of 2nd wk of treatment. The KW value too, higher in the longer period of treatment, support the same notion. In addition, enhanced activities of serum SOD and GPx enzymes of Et-0 group at the end of 4th wk of T3 treatment, without any significant alterations at the end of 2nd wk, indicates time required to produce dominance of antioxidant system. Nevertheless, possibilities of antioxidant dominance created by the T3 treatment at the cellular or tissue level within 2 wks cannot be overruled.

Superoxide and peroxide produced during oxidative stress are neutralized mainly by two pathways – (i) glutathione-dependent pathway with the help of SOD and catalase activities and (ii) glutathione-dependent pathway with the help of SOD and GPx activities. Here, GD-SPHC was found to be significantly altered in Et-0, Et-I and Et-III groups at the end of either 2nd or 4th wk. On the contrary, GI-SPHC of all the groups of rats remained unaltered even after 4 wk of T3 treatment. Interestingly, serum GR activity was found elevated in all the groups of ethanol treatment in $T3_+$ set compared to the respective groups of $T3_0$ set. Even the Et-0 group showed significant difference in serum GR activity between the $T3_0$ and $T3_+$ sets at the end of 4th wk of treatment. This observation in current study corroborates with the reports of high binding energy between GR and T3 *in silico*[22] and enhanced GR activities in liver[22], skeletal muscle[28] and erythrocytes[29] *in vivo*.

Considering the observed changes in the serum level of GSH, activities of GR and GPx in the serum of $T3_+$ animals, maintenance of GSH level is suggested to be a major antioxidant activity of T3. The changes noticed in GD-SPHC also supported the present observation of GSH dependency of T3 to resist oxidative stress.

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