On the other hand, a relatively thin style ensures repeated visits of pollinators to the flower though too thin a style might also discourage pollinator visitation. Hence, the style diameter can be expected to be a tradeoff between these risks and benefits. Thus, our study offers an hitherto unknown parameter that might have shaped the evolution of styles and nectar tubes.

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Nicotinamide and α-tocopherol combination partially protects *t*-butyl hydroperoxide-induced neurotoxicity: Implication for neurodegenerative disease

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In light of evidences of impaired energy metabolism and oxidative damage in neurodegenerative disorders, we have investigated the potential role of compounds which may improve the mitochondrial metabolism and can ameliorate the toxic effects of free radicals, and thus compensate for disease-related defects. Treatments with three doses of *t*-butyl hydroperoxide (*t*-BuOOH) resulted in increased lipid peroxidation in corpus striatum. The superoxide dismutase (SOD, EC 1.15.1.1) activity increased while reduced glutathione content declined. Supplementation with α-tocopherol and nicotinamide and their combination resulted in decline in LPO and showed the tendency towards normalization of SOD activity and GSH content. The results showed that supplementation of a combination of α- tocopherol and nicotinamide provides protection against *t*-BuOOH-induced neurotoxicity.

THERE are considerable experimental evidences to support age-related changes in general[1,2] and aging of central nervous system (CNS) in particular[3,4] which are inflicted by oxygen-free radicals such as superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), peroxyl radical ($RO_2^{\cdot}$), alkoxyl radical ($RO^{\cdot}$) and its non radical derivatives such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl)[5]. The brain is highly vulnerable to attack by oxygen-free radicals during aging owing to high lipid contents and low level of antioxidants to neutralize these molecular renegades along with utilization of large amounts of O_2 (refs 4, 6). Further, strong evidence to indicate that defect in mitochondrial energy metabolism plays an intrinsic role in several neurodegenerative disorders[7–9], is emerging. Reduced complex I activity has been reported in Parkinson's disease patients[10]. In Huntington's disease, multiple enzyme defects have been found[7,11,12]. Reduction in complex IV activity was reported in Alzheimer's disease patients[13–15]. One potential mechanism whereby mitochondrial dysfunction could occur is due to increased generation of free radicals and oxidants[12,16]. Impairments of mitochondrial energy metabolism will result in interruption of oxidative phosphorylation, which results in decreased ATP production[17]. Under circumstances of electron transport chain inhibition or molecular defects, the levels of free radicals production increases[9,10,18,19]. Mitochondrial complex I appears to be the most sensitive. The ability of α-tocopherol (T), a hydrophobic antioxidant to stop the chain reaction initiated by free radicals, has been demonstrated in a wide variety of paradigms[3,20,21].

Nicotinamide (N) is a precursor of NADH which is a substrate for both complex I of the electron transport chain as well as a number of dehydrogenase enzymes involved in the citric acid cycle[12] and thus plays an essential role in electron transport function[18]. It can also block the enzyme poly (ADP-ribose) polymerase, which is activated by H_2O_2 and nitric oxide ($NO^{\bullet}$)[22] and cause cell death. N blocks ATP depletions and lactate increase *in vivo*[23]. Neurons in the striatal targets have been associated with several neurodegenerative diseases notably Parkinson's disease, Huntington's disease and Wilson's disease as well as manganese toxicity[24]. In light of evidence of impaired energy metabolism and oxidative damage in neurodegenerative disorders, we have investigated the potential role of T and N in improving mitochondrial metabolism and ameliorating the toxic effects of free radicals on corpus striatum caused by *t*-BuOOH administration.

Female Swiss albino mice (weighing 25–30 g) were treated with either normal saline or 70% solution of *t*-BuOOH (100–300 mg/kg) by intracerebroventricular (ICV) injections. All mice were anaesthetized with ketamine and xylazine (200 and 2 mg/kg respectively) before the administration of the toxin and throughout the 2 h experiment using the technique described by Adams *et al.*[25]. T (12 mg/kg) (Loba Chemie) and N (200 mg/kg) (Hi Media) were supplemented with food. T, N and T–N combination were given daily for 7 days prior to *t*-BuOOH administration. The animals were examined daily to ascertain that they have consumed the full dose of T, N and T–N combination. Animals were decapitated after 2 h of *t*-BuOOH treatment. The brain was removed quickly and the corpus striatum dissected out and frozen at 0°C. Tissue was homogenized at 0°C in 0.05 M sodium phosphate buffer (pH 7.4) for lipid peroxidation; in 50 mM Tris HCl buffer for superoxide dismutase (SOD) activity and in 0.1 M metaphosphoric acid containing 2 mM EDTA for reduced glutathione (GSH) assay. Homogenization for GSH assay was carried out in the presence of 1.0 M 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to prevent the artifactual oxidation of GSH.

Lipid peroxidation (LPO) was measured by the thiobarbituric acid test[26] in terms of malonaldehyde equivalents (MDA) using a molar extinction coefficient of 1.56×10^5/min/cm as described earlier[27]. Briefly, the homogenate was centifuged at 3000 *g* for 15 min and supernatant was used for assay. Samples of 0.1 ml homogenate were taken and mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% glacial acetic acid and 1.5 ml of 0.8% thiobarbituric acid. The mixture was heated at 95°C for 1 h on a water bath and cooled under tap water before mixing 1 ml of distilled water and 5 ml mixture of *n*-butanol and pyridine (15 : 1 v/v). The mixture was centrifuged at 2200 *g* for 5 min. The amount of MDA formed was measured by the absorbance of upper organic layer at 532 nm using appropriate controls. The results were expressed as nmol MDA/mg protein.

The SOD activity was assayed by the method of Marklund and Marklund[28]. Briefly, the homogenate for SOD activity assay was treated with triton X-100 (1%) for 30 min to ensure that full activity is released. It was then centrifuged at 16,000 *g* at 4°C and the supernatant used for SOD assay. The assay system contained 1 mM DTPA, 5.0 mM Tris HCl buffer (pH 8.2) and tissue homogenate (0.5 ml). The assay mixture was transferred to a 3 ml cuvette and the reaction was initiated by the addition of freshly prepared 2.6 mM pyrogallol solution in 10 mM HCl. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation per 3 ml of assay mixture. The enzyme kinetics was carried out at 27°C (room temperature) in a Perkin-Elmer UV spectrophotometer as described earlier[29]. Calculations were made as units per mg protein.

The GSH content was measured by Ellman's reagent according to the method of Jollow *et al.*[30]. Homogenate for GSH assay was centrifuged at 16000 *g* for 15 min at 4°C. Supernatant (0.5 ml) or freshly made standard using GSH was equilibrated with 4 ml 1 mM DTNB reagent at 412 nm in a Perkin-Elmer UV spectrophotometer as described earlier[31].

Total protein content was determined by the Folin–phenol reaction as described by Lowry *et al.*[32] using bovine serum albumin as a standard.

All data are expressed as mean ± SE. Statistical comparisons were made relative to the appropriate control group by Student's *t* test and analysis of variance. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

Treatment with three doses of *t*-BuOOH resulted in significant ($P < 0.05$) increase in LPO (Figure 1) in corpus striatum. Under these circumstances treatment with either T or N alone had mild attenuation of *t*-

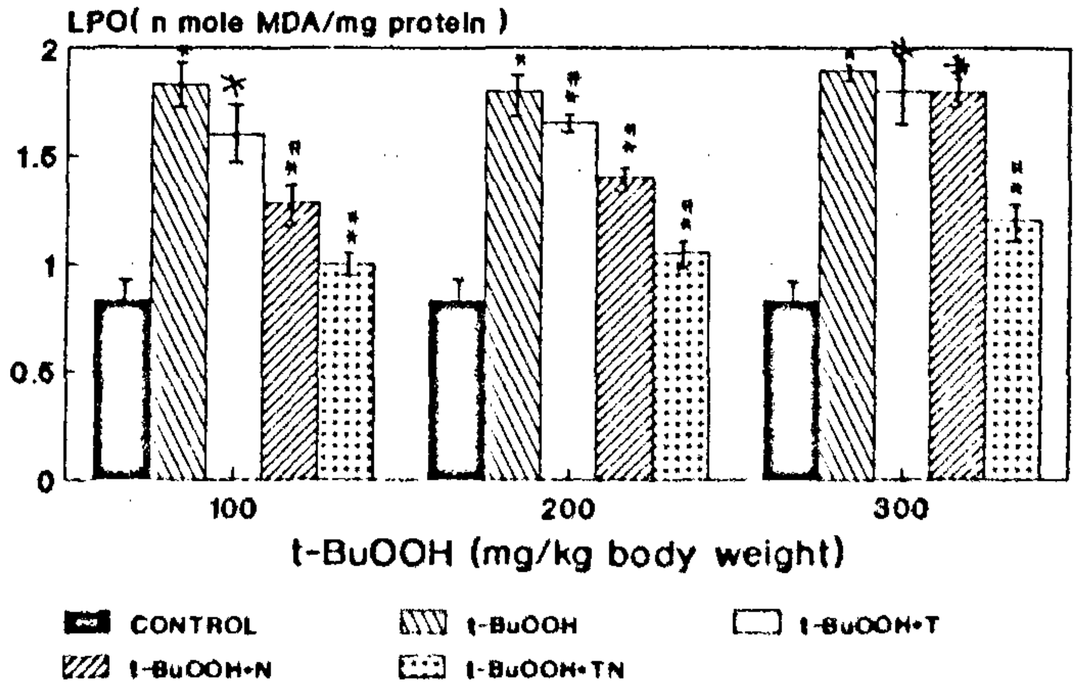

Figure 1. Effects of treatment with α-tocopherol (T), nicotinamide (N) and combination of α-tocopherol–nicotinamide (T-N) on *t*-butyl hydroperoxide (*t*-BuOOH) induced lipid peroxidation (nmole MDA/mg protein) in corpus striatum. $^*P < 0.05$ as compared to control; $\#P < 0.05$ as compared to *t*-BuOOH-treated mice. Error bars indicate SEm.

BuOOH neurotoxicity. Significant ($P < 0.05$) reduction in LPO was noted with T at 200 mg/kg *t*-BuOOH-exposed animals and with N at 100 and 200 mg/kg *t*-BuOOH-exposed animals.

We examined whether three regimens of *t*-BuOOH-induced neurotoxicity might be attenuated by treatment with the T–N combination. The results showed that the T–N combination significantly ($P < 0.05$) protected against three regimens (100, 200 and 300 mg/kg body weight) of *t*-BuOOH-induced neurotoxicity marked by significant decline in LPO in comparison to *t*-BuOOH-treated animals; although the LPO level remained higher than those observed in the control.

The total SOD activity in corpus striatum increased significantly ($P < 0.05$) after *t*-BuOOH treatment (Figure 2). T, N and T–N combination supplementations showed the tendency towards normalization of SOD activity in corpus striatum. In these animals, the SOD activity decreased significantly ($P < 0.05$) compared to *t*-BuOOH-treated animals. A good amount of SOD activity existed in control animals.

The total concentration of intracellular GSH in corpus striatum declined significantly ($P < 0.05$) after treatment with *t*-BuOOH (Figure 3). Supplementations of T and N showed the tendency towards normalization of GSH in corpus striatum. T, N and T–N combination supplementations have resulted in a significant ($P < 0.05$) elevation of GSH content compared to *t*-BuOOH-treated animals.

Many of the brain functions, which deteriorate with advanced age may be partly due to oxidative alterations[33,34] and mitochondrial energy dysfunction[9,16,18]. Lipid peroxidation which is induced by free radicals[35,36] was significantly enhanced after *t*-BuOOH administration in the present study. We have to take into account that neuronal production of free radical could be related to the impairment of brain oxidative metabolism[9,16]. Some elements of electron transport chain such as ubiquinone and cytochrome B_{566}, leak electrons directly into oxygen[37] and produce $O_2^{\cdot -}$ radical. This leads to a micromolecular perturbation in which the impairment of antioxidant enzymatic systems may reflect the inability of mitochondria to maintain a normal functional state[37,38], thus favouring the development of peroxidative damage. Equally interesting is the present finding that the neuronal damage and ROS production can be attenuated by T, N and T–N combination. These findings strongly suggest that the anti-damage action of T, N and T–N combination may indeed play a pivotal role in attenuating free radical damage in brain. The protection conferred by T–N is at least in part due to its free-radical scavenging ability.

Both prokaryotes and eukaryotes are able to dramatically upregulate their armoury of oxidant protections in response to an oxidative stress[39,40]. To adapt against an unfriendly ROS environment, living organisms produce a battery of antioxidative enzymes[1,36,38]. SOD plays an important role in attenuating oxidative damage *in vivo*, thereby delaying not only the onset of age-related degenerative diseases, but also aging itself[34,38]. We observed increased SOD activity in corpus striatum. Similar increase in SOD activity has been reported in the parkinsonian post-mortem brain[41,42]. It is not clear whether the increase of SOD activity in the parkinsonian brain is related to the enhancement of oxidative stress or it is a feedback mechanism to scavenge $O_2^{\cdot -}$ radical. An increase in SOD activity might be useful in preventing neuronal degeneration[43]. On the background of low GSH content however, the high SOD state may produce an increased peroxidative burden in neurons by Fenton reaction[36] (reactions (1), (2)).

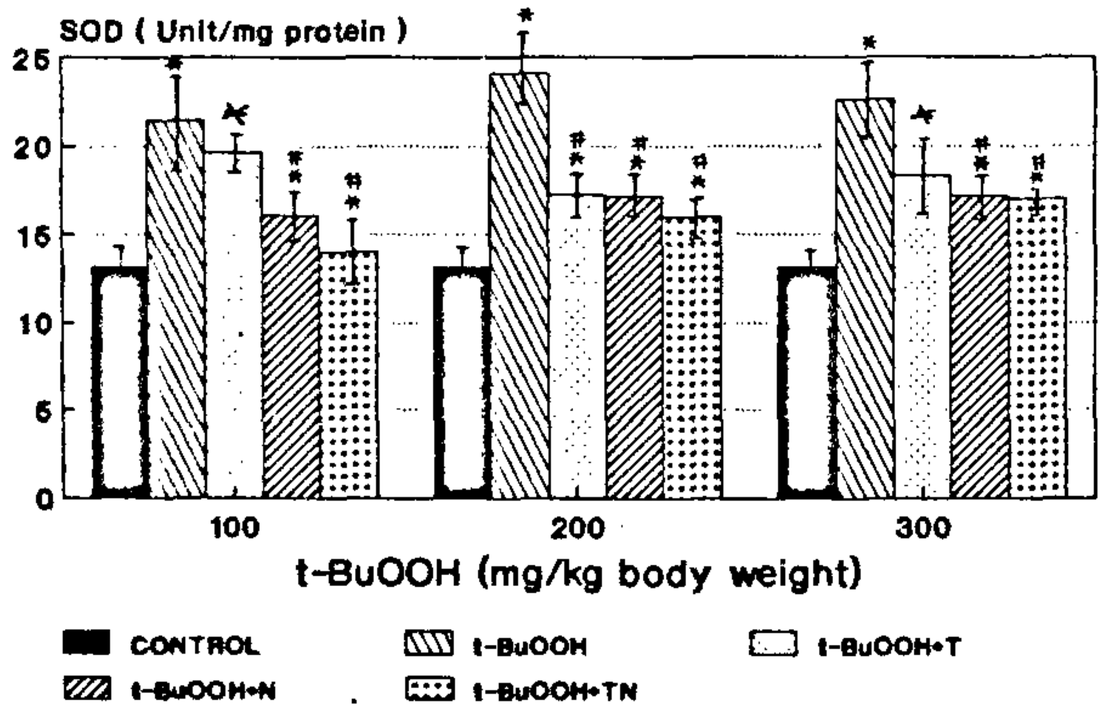

Figure 2. Effects of treatment with α-tocopherol (T), nicotinamide (N) and combination of α-tocopherol–nicotinamide (T–N) on *t*-butyl hydroperoxide (*t*-BuOOH)-induced superoxide dismutase activity (unit/mg protein) in corpus striatum. $^*P < 0.05$ compared to control; $\#P < 0.05$ as compared to *t*-BuOOH-treated mice. Error bars indicate SEm.

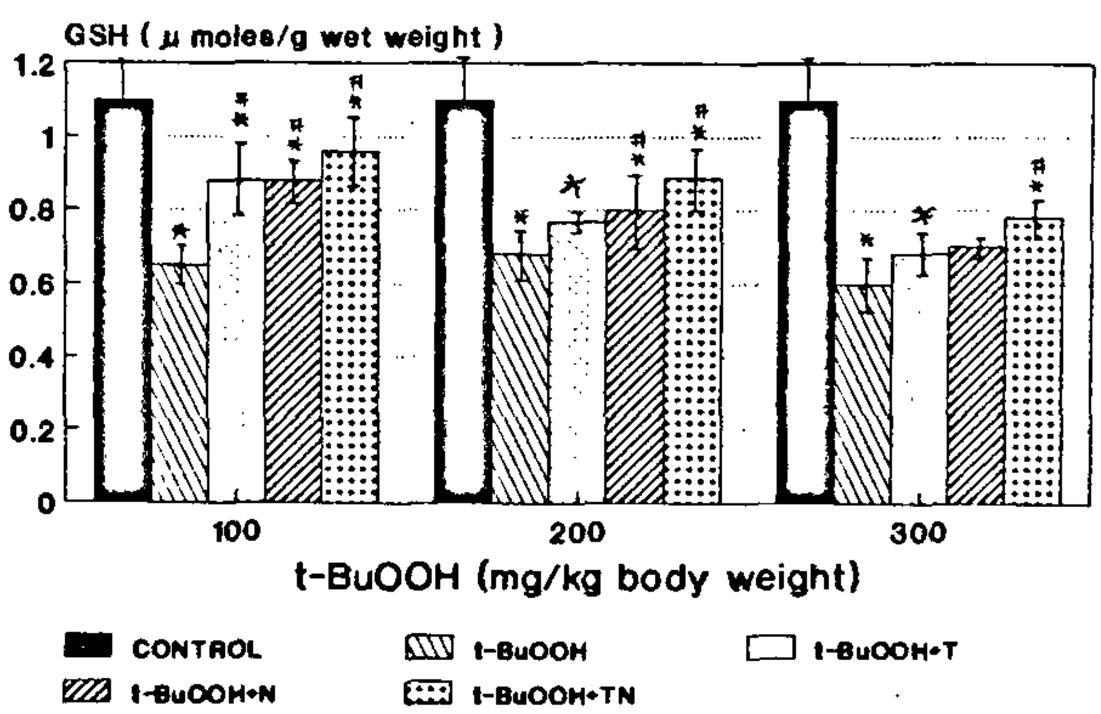

Figure 3. Effects of treatment with α-tocopherol (T), nicotinamide (N) and combination of α-tocopherol–nicotinamide (T–N) on *t*-butyl hydroperoxide (*t*-BuOOH)-induced reduced glutathion (GSH) content (μmol/g wet weight) in corpus striatum. $^*P < 0.05$ compared to control; $\#P < 0.05$ as compared to *t*-BuOOH-treated mice. Error bars indicate SEm.

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \quad (2)$$

In the present study, treatment of T, N and T–N combination prior to the *t*-BuOOH administration showed the tendency towards normalization of SOD activity, although the levels were still higher compared to the control.

The total concentration of intracellular GSH in striatum decreased compared to the control. GSH is a powerful free radical scavenger and also a substrate for glutathione peroxidase (GSH-Px; EC 1.11.1.9)[44] (reaction (3))

$$H_2O_2 + 2\,GSH \xrightarrow{GSH\text{–}Px} GSSG + H_2O. \quad (3)$$

GSH-Px is the most important H_2O_2-removing enzyme in the brain[44]. Studies indicate that in primate brain the total GSH pool is predominantly (99%) in the reduced GSH form[44]. Depletion of GSH may give rise to decreased cytochrome C oxidase (complex IV; EC 1.9.3.1) activity in both whole brain homogenates and purified mitochondrial preparation[45]. The exposure of T, N and T–N combination showed tendency towards increase in the GSH content, however the levels remained lower than that observed in the control. The compensatory increase in GSH by T, N and T–N combination might counteract the H_2O_2 toxicity via redox system.

In conclusion, the treatment of T, N and T–N combination showed neuroprotective effects. We found T–N combination as producing higher degree of neuroprotective effects than either T or N supplementation accompanied by decrease in LPO. Besides this, it also normalized the SOD activity and prevented GSH depletion to afford protection against oxidative processes. Considering the degree of protection afforded by T–N combination, we submised that T–N combination may be working by multiple means to alter the cellular damage that is normally a consequence of the free radicals.

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