Ameliorative effect of a combination of vitamin E, vitamin C, α-lipoic acid and stilbene resveratrol on lindane induced toxicity in mice olfactory lobe and cerebrum

Mehajbeen Bano* & Devendra Kumar Bhatt
Cancer Biology and Toxicology laboratory, Department of Zoology
University College of Science, Mohan Lal Sukhadia University Udaipur 313 001, India

Received 16 June 2009; revised 9 November 2009

Acute dose of lindane (40 mg/kg body weight, ip) caused significant reduction in butyrylcholinesterase (BChE) activity both in olfactory lobe and cerebrum of mice along with reduction in catalase (CAT), total protein and elevation in superoxide dismutase (SOD) and cholesterol contents. Pretreatment by a combination of antioxidants, vitamin E, vitamin C, α-lipoic acid and stilbene resveratrol (125 mg/kg body weight, ip) significantly augment the altered level of BChE and protect the other parameters in both the brain regions. The results were adequately in agreement with the histochemical findings, suggesting the neuroprotective efficacy of combination of antioxidants studied on the lindane induced neurotoxicity.

Keywords: Cerebrum, Lindane, α-lipoic acid, Olfactory lobe, Stilbene resveratrol, Vitamin C, Vitamin E

Lindane (γ-HCH) is an organochlorine pesticide widely used in agriculture, forestry, public health and household applications. Its persistence in the environment, high mammalian toxicity and resistance to biodegradation led to a ban or restricted use in many developed and developing countries1. But continuous use of lindane in agriculture and malaria control programmes is on increase in most of countries including India. Lindane is generally recognized as potent neurostimulant and convulsant, characterized by high insecticidal properties and lipophilic nature2. Lindane induced neurotoxicity especially among infants and young children has been reported3,4.

The distribution of the major cholinergic pathway regulatory enzyme acetylcholinesterase (AChE) has been studied in the brain after lindane poisoning5,6. However, there is no report on the distribution of the closely related enzyme butyrylcholinesterase (BChE). Moreover, the physiological functions of BChE have not been fully elucidated7. BChE has pharmacological and toxicological importance, as it scavenges anticholinesterase compounds before the AChE activity is targeted at synaptic sites8. BChE supplements the action of AChE and plays a role in regulating cholinergic transmission9. Like AChE, BChE inactivates the neurotransmitter acetylcholine (ACh) therefore it may be a viable therapeutic target in pesticide toxicity.

Lindane promotes oxidative stress (OS) by interacting with the cell membrane which triggers the generation of reactive oxygen species (ROS). Enhanced production of ROS also causes oxidative modification of cellular macromolecules such as protein, DNA and lipid. The excess of ROS can be detoxified by the endogenous antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). Endogenous antioxidant system of the body sometimes becomes insufficient because of the continuous exposure to pesticides which is unavoidable. Therefore use of exogenous antioxidants could be one of the effective means to ameliorate the lindane induced neurotoxicity. Although in animal and in vitro studies antioxidants such as vitamin C10, vitamin E11 and quercetin12 have shown their protective efficacy on lindane induced toxicity, information on the effect of antioxidants on lindane induced neurotoxicity is lacking.

Different antioxidants scavenge different type of free radicals, so a combination of antioxidants forms a stronger antioxidant shield to protect the body against the neurotoxicity. Vitamin E works synergistically with other antioxidant nutrients including selenium,
vitamin C, β-carotene and other to quench free radicals, peroxides and other potentially harmful substances. Kontush et al.\textsuperscript{13} revealed that combined administration of vitamin E and vitamin C (400 IU and 1000 mg respectively daily for one month) is better than vitamin E (400 IU) alone in protecting brain from oxidative damage. The neuroprotective efficacy of vitamin C in clinical trial is doubtful when given alone, however it has some benefit when given in combination with other antioxidants\textsuperscript{14}. The combination of resveratrol with vitamin C and E was found more effective in protecting the cell from OS rather than any of these three antioxidants alone\textsuperscript{15}. Thus, in this study combination of antioxidants such as vitamins (vitamin E and vitamin C), thiol containing antioxidant α-lipoic acid and stilbene resveratrol has been tried against lindane toxicity instead of giving a high dose of a single antioxidant.

Materials and Methods

Chemicals—Lindane (γ-HCH) was obtained from Sigma Chemicals St. Louis, Mo, USA (CAS No. 58-89-9, 97% purity). Vitamin E, vitamin C, α-lipoic acid, phenazine methosulfate, and butyrylthiocholine iodide were obtained from Hi Media, India. Dithiobisnitrobenzene (DTNB) and bovine serum albumin were purchased from Sisco Research Laboratories, Mumbai, India. Resveratrol was purchased from Cayman Chemical Company, USA (CAS No. 501-36-0, 98% purity). All other chemicals and solvents used were of analytical grade and purchased locally.

Animals—Healthy, 8-10 weeks old, adult male albino mice weighing 25±5g body weight were used. The animals were kept in polypropylene cages under normal laboratory conditions in 12:12 h L: D periods. The animals were fed on standard balanced diet. The water was made available ad libitum to the animals. Experimental animals were handled according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India (approval no. 1098/AC/07/CPCSEA).

Experimental protocol—The mice were divided into following four groups of 12 animals each:

- **Group I**: Control group [given only olive oil, ip]
- **Group II**: Lindane group [given acute dose of lindane (40 mg/kg body weight ip for 18 h) dissolved in olive oil]
- **Group III**: Antioxidant group [given combination of antioxidants a day (24 h) before and one hr prior to the vehicle (125 mg/kg body weight ip), the combination contained vitamin E: 50mg/kg; vitamin C: 50 mg/kg; α-lipoic acid: 20mg/kg and resveratrol: 5mg/kg body weight]
- **Group IV**: Antioxidant and lindane group [given combination of antioxidants (as mentioned in group III) plus lindane (as mentioned in group II)]

In group IV, antioxidants were administered a day (24h) before and also one hr prior to the lindane administration.

Dose selection—The dose of lindane was selected on the basis of Magour et al.\textsuperscript{16}. The ip dose of lindane is reported to be 125 mg/kg in mice\textsuperscript{17}. The selected dose (40 mg/kg body weight) corresponds to 1/3rd of LD\textsubscript{50} value. The dose of antioxidants was decided after pilot study.

Histochemical localization—The animals were killed by cervical dislocation, and both olfactory lobe and cerebrum were immediately removed and fixed in calcium formol, chilled at 4°C for 18-20 h. The sections (15 µm thick) were cut on the cryostat. The sections were processed for localization of BChE by Karnowsky and Roots method\textsuperscript{18}. Qualitative assessment was performed on the basis of color intensities of enzyme reaction.

Biochemical estimation—The animals of all groups were killed by cervical dislocation after 18 h of treatment. Brain regions were immediately dissected out, washed with ice cold physiological saline and homogenized (10% w/v) in ice cold 0.1M phosphate buffer (pH 7.4).The homogenate was centrifuged at 6000 g for 10 min to obtain the supernatant. In the supernatant the following biochemical estimations were performed at low temperature:

BChE: The activity of BChE (E.C. 3.1.1.8) was assayed as per Ellman et al.\textsuperscript{19} by using butyrylthiocholine iodide as a substrate. The activity was calculated using a molar extinction coefficient of 13.61 mM\textsuperscript{-1} cm\textsuperscript{-1} and expressed as µmol substrate hydrolyzed /min / g tissue weight.

SOD: SOD (E.C.1.15.1.1) activity was determined as per Kakkar et al.\textsuperscript{20}. The assay was based on inhibition of the formation of nicotinamide adenine dinucleotide, phenazine methosulphate, and amino blue tetrazolium formazan. Results are expressed as units (U) of SOD activity/mg protein. One unit of enzyme induced 50% inhibition of NBT (nitroblue tetrazolium) reduction/min.

CAT: CAT (E.C. 1.11.1.6) was assayed by the method of Cohen et al.\textsuperscript{21}. Results are expressed as
units (U) of CAT activity/mg protein. One unit of enzyme activity was defined as µmol of H₂O₂ decomposed/min.

Protein: Total protein was estimated as per Lowry et. al using Folin's reagent, and bovine serum albumin as standard. The value of protein content was expressed in mg/100mg tissue weight.

Cholesterol: Total cholesterol was estimated by the method of Oser. The values were expressed in mg/g tissue weight.

Statistical evaluation—The biochemical results are expressed as mean ± SD. The results obtained from different groups were analyzed by one way ANOVA. Inter group comparisons were performed by using the least significance difference (LSD) test. A probability (P) value of <0.05 was accepted as being statistically significant.

Results

Histochemical results

Distribution of BChE in different layers of the olfactory lobe of mice (Table 1)—Cajal identified following layers in the olfactory lobe of mice (i) Peripherial fibrillar layer (PFL), (ii ) Glomerular Layer (GL), (iii ) External Plexiform Layer (EPL), (iv) Mitral cell layer (ML), (v) Internal Plexiform layer (IPL), (vi) Granular Cell Layer (GRL) and (vii) Ependymal layer (EL).

In the control group of the present study, the non medullated nerve fibers in PFL were devoid of BChE activity, whereas in the same layer neuroglial cells were positively stained. In the GL, the glomeruli (g) were equipped with BChE activity, while the interglomerular fibers showed less intense reaction. The EP layer was more or less negative for BChE except the tufted cells and their processes which showed mild activity. The mitral cell layer was strongly positive for enzyme BChE. The IP layer, which is fibrous in nature, showed less reaction compared to very intense stained cells. GRL showed some mild reaction in fibers running through these regions (Fig. 1a). In the present study, lindane exposure resulted in homogenous reduction in BChE activity (Fig. 1b). The positively stained cells in PFL were devoid of enzymatic activity. The interglomerular region showed complete absence of BChE activity. Besides, all other layers showed reduction in BChE activity due to lindane treatment. The pretreatment with antioxidants in lindane treated group showed the restoration of BChE activity which is comparable with control group (Fig. 1d). Antioxidants supplementation markedly enhanced the BChE activity as compared to control group. Interestingly, in this group elevation of BChE activity was observed in PFL, which was intensely positive, compared to control (Fig. 1c).

Histochemical distribution of BChE in the different nuclei and fiber tracts of cerebrum (Table 2)—The distribution of BChE in the control group is depicted in Fig. 2a.

Cerebral cortex: In the cerebral cortex (CC) negligible BChE reaction was observed. On the other hand, the truncus corpus callosum (TCC) revealed strong BChE reaction. The area cinguli of cortex (CCA) was negative for BChE reaction, whereas cingulum (Cg) was strongly positive. The external capsule (EC) illustrated moderate BChE activity.

Basal ganglia: The BChE preparations of the nucleus caudate putamen (NCP) showed intensely positive, but diffused BChE activity. The internal capsule fibers (CI) were also intensely positive for BChE reaction.

Area septalis: The preparations of the nucleus triangularis septi (NTS) was devoid of BChE activity, whereas both the nucleus fimbrialis septi (NFS) and nucleus lateralis septi (NLS) exhibited intense activity. The commissure anterior pars anterior (CAP) and medial forebrain bundle (mfb) showed strongly positive reaction (Fig. 2a).

In lindane treated group the BChE activity declined in the cerebrum of mice (Fig. 2b). The reduction of BChE activity in CAP, mfb, Cg and TCC as well as in

---

Table 1—Effect of lindane and antioxidants on the distribution of BChE activity in different layers of olfactory lobe of mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>PFL</th>
<th>GL</th>
<th>EPL</th>
<th>ML</th>
<th>IPL</th>
<th>GRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>± +/-</td>
<td>++</td>
<td>±</td>
<td>++</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>LD</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>AA</td>
<td>+ ++</td>
<td>+++</td>
<td>±</td>
<td>++</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>AA+LD</td>
<td>+ -</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>±</td>
</tr>
</tbody>
</table>

Abbreviation used: ++++ = strong; +++ = intense; ++ =less intense; + - = moderate; + - = mild; ± negligible; - - = negative. C = Control, LD = Lindane, AA = Antioxidants, AA + LD = Antioxidants + lindane.
Fig. 1—(a) Distribution of BChE activity in different layers of olfactory lobe of control mice ×100. Note the intensely positive g in GL. Also note the deeply stained ig fibers. Note the negative reaction in PFL, and strong activity in ML. (b) - Distribution of BChE activity in different layers of olfactory lobe of lindane treated mice ×100. Note the reduction of BChE activity in each layer, maximum reduction in ig fibers of GL. ML and IPL also show the reduced activity of BChE. (c) - Distribution of BChE activity in different layers of olfactory lobe of antioxidants group ×100. The elevation of BChE activity, especially in PFL is seen. (d) - Distribution of BChE activity in different layers of olfactory lobe of antioxidants plus lindane treated group ×100. Note the restoration of BChE activity.

Table 2 —Effect of lindane and antioxidants on the distribution of BChE activity in different nuclei and fiber tracts of different groups of mice

<table>
<thead>
<tr>
<th>Nuclei and fiber tracts</th>
<th>GROUPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Cerebral Cortex (CC)</td>
<td>±</td>
</tr>
<tr>
<td>Cerebral cortex area cinguli (CCA)</td>
<td>- -</td>
</tr>
<tr>
<td>Cingulum (Cg)</td>
<td>++++</td>
</tr>
<tr>
<td>Commisure anterior pars anterior (CAP)</td>
<td>++++</td>
</tr>
<tr>
<td>Nucleus Caudatus Putamen (NCP)</td>
<td>++++</td>
</tr>
<tr>
<td>Truncus Corpus Callosum (TCC)</td>
<td>++++</td>
</tr>
<tr>
<td>Nucleus Lateralis Septi (NLS)</td>
<td>++++</td>
</tr>
<tr>
<td>Nucleus Triangularis Septi (NTS)</td>
<td>---</td>
</tr>
<tr>
<td>Nucleus Fimbrialis Septi (NFS)</td>
<td>++++</td>
</tr>
<tr>
<td>Medial forebrain bundle (mfb)</td>
<td>++++</td>
</tr>
<tr>
<td>Area preoptica (AP)</td>
<td>- -</td>
</tr>
<tr>
<td>Capsula Externa (EC)</td>
<td>+++ -</td>
</tr>
<tr>
<td>Capsula Interna (CI)</td>
<td>+++ -</td>
</tr>
</tbody>
</table>

Abbreviation used : ++++ = strong; +++ = intense; ++ =less intense; ++ = moderate; + - = mild; ± Negligible; - - = negative. C = Control, LD = Lindane, AA = Antioxidants, AA + LD = Antioxidants + lindane.
Pretreatment with antioxidants in lindane treated group restored the BChE activity (Fig.2d). The elevated BChE activity due to antioxidants supplementation in TCC, CAP and area septalis region was clearly discernible (Fig. 2c).

**Biochemical results**

BChE activity (Fig. 3): The BChE activity in olfactory lobe of mice exposed to lindane was decreased ($P<0.05$), whereas in antioxidant plus lindane treated group the BChE activity significantly ($P<0.01$) increased compared to lindane treated group. Cerebrum region also showed a significant ($P<0.01$) reduction in BChE activity due to the lindane intoxication, whereas the enzymatic activity significantly ($P<0.01$) recovered after pretreatment with combination of antioxidants plus lindane exposed mice.

Fig. 2—(a) Distribution of BChE activity in different nuclei and fiber tract of cerebrum of control mice ×25. Note the strong reaction in TCC, Cg, CAP, mfb regions. In the area septalis region NTS shows the negative activity, whereas the NLS and NFS show intense activity. NCP and Cl show the intense reaction. (b) Distribution of BChE activity in different nuclei and fiber tract of cerebrum of lindane treated mice ×25. Note the reduction of BChE activity in CAP, mfb, Cg and TCC as well as in basal ganglia. (c) Distribution of BChE activity in different nuclei and fiber tract of cerebrum of antioxidants group ×25. The elevated BChE activity is seen in TCC, CAP, and area septalis region. (d) Distribution of BChE activity in different nuclei and fiber tract of cerebrum of antioxidants plus lindane treated group ×25. Note the restoration of BChE activity.

![Fig. 3](image-url)
SOD and CAT activities (Table 3): The SOD activity increased due to lindane treatment as compared to control \((P>0.05)\), whereas reduction in SOD activity was observed in antioxidants plus lindane treated group \((P>0.05)\). The CAT activity significantly \((P<0.01)\) declined in the lindane treated group compared to control group. In antioxidants plus lindane treated group, the activity of CAT enzyme increased significantly \((P<0.01)\) compared to lindane group.

Protein and cholesterol content (Table 3): The value of total protein decreased significantly \((P<0.01)\), whereas the value of total cholesterol increased in cerebrum of lindane treated mice as compared to control. The pretreatment with antioxidants in the lindane treated group significantly increased the protein \((P<0.01)\), whereas cholesterol level declined \((P<0.01)\).

**Discussion**

Brain is uniquely vulnerable to toxicants due to its inability to replace lost cells, long distances over which neurons must transport cellular products, great surface area, high sensitivity of neurons to energy and oxygen deficits, and tendency for certain parts of the brain to accumulate lipophilic substances. The neurotoxicity of lindane may occur due to its preferential accumulation in brain and its ability to cross the blood brain barrier (BBB), consequently causing functional impairment in BBB. Cholinesterases are located in the BBB. Therefore, any change in BBB permeability may influence the activity of cholinesterases. Thus, the decrease in BChE enzyme activity due to lindane may also be linked to such physiological changes. The pretreatment with combination of certain antioxidants augmented BChE activity in the olfactory lobe and cerebrum of lindane treated mice.

In order to substantiate biochemical results qualitative histochemical study was also carried out. The histochemical results validate biochemical findings that lindane inhibits the activity of BChE in olfactory lobe and cerebrum of mice. To date no studies have specifically been performed to examine BChE using histochemical method on olfactory lobe and cerebrum due to lindane intoxication. Histochemically marked reduction in BChE activity was observed in the synapse rich glomerular layer of the olfactory lobe, consequently the transmission of impulse across the synapses in the glomerular layer may be hampered. Due to lipophilic nature, higher concentrations of lindane were found to be high in white matter and myelinated structures of brain.

In support of this, histochemical results of present investigation revealed that lindane intoxication decreases the level of BChE enzymes mainly at TCC, Cg, CAP and mfb regions of the cerebrum which are composed of white matters. The lindane has been reported to induce oxidative stress. The oxidative stress causes disturbance in antioxidant enzyme system. The activity of antioxidant enzyme system may be increased or decreased depending upon the dose and duration of pesticides exposure as well susceptibility of the exposed species. The effort of antioxidant enzymes is to remove the continuously generated free radicals, initially by increasing the enzymes followed by decrease in their activity. In the present study, an increase in SOD activity was observed which may be linked to the increased production of superoxide radical by lindane. The induction of SOD activity is supposed to be due to adaptive changes following oxidative stress. Lindane exposure decreases the activity of CAT in cerebrum of mice consequently leading to accumulation of \(\text{H}_2\text{O}_2\). On the other hand \(\text{H}_2\text{O}_2\) is also produced at higher level on account of increase in SOD activity. Consequently, the increase
in H$_2$O$_2$ adversely affects the functioning of the brain$^{31}$. The free radicals or ROS that produced during lindane treatment have the ability to modify proteins, lipids, and nucleic acid to develop or enhance lindane mediated manifestations. In the present study, an increase in cholesterol content was observed after lindane treatment which is in agreement with previous studies$^{32,33}$. An increase in cholesterol content may be due to perturbation of membrane phospholipids composition as well as membrane fluidity. The change in cholesterol content suggests the possible involvement of compensatory mechanism that would balance the amount of cholesterol in brain. The possible physiological consequence of increased cholesterol level may lead to atherosclerosis. The long term exposure of workers to organochlorine pesticides makes them susceptible to developing stroke and brain damages$^{34}$.

Antioxidants are potential therapeutic agents against many health disorders associated with aging including neurodegenerative diseases such as diabetic neuropathy, Alzheimer's and Parkinson's diseases. Moreover, the combination of antioxidants seems to be more effective than high doses of a single antioxidant. In view of this, in the present study antioxidants such as vitamins (vitamin E and vitamin C), thiol containing antioxidant α-lipoic acid and stilbene resveratrol, a potent antioxidant in red wine were undertaken to ameliorate lindane induced neurotoxicity. Vitamin E (α-tocopherol), a lipid soluble chain breaking antioxidant plays an essential role in protecting integrity of lipid structure including cell membranes. The protective role of vitamin E is most likely due to its ability to modulate superoxide radical generation$^{35}$ thereby preventing oxidative damage. Although, in vitro study showed that vitamin E can protect lindane toxicity$^{15}$, vitamin E alone at low dose may be unable to annul the neurotoxic effects due to its subdued penetration at the level of BBB$^{36}$ as well as its ability to generate phenoxyl radical. Hence, reduction of phenoxyl radical by other antioxidants to regenerate the active vitamin E could provide an effective means to improve neurological functions. Therefore, the antioxidant potential of vitamin E could possibly be maximized by supplementation with dihydrolipoic acid in presence of ascorbic acid$^{37}$. Vitamin C (ascorbic acid) is water soluble and can directly react with superoxide, hydroxyl radicals, and singlet oxygen$^{38}$. The possible mechanism by which vitamin C reduces the OS in the present study may be attributed to its reducing nature. Vitamin C crosses the BBB in its oxidized form (dehydro ascorbate), retained there as ascorbate form and protect endogenous vitamin E against oxidation by reducing the phenoxyl radicals within the biomembranes$^{39}$. Lipic acid and its reduced form dihydrolipoic acid are potent antioxidants which cross the BBB$^{40}$. The protective effect of lipoic acid and dihydro lipoic acid may involve the recycling of other antioxidants such as GSH, ascorbate and vitamin E in neural membranes. Exogenous lipoic acid is able to enhance the protective effect of ascorbate by regenerating it from dehydroascorbate$^{41}$. Resveratrol, naturally present at high concentration in grape skin, seeds and red wine and also has ability to cross the BBB$^{42}$. Several studies showed the neuroprotective efficacy of resveratrol alone$^{43-44}$. Nonetheless, the resveratrol provides greater degree of protection when given with vitamin C and E rather than administered singly$^{15}$. The neuroprotection by resveratrol may be due to its intrinsic free radical scavenger ability which balances the prooxidant/antioxidant equilibrium possibly by increasing the endogenous defensive capacity of the brain.

In conclusion, the results of present study suggest that neuroprotective efficacy of combination of antioxidants may result of their synergistic action probably by scavenging and sequestering free radicals directly or by improving endogenous antioxidants. Although, present data indicate that the combination of antioxidants as opted in the present study is a promising neuroprotective agent, clinical studies are needed to establish its therapeutic effect in humans.

References


17 National Toxicology Programme (NTP), *NTP chemical repository* (Radiation Corporation, August 1991),*lindane* (58:89-90) 1998.


25 Gerr F, Health effects of exposures to neurotoxic agents used in the Persian Gulf War. A report to the Committee on Veterans Affairs of the United States Senate. 325 (1998).


