Protective effects of different antioxidants against endosulfan-induced oxidative stress and immunotoxicity in albino rats

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Endosulfan exposure (8 and 16mg/kg) to rats significantly decreased the activities of superoxide dismutase and catalase, level of reduced glutathione and increased lipid peroxidation. The primary and secondary antiSRBC antibody titers, plaque forming cells counts and delayed hypersensitivity reaction, and the TH1 or TH2 cytokines levels were significantly suppressed in a dose dependent manner. L-ascorbic acid and α-tocopherol produced a synergistic reversal of oxidative stress parameters following endosulfan exposure. N-acetylcysteine produced significant reversal of altered oxidative stress parameters and immune response after endosulfan exposure. A significant attenuation of the oxidative stress markers and immunotoxicity with a combined therapy of L-ascorbic acid plus α-tocopherol and with N-acetylcysteine was clearly demonstrated by the present results.

Keywords: Attenuation, α-tocopherol, Immunotoxicity, L-ascorbic acid, N-acetylcystein, Oxidative Stress

Pesticide-induced oxidative stress as a possible mechanism of toxicity has been a focus of toxicological research⁴. Endosulfan (6,7,8,7,10–hexachloro-15,5a,6,9,9a hexahydro 6,9 methano 2,4,3 benzoicxanthiepin-3-oxide), a polycyclic chlorinated hydrocarbon of cyclodiene group is a widely used pesticide². Neurotoxicity is one of the major issues of concern in acute endosulfan exposure⁴. Effect of endosulfan poisoning with suspected involvement of central nervous system has been reported⁴.

Dose dependent effect of endosulfan on humoral and cell mediated immune responses in rats is reported⁶. Saiyed et.al⁶ documented a role of endosulfan exposure causing delay in sexual maturity, especially in male children. The subacute and chronic toxic studies of endosulfan in animals have suggested that the liver, kidneys, testes and immune system are the main target organs bearing the major impact of endosulfan toxicity⁵.

The in vivo effect of endosulfan on antioxidant enzymes activity is not yet well evaluated. Oxidative stress is a disruption of pro-oxidant and antioxidant balance as a result of an increase in reactive oxygen species (ROS) generation, impairment of anti-oxidant defense systems or an insufficient capacity to repair oxidative damage⁸. Inactivation and removal of ROS depend on the intracellular antioxidantive defense systems⁹. Induction of various biochemical changes including induction of oxidative stress and immunotoxicity in experimental animals exposed to subchronic doses of organophosphate and its attenuation by N-acetylcysteine have been reported¹⁰. Administration of antioxidants such as ascorbic acid, α-tocopherol and N-acetylcysteine play a protective role against pesticide induced toxicity and immunotoxicity¹¹-¹².

Attempts have been made to evaluate the in vitro toxic effect of endosulfan using different models³,¹³; however its in vivo impact on lipid peroxidation and oxidative stress is poorly elucidated. Therefore this study has been designed to evaluate (i) the dose dependent effect of endosulfan on indices of oxidative stress and immunotoxicity, and (ii) protective effect of various exogenous antioxidants against oxidative stress and immunotoxicity in rats.

Materials and Methods

Chemicals—Endosulfan, technical grade (Cat. No P-435N), α and β stereopisomers, 70:30 ratio was obtained from Accustandard, RFCL™ Limited India. L-ascorbic acid, α-tocopherol, N-acetyl cysteine and
all other chemicals in the present study were of analytical grade and purchased from Sigma–Aldrich Limited St. Louis, MO, USA.

**Animals and treatment**—Wister male albino rats weighing 200-250 g were placed under conventional condition such as light–dark cycle (12:12 h) and temperature (22°C ± 2°C) etc. The study was conducted with the approval of the Institutional Animal Ethical Committee (IEAC) and animal handling was performed as per guideline laid for care and use of animals in scientific research prepared by Indian National Science Academy (INSA) New Delhi. Rats were randomly divided in 9 groups of 8 animals each and treated orally with 4, 8 or 10 mg/kg of endosulfan dissolved in groundnut oil (pharmaceutical quality) once daily for 14 consecutive days and simultaneously antioxidant treatment were given. Control group received daily groundnut oil (orally) or normal saline (ip). Different dose of L-ascorbic acid, α-Tocopherol (emulsified in Tween 20) and N-acetylcysteine dissolved in normal saline were administered ip into the rats of various groups as shown in Table 1.

**Lipid peroxidation**—Melondialdehyde (MDA) level in serum as an index of lipid peroxidation level was determined as per Satoh using thiobarbituric acid reactive substance (TBARS). MDA-TBA adduct formation was measured spectrophotometrically at 532nm. The concentration of melonaldehyde (MDA) was expressed as nmol/ml.

**Antioxidant enzymes**—The activites of superoxide dismutase (SOD) and catalase (CAT) in Tsuchihashi extract were measured spectrophotometrically as described by Nandi and Chatterjee and Sinha respectively.

**Estimation of glutathione levels**—Total glutathione (GSH) content in blood was measured as per Tietze using dithionitrobenzene and expressed as μmoles/ml.

**Humoral immune response**

Humoral immune response was assayed by estimating the following parameters:

**Anti-SRBC antibody titre (Haemagglutination) assay**—Fresh serum antibody titer against SRBC was measured by haemagglutination technique. The antibody titre was expressed as -log₂ of the reciprocal of the first dilution.

**Splenic plaque forming cell (PFC) assay**—The PFC assay was carried out as per Cunningham et al. The endosulfan treated animals were immunized with 0.5×10⁹ SRBC on day 8 and sacrificed on day 14 under anesthesia. The spleen was removed and the plaque forming cells were counted under light microscope and expressed as plaque/million spleen cells.

**Cell mediated immunity**

**Delayed type hypersensitivity (DTH) reaction**—The DTH response was measured as per Liew. Rats were immunized on day ‘0’ with keyhold limpet haemocyanin (KLH) in Fruend’s complete adjuvant (FDA) in equal amounts of PBS, pH 7.4. On day 13 rats were challenged with the same antigen in right hind foot and normal saline was inoculated on the left foot. After 24 h the foot pad edema was measured and difference in the foot pad volume (right-left) was measured by a dial caliper (Mututio Japan). The results were expressed as percentage difference in DTH response. An equal number of animals were maintained unstimulated but pesticide or pesticide plus antioxidant treatment were given to them. Unstimulated rats were treated similarly except they were not immunized with any antigen.

**Cytokines assays**—The cytokines assays were performed using commercially available cytokine assay kits (Diclone, France). It is a solid phase sandwich enzyme linked immuno sorbant assay

<table>
<thead>
<tr>
<th>Groups</th>
<th>Endosulfan (mg/kg)</th>
<th>L-ascorbic acid(L-AA)/α-tocopherol (α-toco)/N-acetylcysteine(NAC)/Vehicle.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle</td>
<td>-</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>Endosulfan + L-AA</td>
<td>8</td>
<td>100 mg/kg of L-AA</td>
</tr>
<tr>
<td>Endosulfan + α-toco</td>
<td>8</td>
<td>30 mg/kg of α-toco</td>
</tr>
<tr>
<td>Endosulfan + L-AA + α-toco</td>
<td>8</td>
<td>L-AA(100mg/kg) + α-toco(30mg/kg)</td>
</tr>
<tr>
<td>Endosulfan + NAC</td>
<td>8</td>
<td>NAC (100 mg/kg)</td>
</tr>
<tr>
<td>Endosulfan + NAC</td>
<td>8</td>
<td>NAC (200 mg/kg)</td>
</tr>
</tbody>
</table>
(ELISA) for measuring the Interferon-γ (INF-γ) and Interluekines-4 (IL-4) levels measured at 450nm using BIORAD 680 and results were expressed in picogram/ml.

Statistical analysis—All data were analyzed by using one way analysis of variance (ANOVA) followed by the Newman-Keul posthoc tests for the multiple group comparisons and $P<0.05$ was considered as the level of significance in all statistical test.

Results
At a dose of 4 mg/kg given for 14 consecutive days, no significant changes in the oxidative stress parameters were observed (Table 2). However, 8 and 16 mg/kg i.e. 20 and 40 % of LD$_{50}$ doses significantly induced changes in the oxidative stress markers. The extent of the lipid peroxidation was found significantly enhanced as evidenced by the increased serum TBARS activity levels ($P<0.001$) and reduced levels of aqueous phase antioxidant, glutathione ($P<0.01$) in comparison with control. The activities of antioxidants enzyme, SOD and CAT in the red blood cells were found significantly decreased compared to the vehicle controls ($P<0.001$).

Endosulfan (8 and 16 mg/kg) significantly induced reduction of both humoral and cell mediated immune responses, ($P<0.001$) (Table 3). The reduction in the primary anti-SRBC antibody response was more consistent as compared to the secondary anti-SRBC antibody titer. A significant reduction in splenic plaque forming cells (PFC) due to endosulfan exposure more so at 8 and 16 mg/kg was observed. Suppression of cell mediated immune response was depicted by reduction in thickness of foot pad following immunization with KLH in rats exposed to higher doses of endosulfan as compared to vehicle treated controls (Table 3). However, endosulfan at 4mg/kg for 14 consecutive days did not significantly influence the humoral and CMI responses.

Endosulfan at 8 and 16mg/kg doses significantly suppressed the INF-γ and IL-4 cytokines levels as compared to vehicle treated controls (Fig.1). The reduction in INF-γ was 11% more as compared to the IL-4 cytokines levels ($P<0.001$).

Table 2— Effect of various doses of endosulfan on oxidative stress parameters in rats
[values are mean ± SE from 8 animals in each group]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nM/ml)</th>
<th>GSH (µM/mL)</th>
<th>SOD (U/g Hb)</th>
<th>CAT (U/g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle)</td>
<td>2.6 ± 0.1</td>
<td>16.0 ± 0.7</td>
<td>860 ± 7.8</td>
<td>3699 ± 59.7</td>
</tr>
<tr>
<td>Endosulfan (mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.4 ± 0.3</td>
<td>12.3 ± 0.7$^b$</td>
<td>750 ± 9.2$^a$</td>
<td>3226 ± 69.4$^a$</td>
</tr>
<tr>
<td>8</td>
<td>5.8 ± 0.4$^a$</td>
<td>8.1 ± 0.5$^a$</td>
<td>428 ± 5.9$^a$</td>
<td>2345 ± 44.5$^a$</td>
</tr>
<tr>
<td>16</td>
<td>6.4 ± 0.5$a$</td>
<td>7.1 ± 1.0$a$</td>
<td>398 ± 7.8$a$</td>
<td>2143 ± 72.9$a$</td>
</tr>
</tbody>
</table>

$P$ values: $^a<0.001$, $^b<0.01$ compared to vehicle control.

Table 3— Effect of endosulfan on humoral and cell mediated immune response.
[Values are mean ± SE from 8 animals in each group]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PAR</th>
<th>SAR</th>
<th>Plaque/10$^6$ spleen cells</th>
<th>Changes (%) in DTH reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Vehicle)</td>
<td>5.8 ± 0.1</td>
<td>7.2 ± 0.2</td>
<td>432 ± 37.9</td>
<td>20.5 ± 0.6</td>
</tr>
<tr>
<td>Endosulfan (mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.0 ± 0.2</td>
<td>6.3 ± 0.5</td>
<td>395 ± 31.8$^b$</td>
<td>16.2 ± 1.3$^b$</td>
</tr>
<tr>
<td>8</td>
<td>3.2 ± 0.3$^a$</td>
<td>5.9 ± 0.9$^b$</td>
<td>305 ± 31.6$^b$</td>
<td>10.1 ± 0.5$^b$</td>
</tr>
<tr>
<td>16</td>
<td>2.9 ± 0.9$^b$</td>
<td>2.14 ± 0.3$^a$</td>
<td>287 ± 32.2$^b$</td>
<td>9.2 ± 0.3$^a$</td>
</tr>
</tbody>
</table>

($P$ values: $^a<0.001$, $^b<0.01$ compared to vehicle control). PAR: Primary antibody response, SAR: Secondary antibody response, DTH: Delayed type hypersensitivity
Endosulfan-induced changes in the oxidative stress marker were significantly attenuated with the combined therapy of L-ascorbic acid and α-tocopherol (Fig. 2A). L-ascorbic acid and α-tocopherol given at a single dose of 100 and 30 mg/kg for 14 consecutive days, showed no significant effect on lipid peroxidation and GSH level (Fig. 2B). However L-ascorbic acid and α-tocopherol when administered in combination significantly attenuate any decrease in the GSH level. The endosulfan induced changes in the red blood cell activities of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) showed significant attenuation when pretreated with combined dose of L-ascorbic acid and α-tocopherol, Fig. 2C.

Endosulfan induced reduction in primary and secondary (P<0.05) anti-SRBC antibody titers was significantly attenuated (P<0.01) by pre-treatment with L-ascorbic acid and α-tocopherol given for 14 consecutive days (Fig. 3A). Pre-treatment with L-ascorbic acid resulted in lowering of endosulfan induced changes in PFC (Fig. 3B). A significant attenuation of any decrease in DTH of rat was observed following antioxidants treatment (Fig. 3C). Moreover the fall in the IFN-γ and IL-4 levels could be reverted with L-ascorbic acid at 100mg/kg and α-tocopherol at 30 mg/kg (Fig. 4). But such protection was not observed at lower doses of L-ascorbic acid and α-tocopherol (data not shown). However, used in combination with L-ascorbic acid, the effect were synergistic and greater then either compound used alone.

Figure 5A shows that extend of lipid peroxidation, attenuated by the treatment with N-acetyl cystein in dose dependent manner. Further, the decrease in endosulfan induced glutathione levels also reverted to normal value (Fig. 5B). Figure 5C shows that N-acetyl cysteine given for 14 days also significantly attenuated the effect of endosulfan on SOD and CAT.

Primary and secondary anti-SRBC antibody titers were attenuated significantly with the pre-treatment with N-acetyl cysteine (100 mg/kg) for 14 consecutive days (P<0.001), which was more pronounced at higher doses (Fig. 6A). A significant reversal of PFC count was observed with pre-treatment with NAC in dose dependent manner (Fig. 6B). Different doses of NAC (100 and 200 mg/kg) produced a significant reversal of endosulfan induced suppression in footpad thickness response in KLH challenged animals (Fig. 6C). Similar observation was documented in attenuation of TH1 and TH2 ratio with both the doses of NAC (Fig.7).

Discussion

Immunological effects of endosulfan exposure in rats have been documented. Enzymatic antioxidant effects under such condition may demonstrate a reduced effect in counteracting the altered parameter of oxidative stress. The results of the present study
evaluated the dose dependent protective effect of ascorbic acid, α-tocopherol and N-acetylcysteine on endosulfan-induced changes in oxidative stress parameter and immunosuppression in rats.

Endosulfan at lower dose (4 mg/kg) did not significantly alter the oxidative stress index, however at higher doses (8 and 16 mg/kg) resulted in alteration in the oxidative stress markers causing significantly
decrease in the activities of antioxidant enzymes, SOD and CAT. An associated increase in the index of lipid peroxidation reaction and decrease in GSH levels was also documented. These changes in oxidative indices suggest that reactive oxygen species (ROS) are involved in endosulfan-induced immunotoxicity in vivo.\(^{20}\)

The present result clearly showed the impact of endosulfan toxicity on humoral immune response resulting in a suppressed primary and secondary antiSRBC antibody titer as well as significant reduction in plaque forming cells counts. This is in agreement with Banerjee and Hussain.\(^{5}\) The cellular immune response was also significantly suppressed following endosulfan exposure as depicted by DTH reaction and altered TH1 verses TH2 response expressed changes in the levels of INF-\(\gamma\) and IL-4 levels respectively. These data further confirm the immunotoxic effect as reported earlier.\(^{10}\)

Endosulfan-induced oxidative stress was differentially, yet significantly attenuated by pre-treatment with various antioxidants as used in the present study. Although L-ascorbic acid and \(\alpha\)-tocopherol produced appreciable effects on endosulfan induced oxidative stress, it produced no apparent change in humoral and CMI. L-ascorbic acid (100 mg/kg) and \(\alpha\)-tocopherol (30 mg/kg) were unable to produce any significant change in TBARS production and reduced GSH level. However, combination of L-ascorbic acid and \(\alpha\)-tocopherol attenuated any increase in the activity of SOD and CAT.

The administration of exogenous antioxidants in presence of endosulfan exposure enhances the activities of endogenous antioxidant enzymes, thus preventing a possible involvement of ROS in the inhibition of these enzymes. This was well depicted in the present study when the two antioxidants, L-ascorbic acid and \(\alpha\)-tocopherol were used in combination. The results of pretreatment with
N-acetylcysteine, which acts not only as a direct free radical scavenger but also promotes the production of GSH, were found to have a significant effect on endosulfan-induced immunotoxicity, more so at a higher doses of 200 mg/kg for the given time periods. The present findings suggest that endosulfan-induced immunotoxicity may be mediated through free radical formation and reduced antioxidant defense mechanism which can be significantly attenuated by the administration of exogenous antioxidants. Hence, exogenous supplement of these antioxidants could delay or prevent xenobiotic induced immunological or biochemical changes, enabling clinical strategies for the prevention and treatment of immunological disorders induced or precipitated by exposure to endosulfan.

References