Effect of acute exposure of triazophos on oxidative stress and histopathological alterations in liver, kidney and brain of Wistar rats

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Acute dose of organophosphorus pesticide Triazophos (O,O-diethyl O-1-phenyl-1H-1,2,4-triazol-3-yl phosphorothioate; Tz) administered orally affects oxidative stress parameters and the histo-architecture of liver, kidney and brain tissues. The results indicate a dose dependent induction of oxidative stress as evident by increased malondialdehyde level and decreased antioxidant defense including glutathione and superoxide dismutase activity in rat liver, kidney and brain. AChE activity was found significantly decreased in the Tz treated groups as compared to the vehicle control (DMSO) group. Histopathological examination of liver, kidney and brain in Tz treated rats revealed medullary congestion and hydropic degeneration of hepatocytes in liver and medullary congestion in kidney. However, no significant histopathological changes were observed in brain tissues.

Keywords: Acetyl cholinesterase, Glutathione, Lipid peroxidation, Superoxide dismutase, Triazophos

Organophosphorus (OP) compounds constitute a heterogeneous category of chemicals specifically designed for the control of pests. Widespread application of organophosphate pesticides being low in their bio-accumulation brings a hazardous risk of exposure to mammalian system because of optimum residual level and steady dissipation rate¹. Results of several studies have shown that OP causes both acute as well as chronic health effects²-⁶. Triazophos (O, O-diethyl O-1-phenyl-1H-1, 2, 4-triazol-3-yl phosphorothioate) being a broad spectrum non-systemic organophosphorus pesticide is used liberally throughout the world for plant protection⁷. General population is exposed to triazophos (Tz) through food products and drinking water but human exposure of acute oral doses of Tz is mostly accidental and suicidal. Thus, Triazophos imposes an important health concern in humans⁸-¹³. The primary mechanism of action of Tz is neurotoxic leading to accumulation of neurotransmitter acetylcholine in synaptic, cholinergic and neuromuscular effects¹¹. The severity of Tz intoxication may vary with dose, route and extent of exposure. Studies on acute toxicity of Tz with respect to oxidative damage, acetylcholinesterase (AChE) inhibition and histo-architectural alterations have not been reported so far. Therefore, in the present study, an attempt has been made to study the effect of Tz on oxidative status along with AchE activity and histopathological changes in liver, kidney and brain tissue after acute exposure in Wistar rats.

Materials and Methods

Chemicals and reagents—Sodium dodecyl sulphate (SDS), butanol, pyridine, acetic acid, trichloro acetic acid (TCA) [E. Merck, India], thiobarbituric acid (TBA) [Merck-Germany], 1, 1, 3, 3-tetramethoxypropane, 5,5-dithionitrobenzoic acid (DTNB) [Sigma Aldrich, USA] and other chemicals of analytical grade were used from Merck, India. Technical grade Triazophos (68%) was received from M/s Hindustan Insecticide, India.

Animals—Adult male Wistar rats weighing 150–200 g obtained from the Central Animal Facility of the Institute were used. Rats were group housed in polyacrylic cages with not more than 4 animals per cage and kept under standard laboratory conditions (natural light/dark cycle at 23 ± 3 °C). Animals were
provided with a nutritionally adequate standard laboratory diet and tap water was provided *ad libitum*. Locally procured sterile paddy husk was used as bedding in the cages. The animals were allowed to acclimatize to the laboratory environment for 7 days prior to the study. This study protocol has been approved by the Institutional Animal Ethics Committee (Approval No. 558/IAEC/10).

**Experimental protocol**—Rats were randomly divided into following four groups of 6 each. Group I (vehicle control) animals were fed 0.5 mL of DMSO. Group II, III and IV animals were fed with Tz (dissolved in 0.5 mL of DMSO) at dose 10.87, 18.11, 21.73 mg/kg body weight corresponding to 15, 25 and 30% of LD₉₀ (72.44 mg/kg) respectively in a similar fashion and sacrificed under light ether anesthesia after 24 hr. Vehicle as well as drug were administered orally.

**Sample collection**—After completion of the treatment period liver, kidney and brain were surgically removed. Small representative slices were fixed in 10% formalin for routine histopathological studies. The remaining was washed with ice-cold saline. Tissues samples of liver, kidney and brain were separately homogenized for 5 min in 4 volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 0.15 M KCl. The homogenates were centrifuged at 9000 g at 4 °C for 20 min. The supernatants were pipette into a clean centrifuge tube and centrifuged further at 104,000 g at 4 °C for 60 min in an ultracentrifuge. The resulting microsomal and cytosolic fractions of liver, kidney and brain were used for the biochemical estimations.

**Biochemical estimation of oxidative stress parameters**

**Liver, kidney and brain tissue lipid peroxidation levels**—Lipid peroxidation was assessed using malondialdehyde (MDA), as an indicator. Briefly, 1.5 mL of 20% (v/v) acetic acid, pH 3.5. 1.5 mL of 0.8% (w/v) of thiobarbituric acid and 0.2 mL of 8.1% (w/v) of sodium dodecyl sulphate were added to 0.1 mL of supernatant, and then heated at 95 °C for 60 min. Five milliliters of n-butanol/pyridine (15:1) was added to the mixture after cooling. The organic layer was separated by centrifugation at 4000 rpm for 10 minutes, and absorbance was measured at 532 nm using a spectrophotometer (UV 5704SS, Electronic Corporation of India limited). The concentration of MDA is expressed in nmol/g wet tissue.

**Liver, kidney and brain tissue reduced glutathione (GSH) levels**—Total Glutathione content was measured following method of Ellman. The homogenate was mixed with an equal quantity of 10% trichloroacetic acid and centrifuged. Hundred micro liter of supernatant (free of proteins) was added to 2 mL of 0.3 M phosphate buffer (pH 8.4), 0.5 mL of 0.04% DTNB in 1% tri-sodium citrate and further 0.4 mL of double distilled water was added in succession and chromophoric product was measured at 412 nm. The concentration of reduced glutathione is expressed as µg/g wet tissue.

**Liver and brain cholinergic status**—AChE is found at mainly neuromuscular junctions and cholinergic brain synapses, where its activity serves to terminate synaptic transmission that’s why only liver and brain tissue were used for AChE activity. The cholinergic markers AChE was estimated in the rat liver and brain according to the method of Ellman et al. Briefly, 10 mM DTNB in 0.1 M Tris–HCl buffer (pH 8.0), 100 µL of supernatant, and 30 mM acetylthiocholine iodide as substrate were added for AChE estimation. Absorbance was measured at 412 nm for 3 minutes at 30-second intervals. AChE activity, expressed as micromoles of acetylthiocholine iodide hydrolyzed per milligram of protein per minute.

**Superoxide dismutase (SOD) activity status in liver, kidney and brain**—SOD activity was measured as per Marklund and Marklund. Buffer consisting of 50 mM (pH 8.2) Tris–HCl buffer, 4 mM pyrogallol and 50µL supernatant were added to study SOD activity. Absorbance was measured at 420 nm for 3 min at 30 sec interval. SOD activity is expressed as U/mg protein. 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.

**Protein estimation**—Protein contents were measured as per Lowry et al. using bovine albumin serum as standard.

**Histopathological analysis**—Liver, kidney and brain sections which were excised from the rats were preserved in 10% neutral buffered formalin solution. Formalin fixed liver, kidney and brain tissue were routinely processed, embedded in paraffin and sectioned at 5 µm thicknesses and stained with hematoxylin and eosin for histopathological studies.

**Statistical analysis**—Results are expressed as mean ± SE. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test. P < 0.05 was considered as significant.
Results

Biochemical measurements

Effect on malondialdehyde levels—In the Tz treated model, a significant increase in liver, kidney and brain MDA levels was observed in group III and IV. However, no significant change in liver and brain MDA levels was observed in group II as compared to vehicle control group (Table 1).

Effect on glutathione content—A significant decrease in liver, kidney and brain GSH level was observed in group III and IV as compared to vehicle control depicted in Table 1.

Effect on Superoxide dismutase (SOD) activity—In the Tz treated model, a significant increase in the activity of SOD was observed in liver and brain of group II as compared to vehicle control group. While, SOD level was decreased significantly in liver, kidney and brain in group IV. However, at the dose of 18.11 mg/kg a significant decrease in brain SOD activity was observed as compared with the control group (Table 1).

Effect on acetylcholinesterase activity—After post hoc analysis, AChE activity was found to be decrease significantly in liver and brain in group II and IV. However, AChE activity in 10.87 mg/kg Tz treated group (group II) showed insignificant results as compared with the vehicle control group (Table 1).

Histopathological analysis—Histopathological changes were observed in the animals of treated groups as compared to the control. Hydropic degeneration of hepatocytes were observed in liver section in groups III and IV at dose 18.11 and 21.73 mg/kg respectively (Fig. 1a) and spotty necrosis was observed in liver section of 21.73 mg/kg Tz treated (group IV) (Fig. 1b). Medullary congestion was observed in kidney (Fig. 1c and d) in all the Tz treated groups. However no significant changes were observed in brain at either dose of Tz with respect to control.

Discussion

Environmental pollution due to indiscriminate use of OPs is an important issue that attracts widespread public concern. Several studies have suggested that OPs produce acute as well as chronic toxicity by inhibiting the cholinesterase enzyme in the nervous system\(^3\)\(^-\)\(^6\). However, there is no consensus with regards to quantitative indices of OP induced toxicity\(^1\). Oxidative stress is defined as a cytological consequence caused by mismatch between the production of free radicals and the ability of antioxidants to scavenge them\(^2\)\(^1\)\(^,\)\(^2\)\(^2\). This imbalance results in occurrence of oxidative modified molecules that can cause oxidative stress\(^2\)\(^3\).

One of the earlier study has shown that Tz caused oxidative stress in rat tissues after chronic doses\(^1\) but effect caused by Tz with acute doses on oxidative stress followed by histopathological alterations is not reported so far. Present study showed acute oral

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (vehicle control)</th>
<th>Group II (10.87 mg/kg)</th>
<th>Group III (18.11 mg/kg)</th>
<th>Group IV (21.73 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver:</td>
<td></td>
<td></td>
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<tr>
<td>MDA</td>
<td>222.83±5.845</td>
<td>247.5±11.512</td>
<td>258.83±5.764*</td>
<td>266.17±3.27**</td>
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<tr>
<td>GSH</td>
<td>279.33±5.948</td>
<td>275.5±7.553</td>
<td>255.5±2.777*</td>
<td>255.5±3.64**</td>
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<tr>
<td>SOD</td>
<td>12.06±0.0587</td>
<td>13.24±0.208***</td>
<td>12.38±0.136</td>
<td>13.28±0.164***</td>
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<td>AChE</td>
<td>0.23±0.00894</td>
<td>0.205±0.005</td>
<td>0.2±0.0073*</td>
<td>0.123±0.00558***</td>
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<tr>
<td>Kidney:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td>229.62±5.778</td>
<td>237.5±9.512</td>
<td>236.83±5.764*</td>
<td>248.67 + 2.65*</td>
</tr>
<tr>
<td>GSH</td>
<td>265.33±5.948</td>
<td>261.5±7.463</td>
<td>258.5±2.777*</td>
<td>258.5±3.777**</td>
</tr>
<tr>
<td>SOD</td>
<td>9.16±0.41</td>
<td>9.34±0.38</td>
<td>11.58±0.136*</td>
<td>7.23 + 0.52**</td>
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<tr>
<td>Brain:</td>
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<tr>
<td>MDA</td>
<td>297.5±4.595</td>
<td>316±8.683</td>
<td>355.33±22.693*</td>
<td>388.67±2.963***</td>
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<td>GSH</td>
<td>300±2.582</td>
<td>297.17±1.815</td>
<td>289.5±3.5*</td>
<td>278.5±0.922***</td>
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<td>SOD</td>
<td>5±0.03686</td>
<td>7.21±0.204***</td>
<td>4.44±0.119*</td>
<td>3.41±0.06313***</td>
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<td>AChE</td>
<td>0.755±0.08156</td>
<td>0.6333±0.00568</td>
<td>0.5267±0.02028*</td>
<td>0.3217±0.02496***</td>
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</tbody>
</table>

MDA = n mol/g-wet tissue, GSH=µg/g-wet tissue, SOD=Units/mg protein, one unit of superoxide dismutase is defined as the amount, which inhibits the INT reaction by 50 %, AChE=µ mole of acetylthiocholine hydrolyzed/mg protein/min. P values: *<0.05, **<0.01, ***<0.001 as compared to control values.
toxicity of Tz caused oxidative stress in experimental animals and its exposure significantly increased the level of MDA in liver, kidney and brain at the dose 18.4 mg/kg and 21.73 mg/kg. In brain, higher lipid peroxidation was observed which could be due to high susceptibility of brain to oxidative insult, because it contains large amount of polyunsaturated fatty acid and consumes 20% of the body's oxygen. Moreover, in spite of a high rate of oxidative metabolism, the brain has a relatively low antioxidant defense system. Increased level of MDA in brain after acute exposure of OP is also supported by one of the study.

Enzymatic antioxidants are essential for the conversion of ROS to innocuous metabolites. The level of endogenous enzymatic antioxidants GSH and SOD was monitored in control and pesticide-exposed groups of rats. Glutathione (GSH), a major endogenous antioxidant, participates in detoxification reactions and counter-balances free radical mediated damage by eliminating the compounds responsible for LPO or by increasing the efficiency of NADPH that protects detoxifying enzymes. In the present study, glutathione levels were found to be decreased significantly in liver, kidney and brain, this reduction could be due to the consumption of glutathione while protecting against the Tz-induced oxidative stress or may be due to the decreased activity of GSH indicating the utilization of this antioxidant in the detoxification of Triazophos. The higher reduction of glutathione observed in liver as compared to brain may be due to higher Tz accumulation in liver. Higher reduction in glutathione level of liver as compared to brain indicates the liver to be more susceptible to Tz exposure.

SOD enzyme present in all eukaryotes contained copper or Zinc which played an important role in the catalyses of superoxide radicals to form hydrogen peroxide, further it is converted in to H2O by Catalase. SOD is a link in the biological defense mechanism through disposition of endogenous cytotoxic O2-, which are deleterious to structural proteins of plasma membrane. The decreased activity of SOD in fresh water fish (C. punctatus) due to acute exposure of Tz is also reported. It is observed that the pesticides produce oxidative stress by inhibiting the activity of SOD. The activities of superoxide dismutase (SOD) in liver and brain of rats were
increased at dose 10.87 mg/kg bw but decreased at dose 21.73 mg/kg bw as compared to control in the present study (Table 1). In kidney tissue, increased SOD activity was observed at dose 18.11 mg/kg acute dose, while decreased SOD activity was observed at 21.73 mg/kg dose. This may be due to an enhanced superoxide production during Tz metabolism at low doses and decreased SOD indicates that the triazophos produces oxidative stress by inhibiting activity of SOD. The activity of GSH in liver of rats was observed as decreased in the present study, the same is supported by the report but using sub chronic doses.

The ability of Tz to inhibit AChE activity in liver and brain of rats was assessed. It was found that oral administration of the Tz at doses of 18.11 and 21.73 mg/kg for 24 hr could inhibit AChE activity in liver and brain (Table 1). The primary mechanism of OPs toxicity is well studied – they function as inhibitors of the enzyme acetyl cholinesterase (AChE). Human exposure to OPs is most frequently assessed by measurement of decrease in AChE activity. This method is relevant for professional exposure, where OP concentrations entering to body are relatively high. AChE activity measurement has been widely used for monitoring the neurotoxicity of pesticides and is a key target for OPs. This enzyme is very sensitive to stress conditions; it can be used as a biochemical indicator of pollutant stress and exposure to such chemicals in chosen species. In their study, the histopathological examination of liver and kidney sections after 24 hr of Tz exposure demonstrated changes like hydropic degeneration of hepatocytes and medullary congestion consistent with spotty necrosis in liver. Histopathological changes are at par with biochemical changes as potential consequence of Tz toxicity. Excessive production of ROS mediates toxic effect of Tz in liver and correlated well with the initiation of diffuse fatty changes which may lead to subsequent hepatic injury. Due to lack of information regarding the acute (24 hr) toxic effect of Tz, the present study was designed to identify the neurotoxic potential of Triazophos. The results indicated that 24 hr exposure to Tz cause neurotoxic effect by induction of oxidative stress in terms of increased MDA and decreased GSH content in liver, kidney and brain followed by significantly decreased activity of SOD in liver, kidney and brain at higher dose (21.73 mg/kg) and decreased AChE activity in liver and brain at all doses. The results therefore suggest that the Tz induce oxidative stress in Wistar rats after acute exposure.

Conclusion
In summary, the present study demonstrated an increase of oxidative stress in Wistar rats tissue exposed to acute doses of Tz. This study clearly showed an inhibition of AChE activity, presenting this enzyme as a good indicator of intoxication of tissue by pesticides. Nevertheless, in addition to in vitro studies, larger clinical studies are required to better understand the triazophos toxic mechanisms in humans.

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