Induction of oxidative stress and histopathological changes by sub-chronic doses of triazophos

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The effect of triazophos (O, O-diethyl O-1-phenyl-1 H-1, 2, 4-triazol-3-yl phosphorothioate), a widely used insecticide was studied on the induction of oxidative stress and histological alterations at sub-chronic doses in male albino rats. Oral administration of triazophos at concentrations of 1.64, 3.2 and 8.2 mg/kg body wt for 30 days produced dose as well as time-dependent increase in the lipid peroxidation (determined by malondialdehyde levels) and glutathione-S-transferase (GST) activity in serum with a concomitant decrease in ferric reducing ability of plasma (FRAP) and blood glutathione (GSH) content. Histopathological examination of liver of triazophos-treated rats showed significant and progressive degenerative changes as compared to control, which could be due to induction of oxidative stress. However, no significant histopathological changes were observed in spleen, kidney and brain at either dose of triazophos with respect to control. These results indicated that oral administration of triazophos was associated with enhanced lipid peroxidation and compromised antioxidant defence in rats in dose and time-dependent manner. Thus the present study demonstrated for the first time the role of oxidative stress as the important mechanism involved in the stimulation of hepatic histo-architectural alterations at sub-chronic doses of triazophos in rats.  

Keywords: Triazophos, Oxidative stress, Lipid peroxidation, Glutathione, Glutathione-S-transferase, Ferric reducing ability of plasma

Triazophos (O, O-diethyl O-1-phenyl-1 H-1, 2, 4-triazol-3-yl phosphorothioate) is a broad spectrum non-systemic organophosphorus pesticide (OP) used liberally throughout the world for plant protection. Like other OPs, the primary mechanism of action of triazophos is neurotoxic and leads to accumulation of neurotransmitter acetylcholine in synaptic, cholinergic and neuromuscular junctions, resulting in hyper-excitability of parasympathetic receptors sites, thus producing muscarinic effects. Moreover, there is a symptomatic rise at sympathetic pre-ganglionic synapses in adrenal medulla and neuromuscular junctions bestowing nicotinic actions respectively. The severity of acute intoxication of triazophos may vary, depending on the dose, route and extent of exposure. General population is exposed to triazophos via different routes through food products, vegetables and drinking water and thus triazophos imposes an important health concern in humans. Moreover, no reports are available on the possible mechanism, suggesting the toxic manifestations of triazophos on the histoarchitecture of organs, some of which are known to be involved in xenobiotic metabolism and detoxification. Hence, in the present study we have investigated the dose and time-dependent effect of sub-chronic doses of triazophos on the induction of oxidative injury and histopathological alterations in liver, kidney, spleen and brain of male albino rats.

Materials and Methods

Chemicals and reagents

NADPH, oxidized and reduced glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), 2,4,6-tripyridyl-s-triazine (TPTZ), 5,5'-dithionitrobenzoic acid (DTNB) and bovine serum albumin were obtained from Sigma Chemicals Co. (St Louis, MO, USA). 2-Thiobarbituric acid (TBA) was obtained from E. Merck (Mumbai, India). All other reagents used were of analytical grade and obtained either from BDH or Sisco Chemicals (Mumbai, India). Technical grade triazophos (71%), as used for agricultural applications, was supplied by M/S Hindustan Insecticide (Delhi, India).

Animals and treatment

Male Wistar albino rats weighing 200-250 g were placed in individual raised bottom, galvanized wire
cages and kept under standard laboratory conditions of light/dark cycle (12-12 h) and temperature (25 ± 2°C). They were provided with a nutritionally adequate standard laboratory diet obtained from M/S Hindustan Lever Ltd. (Mumbai, India). The rats were randomly divided into four groups of six animals each. Technical grade triazophos was dissolved in olive oil (vehicle) and administered orally in three different doses for 30 days in the following manner with food and water given ad libitum. Group I (control) received treatment with vehicle and Groups II, III and IV received 1.64 (1/50th LD₅₀), 3.2 (1/25th LD₅₀) and 8.2 (1/10th LD₅₀) mg/kg body wt of triazophos, respectively.

Selection of dosage and time intervals used in the present study was based on WHO Guidelines Environmental Health Criteria 104 (Principles of Toxicological Assessment of Pesticide Residues) and ßOECD Test Guidelines 407 for 28 days oral toxicity study in rodents in which at least three dose groups (usually some fractions of the LD₅₀ determined from acute toxicity studies) and a control group is selected for any compound, whose acute toxicity data is available with the aim that the highest dose level induces toxic effects, but not death or severe suffering and the descending dosage sequence demonstrate the dose-related response and ßNo observed adverse effect at the lowest dose level (NOAEL). Moreover, two to four time intervals are frequently optimal for setting the dose and time response curve to identify toxic effects of the test compound.

Food consumption, general condition and any other symptoms were observed daily and body weight was recorded weekly. Appropriate permission was taken from Institutional Animal Ethics Committee and care of the animals was undertaken as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India for laboratory animal facilities.

**Samples**

After overnight fasting, blood samples were collected by puncturing retro-orbital vein from all the rats on 15th and 30th day and processed for separation of serum immediately. Serum separated was used on the same day for various biochemical investigations. Heparinised blood samples were also collected for the estimation of glutathione content in the erythrocytes. On 30th day, all the rats were sacrificed by cervical dislocation and liver, kidney, spleen and brain were excised for histopathological studies.

**Estimation of biochemical parameters**

The total protein content in serum was estimated by method of Lowry et al. The lipid peroxidation in serum was measured as thiobarbituric acid reactive substances (TBARS) ßBriefly, 0.5 ml serum was precipitated with 20% trichloroacetic acid (TCA) and the precipitate was suspended in 0.05 N H₂SO₄ and TBA (0.07% in 1 M sodium sulfate) and incubated in boiling water bath for 30 min. The malonaldehyde (MDA)-TBA adduct, thus formed was extracted with butanol and measured at 532 nm. The results were expressed as nmoles/ml.

Total glutathione (GSH) content in whole blood was estimated as described previously. Briefly, the reaction mixture (1 ml) contained 25 µl of hemolysate, NADPH [0.2 µmoles/ml in 0.01 M/0.005 M phosphate EDTA buffer (pH 7.5)] and glutathione reductase (1 unit). On addition of DTNB, the resulting chromophoric product i.e. 2-nitro, 5-thio benzoic acid was measured at 412 nm. The result was expressed as µmol/ml blood.

Serum GST activity was measured as described previously. The assay mixture contained in a total volume of 3 ml, 1 mM CDNB in ethanol (final conc. of ethanol not less than 4%), 0.001 M GSH, 0.1 M potassium phosphate buffer (pH 6.5) and serum sample. The formation of the resulting adduct (CDNB-GSH, S-2,4 dinitrophenyl glutathione) was monitored by noting the net increase in the absorbance at 340 nm against reagent blank for 0-5 min. The enzyme activity was determined by using the extinction coefficient 9.6 mM⁻¹ cm⁻¹ and the result was expressed as µmoles of product formed/mg protein/min.

For ferric reducing ability of plasma (FRAP), the working FRAP reagent was prepared by adding 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L TPTZ in 40 mmol/L HCl and 20 mmol/L FeCl₃₆H₂O in ratio of 10:1:1 respectively. Sample was added and vortexed for 5 min. The absorbance was monitored at 593 nm for 4 min and the values were expressed as µmol/ml. 

**Histopathological analysis**

Liver, kidney, spleen and brain excised from rats of control group as well as triazophos-treated groups were fixed in 10% neutral formalin buffer. Tissue sections (5 µm) were cut and stained with hematoxylin and eosin.

**Statistical analysis**

Results were expressed as mean ± SD. Data were analyzed by ANOVA test using SPSS version
statistical programme and the individual comparison of treatments was obtained by using Tukey's SD multiple comparison procedure at p<0.05.

Results and Discussion
A large number of xenobiotics have been identified as inducers of free radical generation and, therefore, it is of considerable interest to study free radical-mediated damage and subsequent toxicity in biological system. Lipid peroxidation has been suggested as one of the molecular mechanisms involved in OP pesticide-induced toxicity. Erythrocytes have a variety of redox systems, among which GSH is important particularly in the regulation of redox state and prevention of the cell damage induced by oxidative stress. It was, therefore, worthwhile to investigate the GSH level in the erythrocytes together with GST, since they may efficiently scavenge toxic free radicals and be partly responsible for protection against enhanced lipid peroxidation, resulting from sub-chronic exposure to triazophos. Large numbers of antioxidants are present in blood to scavenge reactive oxygen species (ROS) and prevent ROS-mediated damage. Oxidative injury usually occurs when the free radical generation, following xenobiotic exposure exceeds the total number of antioxidant species present in the blood. The total antioxidant power of these antioxidants can be assessed by various methods, of which FRAP is widely used due to its simplicity and sensitivity.

In the present study, sub-chronic exposure to 8.2 mg/kg of triazophos enhanced lipid peroxidation significantly on 15th and 30th day. On the other hand, a significant increase in lipid peroxidation was observed at 3.2 mg/kg only on 30th day. However, the change in lipid peroxidation remained insignificant at either time interval after treatment with 1.64 mg/kg (Fig. 1A). Similarly, the erythrocyte GSH content was reduced significantly after administration of 8.2 mg/kg of triazophos on 15th as well as 30th day. Nevertheless, significant decrease in GSH level was observed only on 30th day at 3.2 mg/kg of triazophos. No significant change was observed on either interval in GSH level at 1.64 mg/kg of triazophos (Fig. 1B). Concurrently, triazophos treatment enhanced serum GST activity significantly on 15th and 30th day at 8.2 mg/kg, but only on 30th day at 3.2 mg/kg, respectively. Serum GST activity showed an insignificant change on 15th and 30th day at lowest test dose of triazophos (Fig. 1C).

Total antioxidant capacity of plasma estimated as FRAP showed maximum decrease on 15th as well as 30th day after treatment with 8.2 mg/kg of triazophos. FRAP was significantly reduced in rats treated with 3.2 mg/kg only on 30th day (Fig. 2). These results suggested that the mechanism of triazophos toxicity might involve a dose and time-dependent induction of oxidative stress, as evident by enhanced lipid peroxidation and compromised antioxidant defense system. The resultant decrease in GSH content and increase in GST activity correlated well with the increase in lipid peroxidation, suggesting the utilization of this antioxidant in the detoxification of triazophos through GST. Reduction in FRAP is indicative of increased free radical generation.

The histopathological examination of liver of triazophos-treated rats revealed mild degenerative changes in hepatocytes characterized by enlarged nuclei with abundant granular cytoplasm.
Comparison to control group (Fig. 3A). Moreover, exposure to higher doses of triazophos progressively increased the intensity of these degenerative changes such as increased dilatation and congestion of sinusoids and central vein and hydropic degeneration with focal disarray of hepatic cords (Fig. 3B-E).

Formation of pyknotic nuclei with acidophilia of cytoplasm preceded the occurrence of occasional single cell necrosis in few hepatocytes and might provide an insight to the genotoxic effects of triazophos at sub-chronic doses (Fig. 3F). These early degenerative changes were found to be dose-dependent and indicated that triazophos might lead to genotoxicity and severe hepatotoxicity by making these hepatic changes completely irreversible. Absence of any significant histopathological change in kidney, spleen and brain of triazophos treated rats at either dose (data not shown) can be explained by the fact that triazophos like other OP pesticides after dietary intake is metabolized primarily and mostly in liver and the metabolites thus formed exert their harmful effects on liver maximally, rather on other organs (brain, spleen and kidney) which are secondarily involved in xenobiotic detoxification.

In conclusion, the present study suggested that the sub-chronic exposure to triazophos produced oxidative injury and tissue damage, especially in liver by exerting a dose and time-dependent effect on the induction of oxidative stress cascade. Our results also implied that the observed early degenerative changes in hepatocytes might have appeared due to significant induction of oxidative stress after sub-chronic exposure to triazophos. Thus, the study offers important information regarding the role of ROS as possible molecular mechanism involved in conferring sub-chronic toxicity and the consequential tissue damage, following triazophos administration. Moreover, these findings pave way for carrying out in-depth studies in future to determine the extent and persistence of these degenerative hepatic changes with an insight into the specific genes, proteins and the molecular cascade involved therein.

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