Subacute toxicity of anilofos, a new organophosphorus herbicide in male rats: Effect on lipid peroxidation and ATPase activity

Archana Hazarika & S N Sarkar*
Division of Pharmacology and Toxicology

and

Meena Kataria
Division of Biochemistry and Food Science
Indian Veterinary Research Institute, Izatnagar 243 122, India

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Effects of anilofos on lipid peroxidation — an index of oxidative stress, ATPase activity — an integral part of active transport mechanisms for cations, GSH level and GST activity were evaluated in blood (erythrocyte/plasma), brain and liver of male rats after daily oral exposure to 50, 100 or 200 mg/kg for 28 days. None of the doses increased lipid peroxidation. The lowest dose, rather, produced marginally significant decrease in peroxidation in liver. Different doses of anilofos decreased GSH content and activities of GST and ATPases. Inhibition of total ATPase (34-44%) and Na⁺-K⁺-ATPase (45-52%) activities was maximum in liver, while that of Mg²⁺-ATPase (46-56%) was more in erythrocyte. Results indicate that anilofos may not cause oxidative damage to cell membrane in repeatedly exposed animals and may cause neuronal/cellular dysfunction by affecting ionic transport across cell membrane.

Anilofos (S-4-chloro-N-isopropylcarbaniloylmethyl-O, O-dimethylphosphorodithioate), is a recently introduced organophosphorus (OP) herbicide, which is selectively used to control annual grassy weeds and sedges in transplanted rice. Like other OP pesticides, anilofos is lipophilic. These intentionally-introduced environmental xenobiotics are known to have a strong affinity for interaction with membrane phospholipids and the phospholipid component of biomembrane is believed to be the site of action of OP insecticides. There are evidences that oxygen-free radical formation can be a factor in the toxicity of organophosphates. One of the targets of reactive oxygen-induced injury is lipid peroxidation. Peroxidation of membrane phospholipids not only alters lipid milieu and structural and functional integrity of cell membrane but also affects activities of various membrane-bound enzymes, including total ATPase, Na⁺-K⁺-ATPase and Mg²⁺-ATPase. Although inhibition of acetylcholinesterase (AChE) is the principal mode of action of OP pesticides, increased lipid peroxidation and altered ATPase activity have been implicated in mediating OP toxicity in animals. But toxicological information with respect to the effect of repeated exposure to anilofos on these important biochemical traits is not available. Therefore, in vivo effects of oral subacute exposure to anilofos on production of lipid peroxidation — an index of oxidative stress, activity of membrane-bound enzyme — ATPase and related biochemical attributes in rats, as a model of mammalian species, have been examined.

Materials and Methods

Chemicals—Technical grade anilofos (94.5%; M/s Gharda Chemicals Ltd., Mumbai) was used. Refined groundnut oil (Postman) was used as a vehicle. All other chemicals used were of analytical grade.

Animals—Adult male Wistar rats (140-160 g) were procured from Laboratory Animal Resource Section of the Institute. All animals were kept in clean plastic cages and allowed to acclimatize to the laboratory environment for 7 days. The animals were allowed free access to balanced ration and drinking water.

Treatment—Adult male rats of approximately same age were randomly divided into 4 groups of 8-10 animals each. Three groups of rats received graded oral doses of anilofos daily @ 50, 100 or 200mg/kg body weight each for 28 days, which was approximately 1/34, 1/17 and 1/8.5 of the LD₅₀ respectively.
in adult male rats. The group, which served as control, received equivalent quantity of groundnut oil orally. On 28th day, 3 hr after administration of the last dose of anilofos, blood was collected from the orbital sinus through heparinized capillary tubes. All the animals were sacrificed on the 29th day under chloroform anaesthesia.

Biochemical analysis—Plasma and erythrocytes were separated by centrifuging at 2000 rpm for 15 min. Plasma was stored at -20°C. Normal sterile saline (NSS) was used for diluting plasma. Erythrocyte pellets were suspended in phosphate buffer saline (pH 7.4) and kept at 4°C. Liver and brain were minced separately and homogenized with NSS under ice-cold condition. The homogenates (10%) were centrifuged for 10 min at 3000 rpm and the supernatants were stored at 4°C for determining different biochemical parameters.

Lipid peroxidation in erythrocytes and tissue homogenates was measured as malondialdehyde (MDA). Activity of glutathione-S-transferase (GST) was assayed by the procedure of Habig et al. Reduced glutathione (GSH) in blood was estimated by the method of Prins and Loos, while GSH levels of brain and liver homogenates were quantified by the method of Sedlak and Lindsay. Determination of erythrocyte and tissue ATPases were carried out as per Yohtalou. For the assay of Mg2+-ATPase, sodium and potassium ions were excluded from the reaction mixture. Inorganic phosphorus in the reaction mixture was measured by the procedure of Yohtalou.

Statistical analysis—All data are expressed as mean ± SE. Data were analyzed statistically by one-way analysis of variance (ANOVA) followed by Dunnett’s ‘t’ test as the post hoc test.

Results

Lipid peroxidation—None of the doses of anilofos produced any significant increase in lipid peroxidation. On the contrary, some marginally significant reduction of lipid peroxidation was observed at the lowest dose in liver (Table 1).

<table>
<thead>
<tr>
<th>Treatment (mg/kg b. wt.)</th>
<th>Brain Lipid peroxidation (μmol MDA/ml packed RBC or g tissue)</th>
<th>Liver</th>
<th>Glutathione level (mM/ml blood or g tissue)</th>
<th>Glutathione-S-transferase activity (μmol of CDNB-GSH conjugate formed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythrocyte</td>
<td></td>
<td></td>
<td>Plasma</td>
</tr>
<tr>
<td>Control (8)</td>
<td>2.68 ± 0.30</td>
<td>24.97 ± 1.77</td>
<td>27.22 ± 1.10</td>
<td>1.26 ± 0.11</td>
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<td>50 (8)</td>
<td>2.63 ± 0.13</td>
<td>23.43 ± 0.62</td>
<td>23.28 ± 1.30</td>
<td>1.00 ± 0.09</td>
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<tr>
<td>100 (10)</td>
<td>2.46 ± 0.19</td>
<td>25.28 ± 0.25</td>
<td>25.64 ± 0.98</td>
<td>0.85 ± 0.11</td>
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<tr>
<td>200 (10)</td>
<td>2.62 ± 0.19</td>
<td>28.40 ± 0.95</td>
<td>28.53 ± 0.67</td>
<td>0.60 ± 0.05</td>
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<tr>
<td></td>
<td>Glutathione level</td>
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<td>Control (8)</td>
<td>2.84 ± 0.20</td>
<td>1.12 ± 0.006</td>
<td>0.18 ± 0.02</td>
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<td>50 (8)</td>
<td>1.58 ± 0.07</td>
<td>0.11 ± 0.003</td>
<td>0.16 ± 0.01</td>
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<td>100 (10)</td>
<td>1.31 ± 0.10</td>
<td>0.08 ± 0.01</td>
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<tr>
<td>200 (10)</td>
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<td>0.05 ± 0.004</td>
<td>0.11 ± 0.01</td>
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<tr>
<td></td>
<td>Glutathione-S-transferase activity</td>
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<td>Control (8)</td>
<td>0.073 ± 0.002</td>
<td>1.26 ± 0.11</td>
<td>1.70 ± 0.16</td>
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<tr>
<td>50 (8)</td>
<td>0.055 ± 0.006</td>
<td>1.00 ± 0.09</td>
<td>1.07 ± 0.13</td>
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</tr>
<tr>
<td>100 (10)</td>
<td>0.048 ± 0.002</td>
<td>0.85 ± 0.11</td>
<td>1.01 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>200 (10)</td>
<td>0.044 ± 0.004</td>
<td>0.60 ± 0.05</td>
<td>0.67 ± 0.08</td>
<td></td>
</tr>
</tbody>
</table>

Values in the same column bearing no superscript common vary significantly in Dunnett’s ‘t’ test (P<0.05)
HAZARIKA et al.: EFFECT OF ANILOFOS ON LIPID PeroxidATION & ATPases IN Rats

Fig. 1 — Effect of daily oral administration of anilofos (28 days) on total ATPase (A), Mg²⁺-ATPase (B) and Na⁺-K⁺-ATPase (C) in male rats. The enzyme activity is expressed as μmol/hr/ml packed RBCs or mg protein. Bars of the same histogram bearing no superscript common vary significantly (P<0.05) in Dunnett’s t-test.

Reduced glutathione (GSH)—Effect of anilofos feeding for 28 days on GSH level is shown in Table I. Significant decrease in blood GSH content was observed in all anilofos-treated groups. Brain GSH level significantly decreased in animals treated with 100 and 200mg/kg of anilofos. The level of liver GSH decreased significantly at 200mg/kg only.

Glutathione-S-transferase (GST) activity (Table I) —Plasma GST activity decreased significantly in all the three anilofos-treated groups. Reduction in liver GST activity occurred in a similar manner as that of plasma GST, except that the per Cent reduction was more in liver. Brain GST activity decreased significantly in animals administered anilofos in 100 and 200mg/kg levels.

Total adenosine triphosphatase (ATPase) activity—Total ATPase activity of erythrocytes showed dose-related insignificant decrease in all the anilofos-treated groups (Fig.1A). Brain ATPase activity was significantly decreased in animals given 100 (17%) and 200mg/kg (22%) doses. Whereas, liver ATPase activity decreased (34-44%) significantly in all the anilofos-treated groups (Fig. 1A).

Mg²⁺-ATPase activity—Effect of anilofos on Mg²⁺-ATPase activity is depicted in Fig. 1B. The enzyme activity in erythrocytes was significantly reduced with 100 and 200mg/kg by 46 and 56%, respectively. In brain and liver, respective percent reduction occurring with 200mg/kg dose was 28 and 32.

Na⁺-K⁺-ATPase activity—Data on activity of Na⁺-K⁺-ATPase are given in Fig. 1C. Na⁺-K⁺-ATPase activity in RBC and brain showed no significant change. Liver Na⁺-K⁺-ATPase activity decreased significantly (45-52%) in all the anilofos-treated groups.

Discussion

Incapability of anilofos to stimulate peroxidation of membrane fatty acids indicates that this herbicide may not cause oxidative damage to erythrocyte, brain and hepatic cell membrane lipids in repeatedly exposed animals. The lowest dose, on the other hand, caused marginally significant reduction in lipid peroxidation in liver. This suggests that anilofos may exert some hepatoprotective effect against oxidative stress through its antioxidative property at low dose. Antioxidative effects of fungicides, ziram, trichlamide and copper terephalate have been demonstrated in isolated rat liver microsomes. However, this effect of anilofos requires further confirmation.

Many OP compounds are reported to inhibit activities of ATPases. Anilofos decreased the activities of different ATPases with varied magnitude and in a tissue specific manner. This indicates that apart from inhibition of AChE, inhibition of Na⁺-K⁺-ATPase and Mg²⁺-ATPase could be a causative factor of neuronal/cellular dysfunction by affecting cationic transport across the membrane and disturbing uptake as well as release of certain neurotransmitters.

Improper functioning of Na⁺-K⁺-ATPase may disturb the membrane Na⁺-K⁺-pump that may increase cellular excitability through prolonged depolarization of cells. Inhibition of this enzyme increases intracellular Ca²⁺ and decreases intracellular Mg²⁺ concentration. Decreased activity of Mg²⁺-ATPase can also lead to alteration in cellular magnesium homeostasis. Elevated intracellular Ca²⁺ displaces Mg²⁺ from its binding sites and, as Mg²⁺ is easily permeable, accentuates its leakage from the cells, leading to diminution in functional availability of magnesium ions. Rise in intracellular Ca²⁺ also increases renal Mg²⁺ excretion by reducing reabsorption of Mg²⁺ in renal tubules. In addition, escalated
intracellular Ca\(^{2+}\) decreases enteric Mg\(^{2+}\) absorption\(^{31}\). The eventual impact of elevated intracellular Ca\(^{2+}\) and reduced intracellular Mg\(^{2+}\) is escalation of free reactive oxygen species generation and oxidative injury to cells\(^{35}\). In the present study, ATPases were significantly inhibited without being associated with augmented MDA production. Magnitude of inhibitory effect of anilofos upon ATPase enzymes suggests the potentiality of this herbicide to induce free radical formation. Thus, absence of biochemical evidence of lipid peroxidation in ATPase-inhibited rats could be attributed to lesser production of reactive oxygen species during the course of daily exposure to anilofos.

The loss of ATPase activity is believed to result from the oxidation of thiol groups\(^{26}\). GSH comprises 90% of the non-protein thiol content of mammalian cells. In the present study, however, inhibition of ATPases in different tissues and dose levels was not always associated with GSH depletion. Cellular depletion of GSH by about 20-30% can impair cell defence against toxic action, which may lead to cell injury and death\(^{37}\). Anilofos-treated rats, experiencing greater GSH depletion (39-64%) in different tissues, exhibited only mild to moderate symptoms without any mortality. Thus, 20-30% GSH depletion may not be related to mortality of animals, particularly during repeated exposure to anilofos.

GST was maximally affected in liver (37-61%), being significantly decreased in all the doses, but liver GSH was significantly depleted (39%) only at 200mg/kg dose. Whereas, the percentages of their significant decrease in brain were almost similar. Besides, anilofos did not alter these biochemical attributes in the same cellular/extracellular components of rats in acute toxicity study (unpublished data). This indicates that GSH-GST metabolic pathway may not have significant involvement in the detoxification of anilofos. Insignificance of GSH-mediated detoxification of dimethyl-substituted organothiophosphate insecticides in vivo may support this view\(^{28-30}\). Thus, reduction of GSH does not appear to be a consequence of GSH consumption by GST. Organophosphates are not likely to have any direct effect on GSH biosynthesis\(^{31}\). Therefore, the possibility of decreased GSH level as a result of its reduced synthesis may be ruled out. GSH level reflects cellular antioxidant status. Reduction in GSH with anilofos failed to increase lipid peroxidation. Anilofos given orally as a single dose (1/2 LD\(_{50}\)) in rat significantly increased lipid peroxidation, up to about 2.5-fold, in these tissues (unpublished data), which indicates capability of anilofos to cause formation of reactive oxygen species and oxidative stress. Smaller doses used in this study may be an important explanation for the absence of peroxidative damage, but the possibility of production of reactive oxygen species at low level cannot be excluded. The concept of free radical generation in consequence of significant inhibition of ATPase enzymes, as discussed above, may support this speculation. Thus, absolute level of GSH reached at the end of experiment may be the cumulative consequence of slow rate of progressive utilization of GSH by antioxidant defence system.

From the results of this study, it may be concluded that subacute oral administration of anilofos may not induce oxidative stress in animals. Inhibition of ATPases suggests electrophysiological disturbances in neurons/cells due to altered cationic transport across the cell membrane. GSH and GST did not appear to play an important role in the metabolism of anilofos in rats.

Acknowledgement

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References


