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1. Introduction

Pesticides are applied to grains during growth, post harvest, and storage, in order to prevent pest infestation that could damage the crops integrity and affect harvest. Recent studies have shown that insecticide residues are present in grains and other foodstuffs (wheat, corn, beans, and chickpeas) stored in Sonora, Mexico (Aldana et al, 2008). Concentrations of the most common insecticides such as DDT were detected to be within safe levels for consumption as established by the World Health Organization (Food and Agriculture Organization of the United Nations [FAO/WHO], 1999), although the use of some detected insecticides such as DDT and metabolites are no longer permitted in Mexico (CICOPLAFEST, 2004). Most grains are milled prior to human consumption. However, processing does not completely eliminate the insecticide (Pimentel & Casadei, 2000; Holland et al., 1994). In Mexico, residues of organochlorine (OC) insecticides are detected in adipose tissue, blood, and breast milk in populations living near agricultural fields. Studies document OC exposures in Comarca Lagunera, Mexico City, Puebla, Veracruz (RAPAM, 1997) and the Yaqui Valley in Sonora (Guillette et al., 1998). Such exposures are of great concern, especially since exposure to insecticides has been shown to alter lipid metabolism and produce liver damage in rats (Shakoori et al., 1998; Narayan et al., 1990; Videira et al., 2001; Aldana et al., 1998; Aldana et al., 2001).

The objective of our study was to evaluate the potential hepatotoxic effects of insecticide exposure at these levels in rats, using serum biochemical indicators of hepatic damage (transaminases, cholesterol, triglycerides, total protein, and albumin), as well as light and electron microscopy to evaluate microstructure hepatic changes.
2. Materials and methods

2.1 Materials
Pluronic F-68 (20% purity) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Malathion (98.5% purity), chlorpyrifos (99.6% purity), and deltamethrin (99.8% purity) were obtained from Sigma-Aldrich (SAF, Deisenhofen, Germany). CYP (91% purity; cis/trans, 49.9:50.1) was from Zeneca Co. (ICI of Mexico) and 4,4'-DDT (99% purity) was from Chem Service (West Chester, PA, USA).

2.2 Animals
Seventy-six male Wistar rats were purchased from the University of Puebla farm, (Mexico), weighing 280–300 g and were acclimated for a minimum of 7 days before treatment. The animals were randomly selected, based on their body weight and housed in individual stainless-steel wire hanging cages during the 7 days of treatment. They were exposed to a 12-h light/dark cycle, at a room temperature of 19–22°C. The animals had free access to a rodent lab diet (Ralston Rations, Purina, St. Louis, MO) and water. Rats were divided into seven groups as shown in figure 1.

Fig. 1. Study outline. Adult male Wistar rats were injected IP with insecticides for 9 consecutive days, Malathion, 63 µg kg\(^{-1}\); Chlorpyrifos, 80 µg kg\(^{-1}\); Cypermethrin, 780 µg kg\(^{-1}\); Deltamethrin, 979 µg kg\(^{-1}\); and 4,4’-DDT 49 µg kg\(^{-1}\). In the insecticide-treated groups 12 animals were included, and in the pluronic control group (administered with Pluronic F-68 as vehicle). Animals were sacrificed on days 0, 3, 6 and 9. The effects on serum levels of cholesterol, triglycerides, total protein, albumin, and hepatic enzymes alanine aminotransferase and aspartate aminotransferase were measured at each time point. Changes in liver cell morphology were also evaluated.
One group was sacrificed after the seven-day adjustment period to light/food (day 0), and was free of any exposure. The second group only received pluronic F-68 (20%) as a vehicle for nine consecutive days (Aldana et al., 1998; Aldana et al., 2001). The five experimental groups were exposed by intraperitoneal injection (IP) for nine consecutive days to malathion (MAL), chlorpyriphos (CHL), cypermethrin (CYP), deltamethrin (DEL) and 4,4’-DDT at dose of 63 µg kg⁻¹, 80 µg kg⁻¹, 780 µg kg⁻¹, 979 µg kg⁻¹, and 49 µg kg⁻¹ body weight per day, respectively. The doses used in the present study were similar to those that induce subacute intoxication and were similar to concentrations detected in stored grains in a previous work. Permitted residual concentrations published in CICOPLAFEST (2004) and concentrations used in this study were included in table 1. Pesticides were administrated by IP injection to assure the total exposure to each insecticide. The side of administration is very close to the liver which is the target organ in this study.

At three-day intervals, four rats from each group, including the pluronic control group, were sacrificed (days 3, 6, 9).

### Table 1. Insecticides concentrations permitted and concentration found in grains in Sonora State.

<table>
<thead>
<tr>
<th>INSECTICIDES PERMITTED</th>
<th>NO PERMITTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mg/kg)</td>
<td>Malathion</td>
</tr>
<tr>
<td>Permitted</td>
<td>8.0</td>
</tr>
<tr>
<td>Found in grains (mg/kg)</td>
<td>0.063</td>
</tr>
<tr>
<td>Grains</td>
<td>Bean</td>
</tr>
</tbody>
</table>

At 3-day intervals, 4 rats from each group were sacrificed (days 3, 6, 9). Food was removed 12 h prior to sacrifice. Blood was collected by cardiac puncture while rats were under ether anesthesia. The animals were euthanized using an overdose of anesthetics. The present study complied with the institution’s guidelines and the Mexican official regulation (NOM-062-ZOO-1999) regarding technical specifications for production, care, and use of laboratory animals. The protocol was also approved by the local animal ethics committee.

### 2.3 Behavioral and physical evaluation

Drowsiness, bristly hair, pruritus and aggressiveness were assessed and recorded as signs of toxicity after insecticide exposure. Animals were observed daily. The animals in each group were weighed daily and prior to sacrifice.

### 2.4 Biochemical assays

Blood samples were collected at pre-determined time points and centrifuged at 800×g for 3 minutes. Serum samples were also collected, and used in the following assays: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, cholesterol, triglycerides (TG), total proteins, and albumin concentration analysis (Figure 3). These assays were performed with the Monotest® Lakeside (Boehringer Mannheim GmbH, www.intechopen.com
Seelze, Germany) and a Hitachi 912 automated instrument spectrophotometer from the Clinical Analysis Laboratory at the Sonora State General Hospital, according to the specifications of the International Foundation of Clinical Chemistry (IFCC, 1986).

2.5 Light and electron microscopy studies
Immediately after euthanizing the animals, livers were removed and representative tissue fragments were placed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) in 0.1M sodium cacodylate buffer, pH 7.2 (Electron Microscopy Sciences, Fort Washington, PA, USA), and processed for embedding in epoxy resin. Sections 0.5 μm thick were stained with toluidine blue and examined by light microscopy (Nikon, Co. Japan). Thin sections were contrasted with uranyl acetate and lead citrate and examined by transmission electron microscopy (Jeol 100SX, Japan).

3. Statistical analysis
Analyses of variance (ANOVA) and linear regression were performed to determine the statistical significance of the effects of pesticides on the biochemical and histological indicators of liver function. All statistical analyses were carried out using JMP version 4.

4. Results
4.1 Behavioral and physical evaluation
Among the insecticides chosen for this study, MAL is classified as slightly hazardous (class III), and CHL, CYP, DEL and 4,4'-DDT are classified as to moderate hazardous (class II) as shown in table 2 (International Program of the Chemical Safety [IPCS], 2009). The sings of toxicity in the animals were related to the concentration of insecticides used. The animals treated with slight or moderately toxic insecticides showed a higher degree of drowsiness. Bristly hair, and pruritus were present in all treated groups, but a higher percent was evident in the moderately toxic compounds. Aggressiveness was mainly noticed in some of the moderately toxic compounds.

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Class</th>
<th>Toxicity</th>
<th>Drowsiness (%)</th>
<th>Bristly Hair (%)</th>
<th>Itching (%)</th>
<th>Aggressiveness (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>none</td>
<td>none</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Malathion</td>
<td>III</td>
<td>slightly hazardous</td>
<td>58</td>
<td>35</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>Chlorpyriphos</td>
<td>II</td>
<td>Moderately hazardous</td>
<td>60</td>
<td>28</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
<td>Cypermethrin</td>
<td>II</td>
<td>Moderately hazardous</td>
<td>1</td>
<td>50</td>
<td>38</td>
<td>13</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>II</td>
<td>Moderately hazardous</td>
<td>0</td>
<td>56</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>4,4'-DDT</td>
<td>II</td>
<td>Moderately hazardous</td>
<td>1</td>
<td>47</td>
<td>47</td>
<td>0</td>
</tr>
</tbody>
</table>

*Classification of active pesticide ingredient (IPCS, 2009).

Table 2. Signs of toxicity following insecticide application
4.2 Body weight
When body weights were analyzed, it was evident that rats exposed to CYP and DEL experienced delayed growth. The observed response was directly related to the insecticide concentration (Figure 2). The animals treated with MAL did not lose weight during the course of treatment were compared to the control group. A slight weight loss was observed in animals exposed to 4,4'-DDT. Animals treated with CHL showed a decrease in weight gain that was statistically significant vs control group. This effect is directly related to the insecticide concentration used in this study (Figure 2A).

![Fig. 2. Weight gain vs time.](image)

4.3 Lipid profile
TG levels increased in all insecticide-treated groups after 6 day and these levels remained high until the end of treatment (Figure 3). This increase mainly reflects liver damage. However,
only the MAL-treated group showed decreased TG levels vs controls. Although the insecticide concentrations chosen for this study were based on levels detected in grains in previous work (Aldana et al., 2008), some of these concentrations MAL and CHL, DEL, CYP and 4,4'-DDT were within permissible limits in food for human consumption (Table 1). As shown in figure 3A, a cumulative effect was observed on day 9 of treatment, when all the insecticide-treated groups exhibited statistically significant increases in levels of TG (p < 0.05).

Fig. 3. Triglyceride and cholesterol levels. In (A), we show triglyceride (TG) levels during 0, 3, 6 and 9 days of consecutive insecticide treatment. Control group with (○), chlorpyriphos (●), 4,4'-DDT (△), deltamethrin (▲), malathion (□), cypermethrin (■). In (B), Cholesterol levels during 0, 3, 6 and 9 days of consecutive insecticide treatment. The different groups are represented by the same symbols used in (A).
Cholesterol levels typically decrease after exposure to insecticides (Aldana et al., 1998; Aldana et al., 2001). However, the reduction in the CHL-treated group was very small and was not observed until day 6 after treatment. The 4,4'-DDT-treated group exhibited the greatest reduction in cholesterol levels and the MAL-treated group showed the smallest decrease. CYP treatment showed a decrease in serum cholesterol on day 3 of treatment and that remained unchanged until the end of treatment (day 9) (Figure 3B). The decrease in serum cholesterol levels and the increase in TG levels confirm these insecticides caused liver damage.

4.4 Liver damage
In the case of the MAL- and CHL-treated groups, the expected decreased in albumin level was absent. On the contrary, albumin levels remained slightly elevated vs the control group (MAL, p < 0.05). Neither MAL nor CHL induced an acute phase response as evidenced by this marker.

Fig. 4. Liver function tests. (A) Albumin levels were measured on days 0, 3, 6 and 9 of insecticide administration. Control is in open bars in the middle; insecticides whose used concentrations were within permitted limits are on its left side; insecticides used in concentrations higher than those permitted by the Mexican Official standard appear on its right side (B) Total protein concentration, (C) Alanine transferase (ALT) activity, and (D) Aspartate aminotransferase (AST).
Total protein levels, exhibited a similar pattern to albumin levels (Figure 4A). Only DEL, CYP, and 4,4’-DDT had decreased protein levels vs the control group (Figure 4B). ALT and ASL are biomarkers of liver damage; that typically increase after insecticide administration. However, both enzymes showed an initial decrease followed by a return to normal levels on day 6 (Figure 4C, 4D). The MAL-treated group, showed minimal changes in enzyme levels vs to the control.

4.5 Electron microscopy studies
The results of electron microscopy included changes in liver structure following treatment that were much more pronounced after 9 days of insecticide treatment. Figure 5A shows the control liver from an animal treated only with pluronic. The central vein is shown surrounded by cords of normal hepatocytes, along with some sinusoids that are occupied by erythrocytes. Figure 5B shows control untreated liver. The ultrastructure of a normal hepatocyte with cytoplasm containing abundant mitochondria, lysosomes and intact rough endoplasmic reticulum are also shown.

Figure 6 shows the ultrastructure of hepatocytes from animals treated with different insecticides. DEL shows lipid droplets of different sizes and mitochondria with varying degrees of damage (Figure 6A). MAL shows liver with abundant clear and irregular vesicles. Severe damage to mitochondria is observed (Figure 6B). CHL shows mitochondria with varying degrees of damage. Lipid droplets and areas with glycogen are also present (Figure 6C). 4,4’-DDT shows extensive cytoplasmic damage and multiple irregular vesicles and mitochondria (Figure 6D).

Fig. 5. Histopathology/Hepatocyte tissue sections. A). Control liver from animal treated only with pluronic. Central vein (cv) is shown surrounded by cords of normal hepatocytes (h). Some sinusoids are occupied by erythrocytes (x 20). B). Control untreated liver. Ultrastructure of a normal typical hepatocyte shows a round nucleus (n) and a cytoplasm with abundant mitochondria (m), lysosomes (ly), rough endoplasmic reticulum and clear areas with glycogen. Scale= 1.5 µm.
Fig. 6. Ultrastructure of hepatocytes from animals treated with different insecticides. A). Nine days after treated with deltamethrin. Lipid droplets (l) of different sizes, and mitochondria (m) with a different degree of damage are shown. Scarce rough endoplasmic reticulum and clear areas of glycogen (g) are observed. B). Animal treated 9 days with malathion. Abundant clear and irregular vesicles (arrows) are shown, along with severe damage of mitochondria (m). Lipid (l) droplet. Nuclei (n). C). Hepatocyte from animal treated with chlorpyriphos for 9 days. Similar changes were observed as in malathion treated animals. Mitochondria (m) with a different degree of damage, and lipid (l) droplets are observed. Areas with glycogen (g) are also shown. Nucleus (n). D). Animal treated with 4,4'-DDT. Extensive cytoplasmic damage is observed. Multiple irregular vesicles (arrows) and damaged mitochondria (m) are seen. Nucleus (n). Scale = 1.5 µm.
5. Discussion

To evaluate the hepatic effects of insecticides in rats, the doses used in the present study were similar to those found in stored grains in the state of Sonora. In some cases, the doses were within or above permissible limits. Still, it is clear that each insecticide affected the markers used, animal behavior, animal weight, appearance, alteration of lipid profile and liver function, and caused hepatocellular damage.

The results of this study indicate that CHL may have a different mechanism compared to CYP and DEL, even though they all belong to the moderately toxic group of compounds. In this context, it is important to consider both the toxicity and the concentration used for each insecticide (Table 1), as noted by other researchers (Manna et al., 2004).

Regarding the symptoms shown by the animals after insecticide exposures, the results of the present research demonstrate that weight gain/loss measurement was a good qualitative parameter to evaluate toxicity as shown in the results obtained with MAL (Naraharisetti et al., 2008), with CHL (Meggs & Brewer, 2007), with pyrethroid CYP by our research group (Aldana et al., 2001; Aldana et al., 1998), and by others (Hussain et al., 2009). The lipid profile also indicated the presence of liver damage, as evidenced by the decrease in cholesterol levels and the increase in TG levels after day 3 of treatment. In a recent study (Lasram et al., 2009) of acute toxicity at higher doses than used here, the same cholesterol pattern was observed with a transient level decrease, followed by a gradual return to normal levels. Plasma TG also increased significantly in their study. TG levels in our study increased progressively until the end of treatment, as has been observed during acute CYP intoxication reported in other studies at higher doses (Eraslan et al., 2008).

In relation to albumin, an acute phase protein, our results indicated that doses of CYP and DEL which were above permissible limits, caused a decrease albumin levels. On the other hand, when MAL and CHL were administered at a lower dose (within permissible limits), in albumin levels did not decrease.

In our study, the CHL-treated group behaved similar to the control group (Figure 4B). MAL caused slight increase in total protein values vs the control group. Total protein served as a good biomarker of acute damage to liver because it partially reflects the decrease in albumin levels.

ALT and AST did not evoke immediate liver damage. Levels of both enzymes began to increase after day 3, indicating that these insecticides were provoking cumulative liver damage by inducing slight necrosis or cell damage.

Severe liver damage was not observed immediately after MAL exposure. The effect of MAL exposure were noticeable after day 6, suggesting a cumulative effect that is manifested more slowly than with other insecticides.

MAL gave rise to an acute phase response at the dose used in this study (within permissible limits), but it was the compound that elicited the lowest degree of liver damage. Our results confirm others studies (Rezg et al., 2008) that observed an increase in AST and ALT following administration of MAL at 100 mg/kg for 32 days, while hepatic proteins and lipid content decreased significantly (Rezg et al., 2007).

These data show that serum albumin is the best biomarker to evaluate liver damage, since its levels remained low during the 9 days of treatment in relation to the concentration of insecticides. The liver damage observed in these studies was also confirmed with histopathological analysis, in which mitochondrial damage, presence of lipid droplets, and rough endoplasmic reticulum fragmentation were noticed as the most significant changes. The observed cell lysis was produced on the last days of treatment. We administered insecticides for 9 consecutive days and this sub-acute treatment more closely mimics human
exposure. Grains and grain products with insecticide residues at low doses are typically consumed over long periods of time. Our results are in agreement with another study in which MAL was administered at 33.051 mg/kg/day in Wistar rats via ip injection for 40 days (Saadi et al., 2008) and observed alterations with cytotoxicity signs in liver, lungs, bone marrow and testicles, caused by the high MAL concentrations.

The typical human exposure to pesticides is by oral consumption of grains. We chose the ip route of administration in this study, because it has a large surface of absorption and great vascularization, thus allowing for a rapid absorption. Also, these pesticides are very irritating to the animal’s gastrointestinal tract when administered orally. Such so this irritation would have interfered with proper feeding.

6. Conclusion

The present study conducted with Wistar rats exposed to insecticides MAL, CHL, CYP, DEL and 4,4’ DDT at levels found in stored grains in Sonora, suggests possible hepatotoxic effects. These effects were manifested by biochemical alterations (decrease in serum cholesterol levels and increase in TG levels) as well as changes in the liver characterized by the presence of lipid droplets and fragmentation of rough endoplasmic reticulum. The most useful biomarker for evaluating hepatic damage was serum albumin. Although we cannot correlate the results observed in rats directly to oral consumption in humans, they suggest the possibility that these insecticides may not be as safe as other studies have previously reported. The insecticides examined in this study can provoke hepatic damage in rats and humans and these effects can be magnified under conditions of chronic exposure. Further studies are necessary to determine the effect of chronic exposure to these insecticides in rats and humans. A critical component of such studies should include monitoring of pesticide residue levels in grains and grain products.

7. References


This book contains 30 Chapters divided into 5 Sections. Section A covers integrated pest management, alternative insect control strategies, ecological impact of insecticides as well as pesticides and drugs of forensic interest. Section B is dedicated to chemical control and health risks, applications for insecticides, metabolism of pesticides by human cytochrome p450, etc. Section C provides biochemical analyses of action of chlorfluazuron, pest control effects on seed yield, chemical ecology, quality control, development of ideal insecticide, insecticide resistance, etc. Section D reviews current analytical methods, electroanalysis of insecticides, insecticide activity and secondary metabolites. Section E provides data contributing to better understanding of biological control through Bacillus sphaericus and B. thuringiensis, entomopathogenic nematodes insecticides, vector-borne disease, etc. The subject matter in this book should attract the reader’s concern to support rational decisions regarding the use of pesticides.

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