Haemato-biochemical and hepatopathological changes in male rats following oxidative stress, after prolonged exposure to Dicofol

By

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SUMMARY

Aim: To investigate the effect of chronic toxicity of dicofol on haematological indices, oxidation status, enzymatic activities and the accompanied histopathological changes of the liver in male albino rats.

Methods: Two equal groups of adult rats were orally administrated two sublethal doses of dicofol (4.19 and 16.75 mg/kg body weight) through drinking water (30 and 120 ppm) in a rate of 5 days/week. The third group was kept as control. After 6 months; blood samples were taken for haematological examinations, transminases (AST and ALT) and bilirubin were determined in the serum. Liver specimens were used for the detection of histopathological changes as well as the determination of total glutathione (GSH) and lipid peroxidation as evidenced by malondialdehyde (MDA) level.

Results: The higher dose of dicofol (16.75 mg/kg b. w) resulted in a significant decrease in RBCs count, Hb concentration and MCHC accompanied by a significant increase in T.L.C, neutrophils and lymphocytes number, with a significant decrease in monocytes and oesinophils were reduced. Lipid peroxidation level in the liver was significantly increased with a concomitant decrease level of total glutathione.

Conclusion: Long term exposure to sublethal doses of dicofol can lead to induction of hepatic injury and haematological alterations.

INTRODUCTION

The question of the safety of post-harvest pesticides and residual pesticides has become an important topic in food hygiene. On the other hand, allergic diseases have been increasing steadily, and this might be related to...
synthetic chemicals in food and environment (Tanaka et al., 2000).

Acute toxicities of pesticides are well documented but little attention has been given to the chronic low dose effects of pesticides, especially as environmental pollutants. Chronic immunotoxic exposures to chemical stressors, which cause direct damage to the immune system, can result in suppression of immune responses and decreased resistance to infections and malignancies, poor response to antibiotics and vaccination failures (Umesh et al., 2004).

Dicofol is an organochlorine acaricide (a chemical that kills mites) structurally similar to DDT, used on a wide variety of fruit, vegetable, ornamental and field crops. Modern manufacturing process can produce technical grade Dicofol which contains less than 1% DDT (Pesticides News, 1999).

The United States, Environmental protection Agency (EPA) has classified dicofol as toxicity class II - moderately toxic, and toxicity class III - slightly toxic (Extoxnet, 1996).

In a number of studies, dicofol residues on plant tissues have shown to remain unchanged for up to 2 years (Tillman, 1992).

Surendranarth and Rao (1991) suggested that the low degradability of dicofol leads to its accumulation in tissues as residues even at sublethal concentration under chronic exposures.

The need for immunotoxicological studies is more urgent in cases of such pesticides which persist in the environment or accumulate in the body as under these conditions living beings are subjected to a continuous exposure to pesticides at a subchronic and sublethal level which may render them, in turn, more prone to diseases, or increase the severity of existing diseases by causing immunodepression (Bahtia and Jasdeep, 1994).

Organochlorines accumulate mainly in adipose tissue then oxidized to more water soluble metabolites such as sulfate, diols, or lactones. The earliest biochemical indicator of exposure to organochlorines is the induction of microsomal enzymes in the liver of experimental animals (Bebe and Panemangalore, 2003).

Carbon tetrachlorides (CCL₄) have been reported to disturb the oxidant/antioxidant balance of the cells by generating oxygen free radicals and reactive oxygen species (ROS) inducing liver cirrhosis (Gustafson et al., 2000 and Fadhel et al. 2002).

In our study we are trying to illustrate the oxidative damaging
Effect of chronic two sublethal doses of Dicofol on hematological parameters, liver oxidant and antioxidants balance, enzymatic activities with the accompanied histopathological changes in male albino rats, as a model closest to the civilization hazards.

MATERIAL AND METHODS

1- Experimental animals:
60 male albino rats, weighing 180 ± 10 g. apparently normal and raised in the farm of general organization of serum and vaccine (Helwan farm) were used. The experimental animals were kept in plastic cages under good hygienic conditions as well as balanced diet and water which were provided ad libitum. Animals were allowed to be acclimatized for a minimum of 2 weeks prior to the experiment.

2- Experimental materials:
Dicofol (18.5 %) emulsifiable concentrate (EC) {trade name: Kelthane "EL Nassr" Comp.} is used as an acaricide. The product belongs to "O Chs", under chemical name: 4-chloro-α (4=chloro-phenyl)- α-trichloromethyl) benzene methanol (CAS).

The median lethal dose (LD50) of dicofol (administered to rat per OS) was determined according to Wiel (1952) and its value was 348.86 mg/Kg body weight. In the present study, the used dosages were chosen according to the maximum tolerated dose (MTD) which suppressed body weight gain slightly: i.e.10 %. Generally, 1/4 and 1/10 MTD, are then selected for testing (Hayes, 1989). Accordingly, dicofol was administered to rats at 4.19 and 16.75 mg/Kg body weight (representing 1/80 and 1/20 LD50). Dicofol 18.5 % EC was emulsified daily in dinking water immediately before the use throughout the experimental period (successive 6 months).

3- Experimental design:
Animals were classified randomly into three groups.
Group 1: Twenty rats received tap water only and considered as the control group.
Group 2: Twenty rats received 4.19 mg/Kg body weight (30 ppm) dicofol through drinking water in a glass bottle (5 days/week) which represent the lowest dose.
Group 3: Twenty rats received 16.75 mg/Kg body weight (120 ppm) dicofol through drinking water (5 days/week), which represents the highest dose.
Throughout the experiment; clinical behaviour, water and food consumption were recorded daily. Animals were weighed weekly and the dose were adjusted accordingly.

4- Sampling for biochemical and hematological examination:
At the end of the experimental
period (6 months), blood samples were collected under ether anaesthesia from orbital sinus vein by heparinized capillary tubes into clean dry Ependorf tubes according to Schalm (1986). Serum was separated by centrifugation at 3500 rpm for 15 minutes and store at -40°C, till the different assays were carried out, including serum transaminases, aspartate aminotransaminase "AST", alanine aminotransaminase "ALT" (Reitman and Frankel, 1957) and total and conjugated bilirubins (Martinek, 1966).

Other blood samples were taken in dry, clean tubes containing EDTA as anticoagulant (1 mg/1 ml blood) (Schalm, 1986) for further haematological examination including; red blood cells (RBC's count) according to Thompson (1980), haemoglobin concentration (Hb) measured according to Crosby et al. (1954). Packed cells volume (PCV) determined by the microhaematocrite method of Schalm (1986). Haematological values were then utilized for calculating of erythrocyte indices (mean corpuscular volume “MCV”, mean corpuscular haemoglobin “MCH” and mean corpuscular hemoglobin concentration “MCHC”).

5- Sampling of tissue:
Five animals/group were sacrificed some of liver specimens were taken then fixed in 10% formal saline for histopathological examination through the light microscope (Bancraft et al., 1996). Other liver specimens were taken and kept in liquid nitrogen in deep freezer at (-40°C) until estimation of liver glutathione content and lipid peroxidation level.

Lipid peroxidation:
It was measured in liver tissues according to the method of Ohkawa et al. (1979), based on the formation of thiobarbituric acid reactive substance (TBARs) and expressed as the extent of malondialdehyde (MDA) production.

Total glutathione (GSH):
It was measured in liver tissues according to the method of Bergmeyer and Grabl (1995), based on the catalytic action of GSH as it undergoes periodical oxidation by DTNB and reduction by NADPH.

Statistical analysis:
The mean ± standard error was calculated for each of the treatment groups of the experiment. Results of the obtained data were computed and analyzed for significance by analysis of variance (ANOVA test) and Duncan multiple range test for comparison of means according to (Duncan, 1955) was computed to compare means using the computer program (SPSS, 2006).
RESULT AND DISCUSSION

In the present chronic toxicity study, male rats administered dicofol at lower and higher doses 4.19 and 16.75 mg/Kg body weight, respectively in drinking water (30 and 120 ppm) for 6 months, showed no visible signs of toxicity and/or mortality allover during the experimental period.

Carbon tetrachloride is a classical heptotoxicant that causes rapid liver damage progressing from steatosis to centrilobular necrosis. Long term administration of CCl₄ causes chronic liver injury as a widely accepted model to produce hepatic fibrosis (Poli, 2000).

In our study, dicofol treated rats with lower dose showed a granular and vacuolar degenerative changes in the cytoplasm of the hepatocytes (Fig.2) with severe dilatation of portal veins (Fig.3).

The higher dose of dicofol showed an extensively disorganization of the hierarchy of liver architecture with severe fatty changes (Fig. 4 – 5).

Concerning serum transaminases, our results revealed that after 6 months treatment with dicofol, serum sample assayed for liver enzymes showed both AST and ALT enzymes activities (Table 1) were similar to those of the control group in accordance with Salazar et al. (2000) and Muriel et al. (2005) who suggested that the damaged liver is no longer able to produce and release enzymes to plasma. This result was previously admitted by Lee et al. (2001) who stated that prolonged CCl₄ treatment did not lead to a higher degree of fibrosis but to a reduction of matrix metalloproteinase.

Glutathione is a tripeptide found in all tissue and at especially high concentrations in the liver. It is not only a substrate for glutathione peroxidase (GPX) but also functions in detoxifying electrophiles, scavenging free radicals, maintaining the thiol status of proteins, providing a reservoir for cysteine and modulating critical cellular processes such as DNA synthesis, gene expression and immune function (Sies, 1999; Lu, 2000 and Bebe and Panemanglore, 2003). The concentration of glutathione is greatly modified in response to a variety of environmental triggers particularly those that causes increased oxidative stress (Silvia Vertuani et al., 2004).

The oxidative damage is one of the essential mechanisms of CCl₄ hepatotoxicity which triggers apoptosis via a differential inhibitory effect on the plasma membranes calcium transport system that are implicated with hepatocellular damage (Hemminas et al., 2002).
In our study, a decrease hepatic total glutathione content along with a concomitant increased in lipid peroxidation levels (Table 1) was found in high dose group, in accordance with (Luckey and Peterson, 2001; Carter et al., 2003; Federick and Myna, 2003 and Afaf and Mogeda 2007). Such effect was explained by Mitcheva et al. (2006) who recorded that CCL$_4$ undergoes dehalogenation in the liver endoplasmic reticulum leading to the formation of trichlormethyl radical ($\text{CCl}_3$) and initiation of lipid peroxidation. So glutathione has been hypothesized to play a role in the rescue of cells from apoptosis by buffering an endogenously induced oxidative stress (Foyer, 2001 and Turkey et al., 2005).

Kent (2000) stated that toxins that cause direct damage to circulating erythrocytes may cause a rapid decline in hematocrit and are generally associated with hyperbilirubinemia with evidence of erythrocytes regeneration.

In the present study the chronic administration of higher dose of dicofol, elicited a significant increase in serum bilirubin concentration (Table 1) in accordance with Bai et al. (1992); Soni and Mehendale (1994) and Muriel and Escobar (2003). This increase in serum bilirubin is obviously related to the recoded, signified decrease in RBCs count (Table 2) due to the expected haemolysis reflected as significant decreased in Hb % and MCHC (Table 2). Similar results was recorded by Agrawal and Sultana (1993); Bahatia and Jasdeep (1994); Dunstan et al. (1996); Pankaj and Deepa (1998) and Ovuru and Ezeasor (2004). This effect was clarified by Bondy et al. (2000) who recorded that organochlorines insecticides are hydrophilic molecules acting on membranes resulting in increasing the erythrocyte membrane fluidity, osmotic fragility and decrease in level of Na$^+$, K$^+$ in erythrocytes. Such increased permeability to cations produces haemolysis. Moreover, the obtained result showed a significant increase in MCV and MCH. (Table 2) which explains the insignificant change in haematocrit value (PCV) due to the decreased RBCs count.

Our hepatic histopathological findings confirmed this hemolytic effect of higher dose of dicofol (Fig. 5), showed as a focal extravasation of red blood cells in between the degenerated hepatocytes.

In a recent epidemiological study, Jamil et al. (2007) stated that chronic exposure to pesticides resulted in chronic myeloid leukaemia (CML) characterized by increased number of WBCs with a
large number of immature cell. These findings are in accordance with ours (Tables 3), as the high dose of dicofol revealed a significant increased in total leukocytic count as a reflection of increased number of lymphocytes.

Our results revealed that both doses of dicofol resulted in a significant decrease in the number of circulating monocytes a fact explained by their migration to the tissues (damaged liver) as Kupffer cells. This was clearly noticed in liver histopathological findings (Fig. 2 and 4) showing mononuclear leucocytic inflammatory cells aggregation as well as diffuse proliferation of the Kupffer cells.

Kupffer cells (hepatic macrophages) that reside in liver sinusoids, accounting for 80 – 90% of body macrophages, are activated in response to chemical administration and have been implicated in the development of liver inflammation and fibrosis (Luckey and Peterson, 2001). Su et al. (2002) clarified that chronic administration of CCL4 significantly increased CD14 gene expression which are protein receptors that resides on the plasma membrane of Kupffer cells and plays an important role in their activation. With the progression of damaged liver Qui et al. (2005) recorded a decreased CD14 gene expression in Kupffer cells suggesting that Kupffer cells were no longer activated or their activation may be independent of CD14.

Han et al. (2003) recorded an enhancement of steroidogenic activity of dicofol in male rats after 6 months exposure. They attributed such effect to hypertrophy and or vacuolation of adrenal cortex (Lindane, 1999). This finding may clarify our recorded significant increase in neutrophils with the associated significant decreased in oesinophils (Table 3).

Finally, our study revealed that chronic exposure to dicofol even at sublethal dose, leads to deleterious effect on the haematological parameters, bilirubin level, and altered oxidant and antioxidant balance, which triggers the need of accurate and controlled applications of pesticides, with increasing demands for the use of safe alternatives to be researched, identified and widely promoted.
Table (1) : Effect of chronic administration (6 month) of two sublethal doses of dicofol on liver lipid peroxidation, glutathion, serum AST, ALT and bilirubin levels of male albino rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>AST (U/L) Aspartate aminotransfarse</th>
<th>ALT (U/L) Alanine aminotransfarse</th>
<th>Lipid Peroxidation (nmol/g tissue)</th>
<th>Total Glutathion (μmol/g tissue)</th>
<th>Bilirubin (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td>Total</td>
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<td>Direct</td>
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<tr>
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<td></td>
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<td></td>
<td></td>
<td>Indirect</td>
</tr>
<tr>
<td>Control</td>
<td>54.60 ± 0.55</td>
<td>4.61 ± 0.42</td>
<td>11.49 ± 0.35</td>
<td>15.68 ± 0.91</td>
<td>7.70 ± 0.30</td>
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<td>0.68 ± 0.05</td>
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<td></td>
<td>7.01 ± 0.65</td>
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<tr>
<td>Lower dose</td>
<td>52.06 ± 2.71</td>
<td>3.38 ± 0.37</td>
<td>12.38 ± 0.63</td>
<td>19.45 ± 2.23</td>
<td>6.03 ± 0.29</td>
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<td>0.54 ± 0.09</td>
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<td></td>
<td>5.49 ± 0.48</td>
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<tr>
<td>Higher dose</td>
<td>54.47 ± 1.20</td>
<td>2.74 ± 0.76</td>
<td>18.66 ± 0.38</td>
<td>3.13 ± 0.42</td>
<td>9.23 ± 0.35</td>
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<td></td>
<td>0.9 ± 0.08</td>
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<td></td>
<td></td>
<td>8.34 ± 0.3</td>
</tr>
<tr>
<td>F calculated</td>
<td>0.678</td>
<td>3.048</td>
<td>69.177*</td>
<td>36.552*</td>
<td>8.339*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.615*</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>8.234*</td>
</tr>
</tbody>
</table>

* Significant at P < 0.05 using ANOVA test.

a, b significant difference between different letters using Duncan Multiple Range test for comparative of means at P < 0.05.
Table (2) : Haematological parameters of the investigated rats after chronic administration of two sublethal doses of dicofol.

<table>
<thead>
<tr>
<th>Group</th>
<th>RBCs ( X10^6/ml)</th>
<th>Hb (g/dl)</th>
<th>PCV (%)</th>
<th>MCV (Ft)</th>
<th>MCH (pg)</th>
<th>MCHC %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.31 ± 1.12^a</td>
<td>14.25 ± 0.31^a</td>
<td>54.25 ± 1.07</td>
<td>73.63 ± 0.90^a</td>
<td>19.34 ± 0.38^a</td>
<td>26.27 ± 0.29^a</td>
</tr>
<tr>
<td>Lower dose</td>
<td>8.11 ± 0.48^a</td>
<td>15.14 ± 0.33^a</td>
<td>56.75 ± 1.11</td>
<td>71.65 ± 2.70^a</td>
<td>19.21 ± 0.58^a</td>
<td>26.84 ± 0.38^a</td>
</tr>
<tr>
<td>Higher dose</td>
<td>4.25 ± 0.54^b</td>
<td>9.87 ± 0.29^b</td>
<td>58.25 ± 1.18</td>
<td>145.78 ± 18.36^b</td>
<td>24.69 ± 2.84^b</td>
<td>16.99 ± 0.61^b</td>
</tr>
<tr>
<td>F calculate</td>
<td>5.819*</td>
<td>69.52*</td>
<td>3.00</td>
<td>16.124*</td>
<td>3.536</td>
<td>141.4*</td>
</tr>
</tbody>
</table>

* Significant at P < 0.05 using ANOVA test.

a, b significant difference between different letters using Duncan Multiple Range test for comparative of means at P < 0.05.
Table (3): Leukogram of the investigated rats after chronic administration of two sublethal doses of dicofol.

<table>
<thead>
<tr>
<th>Group</th>
<th>TLC (X 10^3/ml)</th>
<th>Differential leucocytic count (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total leucocytic count</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Control</td>
<td>7.33 ± 0.23a</td>
<td>30.40 ± 0.94a</td>
</tr>
<tr>
<td>Lower dose</td>
<td>8.11 ± 0.25a</td>
<td>35.11 ± 1.09b</td>
</tr>
<tr>
<td>Higher dose</td>
<td>12.81 ± 2.40b</td>
<td>37.42 ± 1.16b</td>
</tr>
<tr>
<td>F calculate</td>
<td>96.7*</td>
<td>11.23*</td>
</tr>
</tbody>
</table>

* Significant at P < 0.05 using ANOVA test.
a, b, c significant difference between different letters using Duncan Multiple Range test for comparative of means at P < 0.05.
Fig. (1) : Liver of rat in control group showing the normal histological structure of the hepatocytes, central vein and portal area (H & E X 40).

Fig. (2) : Liver of rat administered dicofol by a low dose for 6 months showing mononuclear leucocytic inflammatory cells aggregation in the portal area with granular and vacuolar degeneration in the cytoplasm of the hepatocytes with different diffuse Kupffer cells proliferation. (H & E X 40).

Fig. (3) : Liver of rat administered dicofol by a low dose for 6 months showing severe dilatation of portal veins with fatty change in mostly of the hepatocytes. (H & E X 40).

Fig. (4) : Liver of rat administered dicofol by a high dose for 6 months showing mononuclear leucocytic inflammatory cells infiltration in the portal area with severe fatty change in the hepatocytes. (H & E X 40).

Fig. (5) : Liver of rat administered dicofol by a high dose for 6 months showing extravasation of red cells in between the degenerated hepatocytes. (H & E X 40).
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**Surendranath, P. and Rao , K. V. (1991):** "Kelthame residue in tissues of the tropical penaeid prawn, meta penaeus monoceros (Fabricius) under


الملخص العربي
يهدف هذا البحث إلى دراسة تأثيرات النتائج على التعرض المشترك للكبد الناتجة عن الإجهاد التآكيدي في ذكور الفئران بعد التعرض المزمن للكبد. 

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2) عادل بكير خليل.

3) المركز البحوث الزراعي.

4) قسم الكيمياء – معهد بحوث صحة الحيوان PAL.

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الملخص

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الدليل

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النتائج

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الخلايا FE (4).

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