Adverse Haemato-Biochemical Effects of Chlorinated Insecticide in Adult Male Rats.

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**Abstract**
The 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (p,p’-DDT) is a highly toxic organochlorine pesticide and posing adverse effects on the environment and public health due to its frequent usage in developing countries. The present study is aimed to investigate the effects of p,p’-DDT exposure on some hematological and biochemical parameters, as well as, histopathological changes in liver. Male Wistar rats received an intraperitoneal (ip) injection of the pesticide at doses of 50 and 100mg/kg for 10 consecutive days. The hematological parameters were evaluated by the level of red blood cells (RBC), white blood cells (WBC), platelet count (PLT), hemoglobin (Hb) and hematocrit (Ht). The biochemical parameters such as liver glycogen, blood glucose, triglycerides (TG) and plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) levels were determined. In addition, histological changes in the liver, proliferating cell nuclear antigen (PCNA) and proliferation index (PI) were analyzed. After 10 days of treatment, hematological analysis revealed a significant increase of RBC, Hb and Ht levels while WBC and PLT counts were significantly decreased. Also, the liver glycogen and TG contents were decreased, whereas blood glucose, ALT, AST and LDH levels were increased. Histopathological examination of the liver revealed pronounced morphological alterations with cytoplasmic vacuolation, focal necrosis, sinusoidal congestion, infiltration of inflammatory leucocytes and loss of hepatic structure in periportal areas of treated rats. Immunohistochemical staining showed an increase in PCNA-positive cells in liver of treated rats. In addition, the PI and the percentage of binucleated hepatocytes cells were significantly increased in liver of DDT-treated rats. These results clearly suggest that DDT sub-acute treatment causes extensive changes in hematological and biochemical parameters leading to liver damage.

**Introduction:**
The discovery of 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (p,p’-DDT) in 1939 was followed by its widespread use in medicine and agriculture, its insecticidal properties have played an important role in the prevention of diseases worldwide (Spencer and Schaumburg, 2000). The importance of DDT in public health became apparent in the 1940s, when it was used to successfully arrest a major outbreak of typhus and kill mosquitoes that are vectors of diseases such as dengue fever, filariasis, and malaria. The use of DDT for malaria control was reduced or banned in the 1970s due to the emergence of environmental concerns such as its bioconcentration and low degradability, replacements such as pyrethroid and bendiocarb were introduced. When DDT emissions ceased in 1990, about 634 kt DDT were released into the environment (Stemmler and Lammel, 2009). Even though the Stockholm Convention
on Persistent Organic Pollutants listed DDT as the ‘‘Dirty Dozen’’ in 2001 for the global community (UNEP, 2002), DDT is still currently used in indoor residue spraying in 14 tropical countries and several other countries are preparing to reintroduce it (van den Berg, 2009). High levels of DDT (parts per million levels) were always detected in malaria control area. In South Africa for example, the mean DDT concentration approached 7.3 mg/g in human serum and 240 mg/kg in chicken fat (Van Dyk et al., 2010). Also, numerous analytical studies showed higher levels of DDT and its main metabolite 1,1-dichloro-2,2-bis(4-chlorophenyl) ethane (p,p’-DDE) than the allowable daily intake in food (Muralidharan et al., 2009), adipose tissues (Aulakh et al., 2007) and maternal milk (Malvaranann et al., 2009) all over the world. Evidence accumulated over the years has suggested that chronic exposure to DDT and its derivatives is associated with loss weight, anorexia, sterility, endocrine disruptions, muscular weakness, tremors, hepatic effects, and anemia in humans (Hillman, 1998; Spencer and Schaumburg, 2000). Studies have shown that liver symptoms, associated with DDT poisoning, include hepatomegaly, liver damage and liver function disorder (Kostka et al., 1996; Shimada et al., 2015). However, experiments to elucidate the effects of DDT on the hematopoietic system are extremely limited, despite the knowledge that DDT and/or its derivatives affects membrane fluidity in erythrocytes and induce apoptosis in human peripheral blood mononuclear cells (Antunes-Madeira and Madeira, 1990), this phenomenon is known to be associated with oxidative stress (Alegria-Torres, 2009). In the current study, we have examined the effect of p,p’-DDT subacute treatment on hematological and biochemical parameters as well as liver histopathological changes in rat. To this end, the hematological parameters were evaluated by red blood cells (RBC), white blood cells (WBC), platelet count (PLT), hemoglobin (Hb) and hematocrit (Ht) levels. The biochemical parameters such as liver glycogen, blood glucose, triglyceride (TG) levels and the activities of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were determined. In addition, proliferating cell nuclear antigen (PCNA) was assessed by immunohistochemical staining.

Materials and Methods:-

Animals and reagents:-
Male Wistar rats (50 days of age) were purchased from the Tunisian Company of Pharmaceutical Industries (SIPHAT, Rades, Tunis, Tunisia). The rats were housed under controlled conditions of temperature (25 °C) with a constant day/night cycle (light from 8:00 to 20:00). Food and water were provided ad libitum. DDT (98% pp’) were purchased from Sigma Chemical (St. Louis, MO, USA). Rats were randomized into three experimental groups of approximately similar weight (n=8) as follows: (1) animals received daily an intraperitoneal (ip) injection of DDT diluted with corn oil at a dose of 50 mg/kg body weight (b.wt) during 10 days, (2) animals were administered 10 daily injections of 100 mg DDT/kg b.wt, (3) control group received equal daily volumes of vehicle during the treatment period. The choice of the dosing period and DDT doses was based on the results of previous studies (Ben Rhouma et al., 2001; Harada et al., 2003; Tebourbi et al., 2006, 2010). Rats were fed and observed daily. The body weight of rats was determined daily throughout the experiment. After 10 days of treatment, blood samples were collected via the ocular vein for hematology analysis, then, all animals were sacrificed by rapid decapitation. The liver were dissected and weighed. Animals were cared for in compliance with the code of practice for the Care and Use of Animals for Scientific Purposes. Approval for these experiments was obtained from the Medical Ethical Committee for the Care and Use of Laboratory Animals of Pasteur Institute of Tunis (approval number: LNFP/Pro 152012). The experimental protocols were approved by the Faculty Ethics Committee (Faculté des Sciences de Bizerte, Tunisia).

Hematological Assay:-

Blood samples were collected from rats into heparinized tubes. Red blood cells (RBC), white blood cells (WBC), platelet count (PLT), hemoglobin (Hb) and hematocrit (Ht) were measured on Hematology Analyzer (Coulter CA 620).

Biochemical Assay:-

Serum was collected by centrifugation (4000g for 15 min). The activities of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were assayed spectrophotometrically according to the standard procedures using commercially available diagnostic kits (Chronolab, France). Plasma lipid parameters such as triglycerides (TG) were determined by enzymatic methods using commercial kit from Sigma diagnostics triglycerides (GPO-Trinder). Glycogen was isolated from liver as described previously (Barberà et al., 1994) with modifications (Ong and Khoo, 2000). Briefly, frozen liver samples were homogenized in 10 vol ice-cold 30% KOH and boiled at 100°C for 30 min. Glycogen was precipitated with ethanol, washed and resolubilized in
water. Glycogen content was determined by treatment with anthrone reagent (1g anthrone dissolved in 500 ml H₂SO₄) and measuring absorbance at 625 nm. The blood glucose was determined with an Accu-Chek Go glucometer (Roche Diagnostics, Meylan, France).

**Histological analysis:**
The livers were fixed overnight at room temperature by direct immersion in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. The samples were dehydrated with ethanol and toluene series and embedded in paraffin. Serial sections (4μm) were mounted on gelatin-coated glass slides cut and stained with haematoxylin and eosin.

**Immunohistochemistry of PCNA:**
Sections (4 μm thick) were cut from paraformaldehyde-fixed and paraffin embedded liver samples. After a standard dehydration-rehydration procedure, liver sections were incubated with 3% H₂O₂ for 5 min to quench endogenous peroxidase activity. Sections were then incubated with anti-PCNA primary antibody from Dako (1/100) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin for 1 hour. Horseradish peroxidase conjugated anti-mouse secondary antibodies (Dako) were used for detection. The sections were washed in PBS and incubated with diaminobenzidine substrate (Dako) for 15 min. After immunohistochemical reaction, the samples were stained with hematoxylin, dehydrated and immersed in xylene. The stage of tissue incubation with primary antigen was omitted in the control reaction. Under a light microscope, 10 random fields were selected and the number of PCNA-positive and nuclei in the same fields was counted using Image-Pro Plus version 4.5 software (Media cybernetics Inc., Silver Spring, MD, USA). The ratio of proliferating cells was expressed as the proportion of PCNA-positive cells to total number of cells. The same method was used to quantify binuclear hepatocytes.

**Statistical analysis:**
Data were analyzed using Statistica for Windows version 5.0 Software. Overall differences in mean values between control and treatment groups were measured using one-way analysis of variance (ANOVA) followed by Turkey’s multiple comparison as the post hoc test. The results were expressed as means ± standard errors of the mean (SEM) and differences were considered statistically significant at p < 0.05.

**Results:**
Exposure of rats to DDT for 10 consecutive days caused a significant increase in RBC, Hb and Ht levels (Table 1). This increase reached 14% and 21% in RBC, 11.7% and 18% in Hb and 8.4% and 14% in Ht levels, respectively in rats treated with 50 and 100 mg of DDT/kg compared with the control. In contrast, WBC and PLT count are significantly decreased in treated rats (Table 1). This decrease reached 47% and 56% in WBC and 72% and 80% in PLT counts of control for 50 and 100 mg of DDT/kg, respectively. The biochemical parameters of the experimental groups were presented in Table 2. The liver glycogen content decreased in animals exposed to DDT by 52.2% and 78.8% of control, respectively for 50 and 100 mg/kg (Table 2). While, blood glucose levels were 28.4% and 70.4% greater than that of the control group, respectively for 50 and 100 mg of DDT/kg (Table 2). Administration of DDT induced a dose-dependent decrease in TG level (p < 0.05) compared with the control group (Table 2). Besides, DDT treatment significantly increased ALT (100 % and 170 %), AST (74% and 145%) and LDH (34.5 % and 178 %) levels in plasma of treated rats compared to control group (Table 2). Liver damage was analyzed by histological examination (Figure 1). No morphological abnormality was observed in control rats showing a regular morphology of liver parenchyma with intact hepatocytes, sinusoids, and portal tract (Figure 1, Photo A). In contrast, exposure to 50 and 100mg of DDT/kg caused cytoplasmic vacuolation, focal necrosis, sinusoidal congestion and infiltration of inflammatory leucocyte cells which are indicative of severe damage (Figure 1, Photo B and C). Also, a loss of hepatic structure in periportal areas and massive necrosis were observed with the high dose (Figure 1, Photo D). In addition, DDT was mitogenic to hepatocytes as indicated by an increase in the percentage of binucleated hepatocytes cells (Table 3). This increase was about 163.6% and 231.8% of control, respectively for 50 and 100 mg/kg. The hepatic proliferative responses were evaluated by the expressions of PCNA, a marker of S phase (Figure 2). In control group, positive staining of PCNA was occasionally appeared in the liver sections (Figure 2, Photo A). While, strong positive staining was observed in liver cells of DDT-treated groups (Figure 2, Photo B and C). The proliferation index grew 2.4 fold (p < 0.05) and 3 fold (p <0.05) in treated rats with 50 and 100 mg of DDT/kg, respectively compared to control (Table 3).
Discussion:

Analysis of blood parameters is relevant for risk evaluation, as any changes in the hematological and biochemical systems have a higher predictive value for human toxicity, when data are translated from animal studies (Olson et al., 2000). The hematopoietic system is one of the most sensitive targets for toxic compounds and an important index of physiological and pathological status in man and animal (Mukinda and Syce, 2007). In this study, we investigated the effect of sub-acute treatment of DDT for 10 consecutive days at the doses of 50 and 100 mg/kg b.wt on hematological, biochemical and histological parameters of liver. In the present study, hematological analysis revealed an increase in the levels of RBC, Hb and Ht while WBC and PLT counts were significantly decreased after exposure to p’-DDT. Conversely, previous study revealed a significant decrease in RBC, Hb and Ht levels after oral administration of 0, 5, 50 and 500 ppm of DDT for 2 weeks (Tomita et al., 2013). Other study showed that exposure to pesticide significantly decreased WBC, while RBC, Hb, Ht, and PLT counts remained inched (Gaikwad et al., 2015). These discrepancies are thought to be related to the doses and durations of DDT exposure, as well as to differences in animal species and individual differences. Increased numbers of RBC were an indication of polycythemia. In polycythemia, the levels of Hb, Ht and RBC are elevated when measured in the complete blood count, as compared to normal (Jepson, 1969). Erythropoiesis occurs in the bone marrow. Erythropoietin is one of the important hormones regulating this process (Aapro and Jelkmann, 2012). The majority of erythropoietin is produced and released by kidneys and liver (Eckardt et al., 1992). Under toxic conditions kidney release too much of protein (erythropoietin) that enhance RBC production. Thus, the adverse effect of DDT on liver and kidney may disturb the erythropoietin expression directly or by disturbing other factors which are responsible for erythropoiettin expression. RBC elevation is directly related to increase in Hb levels (Tognella and Bignotti, 1964). Ht, packed cell volume is the volumetric content of the RBC in the blood (Themtli et al., 2004). Ht can be increased in various physiological conditions such as in Dengue Shock Syndrome, polycythemia, myeloproliferative disorders and hypoxia (Gross, 1990). In the present study, the possible cause of increased Ht levels is the increase of erythropoietin produced from the kidneys in response to DDT administration (Ramadori et al., 2010). In addition, decreased WBC and PLT counts observed in the present study were probably due to harmful effects of DDT on bone marrow and hematopoietic organs. Moreover, the decline in PLT counts suggested a possible effect on blood coagulation and haemostasis blood system damage (Abbes et al., 2006). In view of these points, it is considered that adverse actions of DDT targeting immune and hematopoietic systems might also be responsible for thymic atrophy and hypocellularity in the bone marrow and decreased levels of PLT in the present study. The decrease in PLT counts was also observed after exposure to other pesticides such as diazinon and organophosphate (Hariri et al., 2011; Elsharkawy et al., 2013). Besides, our results showed a significant decrease in the liver glycogen and TG contents whereas blood glucose levels was increased in DDT-treated rats. Our findings were in accordance with other studies carried out in rats and which have also reported an hyperglycemia and a decreased content of liver glycogen and TG in rats exposed to pesticides (Kalender et al., 2005; Elsharkawy et al., 2013). The increased levels of blood glucose may be explained by stimulation of glycogenolysis in several organs (Abdollahi et al., 2004; Pournourmohammadi et al., 2005) and gluconeogenesis in liver (Abdollahi et al., 2004), provoking the release of glucose into the blood and which might be the reason of the significant decrease in liver glycogen content. Also, treatment of rats with DDT may increase the activity of the hepatic gluconeogenic enzyme and phosphoenol pyruvate carboxykinase leading to hyperglycemia. The liver is known as the site of cholesterol and triglycerides synthesis. It has been shown that parenchymal liver diseases are associated with decreases in triglyceride levels (Kalender et al., 2005). In the current study, we have demonstrated that exposure of rats to 50 and 100mg of DDT/kg b.wt, during 10 consecutive days leads to a marked elevation in the levels of serum AST, ALT and LDH, which indicates of hepatocellular damage (Shimada et al., 2015). This elevation could potentially be attributed to the release of these enzymes from the cytoplasm into the blood circulation (Williamson et al., 1996). The increased levels of serum enzymes indicate an enhancement of permeability, damage or necrosis of hepatocytes (Kaczor et al., 2005). Furthermore, biochemical observations confirm histopathological studies on the liver. In fact, our results revealed histological changes in the liver of DDT-treated animals, including cytoplasmic vacuolation, focal necrosis, sinusoidal congestion, infiltration of inflammatory leucocyte and a loss of hepatic structure in periportal areas. Similar changes in the hepatic tissue of rats treated with DDT have been reported by previous studies (Kostka et al., 1996; Shimada et al., 2015). Hepatic atrophy has also been observed in rats treated with organophosphate (Elsharkawy et al., 2013). Generally, hepatocellular vacuolation represents a variety of ultrastructural changes such as deposition offat droplets (Watanabe and Yanagita, 1983), excess accumulation of glycogen (Nayak et al., 1996), mitochondrial changes (Vickers, 2009), and multiple vacuoles with poor cytoplasmic architecture (Adewole and Ojewole, 2007). Cytoplasmic vacuolation caused by plasma membrane invagination is known to develop when cellular permeability is increased despite retention of cellular integrity (Reynolds et al., 1984). The appearance of necrosis with vacuolation seemed to be associated with increased levels of hepatic biomarkers such as ALT, AST and LDH (Shimada et al., 2015).

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Moreover, immunohistochemical study of hepatocyte proliferation showed that PCNA-positive cells were increased in liver of DDT-treated rats. Comparative study has shown that PCNA provides a strong correlation with flow cytometric assessment of S phase proliferating cells (Weisgerber et al., 1993). In agreement, our results showed that DDT-induced PCNA expression may be associated with an increase in the number of cells that accumulate in the S phase of the cell cycle. Also, the increase in hepatocyte proliferation may be at least related to regenerative liver response to pesticide, since during liver growth, histological signs of necrosis and vacuolated cytoplasm were present. Thus, these results may suggest both the direct mitogenic effect leading to hepatomegaly (via hyperplasia and/or hypertrophy) and cytotoxic/regenerative liver response in DDT-treated rats (Kostka et al., 1996). It is well established that in liver, in particular, cell proliferation plays a crucial role in the initiation of carcinogenesis (Ou et al., 2003), either by inducing errors in replication or by converting DNA adducts to mutations before DNA repair can occur. However, the type of hepatic proliferation appears to be a major factor in this phenomenon. Moreover, a recurrent regenerative stimuli as well as continued administration of direct mitogens (DDT, phenobarbitone etc.) expand selective growth of initiated hepatocytes at the promotion step (Farber, 1991). The present results also showed an increase in the binucleate hepatocytes cells in the liver of DDT-treated rats. Our findings were in accordance with another study carried out in rats and which has also reported an increase in hepatocyte proliferation and binucleated hepatocytes cells in liver of DDT-treated rats (Kostka et al., 1996). It has been shown that during regenerative liver growth and after mitotic stimulation by tumor promoters the binucleation rate is reduced (Melchiorri et al., 1993). However, in our study DDT induced sustained increase in binucleation during the experimental period. In conclusion, the results obtained from the present study demonstrate that the sub-acute treatment of p,p'-DDT caused changes in hematological and biochemical parameters leading to impairments of liver histological aspects.

Table 1: Effect of DDT treatment on hematological parameters of rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>DDT 50 mg/kg</th>
<th>DDT 100 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10^12/l)</td>
<td>6.30 ± 0.06</td>
<td>7.18 ± 0.09a</td>
<td>7.62 ± 0.10a</td>
</tr>
<tr>
<td>WBC (10^9/l)</td>
<td>12.53 ± 0.20</td>
<td>6.65 ± 0.30a</td>
<td>5.50 ± 0.41b</td>
</tr>
<tr>
<td>PLT (10^9/l)</td>
<td>880.33 ± 4.09</td>
<td>245.66 ± 51.71a</td>
<td>174.28 ± 14.46b</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>111 ± 0.89</td>
<td>124 ± 0.57a</td>
<td>131 ± 0.57a</td>
</tr>
<tr>
<td>Ht (%)</td>
<td>35.96 ± 0.57</td>
<td>38.98 ± 0.53a</td>
<td>40.99 ± 0.63a</td>
</tr>
</tbody>
</table>

Legend: Each value is the mean ± SEM of 8 determinations in duplicate per group. DDT treatment was preformed as described in Methods. a, b: p < 0.05 compared with controls (Tukey’s multiple comparison post hoc test).

Table 2: Effect of DDT treatment on biochemical parameters of rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>DDT 50 mg/kg</th>
<th>DDT 100 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic glycogen (mg/g)</td>
<td>42.1 ± 0.56</td>
<td>20.12 ± 1.12a</td>
<td>8.93 ± 0.93b</td>
</tr>
<tr>
<td>Blood glucose (g/l)</td>
<td>0.88 ± 0.06</td>
<td>1.13 ± 0.02a</td>
<td>1.5± 0.03b</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.71 ± 0.2</td>
<td>1.55 ± 0.1a</td>
<td>1.23 ± 0.06a</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>34.92 ± 1.98</td>
<td>69.84 ± 2.01a</td>
<td>94.28 ± 1.23b</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>34.17 ± 2.85</td>
<td>59.5 ± 2.9a</td>
<td>83.66 ± 3.08b</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>324 ± 10.73</td>
<td>435.66 ± 21.89</td>
<td>900.76 ± 10.76a</td>
</tr>
</tbody>
</table>

Legend: Each value is the mean of 10 determinations with standard error (SEM). DDT treatment was preformed as described in Methods. a, b: p < 0.05 compared with controls (Tukey’s multiple comparison post hoc test).

Table 3: Effect of DDT treatment on proliferation index and percentage of binucleated hepatocytes cells in rat liver tissues.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PI (%)</th>
<th>Binucleated hepatocytes cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.1 ± 2.4</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>DDT 50 mg/kg</td>
<td>41.7 ± 4.3a</td>
<td>5.8 ± 0.2a</td>
</tr>
<tr>
<td>DDT 100 mg/kg</td>
<td>51.9 ± 5.3a</td>
<td>7.3 ± 0.2a</td>
</tr>
</tbody>
</table>

Legend:
Each value is the mean ± SEM of 8 determinations in duplicate per group. DDT treatment was performed as described in Methods. *: p < 0.05 compared with controls (Tukey’s multiple comparison post hoc test).

**Figure 1:** Photomicrographs of liver sections of control (A) and DDT treated rats with 50 mg/kg (B) and 100 mg/kg (C, D).

**Legend:**
The treated rats received 50 or 100 mg/kg body weight of DDT (ip). Livers were fixed by direct immersion in Bouin’s solution. Serial (4µm) sections were mounted on gelatin-coated glass slides and stained with hematoxylin and eosin.

H, hepatocyte; Hb, binucleated hepatocyte; S, sinusoid; Ne, necrosis; P, portal triad; E, erythrocyte; Small arrowhead points to cytoplasmic vacuolation; Great arrowhead points to periportal congestion; Arrow points to loss of hepatic structure. Magnification × 400 (A, B, C, D).
**Figure 2:** Hepatic expression of PCNA by immunohistochemistry in control (A) and DDT treated rats with 50 mg/kg (B) and 100 mg/kg (C).

**Legend:**
- H, hepatocyte; Hb, binucleated hepatocyte; In, infiltration of inflammatory cell; arrowhead points to S-phase nuclei; arrow points to vacuolation of cytoplasm. Magnification × 400 (A, B, C).

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**References:**