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Hepatotoxicity induced by sub-acute exposure of rats to 2,4-Dichlorophenoxyacetic acid based herbicide "Désormone lourd"

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ABSTRACT

"Désormone Lourd" is a 2,4-Dichlorophenoxyacetic based herbicide that includes 600 g/L 2,4-D. In this study we analyzed the toxic effects of 2,4-D on rat liver. Animals were daily treated with 15, 75 and 150 mg/kg, via oral gavage during 4 weeks. Hepatotoxicity was monitored by quantitative analysis of the serum enzymes markers of hepatotoxicity. Oxidative stress markers, catalase and glutathione reductase (CAT and GR), were analyzed in liver. We also investigated liver tissues histopathologically. Our results revealed that, when rats of 2,4-D treated groups were compared with the control group, the body weight decreased and the liver weight increased significantly at the end of the 4th week. The microscopic evaluation showed that 2,4-D induced hepatic cord disruption, focal necrosis, vessel dilation and pycnotic nucleus. Histological effects were found in all treated groups and their severity was dose dependent. Through sub-acute treatment, starting from the low to the high doses of 2,4-D, it was observed that there were effects on the activity of the serum enzyme markers, on TSP, Alb and the glycemia levels. We also observed a significant reduction in the hepatic antioxidant enzyme activities. To conclude, we can suggest that 2,4-D induces hepatoxicity and cellular alterations in rat.

1. Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) is the active ingredient of "Désormone Lourd" which is marked as a selective systemic herbicide. It is widely used throughout the world including Tunisia. 2,4-D is a moderately persistent chemical with a half life ranging from 20 to 200 days. The spraying of 2,4-D often contaminates the ground water systems. About 91.7% of 2,4-D will eventually end up in water, thereby jeopardizing the health of organisms that are exposed [1,2]. The toxicity of 2,4-D and other related compounds was attributed to the free acid form of the chemicals [3]. It has been indicated that the toxicity of this herbicide also showed a direct correlation with the concentration and time exposure [4].

2,4-D is chemically derived from phenoxyacetic acid (Fig. 1). Its herbicidal activity is mediated by an auxin-like capacity to alter normal protein synthesis and cell division in plant meristems and leaves [5]. Yet, Romero-Puertas et al. [6] have recently suggested that the 2,4-D-herbicidal activity may also be due to an increase in the production of oxygen reactive species. The latter's lead to the generation of oxidative stress in the weed.

Pesticide exposure can lead to oxidative stress through unregulated generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, peroxyl radicals and singlet oxygen. ROS are produced during normal process in the cell. Under normal conditions antioxidant systems of the cell minimize damage caused by ROS. When ROS generation increases to an extent that it overcomes the cellular antioxidant systems, the result is oxidative stress. Several studies have shown that 2,4-D produces oxidative stress and/or depletes antioxidants both *in vitro* and *in vivo*. *In vitro* reports have looked, especially, at the effects of 2,4-D on hepatocytes and red blood cells [7,8]. *In vivo* oxidative activity has been studied in many species including yeast, plants, fish and rats [6,9–12]. Celik et al. [11] studied the effects of 2,4-D on serum marker enzymes, erythrocyte and tissue antioxidant defense and lipid peroxidation in rats. The authors found that the administration of 1.5 and 3 mg/day of 2,4-D during 25 days induced *in vivo* oxidation.

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic herbicide; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; CPK, creatine phosphokinase; GGT, gamma-glutamyltransferase; GR, glutathione reductase; LDH, lactate dehydrogenase; TB, total bilirubin; TSP, serum total protein.

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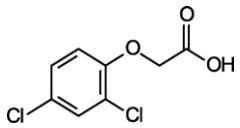

Fig. 1. Chemical structure of 2,4-D.

Being a chemical herbicide that is currently used in more than 1500 commercial formulation [13,14], current studies dealing with methods of studying its toxicity have been reported. Such as the study done by Mountassif et al. [2] in jerboa daily treated with 2,4-D. The results show that 2,4-D induces toxicity which affects energy metabolism, morphological perturbation and oxidative stress. Obidike et al. [14] have demonstrated that exposure to 2,4-Dichlorophenoxyacetic acid exerts toxic effects on the haematological indices of West African dwarf goats. This claim is supported by the fact that 2,4-D toxicity may be medicated by effects associated with the plasma membrane, interference or cellular metabolic pathways involving acetyl coenzyme A or uncoupling of oxidative phosphorylation. Other researchers have reported oxidant effects of 2,4-D, indicating the potential for cytotoxicity or genotoxicity. For example, Bukowska [8] reported that treatment of human erythrocytes *in vitro* with 2,4-D at 250 and 500 ppm resulted in decreased levels of reduced glutathione, decreased activity of superoxide dismutase, and increased levels of glutathione peroxidase. These significant changes in antioxidant enzyme activities and evidence of oxidative stress indicate that 2,4-D should be taken seriously as a cytotoxic and potentially genotoxic agent.

Cholorophenoxy herbicides are structurally related to the hypolipidemic drug ethyl 2-(4-chlorophenoxy)-2-methylpropionate, which is considered the representative of hepatocarcinogenic agents that combine induction of peroxisome proliferation with changes in a number of enzymatic activities in the liver of some mammalian species [10]. Thus, being a phenoxyacid herbicide, we need to further investigate the potential hepatotoxicity of 2,4-D. Certain serum chemistry may be used to identify tissue damage [15]. The damage in liver, the metabolic center for detoxification of chemicals, was confirmed by changes in the activities of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Lactate dehydrogenase (LDH) and Alkaline phosphatase (ALP). The activities of such serum enzymes markers were determined to indicate the metabolic change under the effect of stress. Indeed, changes in the levels of these liver enzymes in serum are actually to be the most relevant signal of liver toxicity; then macroscopic and in particular histopathological observations will allow confirmation of the occurrence of hepatotoxicity and will provide further evidence of the type of liver toxicity [16]. But these evaluations are not sufficient to identify mechanism(s) of liver toxicity. So it raises the necessity of deeper mechanism-oriented studies. These may include: testing using *in vitro* liver models, additional histopathology, immunology-based test, etc. to further characterize the hepatotoxic effects of 2,4-D herbicide.

The aim of this study was to evaluate the biochemical, histopathological alterations and antioxidant status of liver rat exposed to the toxic effects of 2,4-D based herbicide "Désormone lourd".

2. Materials and methods

2.1. Chemicals

2,4-D commercial formulation (Désormone Lourd) consists of 600 g/L 2,4-D Ester butylglycol which register number is *H.96064*. It was available and used in experimentation in Tunisia. The solutions of the 2,4-D formulation were prepared by the addition of appropriate volumes of distilled water.

2.2. Experimental design

2.2.1. Animals

Male Wistar rats, weighing from 150 to 180 g were obtained from Central Pharmacy (Société des Industries Pharmaceutiques Tunis, Tunisia). Before experiments, animals were housed for 2 weeks in polyethylene home cages, with sawdust-covered floors. They were maintained in a colony room at $22 \pm 2\,^{\circ}C$ under conditions of controlled humidity ($70\% \pm 5\%$) and a 12 h light/dark cycle. Access to water and standard commercial pellet chow (SICO, Sfax Tunisia) was free. All the breeding phases and all experiments were conformable to the rules of the Tunisian Society for the Care and Use of Laboratory Animals. All experiments were conducted at the animal facilities of the Faculty of Medicine, Monastir; with the approval of the Faculty of Medicine Ethics committee.

2.2.2. Treatment administration of 2,4-D

After the acclimatizing period, animals were randomly divided into four different groups of 10 animals each. The first group served as a control and received 1 ml of distilled water via oral gavage once a day. The 2,4-D treated groups – (G1) (G2) and (G3) – were respectively given an oral 2,4-D treatment at a dose level of 15, 75 and 150 mg/kg weighing each (oral LD_{50}; 375 mg/kg) in water, daily. All the treatments continued for a period of 4 weeks. 2,4-D solutions were administered in the morning (between 8:00 and 9:00) to non-fasted rats. Before sacrificing the animals, they were fasted overnight and euthanized under light ether anesthesia.

2.3. Measurement of body weight, organ weight, and water and food consumption

Water and food consumption and the individual animal body weight were recorded daily throughout the experiment. At the end of the experimental period livers were dissected out, washed with ice-cold physiologic saline solution (0.9%), blotted dry, and weighed. The relative liver weight (liver weight/100 g of body weight) of each rat was calculated.

2.4. Histopathological analysis

For the histopathological observations at the light microscopic level, fresh tissue pieces of liver were fixed in Bouin liquid. Following two days of fixation, the specimens were washed and dehydrated through a graded series of ethanol. Then, they were embedded in paraffin wax. Blocks were made and sectioned at 5 μm thickness using a rotary microtome. Sections were rehydrated in distilled water and stained with Hematoxylin-Eosin (H-E) then examined under light microscopy.

2.5. Blood and liver tissue collection

At the end of treatments, the rats were anesthetized by the inhalation of diethyl ether and they were sacrificed. The blood samples were obtained from a cardiac puncture using syringe for the determination of serum enzyme levels. So, blood samples were put immediately into ice-chilled silicon disposable glass tubes. They were let to stand for 30 min. Serum samples were obtained by centrifuging blood samples at 3500 rpm for 15 min at $4\,^{\circ}C$ and stored at $-80\,^{\circ}C$ in aliquots until analysis. Liver tissue was homogenized for 30 s in 10 volumes of ice-cold 10 mmol/L phosphate-buffered

Table 1
Body weight, weight gain, absolute and relative liver weight, water and food intake of control and experimental rats.

Parameter	Control	2,4-D treatment		
		G1	G2	G3
Initial body weight (g)	221.12 ± 17.73	223.1 ± 24.19	222 ± 22.27	223 ± 13.89
Final body weight (g)	259 ± 29.10	247.3 ± 26.15	195.8 ± 20.01	180 ± 18.68
Weight gain (%)	17.29 ± 4.06[a]	10.90 ± 2.77[b]	−11.78 ± 3.13[c]	−19.40 ± 3.91[d]
Absolute liver weight (g)	8.03 ± 0.98[a]	9.28 ± 0.69[b]	11.25 ± 0.95[c]	11.49 ± 0.79[c]
Relative liver weight (g/100 g body weight)	3.12 ± 0.28[a]	3.70 ± 0.40[b]	5.02 ± 0.48[c]	5.90 ± 0.13[d]
Food intake (g/day/rat)	20.17 ± 1.20[a]	18.42 ± 0.92[b]	16.72 ± 1.52[c]	12.89 ± 0.78[d]
Water intake (ml/day/jour)	20.01 ± 3.51[a]	18.67 ± 2.00[a]	17.70 ± 1.69[a]	12.78 ± 0.72[b]

Values are means ± S.D. For ten rats each group. Significance at $p < 0.05$. Values on the same row with different letters are significantly different. "control" indicates control rats treated daily with 1 ml of distilled water; "G1", rats treated with 15 mg/kg BW of 2,4-D; "G2", rats treated with 75 mg/kg BW of 2,4-D; "G3", rats treated with 150 mg/kg BW of 2,4-D.

saline (pH 7.4) containing 1.15% KCl. The homogenate was subjected to centrifugation at 6500 rpm for 15 min at 4 °C. The supernatant fractions were collected and stored at −80 °C until analysis.

2.6. The biochemical parameters of liver functioning

The serum samples collected will serve for the measurement of some enzymes levels. The determination of enzyme activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), plasma alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), Total bilirubin (TB), Albumin (Alb), Lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) were assessed with commercially available diagnostic kits supplied by Randox Laboratoires (Ardmore, Northern Ireland, UK). Enzyme activity was expressed in International Units per litre (IU/L).

Protein content in serum and in supernatant fractions was determined according to Bradford method [17]. The absorbance was measured spectrophotometrically at 750 nm.

Plasma glucose was measured by the glucose oxidase and peroxidase using quinoneimine as a chromogen. The amount of plasma glucose is related to the amount of quinoneimine which is measured spectrophotometrically at 505 nm [18].

2.7. The liver activities of antioxidant enzymes

Analyses of antioxidant enzyme activities were made using a Biorad UV–visible spectrophotometer with a "kinetics" program (Biorad, Mares la coquette, France). The measurement of Glutathione reductase (GR) activities in supernatants was performed by using commercially available diagnostic kits supplied by Randox Laboratoires. The assay was adapted from the method of Goldberg and Spooner [19]. Glutathione reductase catalyzes the reduction of oxidized glutathione in the presence of NADPH which is oxidized to $NADP^+$. The decrease in the absorbance is measured at 340 nm. One Unit of activity is equal to the micromole of NADPH oxidized per minute per milligram of protein.

The catalase (CAT) activity was measured at 25 °C according to the method of Aebi [20]. Hydrogen peroxide (H_2O_2) decomposition by CAT enzyme was monitored kinetically at 240 nm. The molar extinction coefficient of 43.6 mol L^{-1} cm^{-1} was used to determine the CAT activity. One unit of activity is equal to the micromole of H_2O_2 degraded per minute per milligram of protein.

2.8. Statistical data analysis

In each assay, the experimental data represent the means of ten independent assays ($n = 10$) ± standards deviations. Data were analyzed using SPSS 11.0 for Windows (Chicago, IL). The statistical significance has been determined using one-way analysis of variance (ANOVA). Differences were considered significant at the level $p < 0.05$ and very significant at the level $p < 0.01$. Means comparison was done using Duncan Test.

3. Results

3.1. The effects of 2,4-D on general rats health

Death was not observed in any of the experimental groups during the experimental period (28 days). Also, no clinical signs of 2,4-D poisoning were observed among treated rats of 15 mg/kg. However, for the group of 75 mg/kg 2,4-D treated rats, some symptoms such as reduced activity, increasing weakness, slight diarrhea and collapse were observed. These clinical signs were more severe for rats treated at 150 mg/kg BW of 2,4-D. We also have noted other symptoms such as molting in the abdominal region, hair loss and nasal hemorrhage.

3.2. The evaluation of body weight, organ weight and food and water consumption

At the end of the 4th week there was a statistically significant decrease in food consumption for the three treated groups compared to the control group ($p < 0.01$). Water intake of the 2,4-D treated groups also reduced. The reduction was, especially, very significant in the 150 mg/kg treated rats, as compared to controls ($p < 0.01$) (Table 1). Furthermore, during the 4 weeks of exposure 2,4-D-exposed animals had significantly decreased body weight versus control group (Fig. 2); and as a consequence, animals gained less body weight than controls ($p < 0.01$). 2,4-D treatment also modified the absolute and relative liver weight. This markedly increased in 15, 75 and 150 mg/kg BW of 2,4-D treated rats when compared with the liver weights of the controls ($p < 0.01$) (Table 1).

3.3. Hepatic histoarchitecture

The histological findings of liver from various treatment groups were presented in Figs. 3–6. Normal control animals revealed clear cut hepatic lobules. They were separated by interlobular septa and traversed by portal veins. Within the lobule, hexagonal array of hepatic plates, radiating towards periphery from a central vein were visible. The hepatocytes were polyhedral. The nuclei were round and the size was roughly the same (Fig. 3).

Intoxicated animals indicated marked alterations of hepatic pathology. After 4 weeks of treatment by 15 mg/kg of 2,4-D the hepatic cords were disrupted at many places (Fig. 4). We also observed focal necrosis, vacuolation of hepatocytes and vessel dilation. These alterations in rat liver were more accentuated after the exposure to 2,4-D at 75 mg/kg dose. The necrotic area was especially more frequent. We have also noted the increase in the glycogen charge (Fig. 5). After 4 weeks of 150 mg/kg 2,4-D treatment, there were several histopathological effects (Fig. 6). These effects were pycnotic

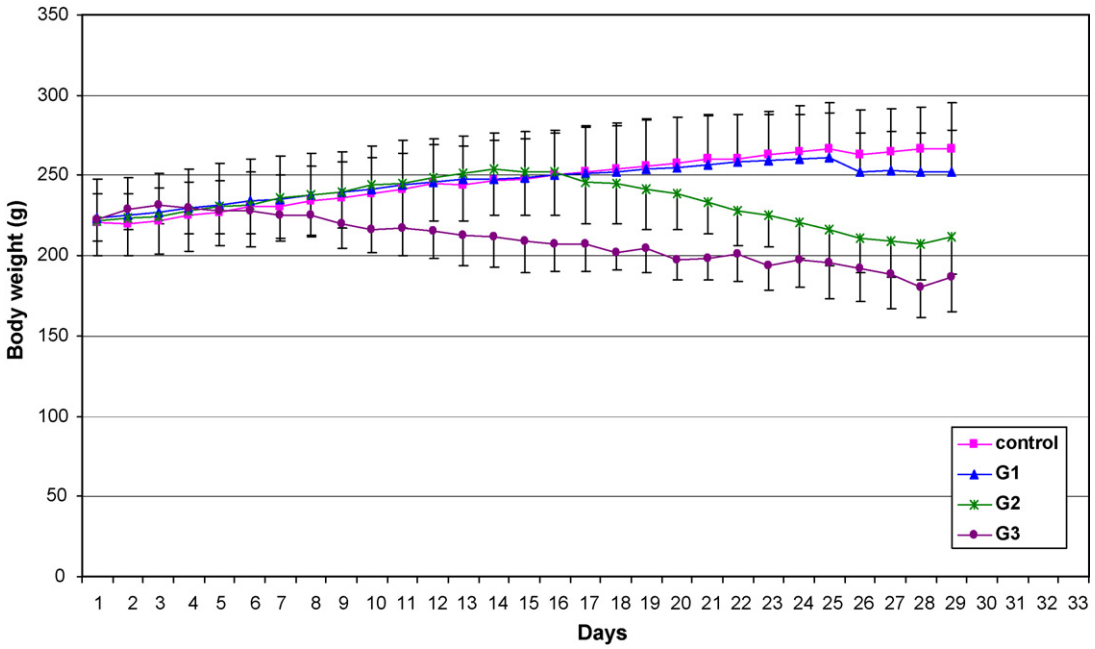

Fig. 2. Effects of 2,4-D treatments on bodyweight of rats. Each value represents the mean ± S.D. of ten rats per group. "control" indicates control rats treated daily with 1 ml of distilled water; "G1", rats treated with 15 mg/kg BW of 2,4-D; "G2", rats treated with 75 mg/kg BW of 2,4-D; "G3", rats treated with 150 mg/kg BW of 2,4-D.

nucleus and congestion of hepatocytes in liver tissue. There was a significant increase in the cells undergoing necrosis. A few hepatocytes were vacuolated and had lost the usual polyhedral shape. The hepatic cords were disrupted at many areas. Hexagonal array of hepatic plates radiating towards periphery from a central vein was not visible any more.

3.4. The evaluation of biochemical hepatic function

Serum total protein (TSP), Alb, TB, AST, ALT, ALP, GGT, LDH and CPK are indicators of hepatic function. Table 2 indicates that 2,4-D strongly decreases Total protein and albumin levels ($p < 0.01$). By contrast, there is a statistically significant increase in TB, LDH and

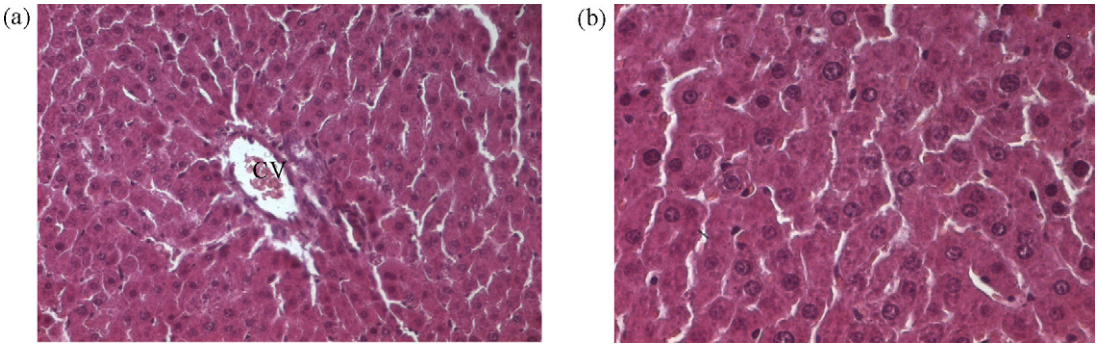

Fig. 3. (a and b) Photomicrograph (magnification: 200× and 400×) of histological sections from a control animal showing an hepatic lobule with the uniform pattern of the polyhedral hepatocyte radiating from the central vein towards the periphery. Sections were stained with Hematoxylin-Eosin (H-E).

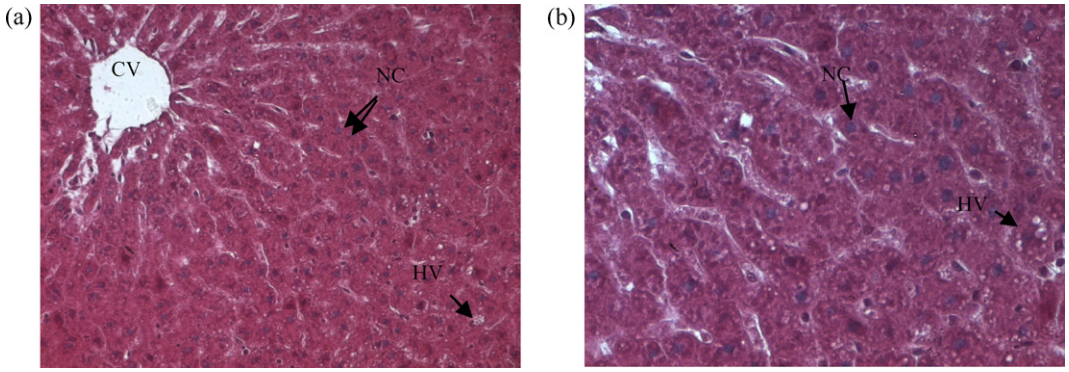

Fig. 4. Liver sections of a 15 mg/kg 2,4-D treated group demonstrating disrupted pattern of hepatic cords, focal necrotic cells (NC) and hepatocyte vacuolation (HV). Liver sections were stained using the Hematoxylin-Eosin method. Original magnifications: (a) 200× and (b) 400×.

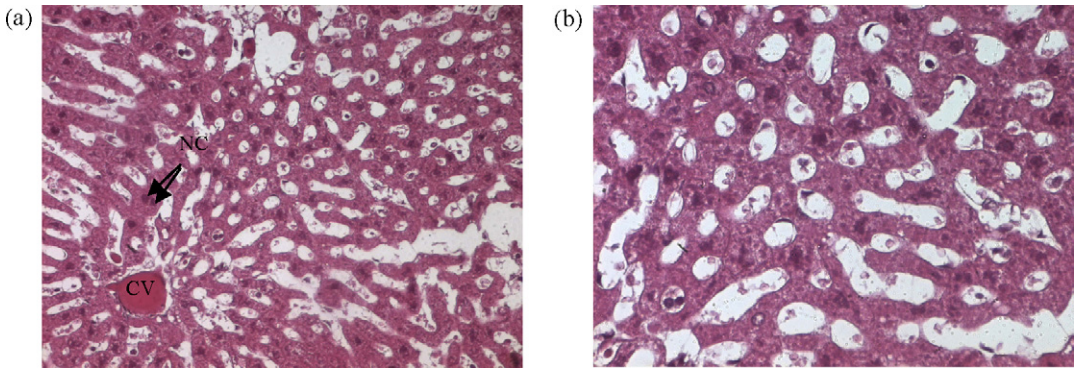

Fig. 5. Photomicrograph of HE staining section of liver from rat of 75 mg/kg 2,4-D treatment group (4 weeks). Regions of necrosis (NC) are evident in many parts of the section. Vessel dilation is more clear. Magnification: (a) 200× and (b) 400×.

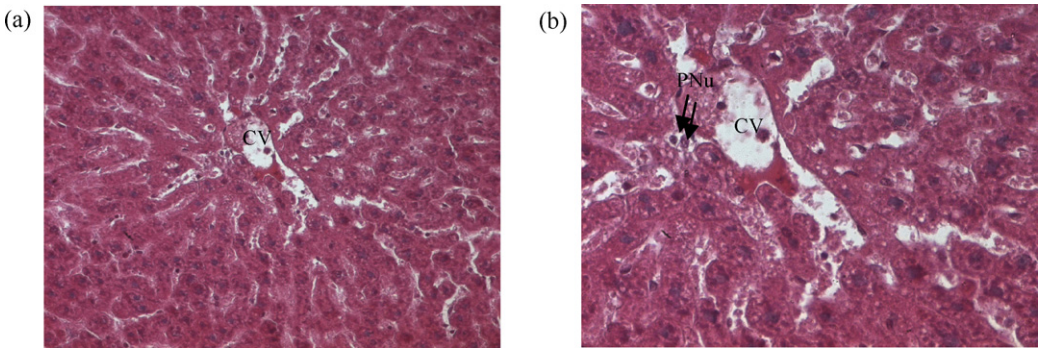

Fig. 6. Histological sections of liver from rat after exposure to 150 mg/kg of 2,4-D showing abnormal cords arrangement, marked vacuolization (HV), necrotic areas and pycnotic nucleus (PNu). Magnification: (a) 200× and (b) 400×.

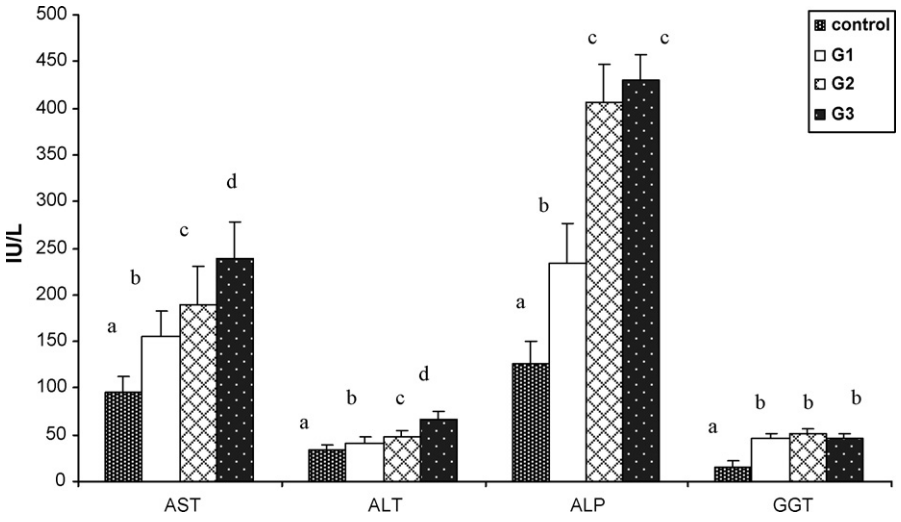

Fig. 7. Serum AST, ALT, ALP, GGT profiles 4 weeks after 2,4-D treatments to rats. Each value represents the mean ± S.D. of ten rats per group. Values with different letters are significantly different at $p < 0.05$. "control" indicates control rats treated daily with 1 ml of distilled water; "G1", rats treated with 15 mg/kg BW of 2,4-D; "G2", rats treated with 75 mg/kg BW of 2,4-D; "G3", rats treated with 150 mg/kg BW of 2,4-D.

CPK activities ($p < 0.01$). No changes were observed for glycemia at 15 and 75 mg/kg. However, for 150 mg/kg 2,4-D-treated group, the increase is very significant at the end of the 4th week ($p < 0.01$). Concerning Fig. 7, it shows that there is a statistically significant increase in AST, ALT, ALP, and GGT activities ($p < 0.01$).

3.5. The activities of liver antioxidant enzymes

Results in Table 3 showed biochemical parameters which indicated the liver oxidative damage. In fact, the treatment of rats with 2,4-D at increasing doses changed activities of antioxidant enzymes such as CAT and GR in the liver. Exposure of rats to 2,4-D produced a very significant decline in CAT and GR enzyme activities by (−16.88, −23.70 and −43.22%) and by (−23.11, −28.25 and −51.36%), respectively for 15, 75 and 150 mg/kg BW of 2,4-D.

4. Discussion

Over the years, crop farmers have always used pesticides to control harmful agricultural pests in their farmlands. Herbicides

Table 2
Effects of sub-acute 2,4-D treatments at three increasing doses during 4 weeks on serum TB, Alb, LDH, CPK, TSP and glycemia profiles of rats compared to control.

Parameter	Control	2,4-D treatment		
		G1	G2	G3
TB (U/l)	7.77 ± 5.20^{a}	9.46 ± 5.10^{ab}	13.89 ± 4.96^{b}	12.80 ± 4.69^{ab}
Alb (g/dl)	4.44 ± 0.44^{c}	3.04 ± 0.38^{b}	2.55 ± 0.54^{a}	2.67 ± 0.34^{ab}
LDH (U/l)	227.10 ± 69.99^{a}	574.00 ± 61.96^{b}	666.30 ± 124.35^{b}	748.00 ± 111.35^{c}
CPK (U/l)	93.70 ± 36.86^{a}	367.30 ± 137.61^{b}	637.10 ± 140.26^{c}	742.00 ± 109.33^{d}
TSP (g/dl)	8.68 ± 0.24^{c}	7.48 ± 0.15^{b}	6.33 ± 0.48^{a}	6.40 ± 0.40^{a}
Glycemia (g/l)	1.18 ± 0.42^{a}	1.62 ± 0.36^{a}	1.79 ± 0.63^{a}	2.4 ± 1.04^{b}

Each value represents the mean ± S.D. For ten rats each group. Values on the same row with different letters are significantly different at $p < 0.05$. "control" indicates control rats treated daily with 1 ml of distilled water; "G1", rats treated with 15 mg/kg BW of 2,4-D; "G2", rats treated with 75 mg/kg BW of 2,4-D; "G3", rats treated with 150 mg/kg BW of 2,4-D.

Table 3
Effects of sub-acute 2,4-D treatments at three increasing doses during 4 weeks on antioxidant enzyme activities in the liver of rats compared to control.

Antioxidant enzyme activities	Control	2,4-D treatment		
		G1	G2	G3
CAT (U/mg protein)	23.42 ± 0.54^{c}	19.47 ± 2.27^{b}	17.87 ± 0.93^{b}	13.30 ± 2.23^{a}
GR (U/mg protein)	0.048 ± 0.007^{c}	0.037 ± 0.004^{b}	0.034 ± 0.003^{b}	0.023 ± 0.001^{a}

Each value represents the mean ± S.D. For ten rats each group. Values on the same row with different letters are significantly different at $p < 0.05$. "control" indicates control rats treated daily with 1 ml of distilled water; "G1", rats treated with 15 mg/kg BW of 2,4-D; "G2", rats treated with 75 mg/kg BW of 2,4-D; "G3", rats treated with 150 mg/kg of 2,4-D.

represent the most prominent class of these chemicals. Phenoxyacetic herbicides constitute one of the largest groups of herbicides sold throughout the world. Among them and for many years 2,4-Dichlorophenoxyacetic acid (2,4-D) has been widely used as a plant growth inhibitor [21]. Unfortunately, this herbicide is not a specific weeds target. It can cause low growth rates, reproductive problems, changes in appearance or behavior and death in non-target species including plants, animals and microorganisms [22].

In the present study, 2,4-D was preferred because of its largely use as a selective herbicide in the North of Tunisia for cereals culture. Besides, it is found in a wide variety of biologically active compounds. Information on its negative effects on higher animals are very limited for *in vivo* oral exposure. So, in this study, we used 2,4-D as a part of a commercial formulation commonly used by Tunisian farmers to determine its possible toxic effects on rats by administering increasing doses of this herbicide.

The oral LD_{50} of 2,4-D in rats ranges from 375 to 666 mg/kg and dermal LD_{50} has a value of 1500 mg/kg. In our study, we used three doses of 15, 75 and 150 mg/kg/day which are lower than the doses applied to the rodents. The treatment of 2,4-D was done orally because the effect of the chemical represents a well-characterized *in vivo* toxicity model system.

The present study showed that exposure of rats to increasing doses of 2,4-D, a chlorophenoxyacetic acid herbicide, induced a marked decrease in food consumption and water intake. Then at the end of 4th week, there was a statistically significant decrease in body weight when the 2,4-D-treated groups were compared to the control one. We hypothesize that the decrease in body weight was the result of reduced food and water intake which may be an account to the alteration of appetite due to 2,4-D exposure. In fact, the net body weight gain of the animals intoxicated with 2,4-D was markedly less as compared to the normal controls. Indeed, there is a dose-dependent decrease in body weight gain in rats exposed to this herbicide. These findings are in line with some previous work showing that there was a positive correlation between the decrease of the BW gain and the dose of pesticide administered [23,24].

In addition to reduced activity, increasing weakness, slight diarrhea and collapse were observed in rats treated by 75 and 150 mg/kg BW of 2,4-D. Also, there was molting in the abdominal region, hair loss and nasal hemorrhage of the 150 mg/kg BW treated rats. Indeed, we observed the same symptoms of poisoning cited in previous studies with glyphosate, its technical formulation and its major metabolite [25,26]. In dogs, large doses of 2,4-D (100 mg/kg of body weight) cause anorexia, inability to stand and death [27]. Similar clinical signs were found in rabbits and rats: 5 mg/kg of 2,4-D cause ataxia, muscular stiffness, decreased motor activity and mortality [28].

Importantly, we found that at the end of 28 days, there was a significant increase in the absolute and relative liver weights of the 15, 75 and 150 mg/kg 2,4-D treated rats. Increases in liver weight might be due to oedema in the tissue. In fact, some pesticides cause an increase in the absolute liver weight [29] and relative liver weights [30] in experimental animals. Amacher et al. [31] reported that the increased liver weight was related to enzyme induction of cytochrome-P 450. In an earlier study [2] there was no effect of 2,4-D at 3 mg/kg body weight neither on the aspect nor on the weight of jerboa livers. The authors have explained this fact by the conclusion, previously reported, that jerboa (*Jaculus orientalis*) shows unique peroxisome properties, the jerboa responds in a moderate manner to peroxisome proliferators, without leading to any increase in liver mass or hepatomegaly in contrast with rat, guinea pig and human [32].

The optic microscopic findings demonstrated that histological effects were found in all treated groups and their severity was dose dependent. These effects were hepatic cords disruption, increase in the glycogen charge, focal necrosis, vessel dilation, vacuolation congestion of hepatocytes, pycnotic nucleus and loss of the usual hepatic histoarchitecture. These results indicated that the three doses of 2,4-D treatment contribute to cellular injury in rat liver. Similar findings concerning histology damage were observed by Mountassif et al. [2], like manifold centrilobular necrotic area in liver jerboa exposed to 2,4-D at 3 mg/kg BW for 4 weeks. Also vacuolation of hepatocytes and changes in its arrangement cords were observed by histological analysis in Silver catfish treated with 700 mg/L of 2,4-D [33]. Similar results concerning histology damage were observed by Poleksi and Karan [34], like vacuolation of hepatocytes and pycnotic nucleus in liver carps exposed to trifluralin herbicide. Another study showed the histological alterations after the exposure to agrochemicals: increased vacuolization, necrosis of the hepatocyte and dilatation of sinusoids were histological changes found in liver rat after exposure to chlorpyriphos insecticide for 8 weeks [35].

So, the optic microscopic findings support the biochemical studies. 2,4-D like some other pesticides may cause alterations in the enzymatic activities of ALP, ALT, AST, GGT, LDH, TB and CPK [36–39]. Changes in the levels of these enzymes may differ depending on the exposure time and dose of 2,4-D [2,10,11]. In our study, 4 weeks 2,4-D administration, AST, ALT, ALP, GGT and LDH activities, strongly increased compared to those of the control group. These changes pointed out to the functional disorder of the liver [40,41]. The significant alteration in these serum enzyme activities in rats treated with 2,4-D herbicide in comparison to those of controls, was magnified according to the three doses studied. Serum hepatic marker enzymes (ALP, AST, ALT, GGT, CPK, TB and LDH) are primarily used to evaluate hepatic damage. Furthermore, GGT and ALP activities increase in case of hepatic cell damage and the obstruction of bile ducts due to proliferation [42,43]. The same situation is also valid for total bilirubin. The increase shown in plasma total bilirubin concentration of rats treated with 2,4-D is in accordance with the previous study of Kossmann and Mangner-Krezel [44,45] in workers exposed to pesticides. Clifford and Rees [46] also reported that the elevation in plasma bilirubin concentration could be due to the onset of periportal necrosis, so indicating malfunction in the liver.

However, it is conceivable that 2,4-D herbicide, as a toxicological agent like other pesticides, might be interacting primarily with the liver and muscle tissue cell's membrane. This results in structural damage and changes in enzyme leakage and in metabolism of the constituents. So, 2,4-D may pressurize this enzyme into plasma as a result of enzyme leakage breakdown. We know that these enzymes are principally localized in the cytoplasm and they will be secreted into the blood after hepatocellular injury, resulting in increasing their levels in the serum. It is known that aminotransferases are very active in the liver and their activity can be detected in small amounts. So they are the most useful in monitoring people exposition to such pesticides [11,47]. Consequently, this study may suggest that any damage in hepatic cells may result in alteration in the serum AST and ALT levels. Similarly, another researcher determined that AST activity in the serum of jerboa has increased following exposure to 2,4-D [2]. On the other hand, Celik and Kara [48] and Celik et al. [11] determined the effects of different plant growth regulators such as 2,4-D, on serum marker enzyme fluctuations as *in vivo* and *in vitro*. Oruc and Üner [10] also Celik et al. [11] found an increase in the serum LDH activity, respectively, in Cyprinus carpio following exposure to 2,4-Diamin, and in rats exposed to 50 ppm and 100 ppm of 2,4-D. So, we noted that our results are partly in accordance with the previous results despite the differences between studies in their settings, materials and experimental designs.

Moreover, 2,4-D exposure caused a significant decrease in total protein and albumin levels. The results of the present study suggest that exposure to 15, 75 and 150 mg/kg of 2,4-D may influence total protein and albumin metabolism. Such cases are indicative of disorders in protein synthesis and metabolism. Albumin is synthesized by the liver and often transports or binds drugs or chemicals. The albumin level may decrease in individuals that develop liver function disorders after some xenobiotic treatment [45].

The mechanisms involved in the blood glucose alterations following pesticides exposure are under investigation in the recent years. In our study, sub-acute 2,4-D exposure causes hyperglycaemia. This can be explained by the stimulation of glycogenolysis in several organs and of gluconeogenesis in liver [49], provoking glucose release into the blood. Also, for researchers have reported the underlying mechanism of the effect of pesticides to be increase in the synthesis of adreno-corticotropic hormone and glucagon in adrenal glands and decrease in the synthesis of insulin resulting from oxidative stress caused by elevated levels of free radicals [50]. According to Mountassif et al. [2], no changes were observed for glycemia in rats exposed to 3 mg/kg BW/day of 2,4-D. Consequently the divergence observed between studies may be due to the difference between the experimental protocols. Although, our results are in agreement with Oruc and Uner [10] who reported that *Cyprinus carpio* exposed to 2,4-D showed elevated serum glucose following acute exposure to herbicide 2,4-D. In the same way, Cattaneo et al. [33] noted a considerable rise in plasma glucose levels accompanied by a decrease in hepatic glycogen and glucose after 2,4-D exposure in silver catfish (*Rhamdia quelen*). The significant increase in blood glucose and the depletion of hepatic glycogen are generally explained by the activation of hepatic cells glycogenolysis and gluconeogenesis. Interestingly, stimulated glycogen metabolism and augmented activities of phosphoenolpyruvate carboxykinase (PEPCK) are seen in most diabetic cases [51]. Also, disturbed glycogen metabolism and glucose transport have been suggested as a cause of insulin resistance in patients with diabetes which could be a primary or acquired defect in the pathogenesis of diabetes [52].

Importantly, we found that in addition to the fluctuated level of serum marker enzymes, the results of the present study have demonstrated that sub-acute treatment with 2,4-D could affect the antioxidant defense systems of animals. Oxidative stress occurred as a consequence of imbalance between the production of reactive oxygen species and the antioxidative process in favor of radical production [53]. In the current study, the significant decrease in the antioxidant enzyme activities (CAT, GR) in liver proved the failure of antioxidant defense system to overcome the influx of reactive oxygen species generated by 2,4-D exposure. CAT is a hemoprotein, localized in the peroxizomes and catalyses the decomposition of H_2O_2 to water and oxygen. GR is an enzyme which reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant. However, the inhibition of enzymes involved in free radical removal led to the accumulation of H_2O_2, which promoted lipid peroxidation and modulation of DNA, altered gene expression and cell death [54,55].

So, with respect to the hepatic histoarchitecture of the 2,4-D-treated animals there were several histopathological effects in comparison to untreated normal controls. These observations indicated marked changes in the overall histoarchitecture of liver in response to an increase in doses of 2,4-D; which could be due to its toxic effects, primarily by the generation of reactive oxygen species (ROS) causing damage to the various membranous components of the cell [35]. Indeed, these histological alterations occur because the liver is the metabolic center for detoxification; concentration of this compound could involve metabolic process and produce pathological lesions. The liver cells necrosis can cause the migration of immunizing cells and the expansion of blood vessels which improve the development of the inflammatory reactions [2]. Moreover the necrotic conditions observed in liver of 2,4-D-treated animals are in corroboration with the observed biochemical changes noted in our study wherein an alteration of the serum activities of liver maker enzymes and a decline in the hepatic antioxidant enzyme activities.

Taking into account prooxidative capacities of 2,4-D, it will be necessary to investigate the direct effect of 2,4-D on the generation of ROS. Bukowska et al. [56] studied the increase in ROS level in human erythrocytes exposed *in vitro* to 2,4-D. To our knowledge, no *in vivo* studies have ever investigated the potential induction of biological ROS and consequently ROS-mediated oxidative damage in rats exposed to 2,4-D, which could be a potentially important toxic mechanism. As a continuation to the exploitation of our results, we are focused on the study of lipid peroxidation (MDA), fatty acid profile and oxidative stress in rat blood sera exposed to the same studied doses of 2,4-D (data no shown).

5. Conclusion

The results obtained allow us to conclude that 2,4-D and its formulation product "Désormone Lourd", might act synergically on the liver metabolism and/or injury. In fact, we have shown that 2,4-D causes sub-acute hepatotoxicity. Using high doses of this commercial formulation of 2,4-D can be a potential risk for the human health. However, to better understand this fact, more studies need to be made to determine the mechanism of the effects of studied commercial formulation on the liver detoxifying enzymes, to clarify its mechanism of toxicity and to develop an understanding of the structure–toxicity relationships.

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