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Biochemical and Histological alterations of cellular metabolism from jerboa (*Jaculus orientalis*) by 2, 4-Dichlorophenoxyacetic acid: Effects on D-3-hydroxybutyrate dehydrogenase

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Abstract:

2, 4-Dichlorophenoxyacetic acid (2,4D) is one of the widely used herbicide of the phenoxy family with possible startling number of adverse effects on species other than the weeds which it is designed to kill. The effects of 2,4D were investigated in jerboa (*Jaculus orientalis*), a wild animal of sub-desert highlands. The Jerboas have been daily treated intraperitoneally with 3 mg/kg body weight for 4 weeks. Plasmatic markers, and antioxidants defences systems were assessed and histological alterations were evaluated. The *in vivo* and *in vitro* effects of 2,4D on the mitochondrial D-3-hydroxybutyrate dehydrogenase (BDH) were also determined. Our results showed a strong decrease of triglycerides level and HDL cholesterol and an increase in GOT level and LDL cholesterol. The microscopic evaluation showed that 2,4D induced necrosis of seminiferous tubules cells in testis, hyperplasia of hepatocytes in liver and presence of multinucleated giant cells in brain. The results show also an inhibitory effect on BDH in terms of activity and kinetic parameters. All of these results show that 2,4D induces toxicity which affect energy metabolism, morphological perturbation and oxidative stress.

Key words: 2,4D, *Jaculus orientalis*, clinical parameters, antioxidant enzymes, subcellular markers, histology, D-3-hydroxybutyrate dehydrogenase.

Abbreviations:

BDH, D-3-Hydroxybutyrate dehydrogenase; CPK, Creatine phosphokinase; DL-BOH, DL-3-hydroxybutyrate ; DCIP, Dichloroindophenol; 2,4D, 2,4-Dichlorophenoxyacetic acid; EDTA, Ethylenediamine tetraacetic acid ; D-G-3-P, D-Glycerol-3-phosphate; GOT, Glutamate Oxalate Transaminase; GPT, Glutamate Pyruvate Transaminase; HDL, High Density Lipoprotein; KCN, Potassium cyanide; NAD(H), LDL, Low Density Lipoprotein; Nicotinamide Adenine Dinucleotide Oxidized (reduced) forms; NADPH, Nicotinamide Adenine Dinucleotide Phosphate reduced form.

1. Introduction:

Pesticides of worldwide application are used in agriculture in vast amounts each year, of which herbicides are the most prominent class. Phenoxyacetic herbicides constitute one of the largest groups of herbicides sold in the world. Among them, for many years 2, 4-dichlorophenoxyacetic acid (2,4D) has been the one most used (Zeljezic and Garaj-Vrhovac, 2004). 2,4D is a plant growth inhibitor. It is absorbed into a plant through the plant's surface. This weed killer circulates through all parts of the plant causing abnormal growth by blockage of the passage of medium and nutrients. Subsequently, the roots starve and the plant dies.

2,4D is a moderately persistent chemical with a half-life between 20 and 200 days (Associate Committee on Scientific Criteria for Environmental Quality, 1994). Unfortunately, this herbicide is not a specific weeds target. It can cause low growth rates, reproductive problems, changes in appearance or behavior, or death in non-target species, including plants, animals and microorganisms.

Additionally, the spraying of 2,4D often contaminates ground water systems. About 91.7% of 2,4D will eventually end up in water (Associate committee on Scientific Criteria for Environmental Quality, 1994). This contamination threatens the vegetation and the animal life that are exposed. The chemical will also be carried by run-off into the local river systems, thereby jeopardizing the health of aquatic life as well.

In the urban setting, it has been proven that households using 2,4D put their dogs at twice the risk of developing canine malignant lymphoma (Hayes and Howard, 1991). Documented health problems related to 2,4D include reproductive damage (i.e. sterility), respiratory difficulties, atrophy, nausea, loss of appetite, skin rashes, eye irritation and chronic headaches. Non-Hodgkin's lymphoma has also been associated with 2,4D exposure (Kogevinas, 1995). Furthermore, there is evidence of teratogenicity (birth defects) and mutagenicity provided by studies involving 2,4D and lab animals. Workers applying chlorinated phenoxy herbicides frequently have nervous system disorders, are

exposed to a higher risk of soft tissue sarcoma, and show symptoms of hormonal and internal organ irregularities (Hayes and Howard, 1991).

A wide number of environmental pollutants exert their toxic action by interfering at different levels with mitochondrial respiration, a fundamental step of energy metabolism in eukaryotic organisms. Mitochondria have been recognized as subcellular targets of many xenobiotic compounds; as a consequence, toxicity endpoints related to respiratory functions have been widely used in recent years to assess the adverse effects of various toxicants and to investigate the mechanisms of their action by means of suitable in vitro systems (Argese et al., 2005).

To approach these questions, we used as model system the mitochondrial inner membrane bound NAD-dependent D-3 -hydroxybutyrate dehydrogenase (BDH, EC. 1.1.1.30) which play a essential role in the redox balance. Any alteration of its activity will affect the energetic metabolism. In liver, the enzyme catalyses the conversion of acetoacetate into D-3-hydroxybutyrate in the presence of NADH. This reduced substrate is then transported through the blood stream to peripheral organs, i.e. brain, heart, kidney...(Williamson et al., 1971). In such extrahepatic tissues, D-3-hydroxybutyrate is converted into acetoacetate in the presence of NAD⁺. This substrate is then used, after its conversion to acetyl-CoA, by the respiratory chain as fuel for ATP production, or after formation of acetoacetyl-CoA, for fatty acid synthesis. A catalytic mechanism of the interconversion of D-3-hydroxybutyrate and acetoacetate in both liver and peripheral tissues has been previously proposed by our group (El Kebbaj et al., 1997). Moreover this enzyme from Jerboa has been extensively studied in our lab (Kante et al. 1990, El Kebbaj et al., 1995; Mountassif et al., 2006).

The jerboa (*Jaculus orientalis*), a nocturnal herbivorous rodent living in the subdesert highland of Morocco is an appropriate organism to study metabolic regulation due to its remarkable tolerance to heat, dry diet and cold. Also, it is one of the small mammals that can undergo hibernation (Hooper and El Hilali, 1972; El Hilali and Veillat, 1979). Some studies also demonstrated the originality of jerboa in the study of peroxisome proliferator-activated receptor (PPAR) (Amsaguine-Safir et al., 2003 and Kabine et al., 2004). Indeed, El Kebbaj et al. (1996), utilizing a different type of rodent

(rat, guinea pig and jerboa), reveal that the jerboa shows unique peroxisome properties and responds in a moderate manner to a peroxisome proliferator such as ciprofibrate, without leading to any increase in liver mass or hepatomegaly in contrast to rat, guinea pig and human.

On the other hand, beside numerous species previously used for testing agrochemical pollutants sensitivity, here, for the first time, we report a study on jerboa as a new model for evaluation of 2,4D toxicity. Indeed, jerboa is a wild animal which can be maintained at the laboratory in order to test such environmental chemicals.

The purpose of this study was to provide the impact of 2,4D on the cellular antioxidants, metabolic pathways, cells histology and their effects on the D-3-hydroxybutyrate dehydrogenase activity, protein level and kinetic parameters in mitochondria.

Répondre à la question du referee

“It is not clear if this work was done with an emphasis upon effects and biomarkers for wildlife exposure or for humans.”

2. Materials and Methods:

2.1. Chemicals:

2, 4-Dichlorophenoxyacetic Acid, Succinate and Cytochrome C were from Sigma (St Louis, USA); DL-3-hydroxybutyrate (sodium salt) was purchased from Fluka (Buchs Switzerland); NAD⁺ (free acid) and NADH were from Boehringer (Mannheim, Germany) and all other chemicals were of analytical grade.

2.2. Animal and administration of 2,4D:

Adult jerboas (120 - 150 g), 4-6 months, were captured in the area of Engil Aït Lahcen (in subdesert East Moroccan highland). They were adapted to laboratory conditions during 3 weeks at a temperature of 22°C with food (salad and rat diet), and water *ad libitum*. The light cycle during the entire experiment was set to 14 hours light and 10 hours dark. The 2,4D was dissolved in 1 ml sterile water and administered daily by intraperitoneal injection for four weeks. Four jerboas were given 2,4D (3 mg/kg/day) while control groups were given sterile water serving as control. All the experiments were done in compliance with the Guide for The Care and Use of Laboratory Animals.

2.3. Blood analysis:

The determination of the total cholesterol, HDL, LDL, triglycerides, CPK and transaminases (glutamate oxalate transaminase (GOT) and glutamate pyruvate transaminase (GPT)) was carried out by Centre National de Transfusion Sanguine, Casablanca, using the conventional methods.

2.4. Liver mitochondria isolation:

The jerboas were decapitated and the liver and brain were rapidly removed for mitochondrial extraction according to Fleischer et al. (1979). This method allowed the preparation of high yield and pure mitochondria.

2.5. Biochemical assays:

All assays were conducted at 37°C using Jenway 6405 UV/Visible spectrophotometer and done with liver homogenate except acetylcholinesterase which was assessed with brain homogenate.

2.5.1. Catalase:

The consumption of 7.5 mM H₂O₂ in 50 mM potassium phosphate buffer (pH 7) was monitored at 240 nm according to Aebi, 1984.

2.5.2. Palmitoyl-CoA oxidase:

The enzyme was assayed according to Lazarow and De Duve (1976) with assay conditions: 50 mM phosphate buffer (pH 7.4), 0.2 mM NAD⁺, 50 μM FAD⁺, 12 mM, 1 mM KCN, 200 μg of BSA and 100 μg of protein. The mixture was preincubated 10 min at 37°C before adding 50 μM of palmitoyl-CoA (Sigma) as final concentration. The measure of activity was done at 340 nm.

2.5.3. Glutathione reductase:

The assay of Di ilio et al. (1983) was used. Assay mixture contained 0.5 mM oxidized glutathione, 1 mM EDTA, 0.1 mM NADPH and 50 mM potassium phosphate buffer (pH 7.4) and NADPH consumption was monitored at 340 nm.

2.5.4. Superoxide dismutase:

The enzyme was assayed according to Paoletti et al. (1986) with assay conditions: 5 mM EDTA, 2.5 mM MnCl₂, 0.27 mM NADH, 3.9 mM 2-mercaptoethanol in 50 mM potassium phosphate buffer (pH 7), monitored at 340 nm. The activity was started by the addition of NADH to 0.27 mM final concentration.

2.5.5. Thiobarbituric acid reactive substances:

Lipid peroxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS) and quantified in terms of malondialdehyde (MDA) equivalents according to the method described by Samokyszyn and Marnett (1990).

One ml of samples was added to 1 ml solution (0.375 % thiobarbituric acid and 15 % trichloroacetic acid in 0.25 M hydrochloric acid). The tubes were heated at 100°C during 15 min. Then, they were cooled in the ice to stop the reaction. One then carried out a centrifugation with 1000g during 10 min. The reading of supernatant was made to 535 nm.

2.5.6. NADPH-cytochrome C reductase:

NADPH-cytochrome c reductase was measured as described by Williams and Kamin (1962). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.7), 1.25 mg/ml cytochrome C, 0.1 mM EDTA and 100 µg of protein were incubated during 2 min at 37°C, then the NADPH at 100µM final concentration was added and the increase in absorbance due to the reduction of cytochrome C was measured.

2.5.7. Glycerol 3-phosphate dehydrogenase:

The enzyme was assayed according to Bentley et al. (1973) with assay conditions: 100 mM glycine - NaOH buffer (pH 9), 5 mM NAD⁺ and 100 µg of protein. The mixture was preincubated 2 min at 37°C before adding 5mM of glycerol-3-phosphate (Sigma) as final concentration. The activity was recorded at 340 nm.

2.5.8. Glyceraldehyde 3-phosphate dehydrogenase:

GAPDH activity was determined by monitoring NADH generation at 340 nm (Serrano, 1991). The reaction mixture of 1ml contained 50 mM Tricine-NaOH buffer pH 8.5, 10 mM sodium arsenate, 1mM NAD⁺ and 2 mM D-G-3-P.

2.5.9. Succinate dehydrogenase:

The enzyme was assayed according to King (1967) with assay conditions: 100 mM potassium phosphate buffer (pH 7.4), 0.3 mM EDTA, 0.053 mM DCIP and 100 µg of protein. The mixture was preincubated 10 min at 37°C before adding 50 µl of KCN-Succinate (containing 3.25 mg/ml of KCN in 0.5 M succinate). The measure of activity was done at 625 nm.

2.5.10. Acetylcholinesterase:

Assay procedure was assessed according to Ellman et al. (1961). The assay mixture of brain homogenate contained 80 mM DTNB, 45 mM acetylthiocholine and 100 mM potassium phosphate buffer (pH 7.4). The absorbance at 412 nm was monitored.

2.5.11. D-3-hydroxybutyrate dehydrogenase:

BDH activity was measured as described by Lehninger (1960) following NADH production at 340 nm ($\epsilon = 6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using 100 μg of proteins from frozen mitochondria in a medium containing: 6 mM potassium phosphate pH 8, 0.5 mM EDTA, 1.27% (v/v) redistilled ethanol, 0.3 mM dithiothreitol, in the presence of 2 mM NAD⁺ (Sigma) and 2.5 μg rotenone (final addition) to prevent NADH reoxidation by the respiratory chain. The activity was started by the addition of DL-3-hydroxybutyrate (Sigma) to 10 mM final concentration.

2.5.11.1. *In vitro* BDH inhibition by 2,4D:

For the determination of 2,4D concentration giving 50% inhibition (IC_{50}) of BDH, the activities were measured as described previously in the absence and the presence of different 2,4D concentrations after preincubation for 5 minutes. For study of time course of inhibition, 100 μg of thawed liver mitochondria protein were preincubated for 5 minutes at 25 °C in the buffered medium (6 mM potassium phosphate pH 8, 0.5 mM EDTA, 1.27 % (v/v) redistilled ethanol). The 2,4D was added at zero time of incubation. Aliquots were removed at different times during the incubation for the enzymatic activity measurement. Aliquots of the control assay were removed at the same time in order to calculate the percentage of BDH activity.

2.5.11.2. BDH kinetic studies:

Initial velocities of the enzymatic reaction (in the presence of 2,4D at 0, 1.66, 3.33 or 5 mM) were performed by varying the concentration of the substrates, BOH (from 2.5 to 10 mM) or NAD⁺ (from 0.5 to 2 mM). Values of the Michaelis constants (K_m), dissociation constants (K_D) and maximal

velocity for the oxidation of BOH and the reduction of NAD⁺ by the BDH were obtained by mathematical analysis according to the method of Cleland (1963).

2.5.11.3. BDH chemical protection:

Two chemical protectors were used in this study: N-Ethylmaleimide (NEM) (Sigma) and Phenylglyoxal (PGO) (Aldrich-chemie) which are known to react specifically with cysteyl (Latruffe et al., 1980) and arginyl residues (El Kebbaj et al., 1980) respectively. 100 µg of thawed liver mitochondria protein were preincubated for 5 minutes at 25 °C in the buffered medium (6 mM potassium phosphate pH 8, 0.5 mM EDTA, 1.27% (v/v) redistilled ethanol) in the presence of 5 µM NEM or 10 µM PGO. The 2,4D (IC₅₀) was added and the enzymatic activity was measured as described previously. For all experiments, aliquots of the control assay were removed at the same time in order to calculate the percentage of BDH activity.

2.5.11.4. Western-blotting:

After SDS-PAGE (12%) (Laemmli, 1970) and subsequent transfer in nitrocellulose (Towbin et al., 1992), the proteins from frozen mitochondria (50 µg) were exposed to 1/100 dilution of monospecific rabbit polyclonal anti-BDH anti-rat antibody and detected with the secondary antibody of anti-rabbit, IgG peroxidase conjugate (diluted to 1/2500) (Promega).

2.5.12. Protein assay:

Protein content was measured according to the Bradford procedure, using bovine serum albumin (BSA) as standard (Bradford, 1976).

2.6. Histological analysis

The jerboas were decapitated and the liver, brain and testicular glands were removed, fixed in Bouin liquid and embedded in paraffin according to Cau and Seite (1996). 4µm sections were stained with hematein-eosin, then examined under light microscopy (Olympus-BH-2).

2.7. Immunofluorescence labeling

4 μm sections were rinsed with PBS, and then blocked with 1% bovine serum albumin (BSA) in PBS (1% BSA–PBS). Sections were then incubated in the primary antibody (1/100) at room temperature for 1h, washed for 5 min with three changes of PBS, and incubated in the fluorescence-labeled secondary antibody solution (Sigma) 1/100 for 1 hr. Sections were then washed for 5 min with three changes of PBS. Fluorescence-stained sections were examined under an epifluorescence microscope (Olympus).

2.8. Statistical data analysis:

In each assay, the experimental data represent the mean of four independent assays \pm standards deviations. Means were compared using the Student t-test. Differences were considered significant at the level $p < 0.05$ and very significant at the level $p < 0.01$. The calculation of the inhibition concentrations (IC) is carried out by the analysis of probit (Bliss, 1935).

3. Results:

3.1. Effect of 2,4D on plasmatic parameters:

The table I indicates that 2,4D strongly decreases triglycerides and HDL cholesterol levels (-81.6% and -30.2% respectively). In contrast, there is an increase in GOT level (183%) and in LDL cholesterol (153%). No changes were observed for glycemia, total cholesterol, GPT, CPK, urea and creatinine.

3.2. *In vivo* effect of 2,4D on stress and metabolic biomarkers:

For the stress biomarkers, the treatment of the jerboa shows that 2,4D induced a significant increase (24.1%) in peroxidized lipid level (malondialdehyde-MDA) and a significant decrease in catalase activity (77.7%) compared with the control (table II). Malondialdehyde (MDA) is a terminal product of lipid breakdown due to peroxidation damage and this (and other aldehydes) can be detected by its reaction with thiobarbituric acid. No changes were observed on superoxide dismutase and glutathione reductase activities.

For the metabolic markers, no changes were observed in the peroxisomal palmitoyl-CoA oxidase activity. Mitochondrial D-3-hydroxybutyrate dehydrogenase was very sensible to 2,4D; it strongly decreases (64.3%). No changes were observed for mitochondrial succinate dehydrogenase.

Microsomal NADPH-cytochrome C reductase activity decreases significantly (46.1%).

No changes were observed for cytosolic glycerol-3-phosphate dehydrogenase (G3PDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Brain acetylcholinesterase activity decreases significantly (63.2%).

In order to explore the possible correlations between the various biomarkers, Principal Component Analysis (PCA) was carried out (figure 1). Principal Components Analysis is a way of identifying patterns in data, and expressing the data in a way that highlights their similarities and differences.

The results show the existence of 2 principal groups: the first one which explained 62% of total variance, made up of the BDH, catalase, acetylcholinesterase and NADPH-Cytochrome C reductase.

The second which explained 27.2% of total variance contains the G-3-PDH, GAPDH, palmitoyl-

CoA oxidase and the MDA. All the components of the 1st group presented a significant reduction caused by the treatment by the 2,4D, whereas the 2nd group did not show any notable variation excepted for the MDA where the rate is increased.

3.3. Morphological and histological study:

The macroscopic analysis of the removed organs during the dissection revealed that the testes were more red and hypertrophied (an increase in 91% of weight compared to controls) (figure 2A). Indeed, the testes weight of the control group of jerboa was 425 mg (\pm 61) in comparison with the testes of the treated ones (812 mg \pm 112). All the other organs from treated animals have normal aspect and weight.

Optical microscopy of the testis from the 2,4D exposed jerboa shows a necrosis of the seminiferous tubules characterized by dissolution of the nucleus and nuclear membrane (karyolysis). Some cells showed hypertrophy or increase in individual cell size. Indeed, they showed moderate to complete pycnosis. This process in which cells undergo necrotic changes in the nuclei is characterized by rounding up and condensation resulting in hyperchromatic staining (pycnosis) (figure 2B).

In liver of the 2,4D exposed jerboa, we observed manifold centrilobular necrotic areas filled by detrits and connective tissue, hyperplasia of hepatocytes, as well as cytoplasmic dystrophy characterized by small vacuoles tightly packed in the cytoplasm. Additionally, sharply dilated central veins were noted (figure 3A).

In brain of the 2,4D exposed jerboa, there is an increase in the number of the nucleus as well as a hypercondensation of the chromatin. Also, we observed the presence of some multinucleated giant cells (figure 3B).

3.4. *In vivo* impact of 2,4D on BDH activity :

In order to know if the differences observed concerning BDH activities (see table II), were due to BDH variations level, western-blotting was carried out (Figure 4A). Its analysis revealed no changes of the BDH band. Also, the immunodetection of BDH by fluorescence in liver sections reveals no changes on BDH expression with jerboa control and treated groups (figure 4B).

This suggests that the BDH inhibition observed in table II is not caused by BDH gene expression inhibition but due to a BDH catalytic inhibition by 2,4D.

3.5. BDH inhibitory concentration:

The figure 5 indicates that mitochondrial BDH was sensitive to the presence of 2,4D and this sensitivity depends on the concentration used. Indeed, the calculation of the inhibition concentrations of 50% of BDH activity (IC_{50}) by the analysis of probit (Bliss, 1935) reveals that IC_{50} was estimated at $6.69 \text{ mM} \pm 0.41$.

The time course of BDH inhibition by 2,4D was reported in figure 6. The results show that BDH is rapidly and strongly inhibited by 2,4D. We observed a complete inhibition after 35 minutes in the presence of 3 mM of 2,4D (data not shown). The plots of log half time inactivation vs. log 2,4D concentration result in a straight line with a slope of 1.17, indicating that the reaction is first order with respect to inhibitor concentration and that one mole of 2,4D per mole of enzyme is sufficient to produce inactivation (data not shown). The second order rate constant was also calculated to be $20 \text{ min}^{-1} \text{ M}^{-1}$ (data not shown).

3.6. Effect of 2,4D on BDH kinetic parameters :

The effects in vitro of 2,4D on BDH kinetic parameters were studied. Apparent BDH kinetic parameters: V_{\max} , $K_M\text{BOH}$, $K_M\text{NAD}^+$, K_i (2,4D vs NAD^+) and K_i (2,4D vs BOH) are reported in table III. Interestingly, they show that the apparent kinetic constants with respect to the NAD^+ and BOH are modified when BDH is exposed to 2,4D: $K_M\text{NAD}^+$ and $K_M\text{BOH}$ were increased (x 11.61 and x 2.46 respectively). Also, K_i (2,4D vs NAD^+) and K_i (2,4D vs BOH) calculated were 3.38 mM and 5.74 mM respectively.

3.7. BDH protection to 2,4D :

The NEM and PGO react specifically with cysteyl and arginyl residues respectively. The choice of their modifiers was due to the presence of the cysteyl and arginyl in the active site of BDH (Latruffe et al., 1980, El Kebbaj et al., 1980).

Figure 7 shows that the fixation of the NEM on BDH caused 34 % of inhibition while when we added 2,4D, the inhibition increased to 73 %. This increase can be due to the fixation of 2,4D on other amino acid residue leading to a local conformational change.

Figure 7 shows also that the fixation of the PGO on BDH caused 59 % of inhibition. The addition of 2,4D, doesn't change the degree of inhibition (62 %). These results are in agreement with the fixation of 2,4D at arginyl residue(s) of the BDH active site and not at cysteyl one(s).

4. Discussion:

Pesticides are worldwide used in agriculture each year in vast amounts and herbicides are the most prominent class. Phenoxyacetic herbicides constitute one of the largest groups of herbicides sold in the world. Among them, for many years 2, 4-dichlorophenoxyacetic acid (2,4D) has been the largely used. In this study we used 2,4D to determine its possible toxic effects on jerboa (*Jaculus orientalis*). Previously, we have reported that jerboa (*Jaculus orientalis*) shows unique peroxisome properties and 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 (El Kebbaj et al., 1996).

We focused our study on pesticide formulation instead on purified active ingredient (**A mon avis c'est l'inverse, nous avons utilisé du 2,4D purifié-> clarifier la suite**) because workers producing and applying pesticides are exposed to such formulations and not only to active ingredients. There are many findings that pesticide formulations with 2, 4D contain by-product of its industrial synthesis as polychlorinated dibenzodioxins (PCDD) (Linnainmaa, 1983), nitrosamines (Ibrahim et al., 1991) and other chlorinated phenols that may express clastogenic activity (Gandhi et al., 1998; Ibrahim et al., 1991; Mustonen et al., 1986). Furthermore, beside active ingredient and its production by products pesticide formulations contain number adjuvants (solvents, dilutants, dispersants, emulgators, potentiators) that are kept secret by manufacturers and also might present risk for genomic stability. Therefore, in occupational exposure

to pesticides, all those chemicals, not only active ingredients, affect human health, so all of them should be considered in cytogenetical research simulating the exposure. Some studies reveal that 2,4D lead to apoptosis of human lymphocytes (Kaioumova et al., 2001), HepG2 cells (Tuschl and Schwab, 2003), and cerebellar granule cells (De Moliner et al., 2002), and are associated with non-Hodgkin's lymphoma and other cancers (McDuffie et al., 2001 ; Ibrahim et al., 1991 ; Bradberry et al., 2000).

Studies sponsored jointly by FAO and WHO (USDA, 1980) showed that 2,4D residues in the grain at harvest was around 0.36 mg/kg when good agricultural practice is followed. Nevertheless, it is estimated that the dietary exposure to 2,4D does not exceed 22% of a chronic population adjusted dose (cPAD) for the general population. cPAD represents the dose at which an individual could be exposed over the course of a lifetime and no adverse health effects would be expected, and for 2,4D it appears to be 0,013 mg/kg/day. To get the total estimated exposure of the general population to 2,4D, which is assumed to be up to 0,006 mg/kg per day besides dietary exposure, the pesticide intake calculated on the basis of estimated environmental concentrations (EEC) of 2,4D in surface water (15 ppb) and ground water (14.8 ppb), as well as residential risk assessment should be taken into account (EPA, 2002). However, it is known that the occupational exposures to chemicals are up to 1000 times higher than those for the general population. The intake of 2,4D, due to occupational exposure during its production and application, was estimated to be 0,1 – 0,4 mg/kg per day when all protective measures are taken, which is often not the case (WHO, 1984).

On the other hand, the oral LD50 of 2, 4D in rats ranges from 375 to 666 mg/kg and dermal LD50 has a value of 1500 mg/kg.

In our study, we used a dose of 3 mg/kg/day which widely lowers than doses applied to the rodents and so a little superior to that of cPAD.

Also, in order to all the dose passes in the blood circulation of the jerboa, we think that most simple method of administration was the intraperitoneal way.

In jerboa, the *in vivo* 2,4D treatment causes an important decrease in HDL and triglycerides levels and an increase in LDL and GOT ones (table I). These results suggest a perturbation of lipid metabolism in jerboa which can develop atherosclerosis and cardiac pathologies after long time. The impact of 2,4D on the liver function was also evaluated by the assays of metabolic enzymes and of stress biomarkers (table II). The results show a moderated oxidative stress in liver cells caused by the 2,4D. This stress is shown on lipidic peroxidation (MDA).

Importantly, we found that 2,4D causes the testis hypertrophy in jerboas (figure 2A). Previous studies show that 2,4D has a genotoxic effect both in the somatic and germ-line cells of *Drosophila* (Tripathy, 1993). Others studies show an important decrease in foetal body weights and increased foetal variations in rats exposed to 2,4D (Jeffrey et al., 2001). Also, Alavanja et al. (2003) demonstrated in human the existence of a significantly association between pesticides such as 2,4D and prostate cancer risk.

Also, the analysis of the histological sections of liver and brain shows cells necrosis for the liver and the beginning of tumor development for the brain. Liver cells necrosis can cause the migration of immunizing cells and the expansion of blood vessels which improve the development of the inflammatory reactions. Moreover, acetylcholinesterase activity in brain decreases significantly. Indeed, 2,4D is one of few herbicides to cause nervous system damage which includes inflamed nerve endings, lack of coordination, stiffness in the arms and legs, inability to walk, fatigue, stupor, coma, and death (Zahm, 1990).

In dogs, large doses of 2,4D (100 mg/kg of body weight) cause anorexia, inability to stand, and death (Hayes and Howard, 1991). Similar clinical signs were found in rabbits and rats; 50 mg/kg of 2,4D cause ataxia, muscular stiffness, decreased motor activity and mortality (Jeffrey et al., 2001).

Moreover, 2,4D exposure has been linked to an increased risk of cancer. A study by the National Cancer Institute found that dogs with lymphoma were more likely than healthy dogs to have owners who treated their lawns with 2,4D (or contracted with lawn care companies). The increased risk of lymphoma doubled for lawns that were treated four times per year (Drill, 1953; Hayes and Howard,

1991). In human, the dermal absorption of 2,4D was proved and can present an important risk to human health (John et al., 2005).

In order to study the impact of the 2,4D on mitochondria, we choose BDH as marker of the redox balance. The results showed an important *in vivo* decrease in the BDH activity (table II). Similar results were obtained in rat and protozoa (*Tetrahymena pyriformis*) (Cherkaoui Malki et al., 1991; El Kebbaj et al., 1995) by using 2,4D and other hypolipemic agents (clofibrate, ciprofibrate and clobuzarit). This decrease is not caused by a modification in BDH expression (figures 6 and 7) but by the inhibitory effect of 2,4D by fixation on BDH active site (figures 5, 6 and table III).

Chemical modification of essential aminoacids residues by specific derivating agents is a well known method to identify the active site aminoacids composition by estimation of the rate of enzyme inactivation and to demonstrate the enzyme conformational change. In our study we used the NEM (specific to cysteine) and the PGO (specific to the arginine) since studies already published by our laboratory showed that the BDH active site contains one essential cysteine and one essential arginine for BDH activity (El Kebbaj et al., 1980, Latruffe et al., 1980). The aim was to know the aminoacid implicated in the fixation of 2,4D at the BDH active site. The results obtained reveal that this fixation is done on the arginyl residues (figure 7). Such effect can lead changes in the NAD⁺/NADH equilibrium through the ketone bodies interconversion and consequently affect the physiological functions of cells.

5. Conclusion:

The present study showed the toxicity of 2,4D which induces karyolysis, hypertrophy, necrosis and oxidative stress. Moreover, the D-3-hydroxybutyrate dehydrogenase (mitochondrial marker) is inhibited after *in vivo* and *in vitro* 2,4D treatment. This inhibition was done at the BDH active site especially at its arginyl residues. Since this enzyme is involved in the ketone bodies interconversion and these compounds play a role in the energy metabolism of extrahepatic tissues, 2,4D can cause perturbation on energetic metabolism. So, the use of herbicides in agricultural fields induces health

problems due to environmental persistence and an increase in concentration during the passage through the food chain. Food is the major route for exposure of human population to the toxic chemicals. The assessment of possible risk to species in the natural environments and the determination of water quality criteria are urgently needed.

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Tables & Figures

Table 1
Plasmatic parameters from control and 2,4D treated jerboa

	Control	2,4D
Glycemia (g/l)	1.25 ± 0.12	1.17 ± 0.09
Total cholesterol (mg/l)	1.11 ± 0.22	1.20 ± 0.25
Triglycerides (mg/l)	1.44 ± 0.46	0.265* ± 0.049 (-80%)
HDL cholesterol (mg/l)	0.745 ± 0.063	0.52* ± 0.056 (-30%)
LDL cholesterol (mg/l)	0.185 ± 0.077	0.525* ± 0.049 (×2.83)
GOT (U/l)	159 ± 5.65	403.5* ± 91.2 (×2.53)
GPT (U/l)	6 ± 2.24	5.5 ± 2.21
CPK (U/l)	1143 ± 280	840.5 ± 410.8
Urea (g/l)	0.38 ± 0.08	0.34 ± 0.11
Creatinine (mg/l)	6.5 ± 0.07	7.1 ± 0.05

Values are given as means of four separated animals ± standards deviations. * $p < 0.05$; ** $p < 0.01$ (Student's *t*-test).

Table 2
Effect *in vivo* of 2,4D on response of oxidative stress and metabolic biomarkers

	Control	2,4D
Superoxide dismutase (μmol/min/mg of protein)	5.54 ± 1.04	3.79 ± 1.47
Gluthation reductase (nmol/min/mg of protein)	6.95 ± 0.47	5.35 ± 1.03
Thiobarbutic acid reactive substances (nmol/mg of protein)	0.29 ± 0.02	0.36 ± 0.02* (+24.1%)
Catalase (μmol/min/mg of protein)	0.162 ± 0.04	0.058 ± 0.016* (-77.7%)
Palmitoyl-CoA oxidase (nmol/min/mg of protein)	8.4 ± 2.4	14 ± 4.6
β-Hydroxybutyrate dehydrogenase (nmol/min/mg of protein)	0.21 ± 0.012	0.075 ± 0.03** (-64.3%)
Succinate dehydrogenase (absorbance/min/mg of protein)	3.08 ± 0.43	2.70 ± 0.73
NADPH-cytochrome C reductase (nmol/min/mg of protein)	104 ± 20	56 ± 9.2* (-46.1%)
Acetylcholinesterase (nmol/min/mg of protein)	81.32 ± 19.27	29.91 ± 6.3* (-63.2%)
Glycerol-3-phosphate dehydrogenase (nmol/min/mg of protein)	1.13 ± 0.09	1.24 ± 0.05
Glyceraldehyde-3-phosphate dehydrogenase (μmol/min/mg of protein)	0.53 ± 0.12	0.6 ± 0.09

Values are given as means of four separated animals ± standards deviations. For experimental conditions, see Section 2. * $p < 0.05$; ** $p < 0.01$ (Student's *t*-test).

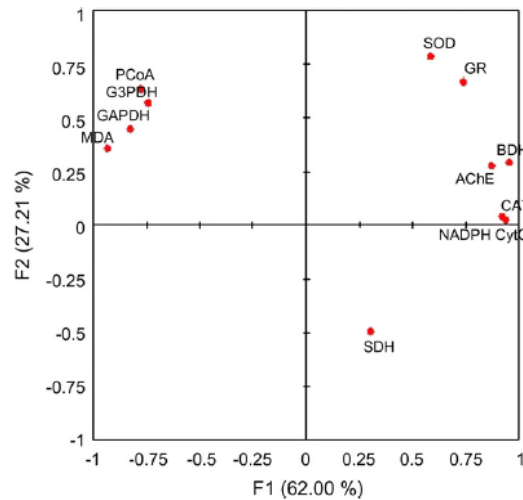


Fig. 1. Principal component analysis including all studied parameters.

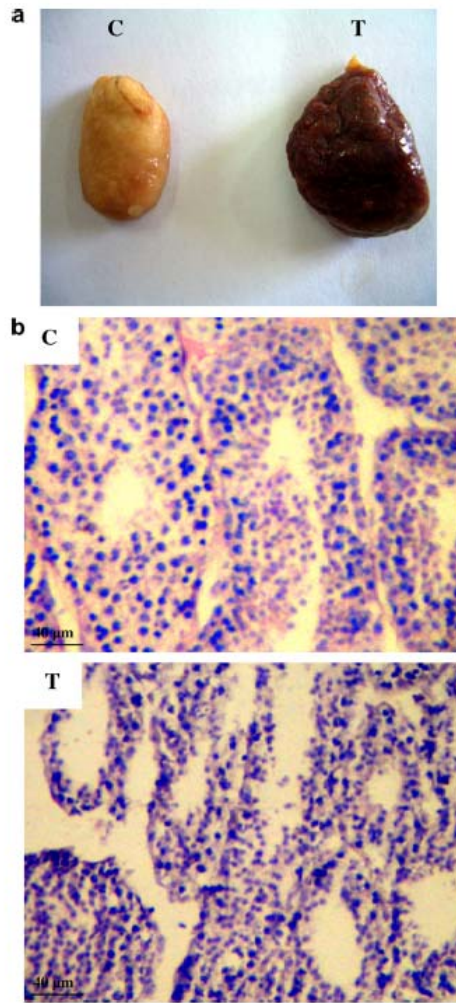


Fig. 2. View of testis organs (a) (magnification: 1×) and its histological sections (b) from control (c) and 2,4D treated (T) typical jерboa. Sections were stained with hematein-eosin (magnification: 400×).

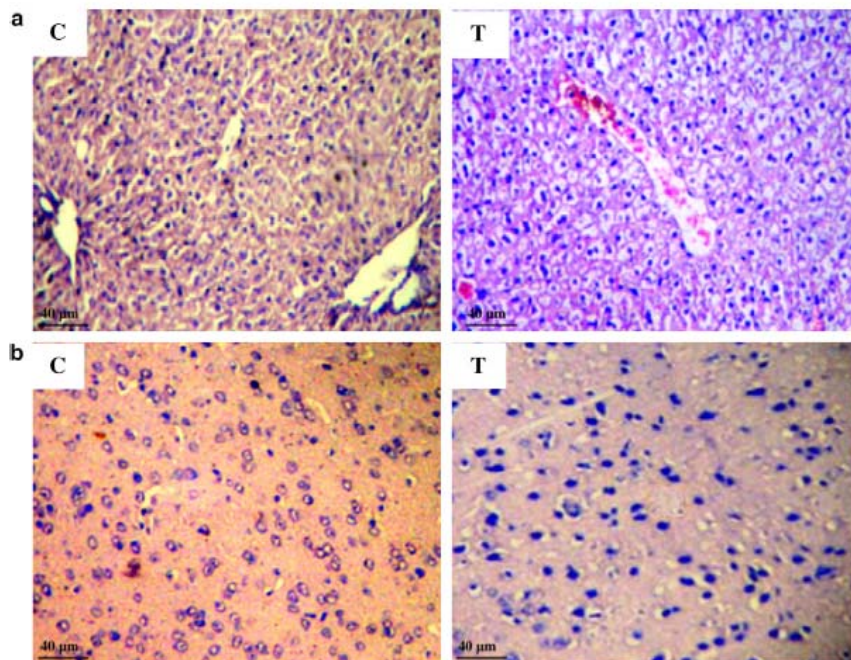


Fig. 3. Histological sections of liver (a) and brain (b) from control (c) and 2,4D treated (T) typical jерboa. Sections were stained with hematein-eosin (magnification: 400×).

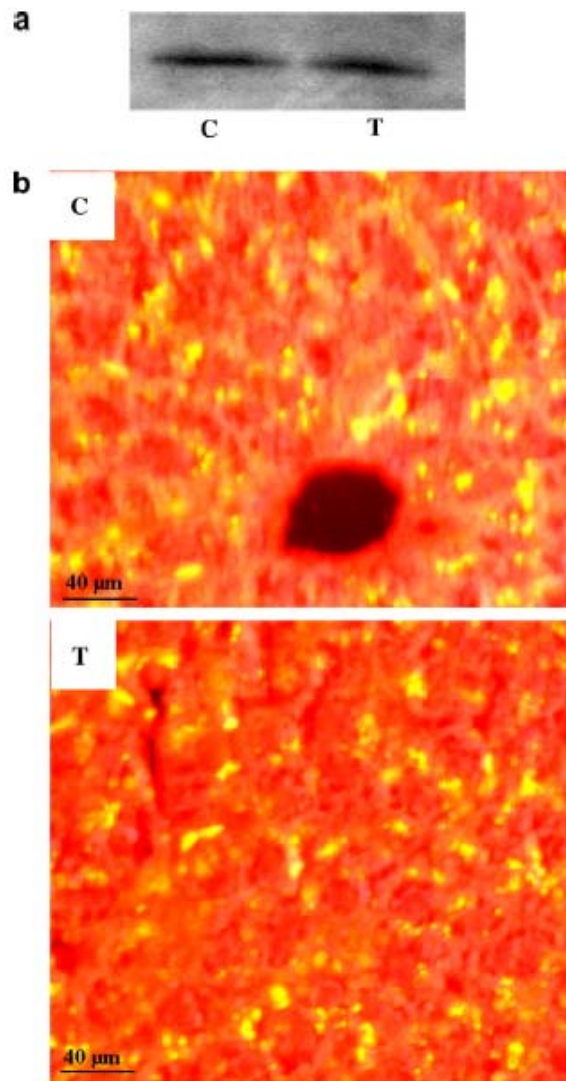


Fig. 4. (a) Western blot of the BDH from control and 2,4D treated jerboa liver. SDS-PAGE was assayed with 50 μ g of proteins from frozen mitochondria. (c and T) Mitochondria extracts from control and 2,4D treated individual jerboas, respectively. (b) Immunofluorescence labeling and subsequent microscopy of BDH of control (a) and treated (b) jerboa liver. Evans blue was used as against dye, and appears in red. BDH labeling appears in yellow. For experimental conditions, see Section 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

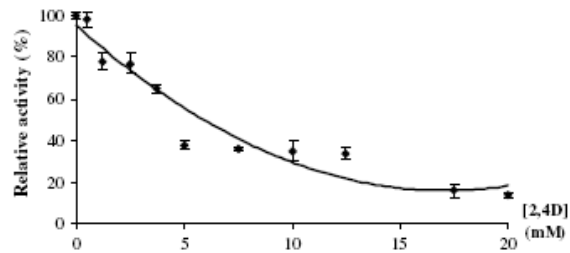


Fig. 5. *In vitro* effect of 2,4D concentration on BDH activity. Values are given as means of four control jerboas. For experimental conditions, see Section 2.

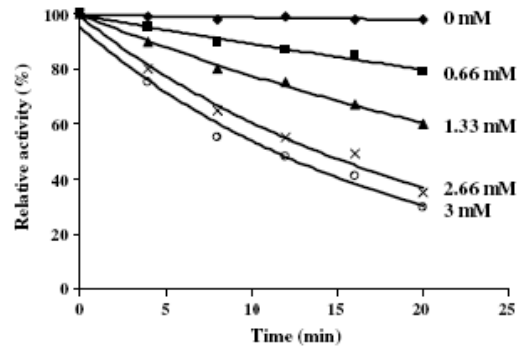


Fig. 6. Inactivation time course of BDH by various concentrations of 2,4D (semi log plot). Values are given as means of four control jerboas. For experimental conditions, see Section 2.

Table 3

Determination of the apparent kinetic parameters of the liver BDH (frozen mitochondrial fractions from control jerboa) in presence and absence of 2,4D

	Control	2,4D
$K_M \text{ NAD}^+$ (mM)	0.21 ± 0.01	$2.44 \pm 0.76^* (\times 11.61)$
$K_i \text{ 2,4D vs NAD}^+$ (mM)	None	3.38 ± 0.6
$K_M \text{ BOH}$ (mM)	1.6 ± 0.22	$3.94 \pm 0.6^* (\times 2.46)$
$K_i \text{ 2,4D vs BOH}$ (mM)	None	5.74 ± 0.7
V_{\max} (nmol/min/mg of protein)	0.44 ± 0.04	0.32 ± 0.02

Experiments were varying NAD^+ concentration (0.5; 1; 1.5 and 2 mM) or BOH concentration (2.5; 5; 7.5 and 10 mM) and 2,4D concentration (0; 1.66; 3.33 and 5 mM). Values are given as means \pm standard deviations from four different animals. $^*p < 0.01$ (Student's *t*-test).

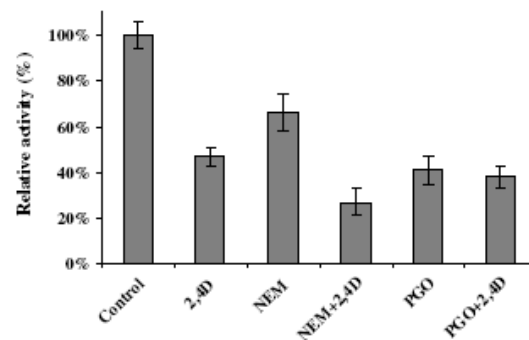


Fig. 7. Protection of BDH against 2,4D. Values are given as means of four control jerboas. For experimental conditions, see Section 2.