Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

Methyl thiophanate-induced toxicity in liver and kidney of adult rats: a biochemical, molecular and histopathological approach

A. Feki[1,2,3], H. Ben Saad[2#], I. Jaballi[1,2#], C. Magne[3], O. Boudawara[4], K. M. Zeghal[2], A. Hakim[2], Y. Ben Ali[5], I. Ben Amara[1*]

[1] Higher Institute of Biotechnology of Sfax, 3000 Sfax, Sfax University, Tunisia
[2] Laboratory of Pharmacology, UR/12 ES-13, Faculty of Medicine, 3029 Sfax, University of Sfax , Tunisia.
[3] EA 2219 Géoarchitecture, University of Western Brittany, UFR Sciences & Techniques, 6 Avenue V. Le Gorgeu, CS 93 837, 29238 Brest Cedex 3, France.
[4] Anatomopathology Laboratory, CHU Habib Bourguiba 3029. Sfax University. Tunisia.
[5] Laboratory of Biochemistry and Enzymatic Engineering of Lipases. BP3038-1173, Sfax University, Tunisia

Correspondence to: ibtissembenamara2010@gmail.com

Received October 30, 2015; **Accepted** February 20, 2017; **Published** February 28, 2017

[#] These authors contributed equally to this work

Doi: http://dx.doi.org/10.14715/cmb/2017.63.2.4

Abstract: The aim of this study was to elucidate the redox effects of Thiophanate methyl (MT) in the rat liver and kidney. Our results showed, after 3 days of MT injection (700 mg/kg), an increase in malondialdehyde (MDA), hydrogen peroxide and advanced oxidation protein products levels. Glutathione peroxidase and superoxide dismutase activities were also remarkably increased in the liver but decrease in the kidney. Glutathione and vitamin C values were significantly reduced. The changes in biochemical parameters were substantiated by histological and molecular data. A smear without ladder formation on agarose gel was shown, indicating random DNA degradation in the liver and the kidney of MT treated rats. The increase in cyclooxygenase-2 gene expression, marker of inflammation, and an increase in genes expression of glutathione peroxidase and superoxide dismutase in liver and their decrease in the kidney were also occurred after MT exposure. These data confirmed the pro-oxidant and genotoxic effects of this fungicide.

Key words: Thiophanate-methyl; Genotoxicity; Rat; Oxidative stress.

Introduction

Environmental pollution plays a crucial role in the occurrence of diseases affecting plants, animals and human beings. The main factor causing environment pollution is the irrational use of pesticides (1, 2). Large-scale and indiscriminate application of these agrochemicals pose human health risks, on especially in developing countries, where the pesticide users are often ill-trained and devoid of appropriate protective devices. The associated health hazards are further extended to those exposed occupationally or inadvertently.

Various pesticides stresses, among Methyl thiophanate (MT) which, may cause oxidative stress. The latter is a broad spectrum fungicide widely used for control of some important fungal diseases of crops (3, 4). This compound is a systemic fungicide affecting the cell division mechanism by inhibiting fungal DNA synthesis (5). MT is a benzimidazole class of fungicide, known as ‘‘likely to be carcinogenic to humans’’ by Environmental Protection Agency carcinogen risk assessment guidelines (6). Being a category-III acute inhalation toxicant, it has been reported to exhibit a dose-related increase in the incidence of follicular and hepatocellular adenomas in male and female F344 rats. It has also been shown to cause skin papilloma at 75 ppm, pituitary adenoma at 200 ppm in male rats and mammary gland fibro-adenoma in female rats at 1200 ppm (7). Besides to the changes shown in cells, MT has been reported to inhibit some metabolizing enzymes, including cytochrome-P450 modulation (8) and affect biological cell activities such as ATP synthesis, signaling, regulation of biosynthetic and catabolic reactions, transport of metabolites and ions (9), due to alteration in the structural and functional integrity of proteins associated with plasma membranes and intracellular organelles membranes. Therefore, it is speculated that the actual risk of genotoxicity from this fungicide might be appreciably higher than that predicted from conventional toxicity tests, as reported by Bolognesi (10) for other pesticides. Furthermore, it was demonstrated that the highest residual levels of MT was occurred in the liver, thyroid, and kidneys (11) and its elimination was rapid, with more than 90% in the urine and feces within 24 h of administration. Moreover, there were several possible routes of metabolism and one of which was via carbendazim. The major urinary metabolite was 5-hydroxycarbendazim sulphate. Since the liver and kidney are metabolically active and the main sites of xenobiotic detoxification, including MT compounds, they are considered, consequently, to be powerful reactive oxygen species (ROS) generators (12).

Up to now, the mechanism of the oxidative stress and DNA damage in detoxifying organ, caused by MT, remains understood. The genotoxic effect may be ex-

plained by DNA bases modified by oxidation which disturbs the balance between lesion inductions from free radical processes and repair effects. In fact, the first frontier of cellular defense against DNA damage consists on endogenous non-enzymatic radical scavengers such as glutathione (GSH), vitamins C and E and antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) as well as sophisticated and highly specified DNA repair pathways. This hypothesis needs further necessary studies in animal models to determine mechanisms by which MT induced liver and kidney cyto- and geno-toxicities and this motivated us to analyze plasma biochemical markers, oxidative stress parameters, histopathological examination and genotoxicity as monitored by genes expression and DNA degradation in liver and kidney of adult rats treated by 700 mg/kg bw of MT during 3 days.

Materials and Methods

Animals and experimental design

Wistar male rats weighing 170±10g, obtained from the Central Pharmacy (SIPHAT, Tunisia), were housed in plastic cages in an acclimate-controlled facility with a constant light dark cycle at a temperature of 22°C ± 2 and humidity of 40%.

In our experimental study, we chose the up and down method designed to estimate the LD50 in rats. MT dissolved in corn oil was i.p. injected at several doses 300, 500, 700, 1000, 1200 and 1400 mg/kg bw (Table 1). LD50 value was determined from cumulative mortality observed 48 h after the injection, although the observation period was prolonged until 14 days.

Once LD50 of MT was evaluated (1000 mg/kg bw), rats were divided at random into two groups of 8 animals each: rats of group 1 (control group) received daily oil corn injection as vehicle and served as negative controls; group 2 (treated A) received daily by i.p injection 700 mg/kg bw of MT.

The experimental procedures were carried out according to the Natural Health Institute of Health Guidelines for Animal Care and approved by the Ethical Committee of Sfax Sciences Faculty. All animal procedures were conducted in strict conformity with the "Institute ethical committee guidelines" for the Care and Use of laboratory animals.

Blood and organ preparation

At the end of experiment (3 days), blood samples were collected in heparined tubes. Plasma was separated from blood by centrifugation (4000 x g for 10 min). Liver and kidney were quickly removed, cleaned from their adhering tissues. Some of them were used for biochemical and molecular analysis. 300 mg of each organ were homogenized in an appropriate phosphate buffer saline (100 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) with an Ultra Turrax homogeniser in ice-cold and centrifuged at 10.000 x g for 15 min at 4°C. The resulting supernatants were used for various biochemical assays. The other ones were immediately fixed in 10 % buffered formalin solution for histological studies.

Biochemical assays

Protein quantification

Protein contents of liver and kidney were measured according to the method of Lowry et al. (13) using bovine serum albumin as standard.

MDA measurement

The MDA concentrations, index of lipid peroxidation (LPO), were determined spectrophotometrically according to Draper and Hadley (14). The MDA values were calculated using 1,1,3,3-tetraethoxypropane as standard and expressed as nmoles of MDA/g of liver and kidney.

Measurement of hydrogen peroxide (H_2O_2)

Measurement of H_2O_2 was carried out by the ferrous ion oxidation xylenol orange (FOX_1) method (15). The amount of H_2O_2 in the supernatant was determined using a spectrophotometer at 560 nm. H_2O_2 levels were expressed as µmoles/mg protein.

Determination of Advanced oxidation protein product (AOPP) levels

AOPP levels were determined according to the method of Kayali et al. (16). The concentration of AOPP for each sample was calculated using the extinction coefficient of 261 cm^{-1} mM^{-1} and the results were expressed as µmoles/mg protein.

Antioxidant enzyme activities determination

* *SOD* activity was estimated according to Beauchamp and Fridovich (17). Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed as units/mg of protein.

* GPx activity was measured according to Flohe and Gunzler (18). The enzyme activity was expressed as nmoles of GSH oxidized/min/mg protein.

Liver and kidney GSH levels

Liver and kidney GSH contents were measured at 412 nm using the method of Ellman et al. (19) modified

Table 1. Cumulative mortality and clinical signs observed after different dose of thiophanate methyl (MT) and different times.

Groups	Dose levels	Mortality	Time of death (hours)	Clinical signs
1	300	0/6	-	Mild tremor
2	500	1/6	48h	Mild tremor
3	700	2/6	36h	Hypoactivity, polyuria, tremor
4	800	2/6	24h	Depression, tremor and dyspnea.
5	1000	3/6	24h	Depression, tremor and dyspnea.
6	1200	4/6	5-6	convulsions
7	1400	6/6	2-3	convulsions

by Jollow et al. (20). Total GSH levels in liver and kidney were expressed as µg/g tissue.

Vitamin C determination

Vitamin C determination was performed as described by Jacques-Silva et al. (21). The data were expressed as µmoles ascorbic acid/mg protein.

DNA fragmentation analysis

The extent of DNA fragmentation in liver and kidney tissues was determined by the method described by Kanno et al. (22). The liver and kidney tissues were lysed by adding lysis buffer (1 % IGEPAL CA-630 in 20mM EDTA, 50 mM Tris–HCl, pH 7.5) and 1 % SDS. They were then digested for 3 h with proteinase K (final concentration 100 µg/ml) at 56 °C. DNA was purified with an equal volume of tris-saturated phenol/chloroform/isoamyl alcohol (25:24:1), incubated on ice for 15 min and centrifuged at 10.000 ×g at 4 °C. The clear supernatant containing DNA was transferred to another tube and mixed. Saturated sodium acetate was added with the same volume of ice-cold isopropanol. The precipitated DNA was separated by centrifugation. It was washed twice with ethyl alcohol (70 %) finally dissolved in 100 ml of Tris EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8). The suspended DNA was incubated with RNase A for 60 min at 37 °C in a water bath. DNA samples (10 mg of DNA/lane) were kept at 80 V for 1 h on 0.8 % agarose gel in Tris-acetate-EDTA buffer, containing 0.5mg/ml of ethidium bromide. The gel was observed under an ultraviolet lamp and photographed.

Total RNA extraction from liver and kidney tissues

Several samples of liver and kidney were collected from both treated (700mg/Kg bw) and control rats. Total mRNAs were isolated from 100 mg of each tissue sample (stored at -80°C before RNA extraction) using the single step guanidine isothiocyanate-phenol-chloroform isolation method as described by Chamczynski and Sacchi (23).

RT-PCR and cDNA synthesis

The cDNA was produced from 2 mg of total mRNA by reverse transcription with MMLv reverse transcriptase using oligo (dT) as a primer in a total volume of 20 µL. Oligo (dT) primed first-strand cDNA was prepared from kidney RNA using MMLv reverse transcriptase at 37°C for 60 min. PCR was performed with gene-specific primers using Taq DNA polymerase. SOD, GPx and COX primer pairs were designed to produce overlapping fragments. The following cycling conditions were used: initial denaturation at 95°C for 5 min followed by denaturation at 95°C, annealing from 65°C and extension at 72°C for 1 min. Expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase served as the control. The number of amplification cycles was determined using individual primer sets to maintain exponential product amplification (30–35 cycles). The amplified PCR products were separated by electrophoresis through 1–1.5% agarose gel. Bands of cDNA were stained with ethidium bromide and visualized by ultraviolet illumination. The primers used for each gene were: SOD Forward : 5'---- ATG GCG ATG AAG GCC GTG TGC----3' / Reverse: 5'---- TTA TTG GGC AAT CCC AAT CAC----3' GPx Forward : 5'---- ATG TCT GCT GCT CGG CTC TCC----3' / Reverse: 5'----TTA GGG GTT GCT AGG CTG CTT----3' COX2 Forward : 5'----ATG CTC TTC CGA GCT GTG CTG----3' / Reverse: 5'----TTA CAG CTC AGT TGA ACG CCT ----3' GAPDH Forward: 5′----CCT CTC TCT TGC TCT CAG TAT----3′ / Reverse: 5′----GTA TCC GTT GTG GAT CTG ACA----3′

Plasma biochemical markers

Plasma levels of creatinine, uric acid, urea, bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by colorimetric methods using commercial reagent kits (Ref: 20151, 20091, 20143, 20102, 20042, and 20046) respectively, purchased from Biomaghreb (Ariana. Tunis. Tunisia).

Histological studies

For histological studies, some portions of liver and kidney tissues were fixed for 48 h in 10 % of buffered formalin solution, dehydrated in an ascending graded series of ethanol, cleared in toluene, and embedded in paraffin. Sections of 5–6 µm thickness were made by using a rotary microtome and stained with hematoxylin and eosin (H&E) for microscopic observations (24). Six slides were prepared from each organ. All sections were evaluated for the degree of injury.

Statistical analysis

Values of each parameter are expressed as the mean ± standard deviation (x± SD). Duncan's multiple range tests provided mean comparisons with the level of statistical significance set at $P < 0.05$. Statistical analyses were performed using SPSS for Windows (Version 17.0).

Results

LD50 value in acute toxicity study by intraperitoneal way

We have adopted the up and down method for the evaluation of i.p. LD50 value of MT in male rats, in order to establish adequate doses of MT to investigate their effects on different organs in this specie. In our study, cumulative mortality was observed during 24, 48 h and even 14 days and shown in table 1. The death time oscillated between 2 h with the highest dose and 48 h for 700 mg/kg bw. From the i.p. way was estimated to be value of MT by i.p. way was estimated to be 1000 mg/kg bw. Clinical signs were registered.

General characteristics of control and MT-treated rats

Food and water consumption by MT treated rats were reduced by 119% and 169%, respectively. Treatment with MT affected also organ weights (Table 2). Our results demonstrated an increase in mean liver weight (+53.7%) of the treated group and a decrease in kidney weight (-42.3 %) of treated rats.

Estimation of LPO, H_2O_2 and AOPP levels

Our results revealed an increase of LPO, ROS production and protein oxidation in the liver and kidney of the MT-treated group as evidenced by the enhanced

Table 2. Morphological parameters, food and water intakes by control and thiophanate methy (MT) treated rats[a].

Parameters & treatments	Absolute liver weight (g)	Absolute kidney weight (g)	Food intake (g/day/rat)	Water intake (mL/day/rat)
Controls	6.01± 1.14 [b]	1.30± 0.33 [b]	20.03 ± 0. 07 [b]	28.28± 3.66 [b]
MT (700 mg/kg bw)	9.24±1.23 [a]	0.75 ± 0.06 [a]	9.14 ± 2.32 [a]	10.51 ± 2.58 [a]
t	5.255	7.654	27.18	35.77
p	0.013	0.0013	<0.001	<0.001

[a] Means not sharing the same letters within a raw are significantly different ($p < 0.05$).Values were expressed as means ± S.D for eight animals in each group. Comparisons were made between two groups: treated with MT versus control group.

Table 3. Liver and Kidney Malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and Advanced oxidation protein product (AOPP) levels in controls and treated with thiophanate methyl (MT) during 3 days[a].

Parameters & treatments		Malondialdehyde (nmoles of MDA/g tissue)	H_2O_2 (µmoles/mg protein)	AOPP (µmoles/mg protein)
Liver	**Control**	49.67 ±2.75[b]	0.32 ±0.11[b]	0.40 ±0.12[b]
	MT(700 mg/kg bw)	72.76 ±8.80[a]	0.67 ±0.01[a]	0.66±0.02 [a]
	T	-6.629	-18.18	-35.15
	P	0.011	0.0015	<0.001
Kidney	**Control**	20.15 ±3.14[b]	0.13 ±0.08[b]	0.23 ±0.30[a]
	MT(700 mg/kg bw)	46.57 ±2.63[a]	0.34± 0.04[a]	0.52±0.07[b]
	T	-89.72	-8.59	-14.72
	P	<0.001	0.0066	0.0023

[a] Means not sharing the same letters within a raw are significantly different ($p < 0.05$).Values were expressed as means ± S.D for eight animals in each group. Comparisons were made between two groups: treated with MT versus control group.

MDA (+46%; +126%), H_2O_2 (+109 %; +161%) and AOPP (+ 56%; +126%) levels in treated rats when compared to controls (Table 3).

Enzymatic and non-enzymatic antioxidant status

GPx and SOD activities increased significantly in the liver and decreased in the kidney homogenates of MT group when compared to those of controls (Table 4).

A significant decrease in liver and kidney GSH and vitamin C values was evident in the MT group compared to these of controls (Table 4).

Plasma biomarkers levels

Compared to the controls plasma bilirubin, AST and ALT levels in MT-treated rats were increased ($p<0.001$), indicating hepatotoxicity induced by MT (Table 5).

Our results also showed a constellation of disorders in the renal function of the MT-treated rats. In fact, Creatinine and urea levels were higher ($p=0.025$) than those of controls (Table 5). Uric acid levels were lower ($p=0.005$) in plasma of the MT-treated group than those of controls (Table 5).

Effects on the DNA fragmentation

As shown in Figure 1 smear (hallmark of necrosis) without ladder formation on agarose gel, indicating random DNA degradation, was observed in the liver and kidney of the MT-treated rats (700 mg/Kg bw) when compared with controls.

Table 4. Liver and Kidney superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, glutathione (GSH) and vitamin C levels in control and treated rats with thiophanate methyl (MT) during 3 days[a].

Parameters & treatments		SOD (units/mg protein)	GPx (nmoles of GSH/min/mg protein)	GSH (µg/g tissue)	Vitamin C (µmol/g tissue)
Liver	**Control**	12.94 ±3.54[b]	10.45 ±1.35[b]	55.40 ±3.33[a]	184.97 ±39.04[a]
	MT group	23.41±4.84 [a]	17.06 ±1.94[a]	27.99 4.31[b]	71.68 ±11.11[b]
	T	-25.16	-5.55	48.44	4.97
	P	<0.001	0.015	<0.001	0.02
Kidney	**Control**	24.58 ±3.44[a]	3.32 ±0.50[a]	19.80 ±4.40[a]	137.44 ±15.70[a]
	MT group	13.21±2.43[b]	1.56± 0.24[b]	8.89±2.63[b]	78.31±22.07[b]
	T	-19.49	-11.085	-10.67	-16.024
	P	0.0013	0.004	0.0043	0.0019

[a] Means not sharing the same letters within a raw are significantly different ($p < 0.05$). Values were expressed as means ± S.D for eight animals in each group. Comparisons were made between two groups: treated with MT versus control group.

Table 5. Effects of thiophanate methyl (MT) on plasma biomarkers of liver and kidney function [a].

Parameters & treatments	Aspartate aminotransferase (U/I)	Alanine aminotransferase (U/I)	Bilirubin (mg/L)	Creatinine (mmol/L)	Urea (mmol/L)	Uric acid (mg/L)
Control	66.5±6.64[b]	36.16±9.36[b]	0.20±0.01[b]	20.51±2.16[b]	7.01±0.23[b]	253.17±45.02[b]
MT(700 mg/kg)	112.29±6.39[a]	72.18±9.73[a]	0.47±0.03[a]	30.50±0.93[a]	13.80±1.51[a]	152.92±26.21[a]
T	-317.24	-168.61	-30.99	-14.06	-9.512	9.23
P	<0.001	<0.001	<0.001	0.0025	0.005	0.0057

[a] Means not sharing the same letters within a raw are significantly different ($p < 0.05$).Values were expressed as means ± S.D for eight animals in each group. Comparisons were made between two groups: treated with MT versus control group.

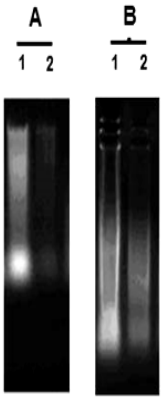

Figure 1. Agarose gel electrophoresis of DNA fragmentation from liver (**A**) and kidney (**B**) of control and MT-treated rats. **A**: Lane 1; control group, lane 2; MT group. **B**: Lane 1; control group, lane 2; MT group.

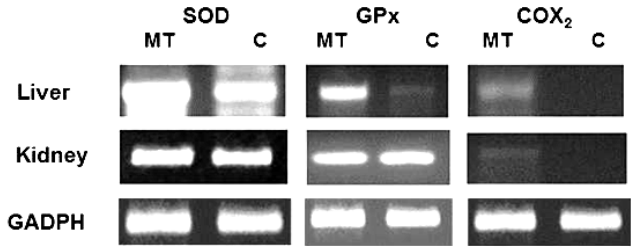

Figure 2. Analysis by RT-PCR of mRNA levels of SOD, GPX and COX_2 in the liver and the kidney. The RT-PCR was performed on mRNA extracted from liver and kidney of rats treated with 700mg/kg of MT and controls **(C).**

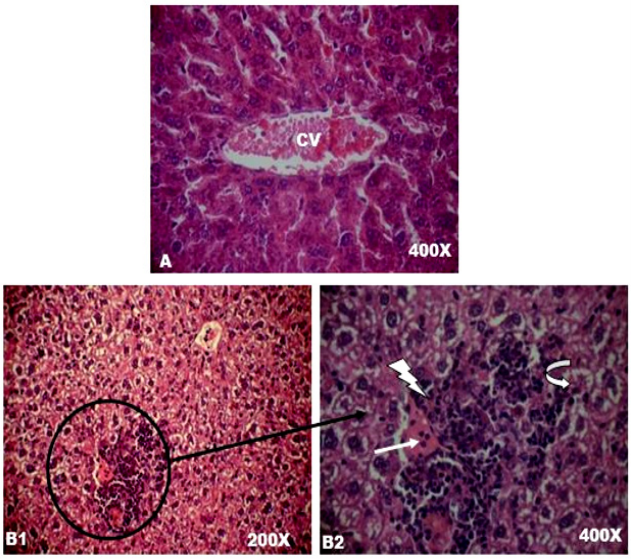

Figure 3. Liver histological sections of adult rats: controls (A) and MT-treated (B1 and B2). Arrows indicate: ↘ infiltration of mononuclear cells, ⊂ necrosis, → hemorrhages **cv:** central venous.

Effects of MT treatment on the SOD, GPx and COX_2 mRNA levels

Antioxidant defences such as SOD and GPx are involved to counteract the toxicity of ROS. Under normal conditions these antioxidants protect the cells and tissues from oxidative damage. Our data revealed that MT provoked a significant increase in SOD and GPx mRNA levels in the liver and no significant changes in kidney of treated rats compared to those of controls (figure 2).

COX_1 is expressed constitutively in most cells, whereas the inducible COX_2 form is usually expressed in response to various inflammatory stimuli. Figure 2 showed results of RT-PCR for COX_2 mRNA expression. Variation of the expression level for this enzyme in liver and kidney associated with MT treatment was examined to establish possible gene co-regulation. COX_2 was slightly expressed in the liver and kidney of MT treated rats as compared to those of controls. No detectable amount of COX_2 mRNA expression was shown in controls.

Histopathological studies

In the MT-treated rats, liver and kidney histological pictures showed numerous abnormalities (Figure 3). Light microscopic examination indicated a liver normal structure in controls (Figure 3A), showing a normal cellular architecture with distinct hepatic cells, sinusoidal spaces, and a central vein. While with MT treatment, severe histopathological changes were observed (Figure $3B_1$ and $3B_2$). MT caused necrosis, hemorrhages, the infiltration of inflammatory leucocyte cells and hepatocyte vacuolization. Kidney exhibited a vascular congestion inside glomeruli and hemorrhages between tubules (Figure 4B). The infiltration of leukocytes cells occurred particularly between tubules in the MT-treated rats (Figure 4B). Furthermore, convoluted tubules were dilated showing vacuoles formation, indicating the beginning of a necrosis step.

Discussion

Little information is available on MT induced liver and kidney oxidative damage. In the present study, we evaluated the pro-oxidant effects of MT on liver and kidney of adult rats administered by i.p way at a dose 700 mg/kg b.w, since the mechanism of its toxicity remains understood. In the current study, the single injection of MT at 700 mg/kg bw in adult rats greatly affected the kidney and liver weights. This could be attributed to the toxicity of the pesticide.

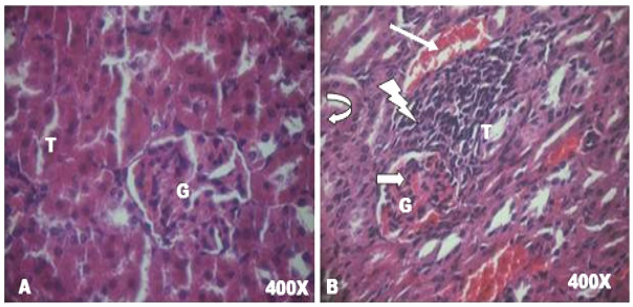

Figure 4. kidney histological sections of adult rats: controls (A) and MT-treated (B). **T:** tubules, **G:** glomeruli. Arrows indicate: ntra-glomeruli hemorrhage, → hemorrhage between tubules infiltration of mononuclear cells, necrosis.

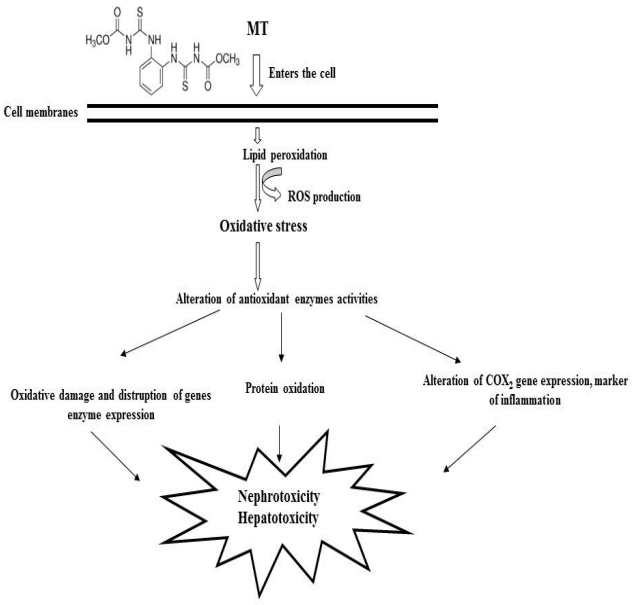

Figure 5. The different level of MT mechanism of action on liver and renal cell metabolism, cellular redox, gene expression and ROS production.

Administration of MT at 700 mg/kg b.w led also to oxidative stress generation in the liver and kidney, with an increase in the levels of MDA levels. The higher level of MDA in the kidney (+56%) might also be related to its more pronounced mitochondria activity compared to the liver (+32%), as explained by Hulbert et al. (25). Furthermore, the high amount of MDA in the kidney might also be due to its involvement in MDA excretion (26). These results indicated MT pro-oxidant effect altering cells membrane integrity and its fluidity.

Generally xenobiotics, such as pesticides, are known to cause oxidative stress through the generation of ROS and can alter the antioxidants or free oxygen radicals scavenging enzyme systems (27). The novelty brought by our study was that MT generated liver and kidney oxygen radical production like H_2O_2, suggesting the dysfunction of the mitochondrial respiratory chain. The latter has been associated with a peroxide production increase in cells causing LPO, DNA damage and protein oxidation (9, 27, 28). Thus, oxidative protein damage may be one of the most putative mechanisms of MT induced toxicity.

Oxidative stress, caused by the imbalance between ROS and biological antioxidant systems, can lead to modifications of macromolecules such as proteins, lipids, and DNA (29, 30, 31). Because the redox status (oxidizing/reducing conditions) of cells is involved in regulating various transcription factors/activators (e.g., activator protein 1 (AP-1), nuclear factor-κB (NF-κB), and p53), thereby influencing cellular target gene expression and modulating cellular signaling pathways, appropriate ROS levels are necessary for normal physiological function of living organisms (32). When, redox-active species are in excess, they may cause DNA damage, repress the activity of cellular enzymes, and induce genotoxicity and cell death (33, 34, 35). In the current study, agarose gel electrophoresis showed undetectable DNA laddering (DNA fragmentation) in the liver and kidney of the control rats. The DNA intact band appeared to be condensed near the application point with no DNA smearing, suggesting no DNA fragmentation, while MT-treatment resulted in massive DNA fragmentations with a subsequent formation of a DNA smear on agarose gel, a hallmark feature of necrosis, confirming MT-induced genotoxicity.

Oxidative stress refers to disrupt redox equilibrium between the free radicals production and the ability of cells to protect against damage caused by these species. Defense against oxidative stress is maintained by using several mechanisms including antioxidant machinery (36). Among the antioxidant enzymes, SOD, CAT, GPx and GST are the first line of defense against oxidative injury. SOD is the primary step of the defense mechanism in the antioxidant system against oxidative stress by catalyzing the dismutation of superoxide radicals (O_2^-) into molecular oxygen (O_2) and H_2O_2 (37). H_2O_2 is neutralized by the combined action of CAT and GPx in all vertebrates (38, 39). These enzymes act in coordination and the cells may be pushed to oxidative stress state if any change occurs in the levels of enzymes. GPx is the general name of an enzyme family with peroxidises activity whose main biological role is to protect the organism from oxidative damage. The biological function of GPx is to reduce lipid hydroperoxides conversion to their corresponding alcohols and free H_2O_2 reaction (40). In the current study, SOD and GPx activities significantly increased in the liver and decreased in the kidney after MT treatment, indicating free radicals production exceeded the capacity of detoxification mechanisms. Interestingly, we have proved, in the present work that MT impaired the transcript coding for the antioxidant enzymes SOD and GPx. Our results suggested that ROS were induced in liver and kidney of MT treated rats and that the antioxidant system was enhanced in order to remove them. So, MT was considered a pro-oxidant and its mechanism was due essentially to the excess production of free radicals.

To substantiate the mechanisms underlying MT-toxicity, the non-enzymatic antioxidant status in liver and kidney was also evaluated. Besides, the last antioxidant system acts as free radical scavenger to complement the activity of the enzymic antioxidant system when oxidative stress is in excess. The depletion of non-enzymatic antioxidants including GSH and vitamin C levels, in the MT injected rats, could be either the result of their increased utilization for conjugation and/or their participation in achieving free radicals products induced by MT toxicity.

Although the detailed mechanism of MT-mediated toxicity is still unknown, our data showed that this fun-

gicide-induced oxidative stress could cause free radical reactions producing deleterious modifications in membranes, proteins, enzymes, gene expression and DNA molecule, which might contribute to oxidative damage. Our results also confirmed the inflammatory effect of MT on liver and kidney tissues. In fact, MT stimulates the transcript coding for COX_2, a marker of inflammation. Presently, there are two cyclooxygenase enzymes which have been identified in humans (with 60% homology): cyclooxygenase-1 (COX_1) and cyclooxygenase-2 (COX_2). Previous research suggests that COX_1 is a housekeeping gene which modulates physiologic responses such as regulation of renal and vascular homeostasis, and gastroprotection. While COX_2 is primarily an immediate early gene whose synthesis can be stimulated rapidly and transiently in response to tumor promoters (41, 42).

The liver plays in the organisms a key role in homeostasis in the organism. Conventional liver test provides information about the hepatocytes integrity, such as plasma transaminases (ALT and AST) considered as the standard biochemistry markers of liver injury. Indeed, the transaminases are well-known enzymes used as biomarkers to predict possible toxicity (43). Generally, damage to liver cells will result in elevations of both these transaminases in the serum (44). Furthermore, measurement of enzymic activities of AST and ALT is of clinical and toxicological importance as changes in their activities are indicative of liver damage by toxicants or in diseased conditions (45). Overproduction of ALT and AST levels, obtained by us, indicated a high risk for cholestatic liver dysfunction and an excess of reactive species production inducing a redox imbalance and hepatotoxicity after short-term MT exposure. Also MT hepatotoxicity was evidenced, in the present study, by an increase of bilirubin levels due to its high production resulting either from hemolysis or the decreased liver uptake, conjugation.

To assess changes in kidney function induced by MT, we choose to monitor uric acid, urea and creatinine. Urea levels were chosen due of its high predictive capability for tissue necrosis, protein catabolism and renal function. The plasma level of urea is directly correlated with protein uptake and inversely correlated with the rate of urea excretion (45). In the present study, the urea level in the plasma was significantly increased after exposure to MT (700 mg/kg b.w.). In the absence of conditions that enhance urea production, such as gastrointestinal bleeding, corticosteroid therapy, and a high-protein diet, elevations in the urea levels may represent a decrease in the glomerular filtration rate (GFR), an increase in the nitrogenous metabolism associated with diminished renal blood flow (46) and results in acute renal failure (47). On the other hand, creatinine is an important analyte with clinical significance that is used for the determination of the renal glomerular filtration rate, as well as the presence of kidney dysfunction and muscle disorder. Creatinine is a by-product of the muscle energy metabolism that is filtered from the blood by the kidneys and excreted into the urine (48). As creatinine is affected very little by liver function, it is thus a sensitive indication of kidney function (49). In this study, the creatinine level in plasma was increased in MT-treated groups compared with the control. The high level of creatinine in the rats treated with MT, as reported in the current study indicates degenerative changes in the kidney function, as described by Salah-Abbès et al. (50). The result clearly showed that MT has a harmful and stressful influence on the kidney tissue. Uric acid is the end product of the purine metabolism in the body and is mainly excreted by the kidney through glomeruli filtration and absorption when renal dysfunction and the blood uric acid are decreased (50). Compared with the control group, the uric acid level in the plasma was significantly decreased after exposure to MT, speaking in favor of a GFR decline.

Our histopathological data substantiated kidney and liver dysfunction in MT-treated rats. There was leucocytic infiltration in hepatic and renal cells considered as a prominent response of the body tissue facing any injurious impacts. Tissue injuries were also characterized by a vascular congestion, indicating the beginning of a necrosis step which was confirmed by DNA fragmentation tests in our experimental study. Histopathological changes could be due to the accumulation of free radicals as the consequence of the increased H_2O_2 products and LPO levels in the kidney and liver tissues of the TM treated rats.

In conclusion, our data indicated, for the first time, 700 mg/kg b.w. of MT revealed genotoxic effects, as indicated by DNA degradation and the impairment of the transcript coding for SOD, GPx and COX_2, affected plasma biomarker parameters and also had a cytotoxic potential effect marked by an increase in H_2O_2, AOPP production, and LPO levels.

Acknowledgments

The present work was supported by DGRST grants (Direction Générale de la Recherche Scientifque et Technique-Tunisie. Appui à la Recherche Universitaire de base UR/12 ES-13).

References

1. Al-Haj M, Nasser A, Anis A. Survey of pesticides used in Qat cultivation in Dhale and Yafe and their adverse effects. J Nat Appl Sci 2005; 9: 103-110.
2. WHO. The WHP Recommended Classification of Pesticides by Hazard and Guidelines to Classification. World Health Organization Programme on Chemical Safety, Geneva, 2009.
3. Hassall. K. A., The Biochemistry and Uses of Pesticides, second ed. Macmillan Press 1990; 49: 323.
4. Saquib Q, Al-Khedhairy AA, Al-Arifi S, Dhawan A, Musarrat J. Assessment of methyl thiophanate–Cu (II) induced DNA damage in human lymphocytes. Toxicol Vitro 2009; 23: 848–854.
5. Seiler JP. Toxicology and genetic effects of benzimidazole compounds. Mut Res 1975; 151-168.
6. Proposed Guidelines for Carcinogen Risk Assessment. Office of Research and Development US Environmental Protection Agency Washington. DC EPA/600/P- 92/003C, Federal Register. Pryor, W.A., 1988. Why is hydroxyl radical the only radical that commonly adds to DNA? Hypothesis: it has rare combination of highelectrophilicity 1996; 61: 17960–18011.
7. Thiophanate-methyl Revised Report of the Hasard Identification Assessment. Revised Toxicology Chapter for Thiophanate-methyl and Carbendazim and retained at 10X in assessing the risk posed by MBC 2000.
8. Palmeira CM, Moreno AJ, Vasco B, Madeira VC. Structural al-

terations in isolated hepatocytes induced by the herbicides paraquat, Dinoseb and 2,4-D. Med Sci Res 1997; 25: 339–342.
9. Li J, Liu X, Ren C, Li J, Sheng F, Hu Z. *In vitro* study on the interaction between thiophanate methyl and human serum albumin. Photochem Photobiol 2009; B 94.
10. Bolognesi C. Genotoxicity of pesticides: a review of human biomonitoring studies. Mut Res 2003; 543: 251-272.
11. Jia L, Wong H, Wang Y, Garza M, Weitman SD, Carbendazim: Disposition, cellular permeability, metabolite identification, and pharmacokinetic comparison with its nanoparticle, J Pharm Sci 2003; 92: 161–172.
12. Ben Amara I, Ben Saad H, Cherif B, Elwej A, Lassoued S, Kallel CH, Zeghal N. Methyl-Thiophanate increases reactive oxygen species production and induces genotoxicity in rat peripheral blood. Toxicol. Mech. Methods 2014; 24: 679-87.
13. Lowry OH, Rosenbrough NJ, Farr AL. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.
14. Draper HH, Hadley M. Malondialdehyde determination as an index of lipid peroxidation. Methods enzymol 1990; 86: 421-431.
15. Ou P, Wolff SP. A discontinuous method for catalase determination at near physiological concentrations of H2O2 and its application to the study of H_2O_2 fluxes within cells. J Biochem Biophys Methods 1996; 31: 59-67.
16. Kayali R, Cakatay U, Akcay T, Altug T. Effect of alpha-lipoic acid supplementation on markers of protein oxidation in post-mitotic tissues of ageing rat. Cell Biochem Funct 2006; 24: 79–85.
17. Beauchamp C, Fridovich I. Superoxyde dimutase: Improved assays and an assay applicable to acrilamide gel. Anal Biochem 1971; 44: 276-287.
18. Flohe L, Gunzler WA. Assays of glutathione peroxidase. Methods enzymol 1984; 105: 114-135.
19. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82: 70-77.
20. Jollow DJ, Mitchell JR. Zampaglione. N., Gillete. J.R., Bromobenzene induced liver necrosis: protective role of glutathione and evidence for 3,4 bromobenzeneoxide as the hepatotoxic intermediate. Pharmacology 1974; 11: 151-169.
21. Jacques-Silva MC, Nogueira CW, Broch LC, Flores EM, Rocha JBT. Diphenyldiselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. J Pharmacol Toxicol 2001; 88: 119-127.
22. Kanno S, Shouji A, Hirata R, Asou K, Ishikawa M. Effects of naringin on cytosine arabinoside (Ara-C)-induced cytotoxicity and apoptosis in P388 cells. Life Sci 2004; 75: 353-418.
23. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156-159.
24. Gabe M. Techniques histologiques. Masson, Paris, 1968 ; 838–879.
25. Hulbert AJ, Turner N, Hinde J, Else P, Guderley H. Howmight you compare mitochondria from different tissues anddifferent species? J Comp Physiol B. 2006; 176: 93-105.
26. Chirino YI, Chaverri JP. Role of oxidative and nitrosative stress in cisplatin-induced nephrotoxicity. Exp Toxicol Pathol 2009; 61: 3223–242.
27. Monteiro DA, Almeida JA, Rantin FT, Kalinin A L. Oxidative stress biomarkers in the freshwater characid fish,Brycon cephalus, exposed to organophosphorus insecticide Folisuper 600 (methyl parathion). Comp Biochem Physiol Part C: Toxicol Pharmacol 2006; 143: 141–149.
28. Nordberg J, Arner ES. Reactive oxygen species, antioxidants and the mammalian thioredoxin system. Free Radic Biol Med 2001; 31: 1287-1312.
29. Shi H, Sui Y, Wang X, Luo Y. Radical production and oxidative damage Induced by cadmium and naphthalene in liver of Carassius auratus. Comp Biochem Physiol 2005; 140: 115-121.
30. Evans MD, Dizdaroglu M, Cooke M.S. Oxidative DNA damage and disease: induction, repair and significance. Mut Res 2004; 567: 1-61.
31. Chen AF, Davies CM, De Lin M, Fermor B. Oxidative DNA damage in osteoarthritic porcine articular cartilage. J Cell Physiol 2008; 217: 828-833.
32. Xie L, Zhu X, Hu Y, Li T, Gao Y, Shi Y, Tang S. Mitochondrial DNA oxidative damage triggering mitochondrial dysfunction and apoptosis in high glucoseinduced HRECs. Inves Ophthal Visual Sci 2008; 49: 4203-4209.
33. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006; 160: 1–40.
34. Cai J, Yang J, Jones DP. Mitochondrial control of apoptosis: the role of cytochrome c. Biochim Biophys Acta 1998; 1366: 139-149.
35. Giannattasio S, Atlante A, Antonacci L, Guaragnella N, Lattanzio P, Passarella S, Marra E. Cytochrome c is released from coupled mitochondria of yeast en route to acetic acid-induced programmed cell death and can work as an electron donor and a ROS scavenger. FEBS Lett 2008; 582: 1519-1525.
36. Archer SL, Gomberg-Maitland M, Maitland ML, Rich S, Garcia JG, Weir EK. Mitochondrial metabolism, redox signaling, and fusion: a mitochondria–ROS–HIF-1alpha–Kv1.5 O2-sensing pathway at the intersection of pulmonary hypertension and cancer. American journal of physiology. Heart Circul Physiol 2008; 294: 570-578.
37. Scherz-Shouval R, Elazar Z. ROS, mitochondria and the regulation of autophagy. T Cell Biol 2007; 17: 422-427.
38. Bergamini CM, Gambetti S, Dondi A, Cervellati C. Oxygen, reactive oxygen species and tissue damage. Curr Pharmacol 2004; 10: 1611-1626.
39. Fattman CL, Schaefer LM, Oury T.D. Extracellular superoxide dismutase in biology and medicine. Free Rad Biol Med 2003; 35: 236-256.
40. Salvi M, Battaglia V, Brunati AM, La Rocca N, Tibaldi E, Pietrangeli P, Marcocci L, Mondovi B, Rossi CA, Toninello A. Catalase takes part in rat liver mitochondria oxidative stress defense. J Biol Chem 2007; 282: 24407-24415.
41. Aly N, EL-Gendy K, Mahmoud F, El-Sebae AK. Protective effect of vitamin C against chlorpyrifos oxidative stress in male mice. Pest Biochem Physiol 2010; 97: 7-12
42. Ran Q, Liang H, Ikeno Y, Qi W, Prolla TA, Roberts LJ, Wolf N, Van Remmen H, Richardson A. Reduction in glutathione peroxidase four increases life span through increased sensitivity to apoptosis, J. Gerontol. Series A: Biol Sci Med Sci 2007; 62: 932-942.
43. Mukinda JT, Eagles FK. Acute and sub-chronic oral toxicity profiles of the aqueous extract of *Polygala fruticosa* in female mice and rats. J Ethnopharmacol 2010; 128: 236–240.
44. Watkins PB. Idiosyncratic Liver Injury: Challenges and Approaches. Toxicol Pathol 2005. 33:1–5.
45. Rahman MF, Siddiqui MK, Jamil K. Effects of vepacide (Azadirachta indica) on aspartate and alanine aminotransferase profiles in sub-chronic study with rats. J Hum Exp Toxicol 2001; 20: 243-249.
46. Mizutani A, Okajima K, Uchiba M, Noguchi T. Activated protein C reduces ischemia/reperfusion-induced renal injury in rats by inhibiting leukocyte activation. Blood 2000; 95:12.
47. Singh NS, Vats P, Suri S, Shyam R, Kumria MML, Ranganathan S, Sridharan K. Effect of an antidiabetic extract of Catharanthus roseus on enzymic activities in streptozotocin induced diabetic rats. J Ethnopharmacol 2001; 76: 269-277.
48. El-Sawi MNM, El-Wassimy MM, Youssef OA. Effect ofVerrucarin J toxin on liver and kidney tissue of male mice. Tex J Sci 2001; 53: 375–384.

49. Aronson D, Hammerman H, Beyar R, Yalonetsky S, Kapeliovich M, Markiewicz W, Goldberg A. Serumblood urea nitrogen and long-term mortality in acuteST-elevation myocardial infarction. Inter J Cardiol 2008; 127: 380–385.
50. Pandey PC, Mishra AP. Novel potentiometric sensing ofcreatinine. Sensors and Actuators B: Chemical 2004; 99: 230–235.
51. Salah-Abbès JB, Abbès S, Houas Z, Ouanes Z, Abdel-Wahhab MA, Bacha H, Oueslati R. Tunisian radish extract (*Raphanus sativus*) enhances the antioxidant status andprotects against oxidative stress induced by zearalenone inBalb/c mice. J Appl Toxicol 2008; 28: 6–14.