Involvement of oxidative stress in the mechanism of p,p'-DDT-induced nephrotoxicity in adult rats

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Abstract. The 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (p,p'-DDT) is an organochlorine pesticide that persists in the environment and has a risk to human health. We investigated whether p,p'-DDT-induces nephrotoxicity in rats and whether oxidative stress and apoptosis are involved in the pathogenesis of this process. Male rats received the pesticide at doses of 50 and 100 mg/kg for 10 days. Renal damage was evaluated by histopathological examination and serum markers. The oxidative stress was evaluated by lipid peroxidation (LPO), metallothioneins (MTs) and protein carbonyl levels. Antioxidant enzymes were assessed by determination of superoxide dismutase (SOD) and catalase (CAT) activities. Glutathione-dependent enzymes and reducing power in kidney were evaluated by glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST) activities. Renal tubular cells apoptosis was assessed through the TUNEL assay. After 10 days of treatment, an increase of serum creatinine and urea levels occurred, LPO and protein carbonyl levels were increased, while MTs level, SOD and CAT activities were decreased. Besides, the GPx, GR, GST, and GSH activities were decreased. Histological alterations in kidney tissue and intense apoptosis in renal tubular cells were observed. These results suggest that DDT sub-acute treatment causes oxidative stress and apoptosis, which may be the chief mechanisms of DDT-induced nephrotoxicity.

Key words: Apoptosis — Histopathology — Kidney — Oxidative stress — p,p'-DDT — Rat

Abbreviations: CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; LPO, lipid peroxidation; MDA, malondialdehyde; MTs, metallothioneins; PBS, phosphate-buffered saline; (p,p'-DDT), 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane; ROS, reactive oxygen species; SOD, superoxide dismutase.

Introduction

Organochlorine pesticides are organic compounds that persist in the environment, bioaccumulate through the food chain and have a risk of causing adverse effects to human health and environment. These pesticides are characterized

by their cyclic structure, number of chlorine atoms and low volatility (Ledirac et al. 2005). The 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (p,p'-DDT), was the most used organochlorine pesticide in the world. It was synthesized in 1874 and was discovered to be an effective insecticide in 1939. It was first used mainly to protect military areas and personnel from vector-borne diseases such as malaria, but later began to be used widely as an agricultural insecticide in the world (Spencer and Schaumburg 2000). The use of p,p'-DDT in the most western countries was banned in 1970s due to its adverse effects on wildlife. However, p,p'-DDT and

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its main metabolite 1,1-dichloro-2,2-bis(4-chlorophenyl) ethane (p,p'-DDE) are still persistent in the environment, both because of their long half life and due to atmospheric drift from countries that continue to use DDT as an insecticide. Approximately 80% of the DDT analogs found in human fat and 40% of DDT analogs found environmentally are DDE (WHO, 1979). Also, numerous analytical studies showed higher levels of DDT and DDE than the allowable daily intake in food (Muralidharan et al. 2009), adipose tissues (Aulakh et al. 2007) and maternal milk (Malarvannan et al. 2009) all over the world. Evidence accumulated over the years has suggested that chronic exposure to DDT and its derivatives is associated with loss weight, anorexia, sterility, endocrine disruptions, muscular weakness, tremors, hepatic effects, and anemia in humans (Hillman 1998; Spencer and Schaumburg 2000). On the other hand, it has been reported that oxidative stress can be used as a biomarker to evaluate damages and a possible mechanism of DDT and DDE toxicity in humans (Frigo et al. 2005; Jin et al. 2014). Furthermore, oxidative stress is one of the best known causes of cellular damage, mostly due to the formation of free radicals that damage cell DNA (Wu and Bratton 2013). Previous studies have indicated that the nephrotoxicity and hepatotoxicity induced by organochlorine pesticides may be associated with the enhanced production of reactive oxygen species (ROS) (Shah and Iqbal 2010; Vijaya Padma et al. 2011). The effects of p,p'-DDT have already been investigated in several studies (Shi et al. 2010; Tebourbi et al. 2010; Jin et al. 2014), but results concerning its eventual nephrotoxic effects are very limited. Therefore, the aim of the present study was to investigate the effect of p,p'-DDT subacute treatment on rat kidney function and the implication of oxidative stress and apoptosis in this organ. To this end, renal biomarkers such creatinine and urea levels were assessed. The status of the oxidative stress was evaluated by biomarkers such lipid peroxidation (LPO), metallothioneins (MTs) and protein carbonyl levels. Antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) activities were measured. In addition, glutathione-dependent enzymes and reducing power in kidney were evaluated. The histological analysis and the detection of apoptotic cells by TUNEL assay in kidney were aimed to be examined.

Materials and Methods

Animals and reagents

Male Wistar rats (50 days of age) were purchased from the Tunisian Company of Pharmaceutical Industries (SIPHAT, Rades, Tunis, Tunisia). The rats were housed under controlled conditions of temperature (25°C) with a constant day/night cycle (light from 8:00 to 20:00). Food and water were provided ad libitum. DDT (98%) were purchased from Sigma Chemical (St. Louis, MO, USA). Rats were randomized into four experimental groups of approximately similar weight (n = 8) as follows: Group 1 (Control), rats received equal daily volumes of vehicle (corn oil) during 10 days; Group 2 (DDT 50), animals received daily an intraperitoneal (i.p.) injection of DDT diluted with corn oil at a dose of 50 mg/kg body weight (b.w.) during 10 days; Group 3 (DDT 100), animals were administered 10 daily injections of 100 mg DDT/kg b.w.; Group 4 (DDT+VitC), animals were i.p. injected by 100 mg of DDT/kg b.w. followed by vitamin C (200 mg/kg b.w.) during the treatment period (Aksoy et al. 2005). The choice of the dosing period and DDT doses was based on the results of previous studies (Ben Rhouma et al. 2001; Harada et al. 2003; Tebourbi et al. 2010). L-ascorbic acid (vitamin C) used in this study were purchased from Sigma chemicals (Aldrich Chemical Company). Rats were fed and observed daily. The body weight of rats was determined daily through the experiment. After 10 days of treatment, all animals were killed by decapitation, the left kidney were dissected and weighed. Animals were cared for in compliance with the code of practice for the Care and Use of Animals for Scientific Purposes. Approval for these experiments was obtained from the Medical Ethical Committee for the Care and Use of Laboratory Animals of Pasteur Institute of Tunis (approval number: LNFP/Pro 152012). The experimental protocols were approved by the Faculty Ethics Committee (Faculté des Sciences de Bizerte, Tunisia).

Tissue preparation

Fractions of kidney (400 mg) from control and treated groups were homogenized in phosphate-buffered saline (PBS, pH 7.2). The homogenates were centrifuged at $600 \times g$ for 10 min and recentrifuged at $13,000 \times g$ for 20 min at +4°C to obtain a postnuclear homogenate and postmitochondrial supernatant fraction (Beytut and Aksakal 2003).

Renal biomarkers assay

Serum was collected by centrifugation ($4000 \times g$ for 15 min). The levels of creatinine and urea in serum were assayed spectrophotometrically according to the standard procedures using commercially available diagnostic kits (Chronolab, France).

Malondialdehyde assay

Lipid peroxidation was measured in the kidney using thiobarbituric acid reacting substance (TBARS) following the method of Buege and Aust (1976) and was expressed in terms of malondialdehyde (MDA) contents.

Measurement of metallothioneins

Determination of MTs was performed according to the technique described by Eaton and Cherian (1991).

Protein carbonyl assay

Protein carbonyl content was assayed by the procedure of Levine et al. (1990) as modified by Parvez and Raisuddin (2005). Soluble protein (0.5 ml) was reacted with 10 mM DNPH in 2 M hydrochloric acid for 1 h at room temperature and precipitated with 6% trichloroacetic acid (TCA). The pelleted protein was washed by resuspension in ethanol/ethyl acetate (1:1). Proteins were then solubilized in 6 M guanidine hydrochloride, 50% formic acid and centrifuged at 16,000 × g for 5 min to remove any trace of insoluble material. The carbonyl content was measured spectrophotometrically at 366 nm. Assay was performed in triplicate and a tissue blank incubated with 2 M HCl without DNPH was included for each sample. The results were expressed as nanomoles of protein carbonyls/mg protein based on the molar extinction coefficient of 21 $M^{-1}·cm^{-1}$.

Determination of antioxidant enzymes activities

Superoxide dismutase activity

The method described by Marklund and Marklund (1974) was used for assay of superoxide dismutase (SOD) activity. Briefly, the assay mixture contained 2.4 ml of 50 mM tris-HCl buffer containing 1 mM EDTA (pH 7.6), 300 μl of 0.2 mM pyrogallol and 300 μl enzyme source. The increase in absorbance was measured immediately at 420 nm against blank containing all the components except the enzyme and pyrogallol at 10 s intervals for 3 min on a Systronics Spectrophotometer. The enzyme activity was expressed as units per mg of protein.

Catalase activity

The catalase activity was measured according to the method of Aebi (1984). Activity was assayed by determining the rate of degradation of H_2O_2 at 240 nm in 10 mM of potassium phosphate buffer (pH 7.0). An extinction coefficient of 0.036 mM/cm was used for calculations. The enzyme activity was expressed as nmol of H_2O_2 consumed per minute per mg of protein.

Glutathione-dependent enzymes and reducing power

Glutathione peroxidase activity

GPx generation was assayed by the method of Paglia and Valentine (1967). The assay mixture contained 1.59 ml of phosphate buffer (100 mM, pH 7.6), 100 μl of EDTA (10 mM), 100 μl of sodium azide, 50 μl of glutathione reductase, 100 μl of reduced glutathione, 100 μl of NADPH (200 mM), 10 μl of H_2O_2 and 10 μl enzyme source. Disappearance of NADPH was measured immediately at 340 nm against blank containing all the components except the enzyme at 10 s intervals for 3 min on a Systronics Spectrophotometer. One unit was defined as 1 nM of NADPH oxidized per minute and the specific activity was reported as units per mg of protein.

Glutathione reductase activity

GR activity was determined as described by Calberg and Mannervik (1985). In this assay, glutathione oxidized is reduced by GR at the expense of NADPH consumption, which is followed at 340 nm. GR activity is proportional to NADPH decay. GR activity was expressed as units per mg of protein.

Glutathione S-transferase activity

GST activity was measured using the method of Habig et al. (1974). Briefly, 1 mM of 1-chloro-2,4-dinitrobenzene (CDNB) was added to buffer containing 1 mM GSH and an aliquot of sample to be tested. Upon addition of CDNB, the change in absorbance at 340 nm was measured as a function of time. The extinction coefficient for this reaction is 9.6 $mM^{-1}·cm^{-1}$. GST activity was expressed as μmol CDNB conjugates/min/mg protein and was reported as units per mg of protein.

Determination of reduced and oxidized glutathione

The levels of reduced and oxidized glutathione (GSH and GSSG) were estimated as described by Hissin and Hilf (1976). Briefly, GSH in the acid soluble supernatant fraction of kidney cells was reacted with o-phthaldialdehyde at pH 8.0 to yield a highly fluorescent cyclic product, while GSSG was determined by the same reagent but at pH 12 and in the presence of N-ethylmaleimide. GSH and GSSG contents were expressed as nmol per mg protein, which allowed the calculation of the glutathione redox ratio (GSSG/GSH).

Histological analysis

Thin slices of kidney tissue with cortex and medulla were fixed overnight at room temperature by direct immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The samples were dehydrated with ethanol and toluene series and embedded in paraffin. Serial sections (4 μm) were mounted on gelatin-coated glass slides cut and stained with haematoxylin and eosin.

Detection of apoptotic cells by TUNEL assay

The kidney tissues were fixed overnight at room temperature by direct immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The samples were dehydrated with ethanol and toluene and embedded in paraffin wax. Serial sections (4 μm thick) were mounted on gelatin-coated glass slides and stained with TUNEL (TdT-mediated dUTP-digoxigenin Nick and Labeling). After deparaffinization and rehydration, tissues sections were incubated with 0.1% (v/ v) Triton X-100 for 2 min on ice, followed by washing of the slides twice in PBS ($CaCl_2 \times 2H_2O$ 0.8 mM, KCl 2.6 mM, KH_2PO_4 1.4 mM, $MgCl_2 \times 6H_2O$ 0.4 mM, NaCl 136 mM, Na_2HPO_4 8 mM, pH 7.2). The specimens were then incubated one hour at 37°C in a solution consisting of 1 mM cobalt chloride, 140 mM sodium cacodylate and terminal deoxyribonucleotidyl transferase (TdT) at a final concentration of 0.1 U/μl to insert biotin-16-dUTP at the 3'-ends of DNA fragments. A streptavidin-peroxidase complex and 3-amino-9-ethylcarbazole served as the detection system for biotin. The slides were washed in PBS, developed with 0.05% diaminobenzidine, and stained for 15 min at room temperature. Sections were lightly counter-stained with hematoxylin and mounted in glycerin jelly. Negative control included omission of TdT from the labelling mixture. The positively labeled cells appear as darkly brown stained. Twenty fields in five sections *per* kidney were sampled randomly in the cortex and outer stripe of the outer medulla. The apoptotic index was calculated, using Image-Pro Plus version 4.5 software (Media Cybernetics Inc., Silver Spring, MD, USA) at ×400 magnification, on the basis of previous report (Luo et al. 2008):

Apoptotic index (%) = (number of positive cells/total number of cells) × 100.

Statistical analysis

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

Results

Renal biomarkers

Renal biomarkers levels of control and treated rats are given in Fig. 1. Exposure of rats to DDT for 10 consecutive days induced a significant increase in serum level of creatinine in treated rats compared to control groups (Fig. 1A). This increase was about 46% and 143% of controls for DDT 50 and DDT 100, respectively. An increase of serum urea level was also observed in treated rats. This increase was of 37.8% and 114%, respectively with DDT 50 and DDT 100 (Fig. 1B).

MDA, protein carbonyl and MTs

DDT treatment significantly raised MDA levels in a dose-dependent manner from 2.16 ± 0.13 nmol/mg protein to 2.29 ± 0.17 nmol/mg prot. and 4.54 ± 0.48 nmol/mg prot., respectively, for DDT 50 and DDT 100 (Fig. 2). Our result indicated that co-administration of vitamin C with DDT modulated significantly the level of MDA concentration to normal control (Fig. 2). The content of protein carbonyl in kidney increased from 1.68 ± 0.22 to 2.93 ± 3.17 and 4.33 ± 0.42 nmol/mg prot., respectively, for DDT 50 and DDT 100 groups (Fig. 3). In contrast, the level of MTs in kidney was significantly decreased in treated rats with the high dose (100 mg of DDT/kg). This decrease was about 51.7% compared with the control group (Fig. 4).
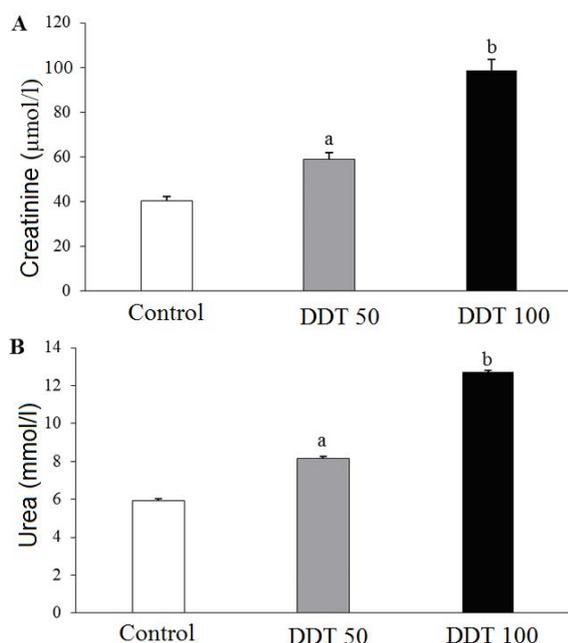

Figure 1. Effect of DDT treatment on serum creatinine (**A**) and urea (**B**) levels of adult rat. Each value is the mean of 8 determinations with standard error (SEM). Rats received an i.p. injection of 50 (DDT 50) or 100 (DDT 100) mg/kg body weight of DDT during 10 days. Control animals received the vehicle. [a,b] $p < 0.05$ compared with controls (Tukey's multiple comparison *post hoc* test).
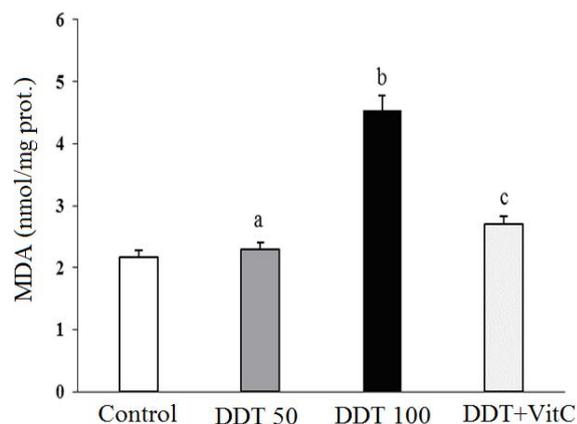
Figure 2. Effect of DDT and vitamin C on the concentration of malondialdehyde in kidney of adult rat. Each value is the mean ± SEM of 8 determinations in duplicate *per* group. Rats received an i.p. injection of 50 (DDT 50) or 100 (DDT 100) mg/kg body weight of DDT during 10 days. DDT+VitC: rats received an i.p. injection of 100.mg of DDT/kg followed by vitamin C (200 mg/kg). Control animals received the vehicle. DDT treatment was preformed as described in the methods section. [a,b] $p < 0.05$ compared with controls (Tukey's multiple comparison *post hoc* test). [c] $p < 0.05$ compared with DDT 100 group (Tukey's multiple comparison *post hoc* test).

Antioxidant enzyme activities

The antioxidant enzymes activities in kidney are presented in Fig. 5. The SOD activity was significantly reduced with the highest dose by 46.5% of control (Fig. 5A). DDT treatment significantly reduced CAT activity (114.8 ± 3.4 and 74 ± 4.8 nmol/H_2O_2/min/mg prot., respectively, with DDT 50

and DDT 100 *versus* 157.3 ± 4.1 nmol/H_2O_2/min/mg prot.) in a dose-dependent manner (Fig. 5B).

Glutathione-dependent enzymes and reducing power

Glutathione-dependent enzymes and reducing power in kidney are presented in Table 1. The GPx activity increased with the dose of 50 mg/kg by 18% and declined with the highest dose by 48.6% of control. Also, the GST activity in kidney increased with the dose of 50 mg/kg by 62.4% and declined with the highest dose by 20.4% of control. The GR activity was not affected in the DDT 50 group but was significantly decreased in DDT 100 group by 44.9% of control. GSH level was significantly decreased in treated rats compared to control groups. This decrease reached 19% and 38.8% of controls for DDT 50 and DDT 100, respectively. In contrast, DDT treatment induced a dose-dependent increase in GSSG levels. This increase reached 51.4% and 122.5% of controls for DDT 50 and DDT 100, respectively. The ratio between concentrations of GSSG and GSH is a valuable marker, characterizing cellular redox status. Thus, exposure to DDT significantly increased the ratio GSSG/GSH in treated rats compared to control groups. This increase reached 87.5% and 275% of controls for DDT 50 and DDT 100, respectively.

Histopathological examination

Histopathological analysis of kidney tissue sections (cortex and medulla) of rats exposed to DDT showed abnormalities as compared to the control. Kidney of control rats exhibited a normal renal cortex with proximal and distal convoluted tubules surrounding renal corpuscles with normal Bowman's space and blood vessels (Fig. 6A, B). In contrast, kidney of
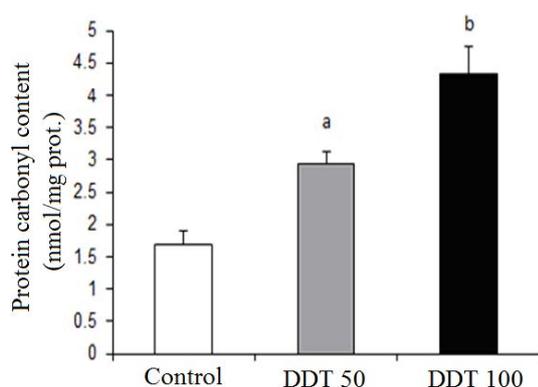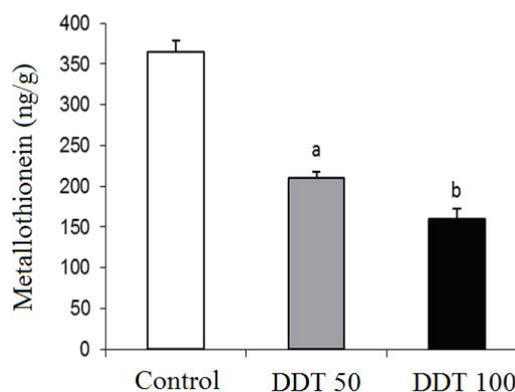

Figure 3. Effect of DDT treatment on kidney protein carbonyl content in adult rat. Each value is the mean ± SEM of 8 determinations. Rats received an i.p. injection of 50 (DDT 50) or 100 (DDT 100) mg/kg body weight of DDT during 10 days. Control animals received the vehicle. [a,b] $p < 0.05$ compared with controls (Tukey's multiple comparison *post hoc* test).

Figure 4. Effect of DDT treatment on kidney metallothionein concentration in adult rat. Each value is the mean ± SEM of 8 determinations. Rats received an i.p. injection of 50 (DDT 50) or 100 (DDT 100) mg/kg body weight of DDT during 10 days. Control animals received the vehicle. [a,b] $p < 0.05$ compared with controls (Tukey's multiple comparison *post hoc* test).

Table 1. Effect of DDT treatment on glutathione-dependent enzymes and reducing power in kidney of adult rat

Parameters	Control	DDT 50	DDT 100
GPx (U/mg Pt)	5.51 ± 0.2	6.51 ± 0.35^a	2.83 ± 0.17^b
GR (U/mg Pt)	3.43 ± 0.16	3.26 ± 0.12	1.89 ± 0.16^a
GST (U/mg Pt)	50.35 ± 1.76	81.79 ± 2.95^a	40.05 ± 2.57^b
GSH (nmol/mg Pt)	98.91 ± 5.66	80.1 ± 4.9^a	60.54 ± 2.51^b
GSSG (nmol/mg Pt)	15.99 ± 0.98	24.22 ± 1.07^a	35.59 ± 2.41^b
GSSG/GSH (%)	0.16 ± 0.01	0.30 ± 0.01^a	0.6 ± 0.05^b

Each value is the mean ± SEM of 8 determinations. Rats received an i.p. injection of 50 (DDT 50) or 100 (DDT 100) mg/kg body weight of DDT during 10 days. Control animals received the vehicle. Gpx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; GSH, reduced glutathione; GSSG, oxidized glutathione. [a] $p < 0.05$; [b] $p < 0.01$ compared with controls (Tukey's multiple comparison *post hoc* test).
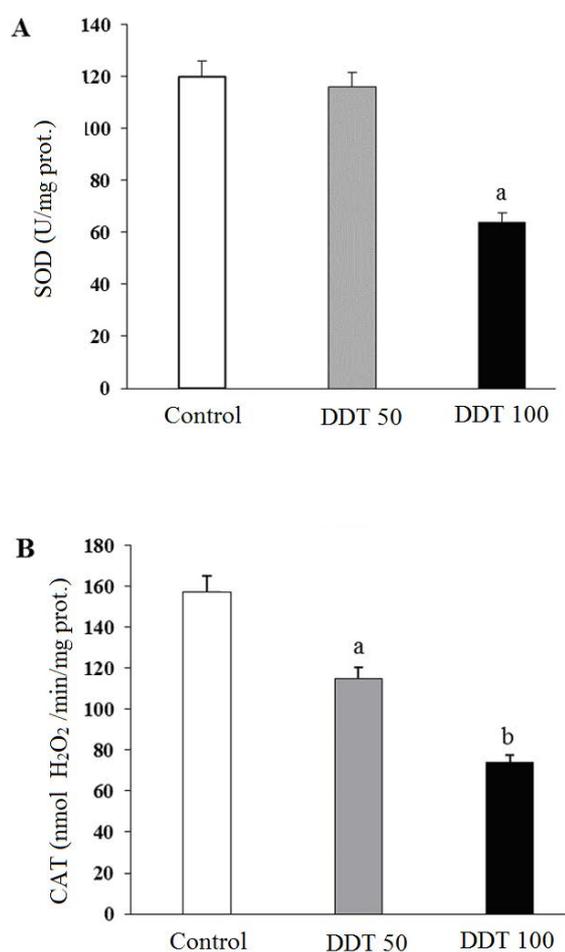

Figure 5. Effect of DDT on antioxidant activities of enzymes superoxide dismutase (SOD, **A**) and catalase (CAT, **B**) in kidney of adult rat. Each value is the mean ± SEM of 8 determinations. Rats received an i.p. injection of 50 (DDT 50) or 100 (DDT 100) mg/kg body weight of DDT during 10 days. Control animals received the vehicle. [a,b] $p < 0.05$ compared with controls (Tukey's multiple comparison *post hoc* test).

rats exposed to 50 mg of DDT/kg showed a congested blood vessels, tubular dilatation due to the accumulation of hyaline droplets in renal tubule, tubular epithelium desquamation and vacuolization of tubular lumen (Fig. 6C, D). Histopathological changes in kidney were more pronounced in the DDT 100 group. We showed atrophy of glomerulus, degeneration of renal tubules, extensive necrosis and increased of hyaline droplets (Fig. 6E, F).

TUNEL assay

Apoptosis was characterized by a TUNEL technique that specifically detects apoptotic cells in kidney. Untreated rats showed no kidney tubular cells apoptosis (Fig. 7A, B), whereas positive staining in renal tubular cells was found after treated with 50 mg of DDT/kg (Fig. 7C, D). With DDT 100, strong positive staining was observed in renal tubular cells (Fig. 7E, F). The apoptotic index grew 35.6-fold ($p < 0.01$) and 118-fold ($p < 0.01$) in cortex and 135-fold ($p < 0.01$) and 247.5-fold ($p < 0.01$) in medulla of treated rats with 50 and 100 mg of DDT/kg, respectively, compared to control (Table 2). Vitamin C administrated in DDT-treated rats improved significantly the apoptotic index compared with control values. The apoptotic index in kidney was returned to control values in DDT+VitC group (Table 2).

Discussion

The aim of this study was to investigate whether p,p'-DDT treatment has the ability to cause nephrotoxicity and if so, whether it is linked to oxidative stress and apoptosis. Kidney is vulnerable to damage due to perfusion and the increased concentration of excreted compounds that occur in renal tubular cells (Mohamed et al. 2003). Plasma levels of urea and creatinine are used as indicators of renal function. The creatinine excretion is dependent almost on the process of
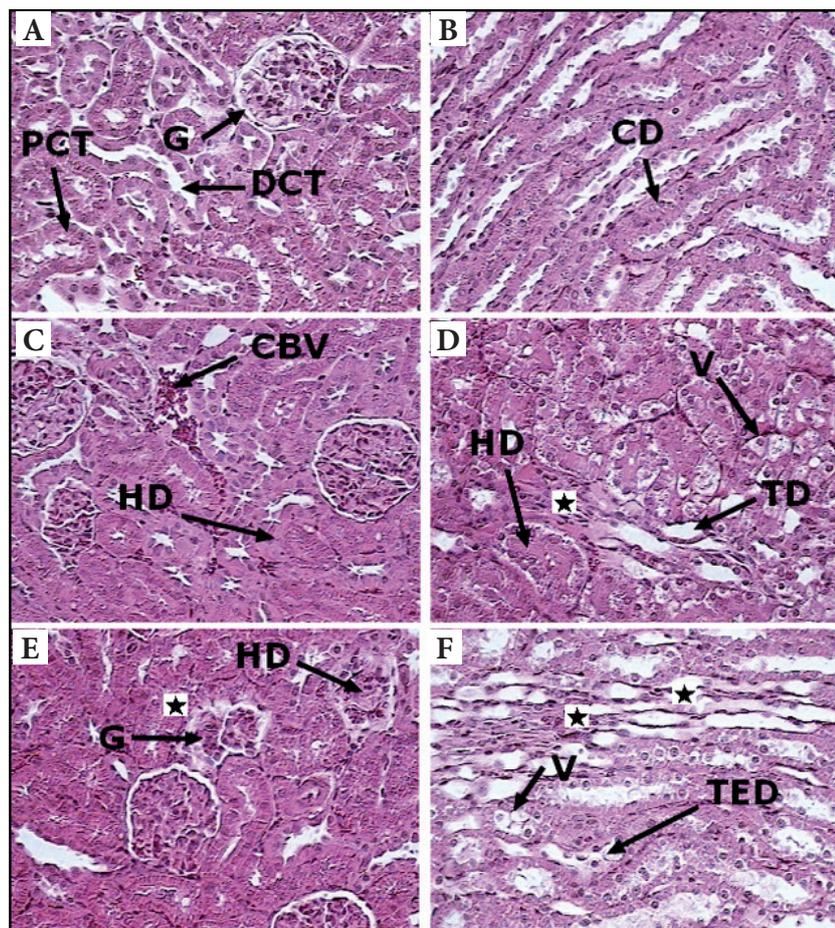
Figure 6. Photomicrographs of kidney sections of control and DDT-treated rats. **A., B.** Kidney of control rats showing normal architecture (A, cortex; B, medulla). **C., D.** Kidney of DDT-treated rats (50 mg/kg) showing congested blood vessels, accumulation of hyaline droplets in renal tubule, tubular epithelium desquamation and vacuolization of tubular lumen (C, cortex; D, medulla). **E., F.** Kidney of DDT-treated rats (100 mg/kg) showing a degeneration of renal tubules with extensive necrosis, increased of hyaline droplets and atrophy of glomerulus (E, cortex; F, medulla). Rats received an i.p. injection of 50 or 100 mg/kg body weight of DDT during 10 days. G, glomerulus; PCT, proximal convoluted tubule; DCT, distal convoluted tubule; CD, collecting duct; HD, hyaline droplets; CBV, congested blood vessels; TD, tubular dilatation; TED, tubular epithelium desquamation; V, vacuolization of tubular lumen; ★ point to necrosis. Magnification: ×400.

glomerular filtration, though tubular secretion contributes slightly (Kassirer 1971). High blood creatinine levels could indicate an inability of the kidney to excrete creatinine, resulting from renal disease mostly impairment of the glomerular function and tubular damage (Marks and Lieberman 2009). So any damage to kidney will show an improper excretion of creatinine. The present study showed that exposure of rats to 50 and 100 mg of DDT/kg b.w., during 10 consecutive days, significantly increased serum creatinine and urea levels. Urea is an end product of protein and amino acid metabolism. Kidney filters excess urea into the urine and in sweat, but some goes into the bloodstream as serum urea. It is a well-known fact that, if blood urea levels are low, a problem centred in the liver might be suspected because urea is produced in the liver. Conversely, high blood levels of urea suggest that the kidney is not excreting urea normally (Vanholder et al. 1992). Thus, in this study increased serum creatinine and urea levels reflect the diagnosis of renal failure. Our study is concomitant with previous reports showing an important increase in serum creatinine and urea levels in rats exposed to 100 mg of DDT/kg/day (Mohammed et al. 2012). Vijaya Padma et al. (2011)

have also reported increased levels of serum creatinine and urea in lindane-exposed rats. Shah and Iqbal (2010) also revealed an increased level of serum creatinine and urea in rats exposed to 10, 15 and 30 mg of diazinon/kg for 8 weeks.

Table 2. Effect of DDT and vitamin C treatment on apoptotic index in kidney of adult rat

	Apoptotic index (%)	
	Cortex	Medulla
Control	0.31 ± 0.03	0.16 ± 0.01
DDT 50	10.1 ± 1.11[a]	21.61 ± 1.91[a]
DDT 100	36.61 ± 2.93[b]	39.6 ± 3.57[b]
DDT+VitC	0.40 ± 0.05[c]	0.22 ± 0.03[c]

Each value is the mean ± SEM of 8 determinations. Rats received an i.p. injection of 50 (DDT 50) or 100 (DDT 100) mg/kg body weight of DDT during 10 days. DDT+VitC, rats received an i.p. injection of 100 mg of DDT/kg followed by vitamin C (200 mg/kg). Control animals received the vehicle. [a] $p < 0.01$; [b] $p < 0.001$ compared with controls (Tukey's multiple comparison *post hoc* test). [c] $p < 0.01$ compared with DDT 100 group (Tukey's multiple comparison *post hoc* test).
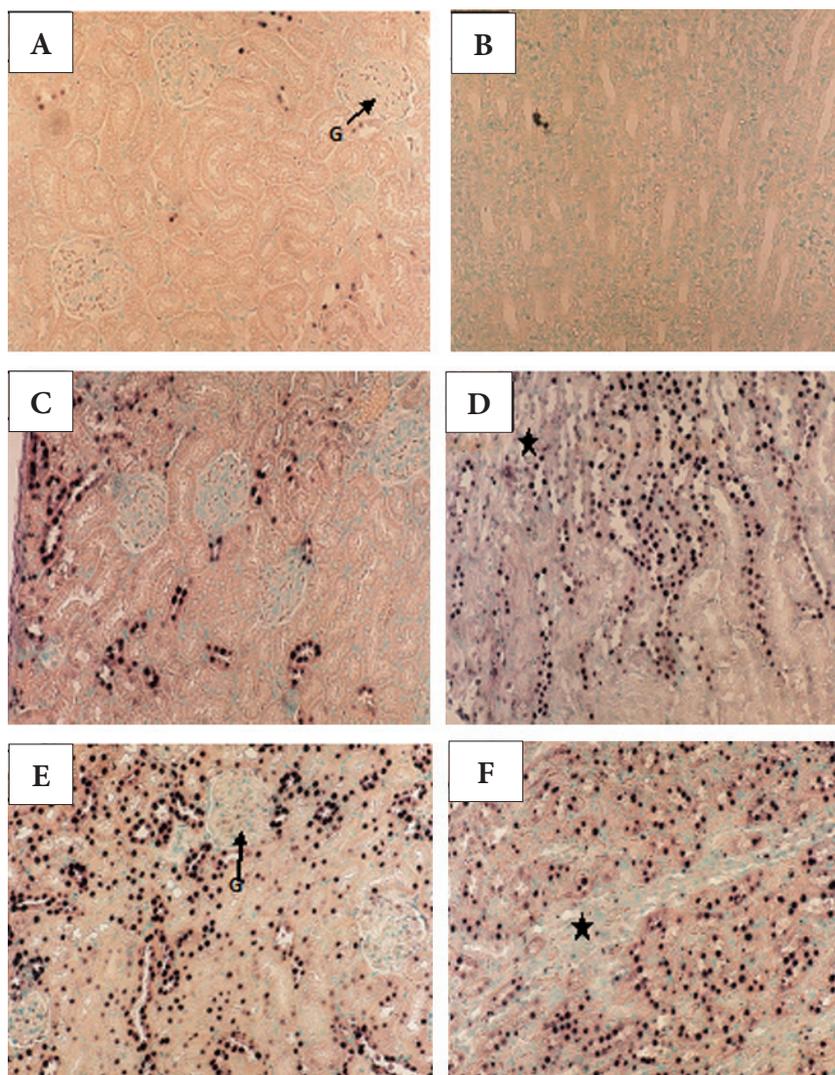
Figure 7. Detection of apoptotic cells in the kidney of rats as revealed by TUNEL assay. Kidney of control rats showed no apoptotic cells in cortex (**A**) and medulla (**B**). Kidney of DDT-treated rats with 50 mg/kg in cortex (**C**) and medulla (**D**), and 100 mg/kg in cortex (**E**) and medulla (**F**) showed TUNEL-positive germ cells. Rats received an i.p. injection of 100 mg/kg body weight of DDT during 10 days. G, glomerulus; ★ point to necrosis. Magnification: ×250.

In the present study, we hypothesized an oxidative stress-like as a potential mechanism inductor of renal injury. ROS have been implicated in several renal diseases induced by some pesticides (Shah and Iqbal 2010; Vijaya Padma et al. 2011; Wei et al. 2014). In turn, ROS are capable of initiating and promoting oxidative damage as LPO (Kovacic and Cooksy 2005). LPO is known to cause cellular injury by inactivation of membrane enzymes and receptors, depolymerisation of polysaccharide, as well as protein cross-linking and fragmentation (Luqman and Rizvi 2006). MDA is a parameter to measure lipid peroxidation and its increase is a direct result of free radical damage to membrane components of the cells. Our data indicated that sub-acute treatment of DDT significantly increased MDA level in kidney tissues of treated rats, which is an indicator of increased oxygen free radical generated by pesticides. Under this situation, stable products like carbonyl groups are produced by ROS action on proteins, also making protein carbonylation a reliable marker of oxidative stress. In the current study, we found that DDT-induced renal dysfunction is associated with oxidant damage, which was evident by increased LPO and protein carbonyl content. Our results confirmed previous data of Mohammed et al. (2012) who have also reported increased level of MDA in the kidney from rats following a single dose of DDT (100 mg/kg). Our finding was in accordance with previous studies using other organochlorines pesticides and has also reported increased MDA level in the kidney of treated rats (Shah and Iqbal 2010; Vijaya Padma et al. 2011; Wei et al. 2014). Induction of cytochrome P450 and other microsomal enzyme by various pesticides such as carbamate, has been reported and it is possible that DDT mediated free radical generation could be through induction of these enzyme (Puatanochokchai et al. 2006). Vitamin C administration to DDT-treated rats resulted in

a significant decrease in LPO level compared to rats treated with DDT alone. These results are similar to the observation of another study where vitamin C was shown to decrease LPO level in deltamethrin-induced damage in rats (Mongi et al. 2011). The protective effect of vitamin C, observed in our study, could be important for protecting the different tissues against the oxidative injury following the use of DDT. MTs are members of a family of low molecular weight proteins rich in cysteine that play a key role in transport of essential heavy metals, detoxification of toxic metals and protection of cells against oxidation stress. We have demonstrated that the levels of MTs decreased in a dose-dependent manner in the kidney of DDT-treated rats. The inhibited level of MTs is closely associated with increased formation of ROS and reactive nitrogen species, respectively. Excessive production of these harmful substances along with a reduction in antioxidants could reduce the level of MTs in kidney (Chin et al. 1993). Antioxidant enzymes, such as SOD and CAT, are essential parts in the cellular defense against free radical-mediated tissue or cellular damage. Our results revealed a decrease in the level of SOD and CAT activities in the kidney of DDT-treated rats. Similarly, recent studies showed that exposure to pesticides (diazinon, lindane and paraquat) decreased SOD and CAT activities in rat kidney (Shah and Iqbal 2010; Vijaya Padma et al. 2011; Wei et al. 2014). SOD plays a vital role in the balance between oxidation and antioxidation. SOD catalyzes the dismutation of superoxide anions into H_2O_2 and eliminates the cytotoxic effects of the superoxide anion (Medinas and Augusto 2010). CAT is a ubiquitous enzyme that prevents cell oxidative damage by degrading H_2O_2 to H_2O and O_2 with high efficiency (Alfonso-Prieto et al. 2009). The balance of theses enzymes systems may be essential to renal health. Hence, the significant reduction in enzymes activities, accompanied by marked increase lipid peroxidation, may reflect adverse effects of DDT on the antioxidant system (Shi et al. 2013). Therefore, the decrease in SOD and CAT activities may explain the early-elevated ROS levels, since it was a crucial enzyme involved in the detoxification of ROS. The decrease in the amount of antioxidant enzymes (SOD and CAT) are marked through their modification in gene expression, decreased uptake or when cells are overloaded with oxidants (Barber and Harris 1994). ROS, on the other hand, possess the ability to sufficiently modify a protein, leading to altered enzyme activity (Bellomo et al. 1983). The decreased enzymes activities in DDT-treated rats may be due to the inactivation of the corresponding gene segment from which the respective enzymes are encoded. Moreover, our results showed that p,p'-DDT administration decreased renal GR and GSH activities while increased the ratio GSSG/GSH, which led to the production of free radicals and causing lipid peroxidation (Nehru and Bansal 1997). GSH is one of the most important non-enzymatic

antioxidant against cellular damage produced by ROS (Luberda 2005). In its reduced form, it is necessary for the detoxification of xenobiotics. Various pesticides have been shown to decrease GSH levels. This reduction in GSH level could be due to direct conjugation of GSH with electrophiles whose increased production may result from pesticide exposure or could be due to inhibition of enzymes, like glutathione reductase, glutathione peroxidase, glucose-6-phosphate dehydrogenase, etc. which are involved in GSH synthesis and regeneration (Reed 1990). GSH is also GST co-substrate. GST is a potent antioxidant that provides cells with a substantial degree of protection against oxidative stress. It catalyzes the conjugation of reduced glutathione with a variety of endogenous compounds and xenobiotics (Romeu et al. 2002). In the current study, GST and GPx activities increased with the dose of 50 mg/kg whereas declined with the highest dose (100 mg/kg). The increased level of GST and GPx may be the adaptive response of the body to ROS attack. However, these increases were not sufficient to protect the membrane lipid which was evident by increased LPO in DDT-treated rats. At the high dose of DDT, a severe oxidative stress may suppress GSH, GST and GPx levels due to the loss of adaptive mechanisms and the oxidation of GSH to GSSG. The decrements in GST and GPx following exposure to DDT may lead to accumulation of peroxides (Shah and Iqbal 2010). Therefore, a depression in GSH levels together with GST and GPx activities makes the cells more susceptible to the attack by toxic compounds (Boesch-Saadatmandi et al. 2008). In the present study, the decrease in GPx, GST and GSH activities, accompanied by the increase of GSSG/GSH ratio and MDA levels, supports that oxidative stress is produced due to DDT administration. Moreover, histological changes were observed in the kidney of treated animals, including congested blood vessels, atrophy of glomerulus, degeneration of renal tubules, extensive necrosis and increased of hyaline droplets. The occurrence of damage to the renal tubular cells and necrosis has been associated with the administration of DDT (Mohammed et al. 2012) and other organochlorine pesticides (Shah and Iqbal 2010; Wei et al. 2014). It is known that when ROS generation overloads the antioxidant defence, the free radicals accumulated in kidney can then interact with endogenous macromolecules, alter the renal cellular function and cause necrotic renal cells (Muthukumaran et al. 2008). For this, oxidative stress seems to be one contributor to DDT-induced nephrotoxicity. Apoptosis plays an important role in the pathogenesis of a variety of renal diseases (Ueda et al. 2000). Intervention of cell apoptosis and apoptosis-related genes may be an effective method to prevent and cure renal diseases (Rana et al. 2001). In the present study, histological examination of kidney tissue by the TUNEL method showed that apoptosis cells occurred in the tubular cells of DDT-treated rats. Also, the apoptotic index was

significantly increased in kidney tissues of DDT-treated rats. However, vitamin C supplementation to the DDT-treated rats decreased the apoptotic index in kidney. Apoptosis is a complex event regulated by a well-tuned balance of inducer and repressor factors, such as the Bcl-2 family, which is a pivotal integrator of survival and death signal. In addition, the Fas system is a widely recognized apoptosis signal transduction pathway in which a ligand-receptor interaction triggers the cell death pathway (Feng et al. 2004). Fas is a surface receptor that triggers apoptotic cell death when cross-linked by FasL (Nagata 1997). Ligation of FasL to Fas in the cell membrane triggers activation of caspase-8. Once activated, caspase-8 transduces a signal to effector caspases, including caspases 3, 6, and 7, and eventually leads to the hydrolysis of cytosolic and nuclear substrates (De Maria et al. 1997). There were many toxicological studies indicated that DDT or its metabolite contributed to cell apoptosis by activation of multiple caspase-mediated mechanisms, including both of the extrinsic and intrinsic apoptosis pathway. In fact, Frigo and co-worker (2005) revealed that DDT induced both the expression of the death ligand TNF-alpha and apoptosis in human embryonic kidney cells through a p38 MAPK-dependent mechanism. Recently, it was reported that p,p'-DDT activated NF-κB/FasL pathway and mitochondrial pathway in human liver cells which were mediated by ROS (Jin et al. 2014). Zhao and co-worker (2012) reported that the enantioselective apoptosis caused by DDT might involve three signalling pathways *via* caspase 3, tumor protein 53 and NF-κB. Previous studies showed that p,p'-DDE led to apoptosis of cultured rat Sertoli cells via mitochondria-mediated or FasL-dependent pathway (Song et al. 2008; Shi et al. 2010). Fewer study elucidated the mechanism of DDT-induced apoptosis in kidney. So, in this study, we showed for the first time that p,p'-DDT treatment induced apoptosis in renal tubular cells. These findings suggested that p,p'-DDT induced apoptosis of renal tubular cells through mitochondria-mediated and FasL-dependent pathway. It has been reported that different stimuli such as DNA damage or increased ROS level caused by DDT might trigger of both Bax activation *via* acting diverse molecules such as p53, and Fas system. Activation of Bax protein leads to the formation of pores in the mitochondria and results in the collapse of the electro chemical gradient across the mitochondrial membrane, then cytochrome *c* is released into cytoplasm where it is associated with procaspase-9/Apaf-1. This complex, in turn, activates a downstream caspase program that ultimately leads to apoptotic cell death. In conclusion, the results obtained from the present study demonstrate that the sub-acute treatment of p,p'-DDT causes renal injury and apoptotic cell death in kidney tissues probably mediated by oxidative stress which may be the chief mechanism of DDT-induced nephrotoxicity in rats.

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