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Histopathological Observations and Biochemical Changes of Rats with Diniconazole's Hepatotoxicity.

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ABSTRACT

Diniconazole is one of azole based fungicides used in agriculture and as medicinal drug. This type have been shown to be hepatotoxic. The particular mode of toxic action for these compounds is not known, however it has been proposed that diniconazole-induced rat hepatotoxicity arise through the specific mechanism of increased liver functions (ALT, AST, ALP & T. bilirubin). The study was designed to identify commonalities of effects across the different diniconazole and to determine unique features of the tissue responses that suggest a toxicity pathway and a mode of action for the observed liver response for diniconazole. Male rats were treated with diniconazole for 4 weeks. The rats were tested for liver enzymes, cholesterol, triglyceride, oxidative stress, anti-oxidants and histopathology. Livers from all diconazole treated rats had highly significant in ALT, AST, ALP, T.bilirubin, cholesterol, triglycerides and MDA. The decrease significant was shown in hepatic and serum anti-oxidants (SOD & CAT). The diniconazole administration caused histopathological changes in the liver such as severe centrilobular necrosis, hepatocyte ballooning, and infiltration of inflammatory cells, hepatocytes vacuolization and vascular congestion. Despite the importance of diniconazole as an anti-fungal, but it causes serious damage to the liver.

Keywords: Diniconazole, hepatotoxicity, oxidative stress, anti-oxidants, Oil Red O.

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INTRODUCTION

During the last years the use of the fungicides and pesticides have increased in developing countries in an effort to control vector-borne diseases and increase production of food. At present, there are more than sixty five thousand chemicals that are classified as the pesticides. Unfortunately, because large amounts of these chemicals are released into the environment daily and many of them affect non-target organisms, this has resulted in some negative side effects on the human health and the environment [1]. According to Repetto and Baliga, [2] the occupational exposures to the pesticides and fungicides occurs due to the misuses of pesticides by concerned individuals and environmental and food residues. Several authors recorded varied grades of conazoles in vegetables and fruits [3]. Navarroa *et al.,* [4] recorded a slow fall in the leftover content of diniconazole after the storage of malt and spent grains; in banana and in soil samples.

Occupational exposures to the fungicides and pesticides occur from skin absorption and inhalations. The exposure of pesticides mainly takes place in the mixing and loading of the equipment, in the spraying of insecticide and improper handling [5]. When handling pesticides, the farmers who use pesticides have no access or only little to information about proper use or the precautions of this pesticides. Therefore, they often do not use even the simplest hygienic and protective measures [6]. Fungicides are pesticides that specifically kill or inhibit fungi underlying diseases important to man. Understanding mechanisms of fungicide action and toxicity is important because humans and domesticated animals encounter these pesticides via a wide variety of applications [7]. Hepatotoxicity is one of many toxic effects of fungicides in exposed workers and experimental animals. Typical symptoms include as hepatic enlargement, dysfunction, focal necrosis, hepatitis and degenerative changes [7]. Conazoles are a class of fungicides with agricultural and pharmaceutical applications. Some conazoles, such as epoxiconazole, cyproconazole, triadimefon and propiconazole induce hepatotoxicity and hepatocellular tumors in mice but with varying potencies and tumor profiles [8].

Diniconazole is a steroid demethylation (ergosterol biosynthesis) inhibitor. It is a highly active triazole fungicide. Diniconazole is widely used for control of a broad range of fungal diseases in many crops. Acute toxicity is classified as WHO class II (Moderately hazardous) and no symptoms for diniconazole poisoning are reported [9, 10].

MATERIALS AND METHODS

Animals and treatment

Male Sprague-Dawley rats were used in Faculty of Science, Damanhour and Fayoum Universities, and obtained from the National Research center breeding farm, Cairo, Egypt, quarantined and acclimated for 1 or 2 weeks before the experiment start. All the experimental rats were kept in the animal house during the study under room temperature, 22±2°C; relative humidity, 56±5%; and manmade lighting, 12 hour cycle. The animals had free to gain a diet and tap water. Rats were divided into two groups of 10 animals each. The 1st group was kept without treatment and served as control. The 2nd group was orally administered with diniconazole at daily dose of 64 mg/kg b.wt. corresponding to (1/10 LD_{50}) for 4 weeks [11]. At the end of the feeding period, the rats were fasted for 16h before sacrifice. Under ether anesthesia, blood was drawn by cardiac puncture and plasma was prepared with EDTA.

Serum Biochemical Parameters

The collected blood was used for the estimation of serum biochemical parameters viz. serum ALT, AST, serum alkaline phosphatase (ALP), total bilirubin (T. BL), total cholesterol and triglycerides contents were estimated by using commercially available reagent kits (Span Diagnostic Ltd., Surat, India). Serum total protein was estimated according to the reported method [12].

Preparation of rats liver homogenates

Liver tissues were placed in a prechilled glass tube and homogenized on ice in potassium phosphate buffer pH 6.7, containing 1 mM EDTA, protease inhibitor cocktail (pancreas extract, 0.02 mg/ml; 0.005 mg/ml; chymotrypsin, pronase, 0.005 mg/ml; papain, 0.33 mg/ml and phosphatase inhibitors (imidazole, 200 mM; sodium fluoride, 115 mM; sodium orthovanadate,100 mM; sodium molybdate, 100 mM and sodium

tartrate dehydrate, 400 mM) and thermolysin, 0.003 mg/ml). The liver homogenates were then transferred to Eppendorf tubes and centrifuged at 10,000 rpm for 15 min at 4°C to prepare the lysates.

Analysis of malondialdehyde (MDA) levels

Malondialdehyde (MDA) concentration was measured in liver homogenates and blood serum by the thiobarbituric acid method [13]. For tissue lipid peroxidation analysis, the chemical kit was purchased from Sigma-Aldrich (St. Louis, MO). The formation of lipid peroxides was measured in the homogenates of the hepatic tissues. The formation of MDA, an end product of fatty acid peroxidation was measured spectrophotometrically at 532 nm by using a thiobarbituric acid reactive substance (TBARS) and compared with values obtained from control MDA, essentially by the method of Genet et al. [14]. The results of malondialdehyde (MDA) were expressed as nmol /mg tissue.

Catalase activity

Catalase activities in rat liver homogenates and serum were determined using the Catalase Assay Kit (Cayman Chemical, Ann Arbor, Michigan) according to the manufacturer's instructions. Assays on hepatic tissues from each of control and diniconazole treated rats were performed in duplicate. Catalase activity was expressed as nmol formaldehyde formed/min/ml [15].

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined in liver homogenates and serum using the Superoxide Dismutase Kit (Cayman, Ann Arbor, Michigan) according to the manufacturer's instructions. Assays on hepatic tissues from each of control and diniconazole treated rats were performed in duplicate. SOD activity was expressed as SOD units/ml [16].

Quantitative evaluation of DNA fragmentation

To measure hepatocytes DNA fragmentation by spectrophotometry, a portion of the hepatic tissues frozen in liquid N_2 was homogenized in chilled lysis buffer (0.5% Triton X-, 20 mM EDTA, 10010 mM Tris–HCl, pH 8.0). Homogenates were then centrifuged to separate intact chromatin in the pellet from DNA cleavage product in the supernatant for 20 min at 27,000 × *g*. Pellets were re-suspended in 0.5N perchloric acid, and concentrated perchloric acid was added to supernatant samples to reach a final concentration of 0.5N. To remove protein, re-suspended pellets and supernatant were heated for 15 min at 90 ◦C and centrifuged for 10 min at 1500 × *g*. The result supernatants were then reacted with diphenylamine for 16–20 h at room temperature. Absorbance at 600 nm was measured using spectrophotometer. The amount of DNA fragmentation in control sample (frag. DNA/(frag. DNA + intact DNA)) is expressed as the percentage of total DNA that appears in the supernatant fraction. Treatment effects on DNA fragmentation are reported as the percentage of control fragmentation.

Histopathology

Livers were collected and fixed in 10% freshly prepared phosphate buffered formalin for histopathologic examination. Fixed tissues were embedded in melted wax, sectioned at 3 µm, and stained with hematoxylin and eosin (H&E) using standard histologic procedures. Three sections per animal were evaluated. Treatment effects on hepatocyte hypertrophy and proliferation have been investigated [17]. Other histologic changes were evaluated here. Digital images of representative liver sections stained with H&E were taken using a Nikon Eclipse microscope and digital camera (Nikon Instruments, Melville, NY). Oil red O staining method was performed on optimal cutting temperature media embedded frozen liver sections by using standard techniques [18].

Statistical analyses

Statistical analyses were carried out using Sigma-Plot (Systat, San Jose, CA). Comparisons of two groups were analyzed and the resulted data are presented as mean ± SD.

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RESULTS

The activities of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) and total bilirubin (T.Bl), were appreciated in serum samples as biomarkers of liver function. In this study, the effects of diniconazol to rats resulted in liver injury in rats as evidenced by a marked significant increase in serum AST, ALT, ALP and T. BL, when compared to control group. Changes in the serum liver function markers suggest increased damage to the hepatocytes by diniconazole as shown in table (1).

Table 1: The statistical analysis of AST, ALT, ALP and T.BL concentrations in different experimental animal groups.

Groups	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	T. BL (mg/dL
Control	23.15±1.05	25.73±1.66	76.17±2.73	0.98±0.10
Diniconazole	80.12±2.59*	180.52±3.45**	110.32±3.09*	1.59±0.18**

Data were expressed as mean+SD; significant differences in control group versus treated groups are *P<0.05, **P<0.01.

Hepatic triglyceride and cholesterol content in the diniconazole group showed a significant rise in hepatic cholesterol and triglyceride respectively (Fig. 1).

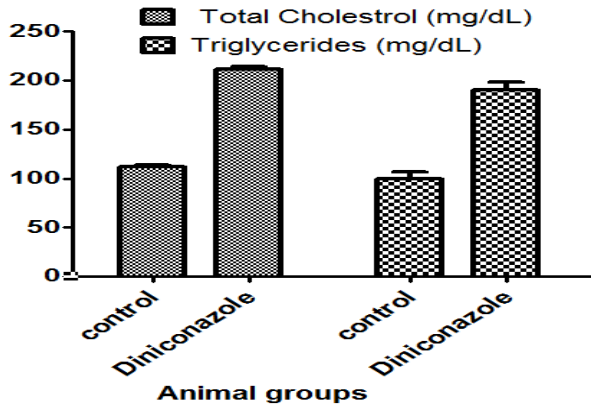

Figure 1: The mean levels of Total Cholesterol and Triglycerides in diniconazole tested group compared with control group.

Blood serum hepatic anti-oxidants and oxidative stress**:** Liver anti-oxidant profile is an overall index of organism's metabolic status. In experimental animals oxidative environment manifests several physiological anomalies if remains untreated. There was a significantly decrease in both CAT and SOD in blood serum in diniconazole group (Figure 2 & 3) when compared with control. But the oxidative stress (MDA) was a significantly increase in diniconazole group when compared with control animal group (Figure 4) .

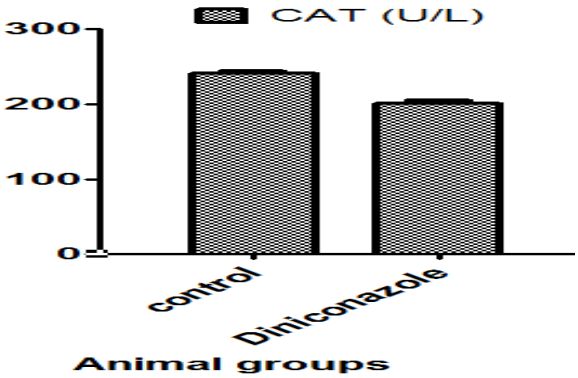

Figure 2: The concentration of blood serum catalase in diniconazole tested group compared with control group.

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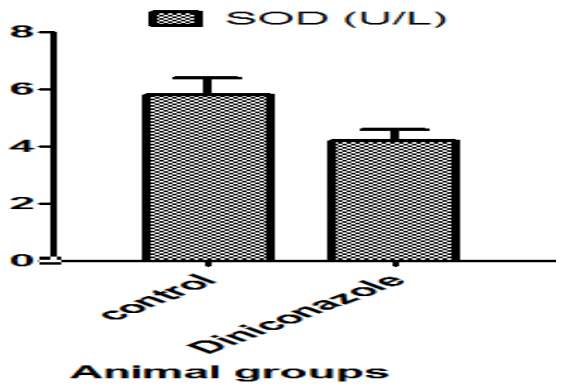

Figure 3: The concentration of blood serum superoxide dismutase in diniconazole tested group compared with control group.

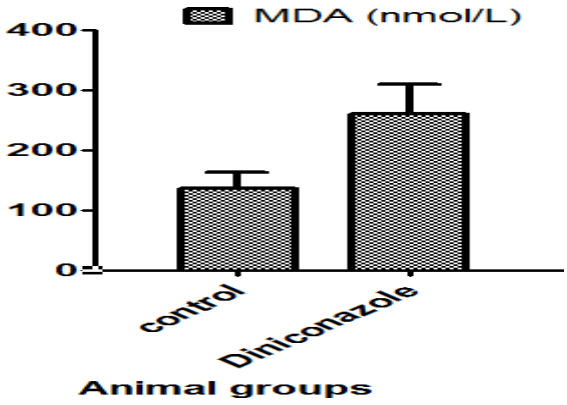

Figure 4: The concentration of blood serum Malondialdehyde (MDA) in diniconazole tested group compared with control group.

The hepatic tissue homogenate MDA was highly significant increase in diniconazole group, but in case of anti-oxidants CAT and SOD, the siginficant decrease was detected when compared with the control group as shown in table (2).

Table 2: The statistical analysis of hepatic homogenate MDA, CAT and SOD concentrations in hepatic tissues in different experimental animal groups.

Groups	MDA (nmol/g)	CAT (U/g)	SOD (U/g)
Control	398.62±15.05	24.60±1.06	80.17±5.73
Diniconazole	600.10±36.40**	17.52±1.45*	25.80±2.16**

Data were expressed as mean+SD; significant differences in control group versus treated groups are *P<0.05, **P<0.01.

Quantitative evaluation of DNA damage based on a sedimentation assay has been presented in figure 5. Fragmentation was assessed by centrifuging liver homogenates and reacting unsedimented DNA fragments with diphenylamine. Control animal did not alter the integrity of genomic DNA. Diniconazole treatment produced significant (P<0.001) DNA damage when compared with the control.

Normal histological structures of liver tissue were observed in the control group (Fig. 6A). The administration of diniconazole caused histopathological changes in the liver such as severe centrilobular necrosis, hepatocyte ballooning, and infiltration of inflammatory cells (such as macrophages and lymphocytes) into the portal tract and sinusoid, hepatocytes vacuolization and vascular congestion (Fig. 6B-F).

In figure 7, Lipid containing vacuoles detected by staining with Oil Red O were predominant in mid-zonal and some periportal hepatocytes.

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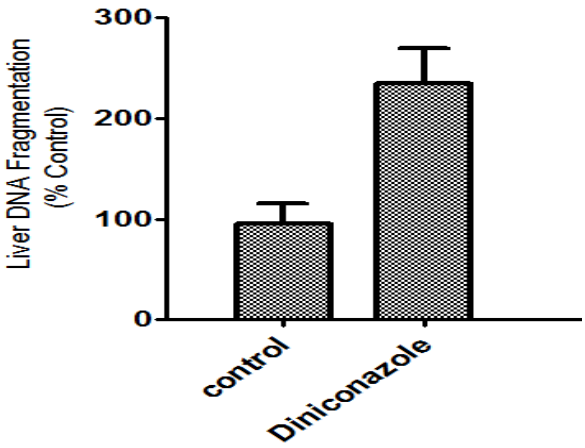

Figure 5: The liver DNA fragmentation percent in control and diniconazole groups.

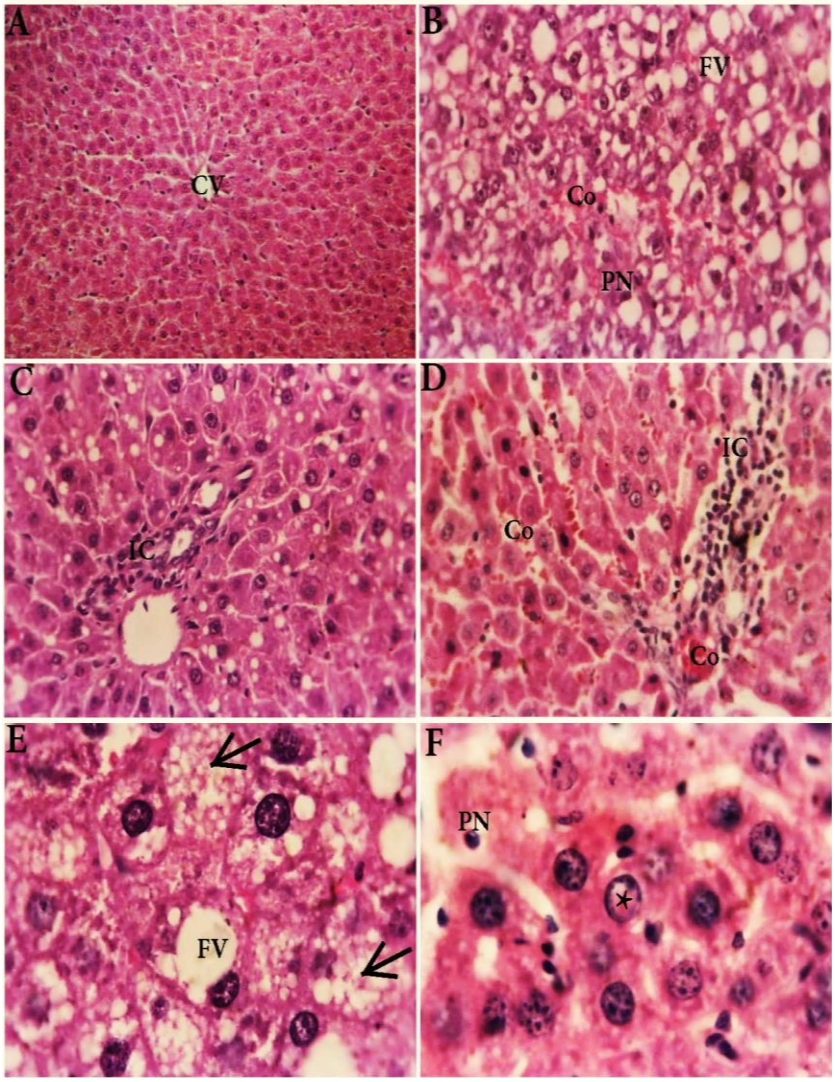

Figure 6: Histopathological effcts of diniconazole intoxication on hepatic tissues. A) The light micrograph of liver section in control group, Central vein (CV). B-F) The light micrographs of hepatic tissues from experimental animals treated with diniconazole, degenerated hepatocytes (Arrows), Fat vacuolization (FV), Inflammatory cell infiltration (IC), Pyknotic nuclei (PN), Congestion of centaral vein and blood sinusoids (Co) and cytoplasmic nuclear inclusion (*). Magnification, A-D X 200, E & F X400.

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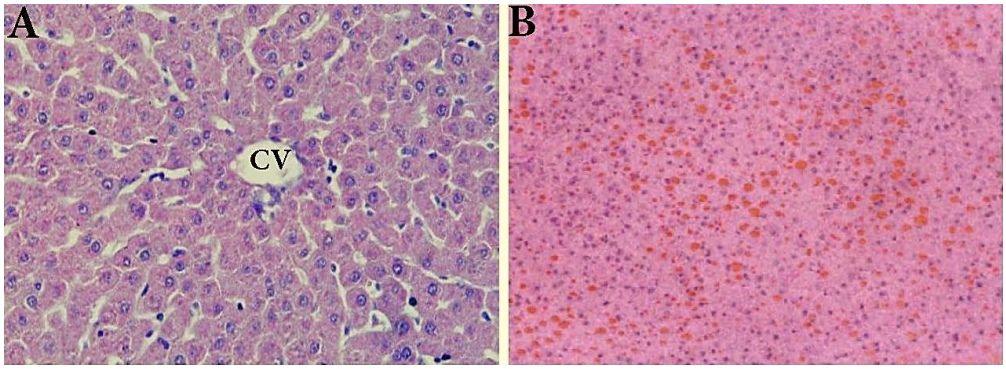

Figure 7: Oil red O stain for lipid droplets in control (A) and treated animals with diniconazole (B) showing the red color for positive stain. Magnification X 200.

DISCUSSION

Diniconazole is one of conazole class and was used to control fungi particularly ascomycetes and basidiomycetes. This fungicide has been widely used in China for many years. The aggregation of diniconazole in field would cause severe hepatotoxicity and acute renal failure for experimatal animal and humans [19].

In the rat feeding study, diniconazole caused hypertrophy of the liver and this result suggested that this chemical induced hepatic drug-metabolizing enzymes. This report deals with biochemical changes in the livers of rats receiving diniconazole at a higher dosage in relation to the metabolic fate and toxicity of the chemical. Moreover, diniconazole has effects on various parameters relating to liver functions including cytochrome P450 and bile flow rate [11]. In the present study, The liver dystrophy and the observed subcutaneous tissue icterus correlated to observed blood biochemical alterations (ALT, AST, ALP and T. bilirubin) in rat injected with diniconazole as report in birds and rabbits [20]. Diniconazole treatment was found to reduce total cholesterol and triglyceride levels in the present study, in disagreement with other reports on the effects of conazole [21, 22].

Liver degeneration induced by diniconazole, may be mediated by depletion of anti-oxidants and elevation of lipid peroxidation [23, 24]. Also, it may be due to diminished expression of multiple genes involved in steroidogenesis in liver and testes of mice which mediated by depletion of anti-oxidants and elevation the rate of lipid peroxidation [25]. Oxidation products of lipids, DNA and proteins are reliable biomarkers of oxidative stress [26]. The diniconazole-induced hepatic damage in this study was accompanied by elevation in MDA. Also, ROS generation and the oxidative damage of lipid membranes mediate diniconazole-induced hepatotoxicity. CAT and SOD are major enzymes that scavenge harmful ROS in hepatic tissues [27].

The diniconazole-induced hepatotoxicity in this study was also accompanied with massive hepatic DNA fragmentation and lipid peroxidation (MDA). DNA fragmentation and lipid peroxidation observed in the present study is the normal consequence of oxidative stress that was demonstrated through decreasing CAT and SOD activities in liver [28].

Diniconazole-induced hepatic toxicity was further confirmed by histological alteration in the liver. The present histological observations correlate well with results from other reports, where short- and long-time diniconazole treatment have been shown to cause adverse effects on liver function in the rat. Many studies suggested that the histological and histochemical alterations observed in mammalian liver under the effect of many conazoles are attributed to induction of several hepatic CYPs. The induction of these CYPs is mediated through the activation of the nuclear receptors [29].

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