

Light and Electron Microscopic Study of the Possible Protective Effect of *Nigella Sativa* on Metalaxyl Induced Hepatotoxicity in Adult Albino Rats

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Abstract

Metalaxyl is a fungicide used to control soil-borne fungal diseases on fruits, cotton, soybean, peanuts and grasses. However, metalaxyl showed hazardous effects in animals. This study aimed to elucidate the histological, ultra structural alterations in the liver tissue caused by metalaxyl and to investigate the possible protective effects of *Nigella Sativa* (NS) against these alterations. Thirty adult male albino rats were divided into three equal groups. Group I (control), Group II (metalaxyl treated) received metalaxyl in a dose of 130 mg/kg/day 3 times per week for continuous 6 weeks. Group III (prophylactic group) received metalaxyl as group II in addition to oral *Nigella Sativa* (NS) in a daily dose of 400 mg/kg. At end of experiment, liver specimens were taken and processed for light and electron microscope examination. The histological study of the liver sections of metalaxyl treated group II showed necrotic and apoptotic changes in hepatocytes. Some central veins were congested and the blood sinusoids were ill-defined in between hepatocyte cords. The bile ducts of some portal tracts appeared with thickened wall and surrounded by cellular infiltrations. The liver sections of prophylactic group III appeared with more preserved histological structure except the presence of slightly congested central veins, and few hepatocytes with apoptotic nuclei. The statistical analysis of biochemical assay of serum and liver tissue of metalaxyl treated animals, showed significant increase in oxidative stress marker malondialdehyde (MDA) with significant decrease in antioxidants glutathione (GSH) and glutathione peroxidase (GPx). However, there was significant increase in GSH with decrease in MDA and increase in GPx nearly to control levels in prophylactic group. Metalaxyl causes histopathological changes in liver most probably through oxidative stress. However, NS therapy could ameliorate these changes in liver most probably through its antioxidant properties. This may indicate the effectiveness of NS in prevention of metalaxyl hepatotoxicity.

Keywords: *Nigella Sativa*; Metalaxyl; Hepatotoxicity; Ultra structure; Rat

Introduction

Pesticides play an important role in the effort to increase food production in today's agriculture but, at the same time, they cause environmental hazards because of their toxicity and sometimes high persistence representing one of the problems of world-wide importance [1]. The presence of these toxic chemicals was recorded in water, air, house dust and in the tissues of non-occupationally exposed people, particularly in the adipose tissue, blood and urine [2].

Metalaxyl is a benzenoid fungicide used to control soil-borne fungal diseases on fruits, cotton, soybean, peanuts and grasses [3]. The problems resulting from metalaxyl come from their high residual level in agriculture crops especially vegetables cultivated under greenhouse conditions and other components of environment [4]. Metalaxyl is very soluble in water and has the potential to reach groundwater and stable under when exposed to sunlight, with a half-life of 400 days [5]. Metalaxyl was reported to have cytogenetic effects on human and animal chromosomes *in vitro* [6] and co carcinogenic potential in Swiss albino mice [7]. The liver is the most sensitive organ to biochemical changes therefore its functions are greatly affected by pollutants resulting in an increase in serum enzymes levels [8].

It was reported that the oxidative stress is the principle manifestations of metalaxyl-induced toxicity [9]. Oxidative stress was reported to lead to increase in the production of reactive oxygen species (ROS). If ROS formation exceeds the capacity of antioxidant, ROS can react with macromolecules such as lipid, protein, and DNA which lead to cell dysfunction and damage. Reaction of ROS with lipid membrane, rich in polyunsaturated fatty acids will cause lipid peroxidation with production of MDA as an end product [10]. Biological defense against

ROS comprises a complex of endogenous antioxidant enzymes, numerous antioxidant factors including glutathione and a variety of nutritional factors [11]. The generation of ROS in experimental animals results in tissue oxidative stress by depletion of tissue glutathione [12]. Hence, restoration of glutathione can prevent the oxidative stress and tissue injury [13].

Medicinal plants play an important role in pharmacology and medicine for many years. Today, it is estimated that about 80% of the world population relies on botanical preparations as medicine to meet their health needs [14]. Clinical and animal studies have shown that the extracts of the NS or black seeds have many therapeutic effects such as bronchodilatation, immunomodulative [15], antibacterial [16], hypotensive [17] antidiabetic [18], hepatoprotective [19], gastro protective [20], antihistaminic and antioxidative [21] and neuroprotective effects [22]. Antitumorigenic effects through induction of cytotoxicity and apoptosis on cancer cells were also previously reported [23]. Moreover, thymoquinone the most abundant component of black seed oil, has been reported to exhibit antioxidant effect [24].

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Materials and Methods

Thirty adult male albino rats (4-6 months old & 150-200 gm) were kept in individual polyethylene cages with stainless-steel tops at the Animal House, Faculty of Medicine, Zagazig University. Rats were subjected to controlled conditions of temperature ($25 \pm 2^\circ\text{C}$) and illumination (12h- light/dark), and allowed free access to normal rat chow diet and water ad libitum. One week after acclimatization rats were randomly divided into three equal groups (ten animals each): **Group I** (control group) was further subdivided into two equal subgroups, one included animals that didn't receive any medication and another subgroup included animals that received daily oral NS dissolved in 2 ml distilled water by orogastric tube in a dose of 400 mg/kg b.w. **Group II** (metalaxyl treated) received metalaxyl dissolved in 2 ml distilled water by orogastric intubation intermittently in a dose of 130 mg/kg body weight/ day 3 times per week for continuous 6 weeks [25]. Metalaxyl was supplied as ready-to-use liquid from Central Agricultural Pesticides Laboratory, ARC, Egypt. **Group III** (prophylactic group) received the same dose of metalaxyl given to animals of group II in addition to NS dissolved in 2 ml distilled water by orogastric tube in a dose of 400 mg/kg body weight daily for 6 weeks [26]. NS were purchased from a local herb market at Cairo, Egypt and added to distilled water after well grinding.

At the end of the experiment, all rats were sacrificed by decapitation under mild anesthesia (anesthetic ether) and the. Midline abdominal incision was done to get the liver. The right lobes were dissected and processed for light and transmission electron microscopes examination.

Histological study

For light microscope, the specimens were fixed in formal saline for 12 hours to prepare paraffin blocks. Sections ($5\ \mu\text{m}$) were prepared and stained by H&E stain [27]. For transmission electron microscope, the specimens were immediately fixed in 2.5% glutaraldehyde in cacodylate buffer at pH 7.4 for 24 hours at 4°C . Then the specimens were washed with the buffer, post fixed in 1% osmium tetroxide in distilled water for 2 hours at 4°C . Specimens were dehydrated with ascending grades of ethanol and then put in propylene oxide to prepare Epon-Araldit resin blocks [28]. Ultrathin sections (70-90 nm) were obtained, stained by uranyl acetate and lead citrate and examined under JEOL 1010 electron microscope, in Histology Department, Faculty of Medicine, Zagazig University.

Biochemical assay

For enzymes determination, blood and liver samples were collected after scarification. The blood was collected in non coated tubes to obtain serum. Sera were obtained by centrifugation at 3000 rpm for 20 min at 4°C , and samples were transferred to polypropylene microtubes and stored at -70°C until analysis. The endogenous non enzymatic antioxidant, glutathione (GSH) concentration (mg/dL) was measured using spectrophotometer [29]. Also, blood malondialdehyde (MDA) as markers for oxidative stress (lipid peroxidation) was determined (nmol/ml erythrocytes) by the double heating method [30].

Liver specimens was removed, washed with ice cold saline to remove extraneous materials and one hundred milligrams of liver tissue were processed biochemically for the assessment of GPx activity (glutathione peroxidase) as an antioxidant enzyme (nmol of NADPH oxidized/min/mg) [31]. Also, the concentration of MDA (nmol /mg liver tissue) as end product of lipid peroxidation was measured [32].

Statistical analysis

All the biochemical data are expressed as Mean \pm SD. Statistical analyses were performed using SPSS software (SPSS Science, Chicago, Illinois, USA). ANOVA with post LSD test was performed for comparison between the different groups. $P < 0.05$ was considered statistically significant.

Results

Histological examination

Light microscope examination of the liver sections of control showed liver lobules consisted of anastomosing cords of hepatocytes radiating from central vein. The hepatocytes were acidophilic and contained central pale stained nuclei and some were binucleated. The hepatocyte cords were separated by blood sinusoids. The control subgroups showed the same findings (Figure1). Each portal area at the corners of the hepatic lobules contained branches from hepatic artery, portal vein and bile duct (Figure 2). Examination of the liver sections of metalaxyl treated group (II) showed enlarged hepatocytes contained pale stained cytoplasm and some contained darkly stained nuclei. Some central veins were congested and the blood sinusoids were ill-defined in between hepatocyte cords. The bile ducts of some portal tracts appeared with thickened wall and surrounded by leucocytic cellular infiltrations (Figure 3 and 4). Examination of the liver sections of prophylactic group III, received NS concomitantly with metalaxyl, revealed preserved structure of hepatic tissue except the presence of slightly congested central veins and portal vein branches. Few hepatocytes appeared with darkly stained nuclei (Figure 5 and 6).

Ultra structural examination of the liver sections of control group showed hepatocytes contained euchromatic nuclei, mitochondria and rough endoplasmic reticulum. Some hepatocytes were binucleated. Bile canaliculi formed by the plasma membranes of adjacent hepatocytes and tightly bounded by junctional complexes. Short microvilli were projecting into the bile canaliculi and long microvilli were seen projecting toward the blood sinusoid (Figure 7 and 8).

Ultra structural examination of the liver sections of metalaxyl treated group revealed the presence of hepatocytes contained shrunken nuclei with more heterochromatin. Some vacuolations and irregular rough endoplasmic reticulum were observed in the cytoplasm of hepatocytes. Some spaces of Disse were narrow with disrupted microvilli (Figure 9). Hepatocytes with electron lucent cytoplasm, few organelles, lysosomes and phagosomes were also detected. Irregular short microvilli toward blood sinusoid were noticed (Figure 10).

Biochemical results

In the metalaxyl treated animals (group II), the levels of serum GSH and liver tissue GPx activity was shown to be significantly decreased ($P < 0.05$) and the levels of serum and liver tissue MDA are shown to significantly increased ($P < 0.05$) when compared to control group. However, NS treatment simultaneously with metalaxyl (group III), showed significant increase in the serum GSH levels when compared to control group and also the serum and liver MDA levels decreased and liver GPx activity increased to nearly that of the control (Table 1).

Discussion

Experimental studies in mice demonstrated that liver is the primary target for metalaxyl-treated animals [33]. The results of the present work demonstrate histological and ultra structural alterations in the liver of albino rats. These alterations seems to follow almost the same

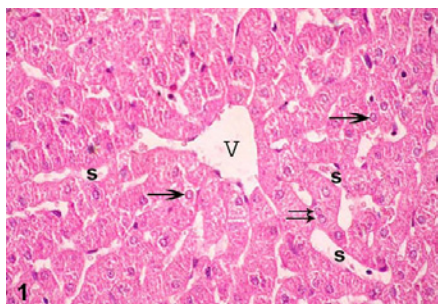


Figure 1: Photomicrographs of sections in the liver of *control group I* showing anastomosing cords of hepatocytes radiating from central vein (V) separated by blood sinusoids (s) and the hepatocytes contains central pale stained nuclei (arrows) and some are binucleated (double arrow).

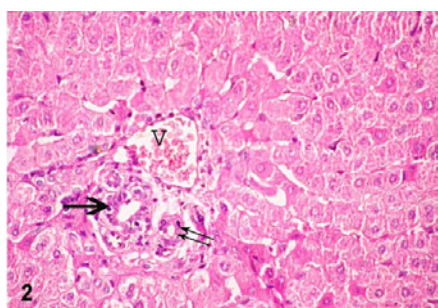


Figure 2: The portal area contains branches from hepatic artery (double arrow), portal vein (V) and bile duct (arrow) (H&E X 400).

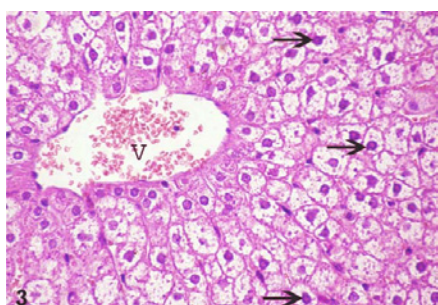


Figure 3: Photomicrographs of sections in the liver of *metalaxyl treated group II* showing enlarged hepatocytes containing pale stained cytoplasm and darkly stained nuclei (arrows), the central vein is congested (V) and the blood sinusoids are ill-defined. Leucocytic cellular infiltrations (thick arrows) around the thickened bile duct (d) are seen (H&E X 400).

pattern as that previously observed by other studies in the liver [34] and kidney [35] of albino mice treated with metalaxyl.

In the present work, examination of the liver of the metalaxyl treated group showed some cells contained shrunken nuclei with more heterochromatin which most probably indicating apoptosis. These observations are in consistence with previous investigators who concluded that metalaxyl induces apoptosis with bax expression in hepatocytes of mice [25]. Also, it has been reported that environmental stressors (metals, particulate matter, and pesticides) can induce apoptotic cell death [36-38]. It is well-known that apoptosis is an important and controlled form of cell death that occurs under a variety of physiological and pathological conditions [39]. The apoptosis induced by organophosphorus pesticide was reported to be induced

by activation of intracellular caspase-3 [40] or by oxidative stress and reactive oxygen species (ROS) which cause DNA damage triggering apoptosis [41].

In the present work, some hepatocytes of metalaxyl treated animals' showed signs of cell necrosis as enlargement in size with pale stained cytoplasm which was presented ultra structurally as electron lucent cytoplasm with few organelles. Congested central veins, irregular disrupted microvilli toward blood sinusoid and leucocytic cellular infiltration were also detected. These findings were previously detected by previous studies in mice [25]. Also, congestion of blood vessels, increase in number of Kupffer cells, cellular infiltration and hydropic degeneration were observed by previous investigators in liver of male rats treated with carbendazim fungicide [42]. These findings could be related to the disrupted microvilli as explained by the fact that the microvilli act as a diffusion barrier between membrane and cytoplasm by its actin-based cytoskeletal core structure, which inhibits the entrance of hydrophilic and lipophilic xenobiotics via microvilli into the cytoplasm. Furthermore, the polarized organization of microvilli to various external signals generates a basal-to-apical lipid flow that clears the plasma membrane from lipophilic xenobiotics [43]. The Leucocytic cellular infiltration of hepatic tissues could be considered as a sign of an immune response as a result of the repeated exposure of the hepatic tissues to the toxic compounds of metalaxyl as these inflammatory cells play an important role to overcome the toxic compounds invading the hepatic tissues [44].

In the present work the histological alterations observed in hepatic tissue due to metalaxyl administration were accompanied by significant

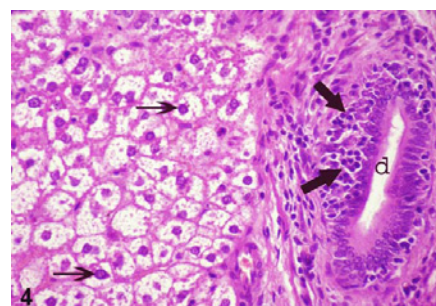


Figure 4: Photomicrographs of sections in the liver of *metalaxyl treated group II* showing enlarged hepatocytes containing pale stained cytoplasm and darkly stained nuclei (arrows), the central vein is congested (V) and the blood sinusoids are ill-defined. Leucocytic cellular infiltrations (thick arrows) around the thickened bile duct (d) are seen (H&E X 400).

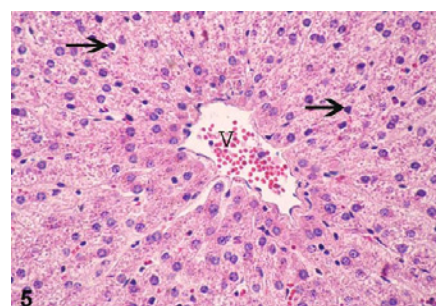


Figure 5: Photomicrograph of sections in the liver of *prophylactic group III* showing congested central vein (V) and few hepatocytes with darkly stained nuclei (arrows), portal area containing congested portal vein branch (V) (H&E X 400).

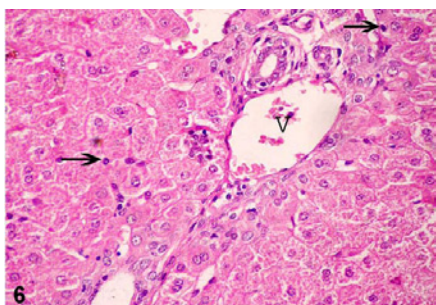


Figure 6: Photomicrograph of sections in the liver of *prophylactic group III* showing congested central vein (V) and few hepatocytes with darkly stained nuclei (arrows), portal area containing congested portal vein branch (V) (H&E X 400).

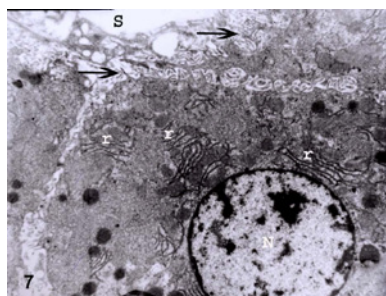


Figure 7: An electron micrograph from a section in the liver of *control group I* showing hepatocyte with euchromatic nucleus (N) and regular rough endoplasmic reticulum (r) and long microvilli (arrows) projecting toward blood sinusoid (S). (TEM X 11000).

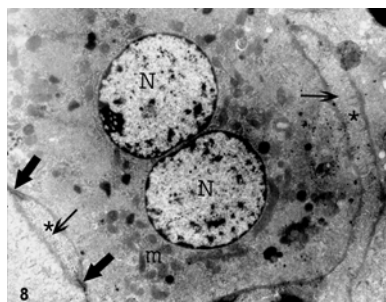


Figure 8: An electron micrograph from a section in the liver of *control group I* showing hepatocyte with euchromatic double nuclei (N). The cytoplasm contains mitochondria (m). Short microvilli (arrows) are projecting into the bile canaliculi (*) sealed by tight junctions (thick arrows) at both sides (TEM X 10000).

increase in serum and liver MDA, the end product of lipid peroxidation with significant decrease in serum GSH and liver GPx indicating that the toxic effect of metalaxyl could be mediated by the process of oxidative stress. This finding is in consistency with previous investigators who suggested that treating animals with metalaxyl induces a significant increase in the oxidative stress, MDA and a significant decrease in the level of serum antioxidant enzymes, superoxide dismutase and catalase [45]. Also, mancozeb fungicide induces cell damage which is closely associated with increase in lipid peroxidation and the decrease in the antioxidant enzymes [46]. Increased intracellular ROS production exceeding the antioxidant defense capacity of the cell leads to lipid peroxidation and generalized oxidative damage to all mitochondrial components [41].

In current study, NS treatment simultaneously with metalaxyl prophylactic, group III leads to preservation of the liver histological structure with reduction of serum and liver MDA enzyme levels when compared to control rats and also increased the GSH and GPx activity to nearly that of the control. These findings are supported by previously performed clinical and experimental investigations that have shown that NS has a protective effect against oxidative damage in isolated rat hepatocytes [47]. Furthermore, NS has antioxidant activity by suppressing the phagocytes [48]. Also, previous investigators demonstrated that crude extracts of the black seeds and some of its active constituents (volatile oil, TQ) might have a protective effect against nephrotoxicity and hepatotoxicity induced by either disease or chemicals [49]. NS was reported to have a significant hepatoprotective effect in carbon tetrachloride-treated administrated rabbits, and that hepatocellular degenerative and necrotic changes are slight without advanced fibrosis and cirrhotic process possibly through immunomodulation and antioxidant activities [50].

In the present study, some fat globules were observed in the cytoplasm of some hepatocytes of prophylactic group III. This may be explained by previous researchers who attributed this fat infiltration as a defense mechanism by which hepatocyte attempt to collect all toxic compounds invading the cell in these vacuoles prior to excretion [51]. During lipid peroxidation caused by ROS, the mitochondria are often associated with fatty acid-containing oil droplets from which they derive raw materials for oxidation and lipid synthesis and metabolism occurs primarily in mitochondria [52].

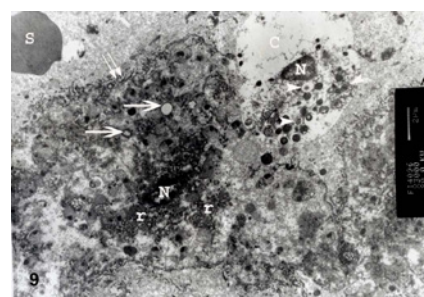


Figure 9: An electron micrograph of a section in the liver of *metalaxyl treated group II* showing hepatocyte with electron lucent cytoplasm (C) containing few organelles and heterochromatic nuclei (N). Cytoplasmic vacuolations (arrows), irregular rough endoplasmic reticulum (r) and phagosomes (arrow heads) are noticed. Irregular short microvilli (double arrow) toward blood sinusoid (S) and ill-defined cell boundaries are also noticed (TEM X 7000).

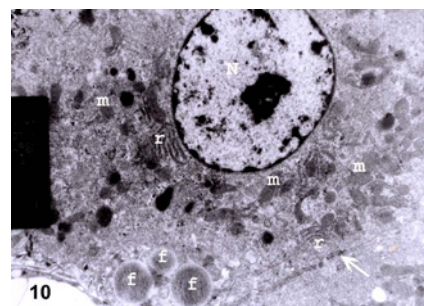


Figure 10: An electron micrograph from a section in the liver of *prophylactic group III* showing hepatocyte with euchromatic nucleus (N), regular rough endoplasmic reticulum (r) and mitochondria (m). Cytoplasmic fat globules (f) are noticed. Tight junction (arrow) between two hepatocytes is also noticed (TEM X 11000).

Group	Serum GSH (mg/dL)	Serum MDA (nmol/mL) erythrocytes	Liver GPx (μmol of NADPH oxidized/min/ protein)	Liver MDA (nmol/ mg) Liver Tissue
Group I (Control)	50.63 ± 3.41	1.18 ± 0.14	9.44 ± 0.92	0.010 ± 0.002
Group II (Metalaxyl)	40.21 ± 2.38*	2.81 ± 0.05*	4.41 ± 0.51*	0.033 ± 0.007*
Group III (Metalaxyl & NS)	61.12 ± 8.26*	1.21 ± 0.12	8.97 ± 0.67	0.011 ± 0.005

*Significant

Table 1: Serum GSH & MDA and Liver tissue GPx & MDA among studied groups.

Conclusion

Metalaxyl causes histological and biochemical changes in liver most probably through oxidative stress. NS therapy could ameliorate these changes in liver and this may be attributed to its antioxidant and free radicals scavenging properties. This may indicate the effectiveness of NS in prevention of metalaxyl hepatotoxicity. Further studies are required to evaluate the possible hepatoprotective effect of NS which is traditionally used as a medicine for many complaints including liver diseases.

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Aim of Work

This study aimed to elucidate the histological, ultra structural and hence, biochemical alterations in the liver tissue caused by metalaxyl and to investigate the possible protective effects of NS against these alterations.

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