

Hepatoprotective Effects of Chitosan and Chitosan Nanoparticles against Biochemical, Genetic, and Histological Disorders Induced by the Toxicity of Emamectin Benzoate

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Abstract

Background: Emamectin benzoate (EMB) is a biopesticide which used in agriculture as an insecticide. It is easier to reach ecologically and affects human health. This study aims to evaluate the protective effect of chitosan and chitosan nanoparticles against EMB-induced hepatotoxicity.

Methods: Male mice were distributed into four groups: G1: the negative control, G2: EMB group (5 mg/kg diet), G3: EMB with Chitosan, (600 mg/kg diet), and G4: EMB with Chitosan nanoparticles (600 mg/kg diet). The experiment continues for 8 weeks, and the animals were sacrificed, and their organs were removed and immediately weighed after sacrifice. The liver was quickly removed and processed for histopathological and genetic studies.

Results: Emamectin benzoate (EMB) treatment induced oxidative stress by increased levels of Malondialdehyde (MDA), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) with inhibition of acetylcholinesterase (AChE), Superoxide dismutase (SOD) and Catalase (CAT) levels. EMB produced several histopathological changes in the liver. Relative expressions of studied genes elevated in the liver with increase in DNA damage. Co-treatment with chitosan and chitosan nanoparticles reduced EMB related liver toxicity that belong to biochemical, histopathological, gene expression, and DNA damage by increasing antioxidant capacity.

Conclusions: This study offers insight into the potential for Chitosan and chitosan nanoparticles as a novel natural material against the oxidative stress induced by EMB.

Keywords: Chitosan Nanoparticles, DNA Fragmentation, Emamectin Benzoate, Gene Expression, Hepatotoxicity.

Introduction

Pesticides used in public health and agriculture have various actions on metabolic mechanisms. It affects on non-target animal and human health (1). One of macrocyclic lactone is Emamectin benzoate (EMB). Although the data about the effects of EMB on antioxidant status is insufficient, many studies have confirmed that EMB insecticides created oxidative stress in intoxicated animals (2). Chitosan, a deacetylated chitin derivative. It has attracted

interest as a biomedical plant due to its rare biological activities. The biological activities include anti-tumor, antioxidant, immuno-enhancing effects (3, 4). On the other hand, chitosan nanoparticles (CNPs) display greater behavior than chitosan (5). It increases the immune-enhancing effects, anticancer, and antimicrobial activity than those of chitosan. In addition, compared to large particles, chitosan nanoparticles have a greater surface curvature;

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Received: 15 May, 2021; Accepted: 26 Jul, 2021

this creates more dissolution pressure with a resulting rise in solubility in saturation (5).

Consequently, this study aims to evaluate the protective role of chitosan and chitosan nanoparticles against oxidative stress and hepatotoxicity induced by EMB in mice.

Materials and Methods

Ethics approval

This experiment was carried out under Egyptian ethical codes for studies on experimental animals and approved by the Ethics Committee of Al-Azhar University. The experimental protocol was approved by

the Biological and Environmental Sciences Department, Faculty of Home Economics, Al-Azhar University, Egypt.

Preparation nanoparticles of chitosan

Chitosan with molecular weight 100,000-300,000 was obtained from Cornell Lap Company, Egypt. For preparing chitosan nanoparticles, we used a laboratory mortar. Chitosan was ground in a laboratory mortar for 9 hours until it turns into a very fine powder (up to down method) (6). The nanoparticles size was measured by transmission electron microscope (Fig. 1).

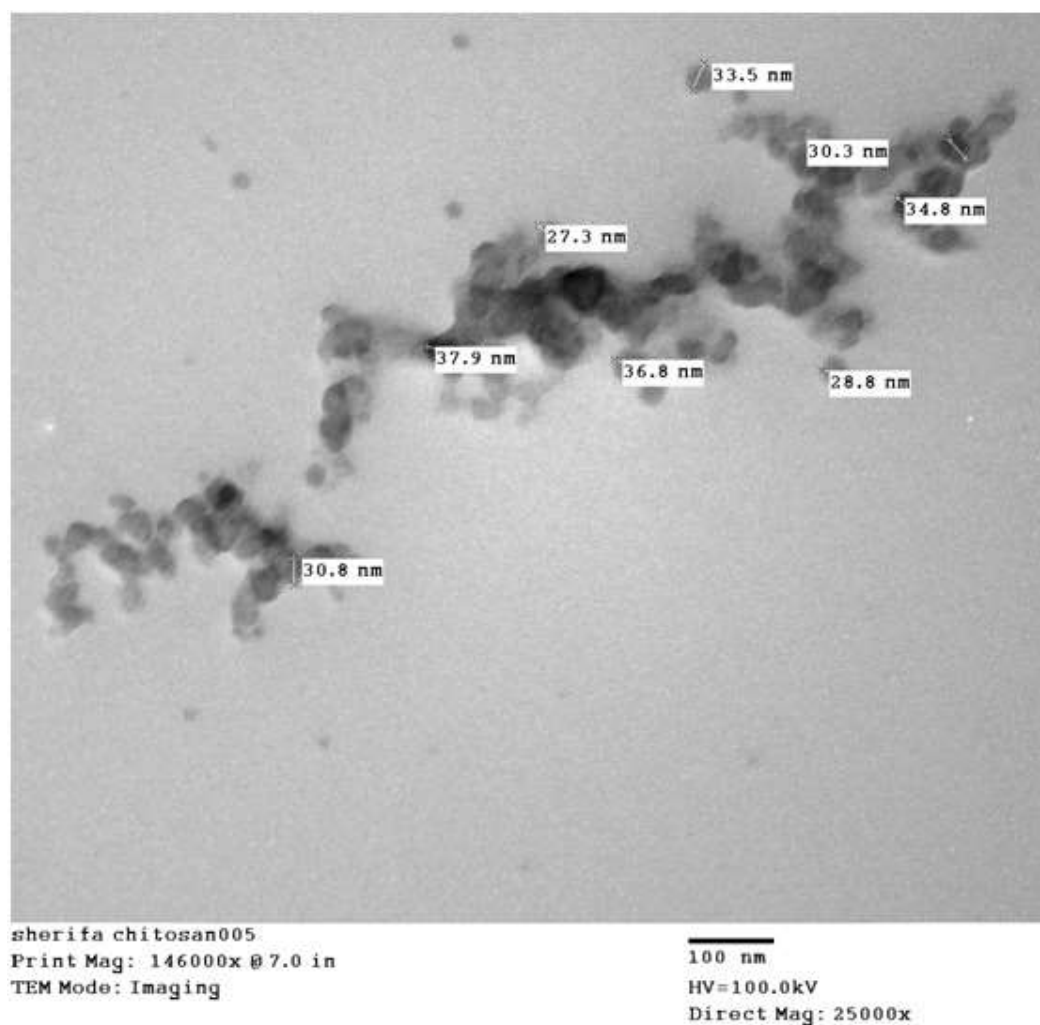


Fig. 1. Transmission electron image of chitosan nanoparticles. The average size of particles is 32.5 nm.

Animals and groups

Thirty-six male albino mice (25 g) were used. The mice were left one week for acclimatization before the beginning of the experiment, then

were divided randomly into four groups, with nine animals for each group. They were treated as follows:

G1: mice are given a diet without any additives.
G2: mice are treated with EMB (Speedo 5.7%) at a dose of 5 mg/kg diet (equivalent to 1/10 LD50) (7).

G3 (EMB+ ChS): mice have treated chitosan (600 mg/kg diet) with EMB (8).

G4 (EMB+ChS.Np): mice have treated chitosan nanoparticles (600 mg/kg diet) with EMB.

Eight weeks later, mice have fasted overnight. They were sacrificed after anesthetized using diethyl ether and their organs were removed and immediately weighed. The liver was quickly removed and processed for histopathological and genetic studies.

Antioxidant's activities and oxidative markers in the liver tissues

Malondialdehyde (MDA) (9), Catalase (CAT) (10), and Superoxide dismutase (SOD) (11) were quantified in Liver tissues homogenates.

Biochemical analysis

Blood samples were taken from hepatic portal vein and centrifuged after coagulating. Serum samples were kept at -20°C until used for biochemical assays, AST and ALT (12).

Determination of Acetylcholinesterase (AChE) level in the blood

The level of AChE was measured through the

acetylthiocholine hydrolysis. The incubation mixture consists of 1 mL contained Tris-HCl (50 mM) with pH 8, NaCl (120 mM), sucrose (240 mM), and a protein concentration of 80–100 $\mu\text{g}/\text{mL}$. With adding 5,5'-dithionitrobenzoic acid (DTNB) (0.03 mL) to acetylthiocholine iodide (0.05 mL) the reaction was started. The final DTNB concentrations and acetylthiocholine iodide were 0.125 and 0.5 mM, respectively. We used Spectrophotometer to measure the reaction at an absorbance of 412 nm (13).

Molecular analysis

Total RNA from the liver was isolated using the RNeasy kit (Qiagen). The purity and integrity of RNA were assessed by NanoDrop, and 1% agarose gels electrophoresis, respectively. The Quantiscript reverse transcriptase (name of company) is used in RNA reverse transcription to cDNA. Real-time PCR reaction contains cDNA as a template in the presence of QuantiTect SYBR Green qPCR Master Mix and gene-specific primers, designed by the *Primer 3 web-based tool* (Table 1), along with Step One Plus real-time PCR system (Applied Biosystem, USA) (14). The critical threshold (Ct) quantities for the target genes were normalized with quantities of the Ct of the internal control (β -actin).

Table 1. Forward and reverse primers used for real-time PCR reaction.

Gene	Forward primer (5'____3')	Reverse primer (5'____3')
<i>Mgst1</i>	TTTTGCCAACCCGGAAGACT	GAGGCCGATACCGAGAAAGG
<i>Cyp2E1</i>	CTCCTCGTCATATCCATCTG	GCAGCCAATCAGAAATGTGG
<i>Caspase 3</i>	GGTATTGAGACAGACAGTGG	CATGGGATCTGTTTCTTTGC
<i>IL1b</i>	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC
β -actin	AAGTCCCTCACCTCCCAAAG	AAGCAATGCTGTACCTTCCC

Comet assay

To perform Comet assay, a cell pellet was placed in 1 mL ice-cold PBS containing 20 mM EDTA/10% DMSO and were then minced into fine pieces and stirred for 5 min and filtered. After stirred and filtered, 100 μl of cell suspensions were integrated with 600 μl of agarose (13). This mixture was spread on

slides and immersed in lysis buffer for 15 min. Then they were placed in the electrophoresis chamber. The DNA fragment migration patterns were evaluated with a fluorescence microscope at a magnification of 40x and with excitation filter 420-490 nm. Komet 5 image analysis software (Kinetic Imaging, Ltd., Liverpool, UK) was used for images analysis.

Histopathological examination

Small parts of the liver were fixed in formalin solution (10%), dehydrated in ethanol from 70% and 100%, then, cleared in xylene, and immersed in paraffin. The sections of the liver stained with Eosin and Hematoxylin dyes (15).

Statistical analysis

The statistical significance was evaluated by one-way ANOVA using SPSS, 20 software, and the individual comparisons were obtained by Duncan's multiple range test.

Results**Body and organs weights.**

EMB group showed a significant decrease in the bodyweight compared to the negative control. However, the treatment with ChS and ChS.Np enhances body weight and removes the side effects of EMB. The weight of organs in EMB showed a decrease in all organs, this decrease was significant in the kidney and spleen compared to the negative control. There is a recovery in the organ's weight when treated with ChS and ChS.Np (Table 2).

Table 2. Effect of ChS and ChS.Np on the body and organs weight of mice exposed to EMB (mean±SD).

Treatment	Body weight (g)	Organ's weight (g)				
		Kidney	Testis	Liver	spleen	heart
G1	33.78±3.25 ^a	0.29±0.04 ^a	0.13±0.02	1.89±0.36 ^a	0.28±0.02 ^a	0.19±0.03
G2	24.08±3.61	0.21±0.05 ^c	0.12±0.03	1.23±0.49 ^b	0.18±0.04 ^b	0.16±0.03
G3	30.50±5.99 ^a	0.27±0.03 ^{ab}	0.13±0.03	1.55±0.31 ^{ab}	0.17±0.06 ^b	0.18±0.03
G4	30.42±4.14 ^a	0.23±0.06 ^{bc}	0.13±0.01	1.52±0.24 ^{ab}	0.18±0.04 ^b	0.17±0.01
Sig.	0.00	0.00	0.87	0.09	0.012	0.42

G1: control, G2: EMB, G3: ChS: G4: ChS.Np: sig: significant at $p < 0.05$, the values with different letters in each column showed a significant difference.

Antioxidants levels and oxidative markers

In EMB, an increase in MDA and decrease activities of SOD and CAT when compared to levels in the control (Table 3). The SOD levels were significantly elevated when treated with ChS and ChS.Np compared to the mice that received the EMB alone. There is considerable protection in reducing the levels of MDA in the treated groups by ChS and ChS.Np.

Biochemical analysis

Table 3 also shows the results of liver biomarkers alanine aminotransferase (ALT) and aspartate aminotransferase (AST). All liver biomarkers were significantly increased in the EMB-treated mice as compared to the negative control. Co-treatment EMB with ChS, and ChS.Np, resulted in a significant recovery of the liver biomarkers.

Table 3. Effect of ChS, and ChS.Np on MDA, CAT, SOD, alanine aminotransferase and aspartate aminotransferase in mice exposed to EMP (mean±SD).

Treatment	MDA u/mg	CAT u/mg	SOD u/mg	AST u/mg	ALT u/mg
G1	2.76±0.24 ^b	86.76±1.45 ^a	2.76±0.57 ^a	32.00±2.64 ^b	42.46±3.36 ^b
G2	3.70±0.26 ^a	82.02±1.18 ^b	1.90±0.28 ^b	59.00±6.55 ^a	71.37±3.24 ^a
G3	2.80±0.19 ^b	84.76±1.66 ^{ab}	3.30±0.40 ^a	32.75±4.57 ^b	44.06±3.35 ^b
G4	2.93±0.25 ^b	81.53±3.04 ^b	3.42±0.41 ^a	35.50±4.50 ^b	41.48±5.38 ^b
Sig.	0.00	0.02	0.02	0.00	0.00

MDA: malondialdehyde, CAT: Catalase, SOD: Superoxide dismutase, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase. Means followed by a similar letter within a column for each parameter are not significantly different at the 0.05% of probability by Duncan's Multiple –Range Test.

Acetylcholinesterase (AChE) activity in the mice liver

The mice treated with EMB showed a significant ($p \leq 0.05$) lower activity of AChE compared with the negative control. The treatment with ChS,

and ChS.Np has recorded a significant recovery for the activity of AChE. Consequently, the treatment with ChS, and ChS.Np alleviated the harmful effects of the pesticide concerning acetylcholinesterase (Fig. 2).

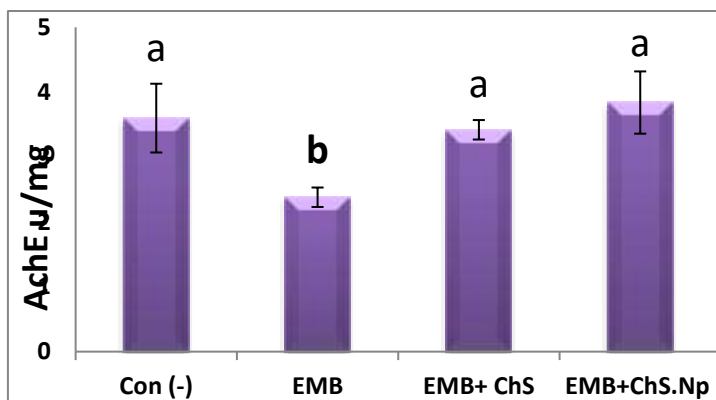


Fig. 2. AChE level in mice liver. Values with different letters in each column showed a significant difference.

Molecular investigation

The qPCR results showed a significant upregulation in the expression levels of *Mgst1*, *Cyp2E1*, Caspase3 and *IL1b* genes in the mice liver treated with EMB as compared to the

negative control group (Fig. 3). This elevation expression was significantly downregulated following treatment with ChS and ChS.Np with the lowest expression in ChS.Np.

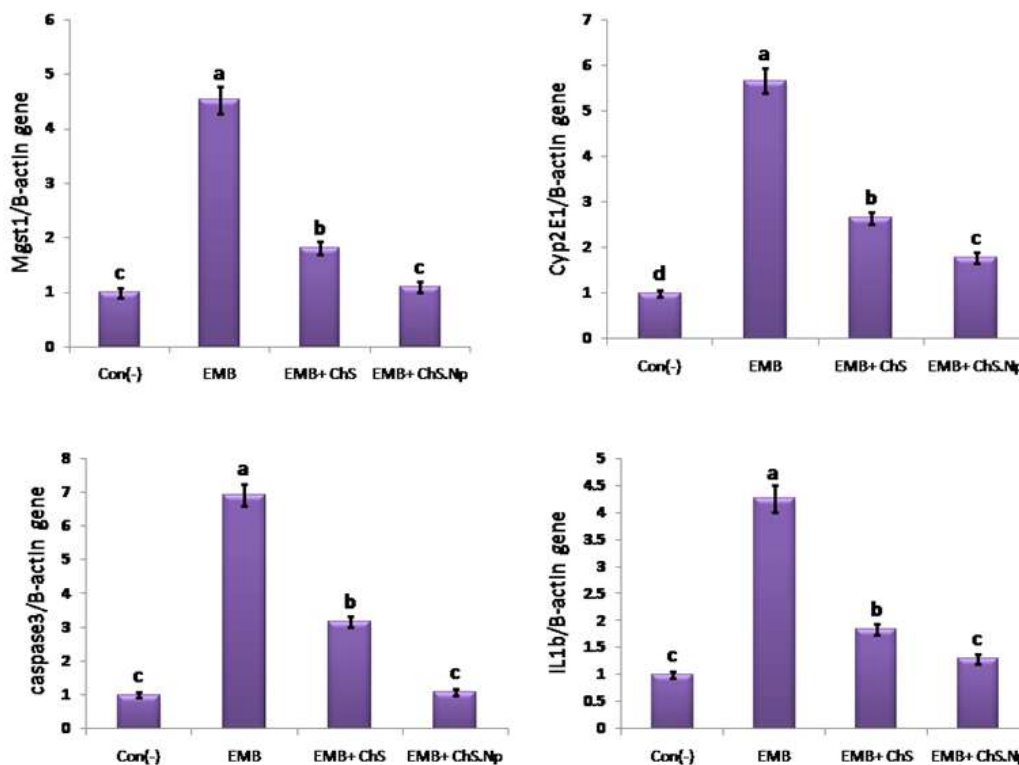


Fig. 3. Relative expression of *Mgst1*, *CYP2E1*, Caspase 3, *IL1b* (compare to β -actin as internal control) genes in the liver of mice treated with EMB only or combination with chitosan or chitosan nanoparticles.

Comet assay

The results of comet assay were shown in Fig. 4 and Table (4). Data showed that EMB had a significant increase in DNA damage as indicated by an increase in tail length, tail

DNA% and tail moment as compared to the normal control. This elevated DNA damage was reduced following treatment with ChS and ChS.Np with the lowest damage in ChS.Np.

Table 4. Comet parameters in mice treated with EMB only or combination with ChS and ChS.Np.

Group	Tailed %	Untailed %	Tails length μm	Tail DNA%	Tail moment
G1	1.75	98.25	1.45 \pm 0.53 ^d	1.36	1.97 \pm 0.69d
G2	32	68	9.63 \pm 1.27 ^a	7.41	71.36 \pm 9.10a
G3	11	89	5.49 \pm 0.74 ^b	3.87	21.25 \pm 3.23b
G4	8	92	4.52 \pm 0.63 ^c	3.02	13.65 \pm 1.67c

Different superscript letters in the same column of tail length showed significance difference at $p < 0.05$.

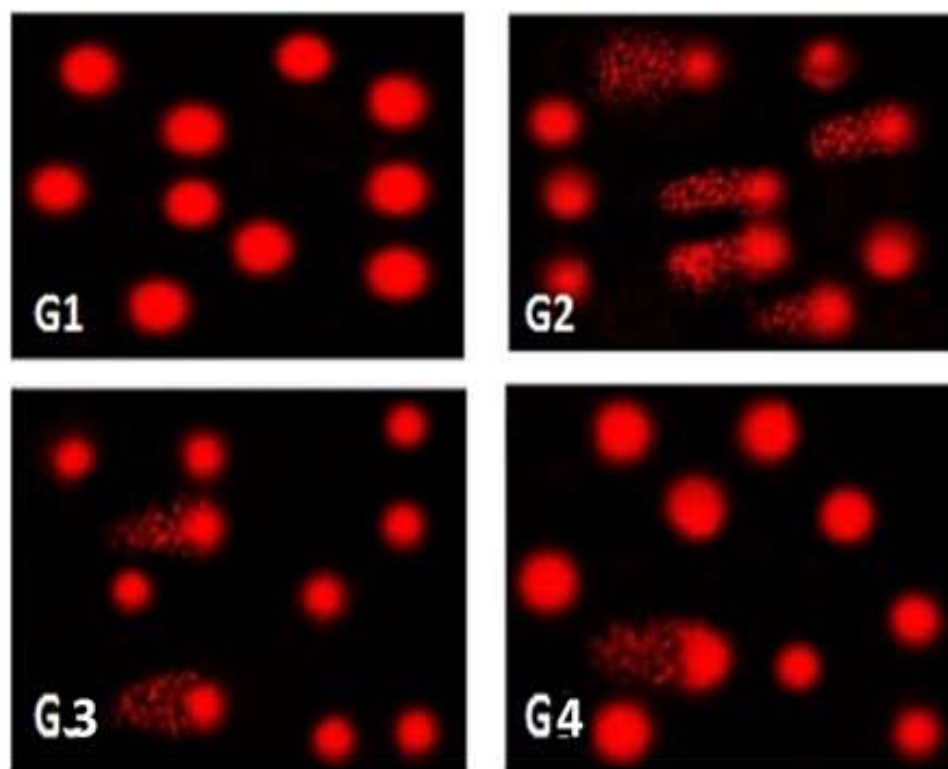


Fig. 4. Representative images of the cells assayed by the comet assay. where G1= negative control; G2= mice treated with EMB; G3= EMB+ChS; G4= mice treated with EMB+ChS.Np.

Histopathological findings

The most histological effects related to the treated mice with EMB were dilatation and congestion of blood vessels and sinusoids, degenerated cytoplasm of the hepatocytes,

karyolysis and pyknotic of some nuclei, infiltration of lymphocytes, vacuolation, and degeneration of cytoplasm, and proliferation of Kupffer cells (Fig. 5, G2). ChS and ChS.Np enhanced the architecture of liver.

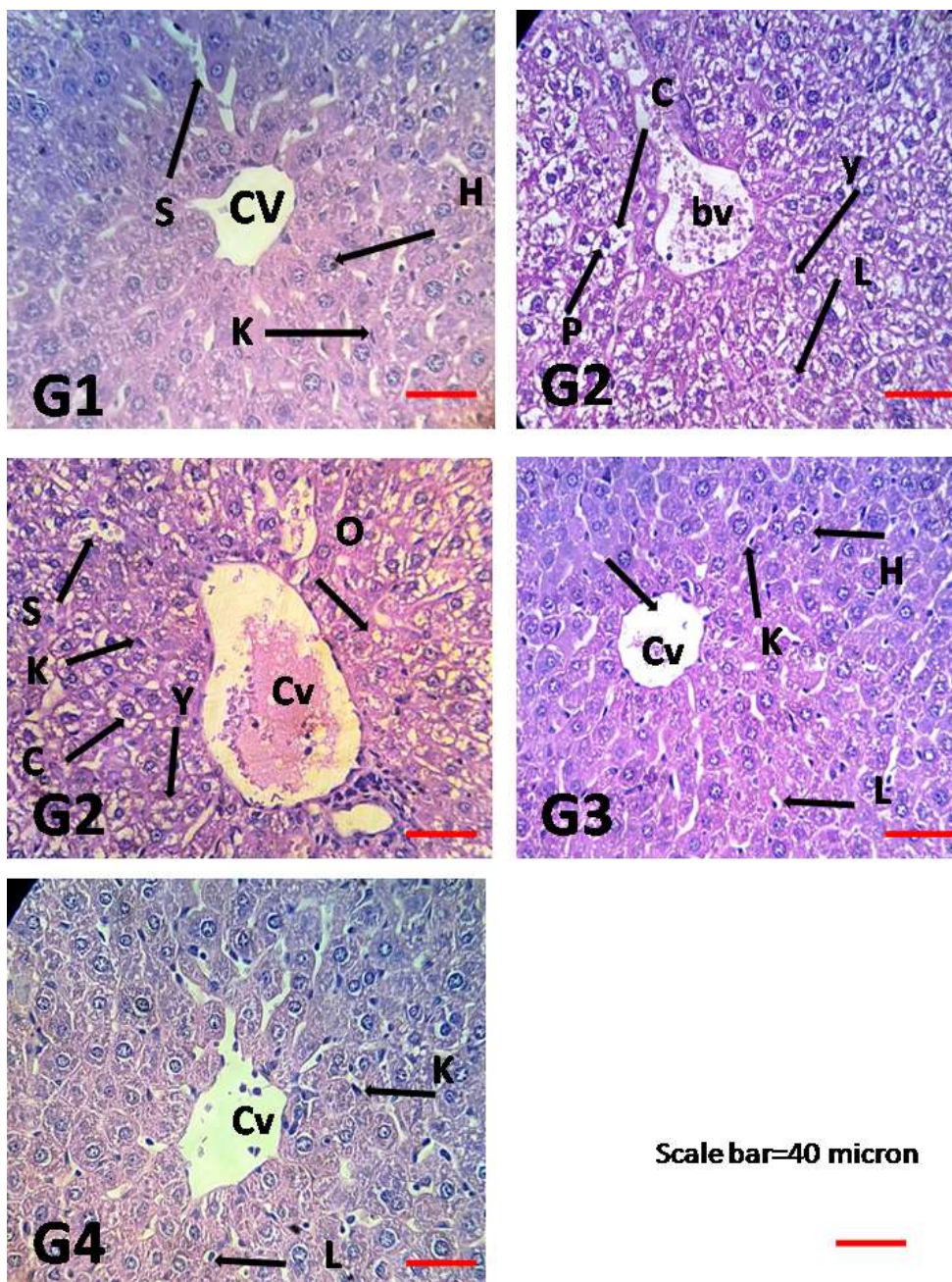


Fig. 5. G1: Section in the liver of control mice showing normal appearance of hepatic cells (H) radiating from a central vein (cv). Also, sinusoids (S) and Kupffer cells (K) are noticed. G2: the liver of mice treated with EMB showing dilatation and congestion of blood vessels (bv), degenerated cytoplasm (c), karyolysis (Y) and pyknotic (p) nuclei, and infiltration of lymphocytes. G2: another section in the previous treatment showed congestion of portal vein (PV), vacuolation and degeneration of cytoplasm (v), karyolysis of nuclei (Y), proliferation of Kupffer cells (K); dilatation and congestion of sinusoids (S). G3: the liver of mice co-treated with EMB and chitosan showing normal central vein (CV), normal hepatic cells (H), normal sinusoids (S), slightly of Kupffer cells (k) and slightly infiltration of lymphocytes (L). G4: the liver of mice treated with EMB and chitosan nanoparticles showing normal central vein (CV) slightly of Kupffer cells (K), and slightly infiltration of lymphocytes (L).

Discussion

Body and organs weights

Our results belong to body weight (Table 2) agree with Khaldoun *et al.* (16). They found a decrease in the body weight after treatment

with EMB. These decrease due to overstimulation of cholinergic. It causes rise in gastric motility and a reduction in food

absorption. Treatment with ChS and ChS.Np effectively alleviates EMB-induced body weight decrease and improves bodyweight. Interestingly, ChS or ChS.Np-treated mice showed no major difference in body weight when compared to the control because they have antioxidant, anti-inflammatory, and antifibrotic characteristics (17).

Antioxidants and oxidative markers

EMB-treated mice showed inhibition of SOD and CAT activity. This could lead to the creation of reactive oxygen species (ROS), namely H₂O₂. The accumulation of H₂O₂ can activate some signaling pathways and lead to oxidative stress (18). On the other side, EMB significantly amplified MDA concentration that considers a biomarker for oxidative stress (19). This increase in MDA agrees with the decrease in CAT and SOD in EMB-treated mice. Besides, the elevated level of MDA interacts with DNA causing mutagenic effects (20). Subsequently, DNA damage in current results compatible with MDA results (Fig. 4). These findings are parallel with those recorded by (21). They notice avermectin inhibited the activity of SOD and increased the level of MDA. Many substances can ameliorate ROS induction by EMB. Abou-Zeid *et al.* (22) used pumpkin seed oil with EMB to mice that ameliorated the toxic effects including oxidative stress. Our present study revealed that co-administration of EMB with ChS and ChS.Np ameliorated the toxic effects including MDA and SOD activities, while there is no significant effects belong to CAT activities. Subhadrappa *et al.* (23) confirmed a significant increase in CAT and SOD activity in CCl₄-induced rats when treated with β -chitosan. These findings indicated that chitosan reduced tissue damage because of involving in reducing both oxidative stress and free radicals. It may be possible that the mechanism of ChS and ChS.Np protection is due to its antioxidant effect belong to SOD not CAT (23). The effect of ChS and ChS.Np on MDA and didn't affect on CAT activities lead to say that there is another mechanism in reducing

MDA don't depend on CAT and depend on SOD only.

Biochemical analysis findings

Liver enzymes (ALT and AST) are widely used to evaluate liver function (24). With a hepatocellular injury, the secretion of these enzymes increases in the blood. ALT and AST noticeably increase with EMB. This increase is sustaining the hypothesis that exposure to pesticides results in biochemical liver toxicity (25). The results agree with (26) who reported a marked elevation of AST and ALT levels in the plasma of EMB-treated rats. These alterations of AST and ALT activity may be due to necrotic changes of hepatic tissue that appear in histopathological examination (Fig. 5). Also, the damage attributed to the EMB-toxic effects because the production of ROS causing damage to the diverse membrane components of the cell. This damage leads to the infiltration of cytoplasmic enzymes (27). Amelioration of the adverse effect of EMB on ALT and AST appears when ChS, and ChS.Np is treated with EMB. In addition, our histological data further supported the AST and ALT results (Fig. 4). Subhadrappa *et al.* (23) said that the rats which received chitosan accompanying with the CCl₄-administration showed markedly decreased tissue and plasma AST and ALT activities. It means that chitosan can stabilize the cell membrane and avoid infiltration of intracellular enzymes within the blood. Administration of the EMB-treated mice with ChS.Np could markedly inhibit the serum level increase of liver enzymes it agrees with (28).

Acetylcholinesterase (AChE) activity in the mice liver

EMB inhibits the activity of AChE, consequently, the acetylcholine doesn't decompose into acetic acid and choline and aggregation in acetylcholine form. So, the excitement doesn't stop and will lead to death (29). Our results (Fig. 2) agree with (16) who observed a significantly lower activity of AChE in EMB-treated groups compared with

the normal control group. Co-treatment EMB with ChS and ChS.Np resulted in a significant recovery of AChE.

Molecular investigation findings

These results agree with (22) they reported that the Mgst1 and CYP2E1 gene expression markedly increased in the kidney and liver of Emamectin-treated mice. Also, these results agree with those of (30) they indicated that the CYP gene expression was upregulated by the treatment with EMB in trout fish. As a result, this suggests that Emamectin changes the transcriptional process of proteins implicated in the distribution, metabolism, and elimination of xenobiotics (22). Similarly, this gene activity has been observed to be increased with treatment by many toxicants (31). Mgst1 activation may be contributed to the depletion of glutathione and oxidized glutathione accumulation. The different measurements refer to the activation of ROS related to the activation of Mgst1. This correlates with our results where Emamectin activated the CYP2E1 expression and subsequently increased the Mgst1 expression. Following our findings, the activation of CYP2E1, which causes extensive ROS release in a hepatoma cell line, was shown to be improved the expression and activation of Mgst1 (32).

Our findings revealed that subchronic treatment of EMB to mice caused a markedly upregulation in the caspase3 gene expression levels in the liver. These results agree with (33) they observed the caspase-3 gene expression obviously increased after administrated rats with EMB. The apoptosis is activated by EMB linked to ROS generation. In turn, ROS triggers the activation pathway of the mitochondrial-dependent intrinsic. It leads to disruption function of mitochondria and consequent potential mitochondrial membrane breakdown then the release of cytochrome-c (34).

Our results revealed mice are treated with EMB produces a significant upregulation in the IL1b gene expression levels in the liver. These outcomes are in parallel to that reported

by Liu (35). They found avermectin causes immune suppression by increasing the IL-1 β -mRNA levels in the pigeon. Also, our results agree with Duzguner and Erdogan (36) who said that imidacloprid stimulated IL-1b expressions in the liver. Chitosan possesses many biological activities like antioxidant activity (37). Also, chitosan nanoparticles have various activities like anti-inflammatory activities, and antioxidants (38). The results refer to treatment with ChS. and ChS.Np make a significant downregulation in the levels of genes expression in the liver. Increased CYP2E1 expression levels in EMB-treated mice indicated that EMB induced oxidative stress also; chitosan beside chitosan nanoparticles ameliorated the side effects of EMB by decreasing the CYP2E1 expression levels (39). The results agree with Sudjarwo *et al.* found that nanoparticle of chitosan-P. merkusii reduced caspase-3 expression in the liver, significantly (40).

Comet assay

Emamectin administration to mice induced a marked increase in liver DNA fragmentation. These results agree with (34), they detected apoptosis in cells of human liver exposure to EMB by enhanced caspase-3 level and increased DNA fragmentation. Our results agree with (33) they showed that EMB oral intake for rats causes an increase in DNA damage. Insecticide's exposure is reported to produce DNA break that leads to the process involved in cells genotoxic (41). The fragmentation of nuclei and condensation of chromatin were noticed resulting from EMB exposure in Sf-9 cells (42).

Otherwise, the treatment with ChS and ChS.Np reduces the tail length, tail DNA%, and tail moment. High activity in scavenging free radicals is one of operation in reducing the DNA damage (43). Chitosan nanoparticles decreased DNA fragmentation (44) this agrees with the results regarding chitosan nanoparticles. The ability of ChS and ChS.Np to inhibit DNA damage is because of containing high flavonoid and polyphenol content (45).

Histopathological findings

The results belong to histopathological studies with EMB treatment agree with Khaldoun (26) and Abou-Zeid (22). They indicated EMB administration produced pathological changes in the liver. The EMB-side effects cause by the production of ROS that causes many changes in the cell. This clearly appears in increasing MDA (Table 3). ROS generated by EMB can damage membrane components of the cell and lead to the leakage of cytoplasmic enzymes. This appears in ALT and AST activities (Table 3). Also, the oxidative stress can lead to apoptosis (46). This clearly appear in gene expression of caspase3 (Fig. 2). It is the first report that proved the treatment with ChS is effective in the prevention side effects of EMB

that causes hepatic damage in rats. These results agree with Subhapradha (23). The mice liver treated with EMB plus ChS.Np showed a marked decrease in hepatocytes degenerative changes induced by EMB. El-Denshary et al. (47) showed the same results with ChS.Np on rat's liver treated with CCl₄. ChS.Np consider a scavenger for the free radicals that inhibit free radical formation (48).

Acknowledgements

The researchers are truly thankful to the members of Biological and Environmental Sciences Department, Al-Azhar University which they provided enormous facilities and support to accomplish this work.

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