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The Effect of Antioxidant Nutrition against Fenvalerate Toxicity in Rat Liver (Histological and Immunohistochemical Studies)

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Author's contribution

The only author performed the whole research work. Author AMA designed the study, wrote the protocol, managed the literature searches and approved the final manuscript. Author AMA read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: Fenvalerate (FEN) is a type II synthetic pyrethroid that has replaced other groups of insecticides due to its improved insecticidal potency. The objective of this study was to investigate the possible role of antioxidant nutrients as a protective agent against alterations of FEN in liver tissue of male albino rats.
Study Design: Histological and immunohistochemical studies.
Place and Duration of Study: Zoology Department, College of Science, Alexandria University - Egypt, between May 2010 and February 2013.
Methodology: Forty animals were divided into four groups of 10 rats each. The first group served as control which received corn oil , second group received a single dose (20mg FEN/kg) 24hours prior to decapitation , third group received (20 mg fish oil (ώ3) /kg/48h) and (4.1 mg selenium (Se) /kg/48h) for 20 days and fourth group received FEN following the supplementation with ώ3 and Se .
Results: Histopathological changes in the FEN group illustrated as degeneration and proliferation of hepatocytes forming acinar and pseudoglandular pattern. The previous changes disappeared from FEN+ (ώ3 and Se) group. The histochemical staining of catalase enzyme revealed increased activity in FEN, FEN+ (ώ3 and Se) groups while activity of glutathione reductase enzyme was decreased in compare with control group. Immunohistochemical staining of Bcl-2 oncoprotein increased in the cytoplasm of periportal and centrilobular hepatocytes in FEN and FEN+ (ώ3 and Se) groups, while it

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decreased in (ώ3 +Se) group.
Conclusion: It was suggested that FEN-induced dysregulation of architecture, antioxidant enzymes and expression of Bcl-2 oncoprotein which might be ameliorated by the effect of antioxidant nutrient.

Keywords: Fenvalerate; antioxidant nutrient; Bcl-2 oncoprotein; catalase and glutathione reductase enzymes.

1. INTRODUCTION

Synthetic pyrethroids are generally viewed as safe insecticides due to their low acute toxicity to mammals .The primary mechanism of toxic action of pyrethroids has generally been considered to be interference with the sodium gate in the nerve membrane [1]. Type II pyrethroids cause lower amplitude of the action potentials and, eventually, total blocking of neural activity because of a marked depolarization of the membrane. Fenvalerate (FEN) (cyano(3-phenoxyphenyl)2-(4chlorophenyl)-3-methylbutyrate) is a type II synthetic pyrethroid has replaced other groups of insecticides due to its improved insecticidal potency [2]. FEN displays moderate toxicity in mammals and is rapidly hydrolyzed in experimental animals to yield fenvaleric acid as a major metabolite [3]. The demonstration of oxidative stress in various tissues of rats was seen following administration of single and multiple FEN sublethal oral doses and suggests fenvalerate to be a potential tumour promoter [1].

Antioxidant nutrients are being widely studied for their possible beneficial properties in the prevention of human diseases including cancer, arthritis and cardiovascular diseases [4]. Selenium is an essential nutrient which is currently receiving much publicity for its anticarcinogenic potentials. Selenium is a structural component of several enzymes with physiological antioxidant properties including glutathione peroxidase (GSH-Px) and thioredoxine [5]. It plays an important role in the protection of tissues from oxidative damage and can antagonize the toxic effects of some chemical substances [6]. Because of the health problems induced by many environmental pollutants, many efforts have been undertaken in evaluating the relative antioxidant potential of selenium [7].

Unsaturated fatty acids (n-6 and n-3) have significant effects on cell growth, proliferation and trigger apoptosis in a number of cell types [8]. Omega-3 (ώ3) rich fish oil feeding may enhance the hepatic antioxidant defense by several mechanism(s) [9].

Bcl-2 is an integral membrane protein targeted to the outer mitochondrial membrane [10]. The Bcl-2 protein was found to play role in cell survival and inhibition of apoptosis rather than regulation of cell replication in species and cell types [11].

Intrinsic antioxidant defense systems such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) may participate in regulating the lipid peroxidation [12] and play a fundamental role in protecting cells and tissues from oxidative damage [13]. Nonenzymatic oxidation may also occur, and several oxidized fatty acid species are known to have biological activity [8].

Because of the potential of FEN to induce oxidative damage in various mammalian tissues in vivo had been comprehensively investigated. The present study was conducted to investigate the possible role of combined antioxidant nutrients as a protective

supplementation for FEN induced alterations in male albino rats liver by histological, histochemistical and immunohistochemical studies.

2. MATERIALS AND METHODS

2.1 Chemicals

Fenvalerate (FEN) (purity = 98%) was purchased from Chem. Service Inc. (West Chester, PA).Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH) and sodium salenite were purchased from Sigma Chemical Company (Saint Louis, USA). Rabbit antihuman Bcl-2 polyclonal antibodies Kites were purchased from (Dako*).* Omega-3 was purchased from (Pharm assure, USA).

2.2 Animals

Forty albino male rats with average body weight 200±50 g. were obtained from National Research Institute, Cairo, Egypt and acclimatized for 2 weeks prior to the experiment.They were housed in universal galvanized wire cages at room temperature (22-25ºC) in a photoperiod of 14 h light/10 h dark. Animals received standard laboratory balanced commercial diet and water *ad libitum.*"Principles of laboratory animal care" (NIH publication No.85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee"

2.3 Experimental Design

The animals were divided randomly into 4 groups 10 rats each. The first group served as control group which received corn oil. Group two received a single oral dose of (FEN) (20 mg/kg, dissolved in corn oil) by gavage 24 hours prior to cervical dislocation. Group three was orally supplemented with combined antioxidant nutrient of Fish oil (ώ3) (20 mg/kg/48h) and selenium (Se) (4.1 mg/kg/48h) for 20 days. Group four received both FEN and combined antioxidant nutrient (ώ3 +Se).

2.4 Tissue Preparations

Rats were fasted 24 hours prior to each designated time point and then sacrificed by cervical dislocation. Liver was removed immediately; one part was frozen at -30ºC for processing the frozen sections and other part was fixed in buffered formalin for processing the paraffin sections.

2.4.1 Histological technique

Paraffin sections (5µm thick) were deparaffinized, dehydrated and stained with haematoxylin and eosin for light microscopical examination.

2.4.2 Immunohistochemical technique

Paraffin sections (5µm) were deparaffinized, dehydrated, incubated in 3% (vol/vol) H_2O_2 in methanol (45 seconds) and washed in phosphate buffered saline (PBS). Sections were heated in a pressure cooker at 116ºC in 10 mM citric acid buffer (pH 6.0), followed by rinsing in tap water, washed in Tris buffered saline (TBS), pH 7.8, followed by blocking treatment

with 2% (wt/vol) bovine serum albumin (BSA).Rabbit antihuman Bcl-2 polyclonal antibodies was diluted in PBS and dropped on the slides, incubated overnight in a humidified chamber placed at 4ºC.They were washed with PBS and incubated at room temperature with biotinylated goat antirabbit antibody (diluted by Tris buffer saline) in a humidified chamber, followed by washing with PBS. Incubation at room temperature with streptavidin horseradish peroxidase diluted in Tris buffer saline) was then performed and the slides 155 were stained with chromogen (solution containing DAB and H2O2 (vol/vol)) [14]. Nuclear fast red was used as counterstain; the slides were left to dry and mounted by glycerin jelly.

2.4.3 Histochemical techniques

By using cryostat**,** the frozen liver was cut under -18ºC to detect the histochemical enzyme reaction. Control sections were boiled before incubation in the following medium:

2.4.3.1 Glutathione reductase:

The frozen sections (8µm thick) were incubated in a media containing NBT, NAD and glutathione dissolved in phosphate buffer pH 7.2 at 37ºC. Then washed in distilled water, left to dry, covered and mounted by glycerin jelly [15].

2.4.3.2 Catalase

The frozen sections (8 µm thick) were incubated in a media containing dimethylbenzedine (DAB) and H2O2, HCl –Tris buffer pH 7.2 at 37^{o}C, then washed in D.W., left to dry, covered and mounted by glycerin jelly (16).The intensities of staining were represented in a semi quantitative manner (+weak, ++ moderate, +++ strong, ++++ intense reaction) .

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Histological findings

Light microscopic examination of control rat liver revealed normal portal tracts at the periphery of the lobules having blood vessels and bile duct radicals. The radiating cord composed of hepatocytes. The polyhedral hepatocytes with acidophilic cytoplasm showed round, vesicular and centrally located nuclei. The hepatic blood sinusoids appeared as narrow spaces lined by endothelial and kupffer cells (Fig. 1a).

The histological examination of rat liver after oral administration of FEN showed several areas with marked hepatocytes proliferation forming acinar pattern, degenerated hepatocytes with dense pyknotic nuclei, marked sinusoid dilatation and sever cellular infiltration (Fig. 1b).Animals supplemented with the combined antioxidant nutrient (ώ3 + Se) showed nearly normal liver architecture had sinusoid lined with endothelial and kupffer cells (Fig. 1c).Animals supplemented with FEN and the combined antioxidant micronutrients (ώ3 + Se) showed areas of nearly normal hepatic parenchyma ,mild sinusoidal dilatation, disappearance of acinar pattern, reappearance of vesicuolated large nuclei and eosinoplillic cytoplasm. The bile ducts appeared as cuoboidal proliferating epithelial cells with low periportal cellular infiltration (Fig. 1d).

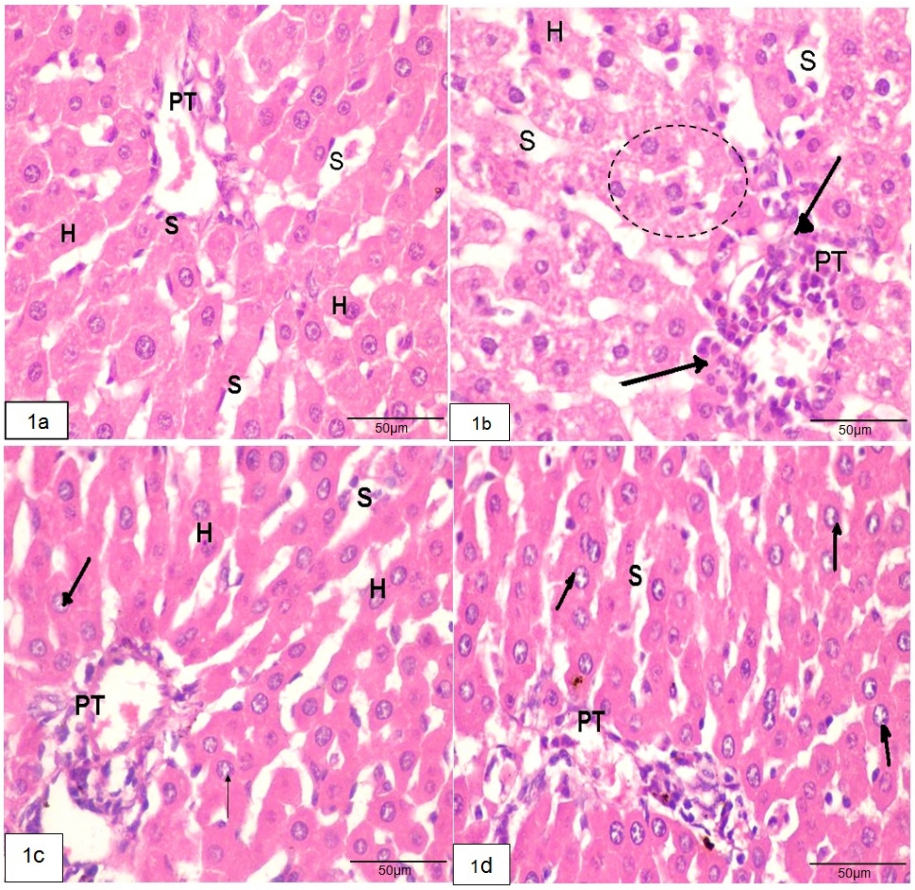

Fig. 1a) Paraffin section photograph of rat liver received corn oil showing: polyhedral hepatocytes (H) with acidophilic cytoplasm and round vesicular nuclei forming cords in-between hepatic sinusoids(S).Note, portal tract (PT) (H&E, Bar=50µm). Fig. 1b) Paraffin section photograph of rat liver received FEN showing: degenerative portal tract (PT) with strong cellular infiltration (arrows). Proliferating hepatocytes (H) forming acinar pattern (dashed line), vacuolated hepatocytes limited dilated sinusoid (S) (H&E, Bar=50µm). Fig. 1c) Paraffin section photograph of rat liver received combined nutrients showing: mild dilation of sinusoids(S), week periportal cellular infiltration (PT). Hepatocytes with vesicular centrally located nuclei (arrows) (H&E, Bar=50µm). Fig. 1d) Paraffin section photograph of rat liver received combined FEN and nutrients showing portal tract (PT) with proliferating cells, large eosinophilic hepatocytes (H) with round nucleoli (arrows).Normal sinusoids (S) (H&E, Bar=50µm)

3.1.2 Immunohistochemical findings

With immunohistochemical stain for Bcl-2 oncoprotein, positive cells stained with brown colure, both the nucleus and the cytoplasm stained purple. The control section was similarly processed by incubation in Bcl-2 polyclonal antibodies and appeared as negative staining. Immunohistochemical staining for Bcl-2 oncoprotein in liver sections of control animals showed small brown granules of positive reaction in few hepatocytes cytoplasm (Fig. 2a). The animals which received FEN showed increased Bcl-2 reaction where the intense

positive staining was observed in the form of frequent wide patches of positive immunostaining particularly seen in pericentral-zone of hepatic lobules (Fig. 2b). (ώ3+ Se) group showed a little increased reaction than control group (Fig. 2c).Animals which received FEN +(ώ3+ Se) showed positive Bcl-2 oncoprotein reaction which appeared as patches of positive reaction but with decreased activity compared to the FEN group (Fig. 2d), also the reaction was increased than the control group (Table 1).

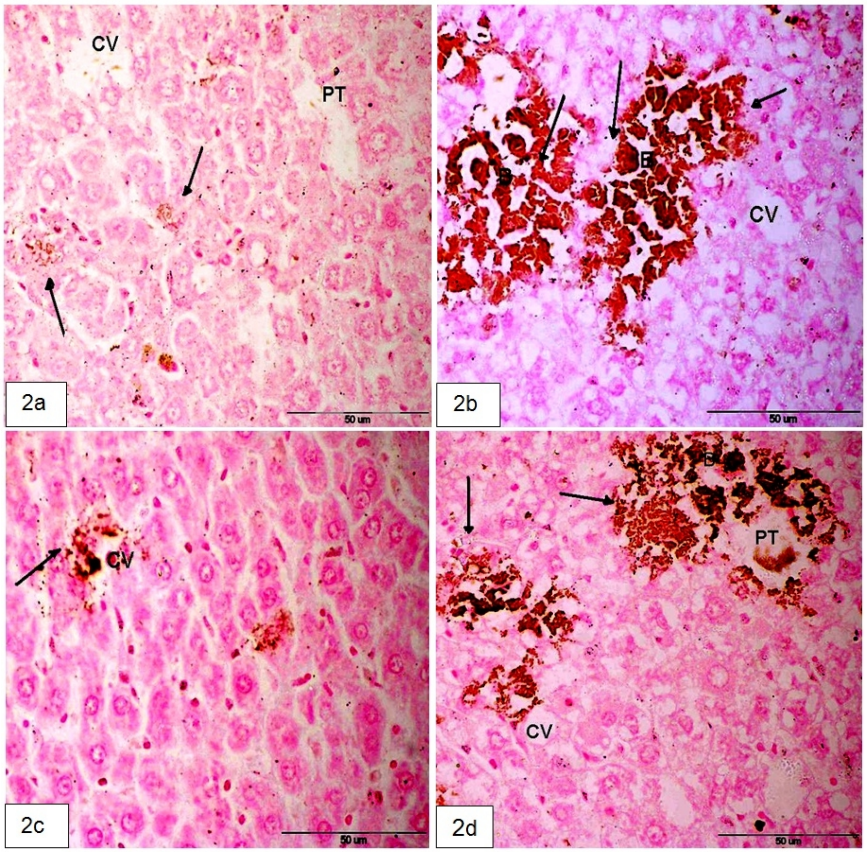

Fig. 2a) Paraffin section photograph of rat liver received corn oil showing: few small granules of Bcl-2 immunostaining in few hepatocytes in the middle zone (arrow), portal tract (PT) and central vein (CV) (DAB bar=50µm).Fig. 2b) Paraffin section photograph of rat liver administrated FEN showing: large areas of dark coarse granules of Bcl-2 immunostaining in the middle zone-B (arrows) and central vein(CV) (DAB bar=50µm). Fig. 2c) Paraffin section photograph of rat liver received combined nutrients showing: decreased reaction of Bcl-2 immunostaining granules (arrow) around central vein (CV) (DAB bar=50µm). Fig. 2d) Paraffin section photograph of rat liver received FEN and combined nutrients showing : moderate reaction of Bcl-2 immunostaining in hepatocytes in the middle zone and portal tract (PT) (arrows). Central vein (CV) (DAB bar=50µm)

3.1.3 Histochemical finding

3.1.3.1 Glutathion reductase

The NBT stained the formazan granules in the cytoplasm with blue color and negative color for the nuclei. An intense positive glutathione reaction was observed in all control hepatocytes at periportal space (Fig. 3a).Whereas a reduction of enzyme activity was observed in the other experimental groups.FEN group showed decreased glutathione reductase activity with small sized granules compared to the other groups (Fig. 3b). The group which received combined nutrient (Fig. 3c) showed decreased glutathione activity than the control group. The group which received combined nutrients plus FEN showed increased glutathione activity incompare with FEN group and showed decreased activity comparing with control group (Fig. 3d) (Table 1).

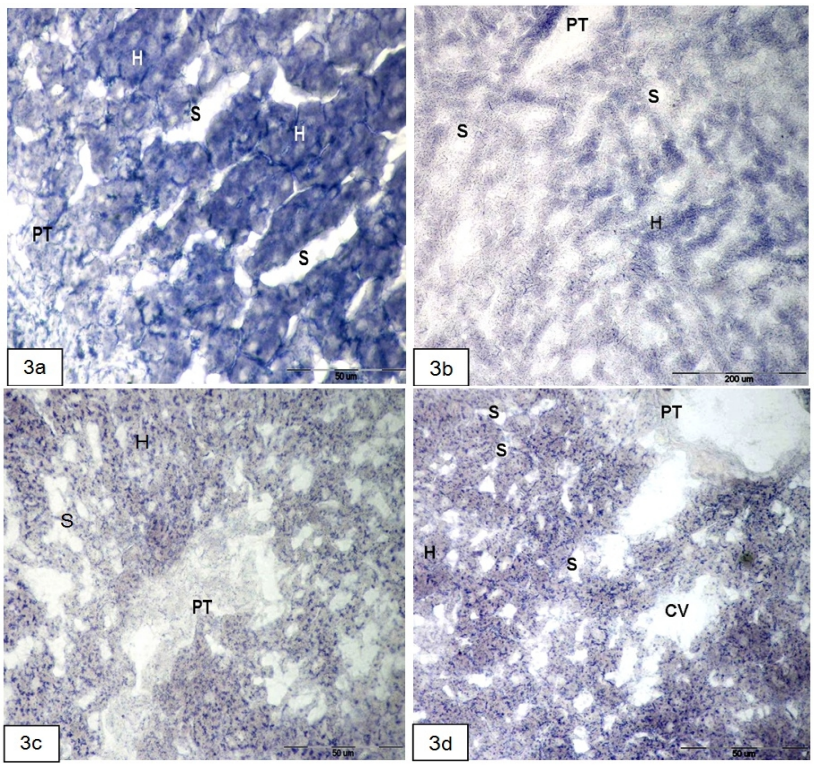

Fig. 3a) Frozen section photograph of rat liver received corn oil showing: intense glutathione reductase activity in hepatocytes cytoplasm (H) and moderate activity in epithelial cell of portal tract (PT) .Note, sinusoid (S) (NBT bar=50µm). Fig. 3b) Frozen section photograph of rat liver administrated FEN showing: decreased activity of glutathione reductase. Note, sinusoid (S), portal tract (PT) and hepatocyte (H) (NBT bar=200µm). Fig. 3c) Frozen section photograph of rat liver received combined nutrients showing: increased glutathione reductase activity which appeared as large sized granules in hepatocytes cytoplasm(H) .Note, portal tract(PT) (NBT bar=50µm). Fig. 3d) Frozen section photograph of rat liver received combined nutrients and FEN showing: increased glutathione reductase activity identified as large granules , sinusoid (S), portal tract (PT), hepatocyte (H) and central vein(CV) (NBT bar=50µm)

3.1.3.2 Catalase

The DAB gave brown granules in the cell cytoplasm while negative in both nuclei and control section. The control group showed moderate catalase activity in few hepatocytes (Fig. 4a). The reaction increased in FEN-group in the centrilobular and periportal areas of liver tissue (Fig. 4b). Animals received the combined antioxidant nutrients showed little increased reaction than control, mostly in many hepatocytes in the middle zone (Fig. 4c). While in the group in periportal and centrilobular area of liver tissue (Fig. 4d) (Table 1).

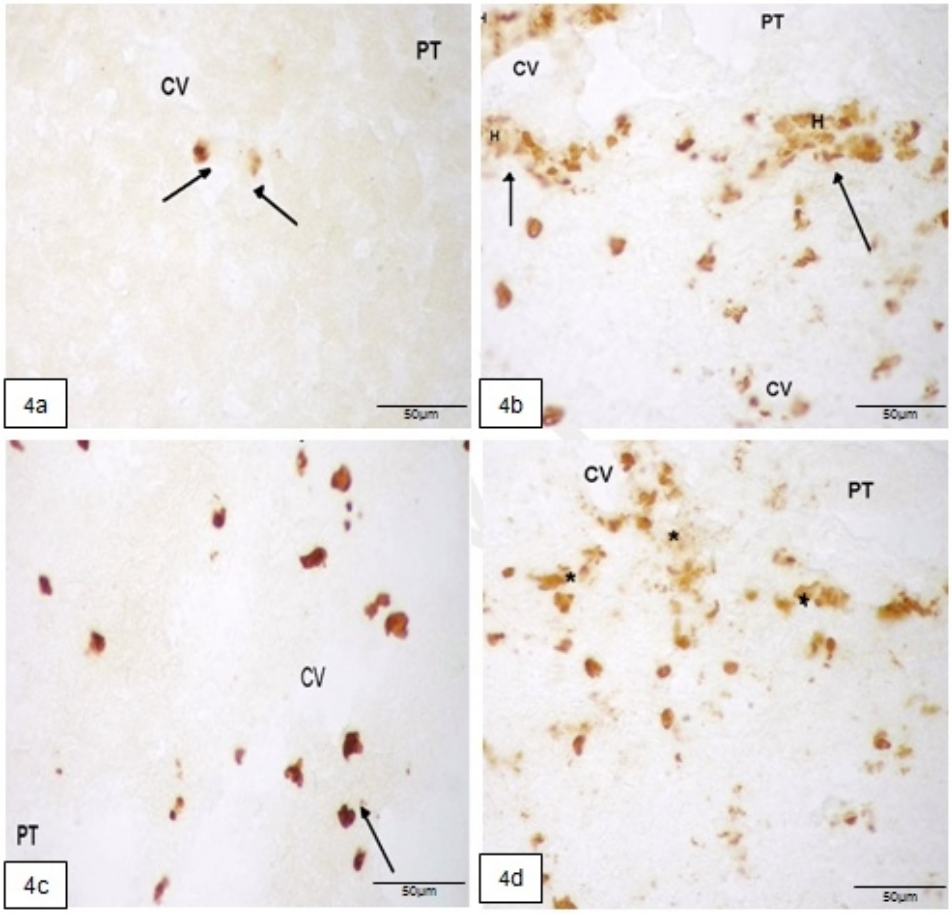

Fig. 4a). Frozen section photograph of rat liver received corn oil showing : moderate catalase activity in few hepatocytes(arrows).Note, portal tract (PT) and central vein(CV)(DAB bar=50µm).Fig. 4b) Frozen section photograph of rat liver administrated FEN showing: increased catalase activity in most hepatocytes of periportal and centrilobular areas (arrows) .Note, portal tract (PT), hepatocyte (H) and central vein(CV) (DAB bar=50µm) . Fig. 4c) Frozen section photograph of rat liver received combined nutrients showing:decreased catalase activity in many hepatocytes(arrows) .Note, portal tract (PT) and central vein(CV) (DAB bar=50µm). Fig. 4d) Frozen section photograph of rat liver received combined nutrients and FEN showing: decreased catalase activity in periportal and centrolobular hepatocytes(stare) .Note , portal tract (PT) and central vein(CV) (DAB bar=50µm)

Table1. Illustrated the semi quantitative intensity of histochemical and immunohistochemical reactions in the experimental groups

Groups Reactions	Control	Fenvalerate	ώ3+ +Se	Fenvalerate ώ3+ +Se
Catalase	+	++++	++	+++
Glutathione reductase	+++	+	++	++
Bcl-2	+	++++	++	++

3.2 Discussion

Several case-control studies have associated parenteral exposure to insecticides or insecticide use in the home with childhood brain tumors, leukemia, lymphomas, testicular cancer and other types of cancer [17].

Histological studies of liver have reported that single oral administration of FFN at 5 mg/kg produced marked degenerative changes in goat liver after 60 days [18]. Oral administration of FEN (200mg/Kg b.wt.) revealed liver congestion, degenerative changes, haemorrhages, mild fatty changes, infiltration of mono nuclear cells, loss of hepatocytes and proliferation of bile ducts [19]. Also oral administration of FEN to animals produced microgranulomatous changes in liver comprising various mononuclear phagocytic cells (microgranuloma) and varying number of multinucleated giant cells [20]. The present results showed areas of proliferation and increased number of hepatic cells forming acinar and pseudo glandular pattern. Also, the liver lost its lobular architecture with more dilatations of sinusoids after 24 hours in animals had given a single dose of FEN. Moreover, focal areas of necrosis and proliferation of bile ducts along with fibrosis after 28-day oral administration of FEN to broiler chicks were found [21]. In addition, rats exposed to formulated FEN by inhalation for a duration of three months showed fatty degenerative changes in centrolobular as well as middle zonal area, most nuclei appeared with karyolysis , Kupffer cell were prominent and lesions in bile duct were observed [22].

Selenium in the form of glutathion peroxidase (GSH-Px) played an important role in the protection of tissues from oxidative damage and could antagonize the toxic effects of some chemical substances [23] and this was clear in the present study where animals received FEN with antioxidant nutrients (ώ3 and Se) showed improved liver with mild sinusoids dilatation and normal arrangement of hepatocytes with vesiculated large nuclei. This results confirmed by previous study [24] where Se could diminish liver injury fibrosis and cirrhosis induced by pesticides. Fenvalerate is an oxidant stress inducer in rat liver, and its potential effects on pro-oxidant/antioxidant balance may also be important for human populations, particularly with selenium deficiencies [25].

Previous studies have shown that Bcl-2 gene encoded a mitochondrial protein (Bcl-2 oncoprotein) that contributed to neoplastic cell expansion primarily by promoting cell survival through interference with “programmed cell death“(PCD)”apoptosis. [26]. Bcl-2 oncoprotein cooperated with oncogenes activated in initiated cells and the changes in gene expression induced by non-genotoxic carcinogens may increase the susceptibility of tumor promotion by altering regulation of cell replication and apoptosis [27]. In the present study immunohistochemical staining of Bcl-2 revealed that FEN induced apoptosis which was evidenced by highly positive staining of hepatocytes cytoplasm on FEN-group and moderate staining in FEN+ combined antioxidant nutrients group where Bcl-2 expression causes some resistance to apoptosis in altered hepatocytes [28]. Also, dysregulation of apoptosis might

result in decreased ability of cell to undergo apoptosis and provide a selective survival advantage of these cells, thus leading to altered growth cellular transformation and tumor progression [29]. Bcl-2 expression increased cell resistance to antioxidants pathway, augmented the expression of intracellular defenses against reactive oxygen species and against both apoptotic and necrotic cell death induced by a variety of agents including chemotherapeutic, drugs, irradiation, and glutathione depletion [30]. Therefore, Bcl-2 oncoprotein expression which observed in the hepatocytes cytoplasm in the present study might be activated by the free radical produced by the pesticide where Bcl-2 might be able to affect the apoptotic threshold of cells by multiple mechanisms and only some of which are dependent on its antioxidant function [31].

Many studies have shown that reduced glutathione (GSH) level and glutathione S-transferase (GST) activity could reduce the covalent binding of the activated forms of well known carcinogens e.g. N-nitrosodimethylamine, aflatoxin B1 to DNA and other macromolecules [32]. Fenvalerate exposure (100 mg/kg/d, i.p., for the last 7 days) in normal rats increased hepatic glutathione peroxidase activity and lipid peroxidation [25]. The higher activity of glutathione S-transeferase in the FEN administrated group suggested that GST might play an important role in the detoxification of FEN [33]. In the present study histochemical staining of glutathione reductase (GSR) showed a decreased activity of this enzyme in FEN- group and this might be due to its consumption during the oxidative stress induced by FEN. Furthermore, the combined nutrient (ώ3 and Se) supplementation before EFN administration might induced the stimulation of several antioxidant enzymes involved in liver cells.The up-regulation of glutathione or a shift in relative glutathione concentration within mitochondria could prevent cellular damage caused by oxygen free radicals through direct detoxification [29]. Se and garlic supplementation lead to increased activities of glutathione S- transferase and glutathione peroxidase which enhance the detoxification mechanism [30]. Some nutrients could be considered to operate in combination rather than work in isolation which could protect human health and minimize the effects of radiation and environment pollution [34]. In this study, the interaction of the combined nutrients (ώ3 and Se) might be even more pronounced and useful as recorded before [35] where selenium has been shown to enhance the ability of fish oil by minimizing lipid peroxidation and stimulating antioxidant enzymes in rat liver .ώ3 and Se were acting in concert, omega-3 had a potent antioxidant effect which might result from the radical scavenger action of the organic toxic substance and additional antioxidant effect of selenium enhanced cellular glutathione levels and induced ROS scavenger enzymes [36].

Se supplementation proved to be beneficial as an adjuvant therapy for increasing the activity of endogenous antioxidative systems and maintaining the integrity of cellular membranes in liver [33]. This was attributed to its ability to decrease lipid peroxidation in different organs [37]. It had been suggested that lipid peroxidation of polyunsaturated fatty acid resulting from the production of toxic radicals migt induce inhibition of cell death by necrosis and cause enhancd apoptosis in some cancer cells [38]. The mechanism of apoptosis induced by Fish oil and selenium might be due to lipid peroxidation, thus it was suggested that lipid peroxidation played an important role in mediating EPA (n3 fish oil) induced apoptosis [39]. In addition, the evaluation of relative glutathione concentrations in the mitochondria and other cellular membranes of control and Bcl-2 expressing cells suggested the ability of Bcl-2 protein for affecting cellular redox balance as antioxidant mechanism [40].

Antioxidant enzyme CAT was the first line of defense against free radical induced oxidative stress. Fenvalerate exposure (100 mg/kg/d, i.p., for the last 7 days) in normal rats did not change the activities of catalase [25] (2011).However, the present histochemical staining of

catalase enzyme revealed increased activity of catalase in FEN group and moderate activity in FEN + (ώ3and Se) compared to control group. The immunogold electron microscopic study of isolated rat liver mitochondria exposed to H2O2 confirmed the presence of catalase in the inner mitochondrial compartments. In the presence of large amounts of ROS, catalase became the most important scavenger of H2O2 in the cytosol [41] where CAT was responsible for the catalytic decomposition of hydrogen peroxide to molecular oxygen and water [42].

4. CONCLUSIONS

This study showed that pretreatment with ώ3 and Se might attenuate FEN- induced oxidative damage by decreasing lipid peroxidation and increasing antioxidant defense system in rat liver. Thus, more attention is needed to limit the use of insecticides, particularly FEN used in the developing counties to reduce their entrance into the human food cycle via environmental pollution.

In addition, the present study found a correlation between the high expression of Bcl-2 staining and depletion of glutathione staining in FEN-group, as well as, its expression in the same area of catalase positive staining in liver tissue. This finding needed further work to investigate the relationship between both mitochondrial membrane proteins (Bcl-2 and catalase) and the antioxidant pathways.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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