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Imidacloprid enhances liver damage in Wistar rats: Biochemical, oxidative damage and histological assessment

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ABSTRACT

Objective: To investigate the potential adverse effects of imidacloprid on biochemical parameters, oxidative stress and liver damage induced in the rat by oral sub-chronic imidaclopride exposure.

Methods: Rats received three different doses of imidacloprid (1/45, 1/22 and 1/10 of LD_{50}) given through gavage for 60 days. Two dozen of male Wistar rats were randomly divided into four experimental groups. Liver damage was determined by measuring aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and lactate dehydrogenase leakages. The prooxidant-antioxydant status in hepatic tissue homogenate was evaluated by measuring the degree of lipid peroxidation, the antioxidant enzymes activities such as catalase, superoxide dismutase and glutathione peroxidase (GPx).

Results: The relative liver weight was significantly higher than that of control and other treated groups at the highest dose 1/10 of LD_{50} of imidacloprid. Additionally, treatment of rats with imidacloprid significantly increased liver lipid peroxidation ($P \leqslant 0.05$ or 0.01) which went together with a significant decrease in the levels of superoxide dismutase and catalase activities. Parallel to these changes, imidacloprid treatment enhanced liver damage as evidence by sharp increase in the liver enzyme activities of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and lactate dehydrogenase. These results were also confirmed by histopathology.

Conclusions: In light of the available data, it is our thought that after imidacloprid sub-chronic exposure, depletion of antioxidant enzymes is accompanied by induction of potential oxidative stress in the hepatic tissues that might affect the function of the liver which caused biochemical and histopathological alteration.

1. Introduction

Many of the pesticides can be damaging following acute or chronic exposures through their residues in water and food[1]. Systemic pesticides, particularly neonicotinoids, are utilized in developed countries, often replacing carbamates, pyrethroids and organophosphates[2] and whose usage has increased globally in the last decades. Neonicotinoids protect against a wide range of insect species. They are effective at low concentrations and can be applied using a variety of methods[3]. They are becoming the most widespread and popular group of insecticides owing to their lower neurotoxicity for mammals[4].

To select among these neonicotinoids, imidacloprid (IMI) is a newer neonicotinoid available in the market since 1991[5], becoming the most successful and the best-selling pesticide throughout the world with global application at a rate of 20 000 tons each year as it was the case in 2010[6]. It is especially efficient against sucking and mining pests in vegetables, fruit, sugar beet, cotton and rice and is an effective seed treatment[7]. It has low mammalian toxicity and also high effectiveness against target insects[8] and acts by binding to the postsynaptic nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insect and eventually causes the impairment of normal nerve function.

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A pesticide was associated with oxidative stress[9], generation of free radicals, alteration in antioxidant system and lipid peroxidation resulted into the tissue damage[10]. Several tests concerning the toxicity and the genotoxic capacity of IMI have been conducted[11]. Liver has been considered as the toxic organ for the toxic effects of xenobiotic and the principal target of IMI toxicity[12]. It has mostly been noticed that IMI rapidly absorbs via the gastrointestinal tract and its metabolism happens principally in the liver. Thus, physiologically the liver will be affected in a straight line by this toxin in the different period of consumption[13].

Due to the increasing application of IMI in agriculture to control insect pests and its likely hazard for consumers by intake of fruits and vegetables with pesticide remains, it looked pertinent to investigate the subchronic effects of commercial product of IMI on rats by means of biochemical parameters, increment oxidative stress and histopathological alterations to spot the potential adverse effects of xenobiotic such as environmental contaminants.

2. Materials and methods

2.1. Chemicals

Confidor 200 SL commercial formulation of IMI contained 200 g/L of the active substance was used in this study. It is produced by Bayer CropScience AG, Monheim, Germany.

2.2. Animals and experimental protocol

A total of 24 adult male Wistar rats aged of 4 months were purchased from Central Pharmacy of Tunisia SIPHAT (Tunisia, Ben Arous). Before experiments, animals were housed for at least 1 week and were kept under conditions of controlled temperature (25 ± 2 ˚C) and relative humidity (55%) with a 12:12 h of light: dark cycle. Rats were fed on standard commercial pellet diet from Sico Sfax, (Tunisia) and tap water *ad libitum* during the whole experiment.

All experimental procedures involving animals were conducted in accordance to the guidelines for animal care and approved by the Faculty of Medicine of Monastir, Tunisia.

The animals were randomly divided into a control group and three treated groups of six animals in each. Control group (n = 6) received orally corn oil via intra gastric gavage at 5 mL/kg of dose volume. All treated groups received the same dose volume. Different dilutions for the doses of insecticide were made with corn oil to obtain the test concentrations. The test concentrations were calculated from the percentage of the active substance of the commercial formulation. Administration of each solution was adjusted based on body weight measured each week.

The rats were given IMI at a dose of 10, 20 and 45 mg by kilogram of body weight corresponded to 1/45, 1/22 and 1/10 of the respective lethal dose 50 (LD_{50}) daily for 60 days to evaluate the subchronic toxicity of IMI on rats.

2.3. Sampling

At the end of the experimental period, blood samples of the rats were taken from the animals by cardiac puncture and collected into sterile tubes. Blood samples were centrifuged at 3 000 r/min for 15 min at 4 ˚C. The plasma was harvested and stored at –20 ˚C until the determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and total protein.

Liver was quickly removed, weighted and divided into 2 portions. The first was fixed in 10% buffered-neutral formalin and embedded in paraffin wax for histopathology. The second portion was homogenized in 10% (w/v) ice cold phosphate buffer (pH 7.4) using an Ulta Turrax homogenizer T25 (Cole-Parmer), and then centrifuged at 10 000 r/min for 20 min at 4 ˚C. The supernatant was collected and stored at –80 ˚C until use for oxidative stress biomarkers studies.

2.4. Measurement of body weight and liver weight

Animal body weight was measured every week, whereas whole liver was quickly removed and weighted after sacrifice for establishing:

Body weight gain (%) = (Final body weight – Initial body weight × 100)/Initial body weight

Relative liver weight (%) = Organ weight × 100/Final body weight

2.5. Histopathological assessement

For histopathological examination, the liver was embedded in paraffin, cut into sections using a rotary microtome and stained with haematoxylin and eosin (H & E) for light microscopic examination. The H & E-stained sections were viewed and photographed using a Leika light microscope (Leica DM750), equipped with an attached camera Leica ICC50.

To evaluate lesions such as hypertrophy of central vein, dilated portal triad, lymphoid infiltration, dilatation of sinusoids, vacuolization and necrosis, six microscopy stained slides per animal were examined. Each liver slide was examined and the severities of histopathological alterations in liver tissue were scored as follows: none (–), mild (+), moderate (++) and severe (+++) damage.

2.6. Biochemical analysis

Plasma AST, ALT, ALP, total proteins and LDH activities were performed from plasma samples by spectrophotometric method according to the instructions supplied with kits from Roche Diagnostics Gmbh (Mannheim, Germany).

2.7. Measurement of lipid peroxidation

Degree of lipid peroxidation (LPO) in hepatic tissue homogenate was measured using the method of Buege and Aust[14] as thiobarbituric acid reactive substances (TBARS). Malondialdehyde (MDA) is a degradation product of peroxidized lipids. The development of the pink-colored complex with the absorbance formed by the reaction of TBA-MDA was measured spectrophotometrically at 530 nm. Results were expressed in terms of MDA per mg protein.

2.8. Assay for antioxidative status in liver tissue homogenate

2.8.1. Estimation of catalase (CAT) activity

CAT activity was performed according to the method of Clairbone[15] using hydrogen peroxide (H_2O_2) as substrate.

2.8.2. Estimation of superoxide dismutase (SOD) activity

The activity of SOD in the liver was carried out with the method of Zhang *et al*.[16] using a ready-made commercial kit manufactured by RANDOX Laboratories (Ransod Kit, Crumlin, UK).

2.8.3. Estimation of glutathione peroxidase (GPx) activity

GPx activity was assayed according to the protocol described by Flohe and Gunzler[17]. The enzyme activity was defined as μmol of GSH oxidized/min/mg of protein, at 25 ˚C.

2.9. Statistical analysis

Results were analyzed using One-way ANOVA followed by Tukey's *post hok* test and were expressed as the mean ± SD of at least six rats in the group. All statistical analyses were performed using SPSS 18.0. All parameters were considered statistically significant if $P \leqslant 0.05$.

3. Results

3.1. The evaluation of body weight gain and relative liver weight

At the end of the experimental period, IMI treatment had no significant effect on body weight gain (Figure 1). Relative liver weight increased significantly in Group 3 in comparison with control and other treated groups ($P \leqslant 0.05$ or 0.01) (Figure 2).

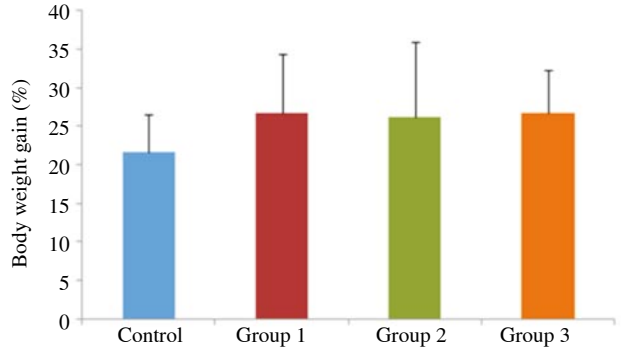

Figure 1. Effect of IMI on body weight gain in the treated and control groups.

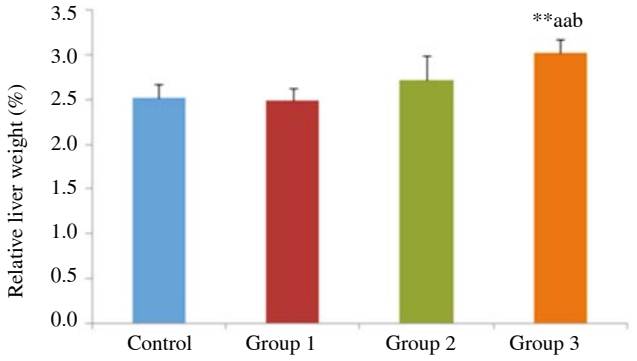

Figure 2. Effect of IMI on relative liver weight in the treated and control groups.

Values represent means ± SD. [**]: Significantly different from the control at 1% confidence level; [aa]: Significantly different from the Group 1 at 1%. [b]: Significantly different from the Group 2 at 5%.

3.2. The evaluation of changes on biochemical parameters

Biochemical changes are presented in Table 1. AST, ALT and ALP activities increased statistically significant after the exposure to 20 and 45 mg/kg body weight of IMI as compared to control ($P \leqslant 0.05$ or 0.01). In addition to this, there was statistically significant increase in LDH for all IMI-treated groups as compared to control ($P \leqslant 0.05$ or 0.01) while a significant decrease was observed in total protein level in groups exposed to higher doses ($P \leqslant 0.01$).

3.3. Lipid peroxidation and antioxidant enzymes

Results of hepatic lipid peroxidation and antioxidant enzymes (LPO, SOD, GPx and CAT) were shown in Table 2.

As a result, LPO in terms of MDA was estimated in liver and was found to be significantly increased in all treated groups compared to control group ($P \leqslant 0.05$ or 0.01).

Treatment with the highest dose also revealed a significant increase in MDA compared to the other treated groups ($P \leqslant 0.01$). This increase was dose dependent.

There was statistically significant decrease in the activity of SOD in Group 3 compared with control group and other treated groups ($P \leqslant 0.05$ or 0.01).

Table 1

Effect on plasma marker enzymes after subchronic treatment with IMI.

Groups	IMI (mg/kg body weight)	AST (UI/L)	ALT (UI/L)	ALP (UI/L)	LDH (UI/L)	Total protein (g/L)
Control	0	67.94 ± 10.47	23.65 ± 3.17	52.17 ± 1.53	165.66 ± 26.91	61.85 ± 4.10
Group 1	10	80.70 ± 25.76	30.22 ± 6.18	59.50 ± 8.74	256.33 ± 29.30^{a*}	56.41 ± 3.28
Group 2	20	135.38 ± 26.25^{a*}	40.16 ± 5.42^{a**,b*}	111.00 ± 3.68^{a**,b**}	319.25 ± 28.00^{a**}	54.56 ± 2.24^{a**}
Group 3	45	188.80 ± 69.10^{a**,b**}	49.40 ± 7.79^{a**,b**}	146.75 ± 23.63^{a**,b**,c**}	396.66 ± 95.17^{a**,b**}	45.03 ± 4.39^{a**,b**,c**}

All values are expressed as mean ± SD. [a*]: Significantly different from control at 5% confidence level, [a**]: Significantly different from control at 1% confidence level, [b*]: Significantly different from the Group1 at 5%, [b**]: Significantly different from the Group 1 at 1%, [c*]: Significantly different from the Group 2 at 5%, [c**]: Significantly different from the Group 2 at 1%.

Table 2

Effect on oxidant and antioxidant enzyme activities in the liver of rats after subchronic treatment with IMI

Groups	IMI (mg/kg body weight)	MDA (nmol MDA/mg protein)	GPx (μmol GSH oxidized/min/mg protein)	SOD (unit/mg protein)	CAT (μmol/min/mg protein)
Control	0	0.38 ± 0.03	2.36 ± 0.16	31.48 ± 4.03	604.15 ± 38.24
Group 1	10	0.71 ± 0.03^{a*}	2.59 ± 0.81	30.66 ± 3.02	417.61 ± 71.65^{a**}
Group 2	20	0.99 ± 0.26^{a**}	1.97 ± 0.50	29.44 ± 2.46	341.19 ± 60.79^{a**}
Group 3	45	1.48 ± 0.25^{a**,b**,c**}	2.43 ± 0.32	23.96 ± 1.90^{a**,b**,c*}	218.64 ± 50.38^{a**,b**,c**}

All values are expressed as mean ± SD. [a*]: Significantly different from control at 5% confidence level, [a**]: Significantly different from control at 1% confidence level, [b*]: Significantly different from the Group 1 at 5%, [b**]: Significantly different from the Group 1 at 1%, [c*]: Significantly different from the Group 2 at 5%, [c**]: Significantly different from the Group 2 at 1%.

Based on the data in Table 2, there was no significant change in GPx activity while CAT activity decreased significantly compared to control group ($P \leqslant 0.01$). Also, that decrease was significant in Group 3 compared to the other treated groups ($P \leqslant 0.01$). This decrease was dose dependent.

3.4. Histopathology of liver tissue

Histopathological findings of the liver were evaluated by light microscopy and representative histopathological sections from control and treated rats were presented in Figure 3. The incidences of lesions in the experimental groups were scored in Table 3.

3.4.1. Control rats

Microscopic examination of the control rat liver sections revealed normal preserved histoarchitecture. The liver parenchyma showed a normal arrangement of hepatocytes in anastomosing cords with visible capillary sinusoids in most places. Each hepatocyte had distinct nuclei with normal vesicular structure and the cytoplasm is faintly granular (Figure 3a and 3d). No histological abnormality was detected in the hepatic tissue.

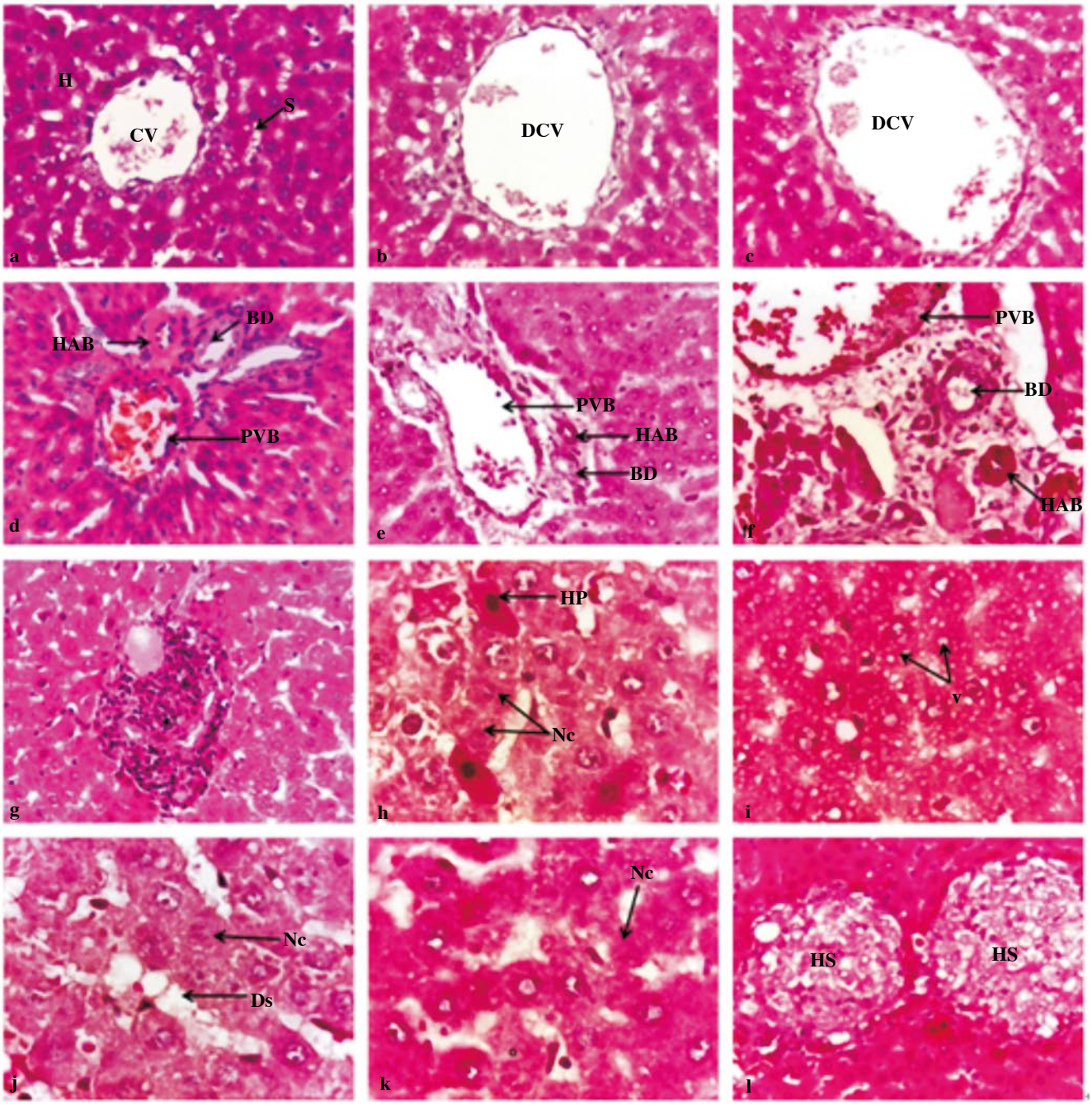

Figure 3. Light microcopy of histological sections of H & E stained liver from control and treated groups.
Histological sections of control animals showed normal liver parenchyma and a normal portal triad (a, d) (400×). Liver sections of Group 2 (rats treated with 20 mg/kg IMI) demonstrated hypertrophied central vein (b) (400×) and dilated portal triad (e) (400×). Liver sections of Group 3 (rats treated with 45 mg/kg IMI) exhibited severe dilated central vein (c) (400×) and severe dilated portal triad (f) (400×). Liver sections of rats treated with 45 mg/kg (Group 3) revealed severe alterations in the parenchyma (g, h, i, j, k and l) (×400, 1 000×, 1 000×, 1 000× and 1 000×, respectively). CV: Central vein; S: Sinusoids; H: Hepatocyte; V: Vacuoles; Nc: Necrotic cell; HP: Hepatocyte's proliferation; O: Oedema; DCV: Dilated central vein; DS: Dilated sinusoid; Portal triad (PVB: Portal vein branch; HAB: Hepatic artery branch; BD: Bile duct); HS: Hepatic steatosis; LI: Inflammatory leucocyte infiltrations.

Table 3
Grading of the histopathological alterations in the liver of rats, exposed to different concentrations of IMI

Histopathological alterations	IMI (mg/kg body weight)			
	0	10	20	45
Hypertrophied central vein	–	+	++	+++
Dilatated portal triad	–	+	++	+++
Dilatated sinusoid	–	–	+	+++
Lymphoid infiltration	–	–	+	+++
Vacuolization	–	–	+	+++
Necrotic cells	–	–	–	++
Hepatocyte's proliferation	–	–	–	+
Oedema	–	–	–	++
Steatosis	–	–	–	+++

(–) none, (+) mild, (++) moderate, (+++) severe.

3.4.2. Group 1 (10 mg/kg body weight)

There were no marked changes after the administration of the lowest dose except mild dilated central vein and portal triad.

2.4.3. Group 2 (20 mg/kg body weight)

Moderate histopathological alterations including hypertrophied central vein (Figure 3b) and a dilated portal triad (Figure 3e) were noted in the liver of rats exposed to 20 mg/kg body weight. We have also observed mild lymphoid in the parenchyma liver, as well as cytoplasmic vacuolation of some cells with mild dilatation of sinusoids capillary.

2.4.4. Group 3 (45 mg/kg body weight)

Intoxicated rats indicated marked alterations of hepatic pathology by severe dilated central vein (Figure 3c), severe dilated portal triad (Figure 3f) and an increase in inflammatory cell infiltration (Figure 3g). We have noted a severe dilatation of sinusoids capillaries (Figure 3j) accompanied with cytoplasmic vacuolation (Figure 3i). In addition, we observed a variation in the size of some hepatocytes with deeply stained nuclei and homogeneous and acidophil cytoplasm (Figure 3h). We have also noted hepatocellular necrosis (Figure 3h, j, k), oedema (Figure 3k) and hepatic steatosis as seen by several lipid droplets accumulation (Figure 3l). Our finding showed that severity of these changes were dose dependent.

4. Discussion

In this investigation, effects of oral administration of three different doses of IMI were applied to rats for 60 days. The liver plays a major role in the detoxification of xenobiotic and environmental chemicals[18].

The results showed that IMI treatment did not affect body weight gain. This verdict is supported by the results of Ince *et al.*[19] and Vohra *et al.*[12] who reported that treatment with IMI did not induce any significant change in body weight gains. However, these results are inconsistent with the finding[20,21] which suggested that IMI induced a significant reduction in the body weight gain in rats.

Relative liver weight increased significantly only in the group treated with the highest dose. Bhardwaj *et al.*[22] noted a significant increase in relative weight of liver at 20 mg/kg/day of IMI for a period of 90 days while studies of Vohra *et al.*[12] revealed that oral administration of IMI did not induce significant changes in the relative weight of liver. The increase in the relative liver weight could be due to the oedema induced by this insecticide. This probability is supported by our histopathological analysis which revealed oedema in the liver tissue of rats treated with 45 mg/kg body weight of IMI.

In the present study, our histopathological analysis also revealed lymphoid infiltration as a sign of inflammation and cell irritability in rats treated with higher doses of IMI. We did find out others histopathological lesions of the rat liver including dilatation of central vein, portal triad and sinusoids capillary. The hepatocyte exhibited loss of their normal architecture, intense cytoplasmic vacuolation, necrosis and hepatic steatosis. In fact, increased lipid peroxidation and depressed antioxidant status in the liver of rats receiving IMI caused oxidative stress which is known to induce endoplasmic reticulum (ER) stress[23]. Hepatic ER stress is accompanied by steatosis conforming to literature[24].

Toor *et al.*[25] demonstrated similar histological changes, by reporting leucocyte infiltration, dilatation of central vein and blood sinusoids that were observed in hepatic tissue of female rats exposed to IMI at 45 mg/kg/body weight for four weeks.

Vohra *et al.*[12] carried out another study on female rats and revealed that oral administration of IMI showed lymphoid infiltration, marked dilatation of central vein and degeneration of hepatocytes. Furthermore, Bhardwaj *et al.*[22] have pointed out that the administration of IMI to female rats for a period of 90 days induced mild focal necrosis and hepatocellular damage.

However, a recent research[26] documented that loach (*Misgurnus anguillicaudatus*) exposed to the IMI revealed no significant histopathological changes in liver at any dose level.

The levels of LDH, ALP, ALT and AST are indicative of the functional efficiency of the liver and hepatoxicity[27]. In fact, when the liver cell membrane is damaged, these different enzymes located in the hepatocyte cytosol are secreted in the blood[28].

The current study reveals that oral administration of IMI caused liver damage to rats as evidence by significant increases in plasma levels of AST, ALT, ALP and LDH in Groups 2 and 3 when compared to control group. It has already been shown in previous studies[22,29] that IMI induced elevation of hepatic enzymes. Thus, the observed elevation in these enzymes could be due to liver dysfunction attributed to the damaging effect of IMI on liver cell membrane, which is in line with the study of Awad *et al.*[30] who found a good correlation between cell damage and the enzyme leakage.

Moreover, the overall secretion of enzymes into blood following hepatocellular necrosis could be an explanation for the elevation in plasma LDH activity[31].

These changes are entirely consistent with liver cell damage in the IMI-treated rats.

Supporting these findings[22,25,32,33] reported a marked elevation in AST, ALT and ALP of IMI treated rats. Results are also in accordance with those of Balani *et al.*[34] in a study of male White Leghorn (WLH) chicks treated with different concentration of IMI.

A study carried out by Vohra *et al.*[12] on female albino rats following oral administration of two doses of IMI for 60 days revealed no significant increase in ALT, AST and ALP activities.

Total protein is a routine test to assess the toxicological nature of diverse chemicals[35]. Decrease of total protein was observed in the present study following IMI treatment in Groups 2 and 3 when compared to control group. This decrease can be caused by protein synthesis shortage as a result of liver dysfunction induced by the existence of IMI[36].

Kaur *et al*.[37] and Vohra *et al*.[12] observed no significant change in total protein concentration after oral administration of IMI in calves and rats.

It has been shown through experiments that liver damage is followed by drop in antioxidant defenses in the liver[38]. Reduced activities of antioxidant enzymes after treatment of pesticides are important indicators for the toxicity of these chemicals[39]. Moreover, enzymatic antioxidants including SOD and CAT are important parameters of antioxidant defenses system to inactivate the reactive oxygen species (ROS) produced by environmental contaminants[40]. They constitute the primary antioxidant enzymes and first lines of defense against the oxyfree radicals in cell[41,42]. In this investigation, it has a statistically significant decrease in SOD and CAT activities in hepatic tissues, which may be related to response to increased oxidative stress after IMI treatment.

In this study, the IMI-treated rats also exhibited significantly increase in MDA level in rat liver, which is taken as an index of LPO. An increased hepatic MDA level is well known as an indicator of tissue damage, and alteration of cell function[43]. In fact, these pathological changes appear due to the formation of ROS or free radicals[44].

Moreover, the decrease in the activities of SOD and CAT concomitant together with increase in lipid peroxidation may be considered as one of the leading reasons to an imbalance in oxidant/antioxidant status and LPO by enhancing ROS generation in liver tissue of male rats. These results are consistent with the findings[42] after oral administration of 20 mg/kg body weight/day of IMI for 90 days to female rats. Moreover, Ince *et al.*[19] indicated that IMI treatment significantly increased MDA levels accompanied by concomitant depletion in liver SOD and CAT activities in mice. A marked increase in the hepatic LPO and in the activities of these antioxidant enzymes were observed after the acute toxicity of IMI toward male mice[41].

Other results of Duzguner and Erdogan[45] have shown an increase in lipid peroxidation, but neither SOD nor CAT activities were affected in the liver of rats following chronic exposure to IMI. An earlier study of Wang *et al*.[46] was observed a significant increase in MDA levels, while the SOD activity was significantly induced and CAT activity was inhibited at most exposure intervals of IMI in earthworm *Eisenia fetida*.

Considering the results and data mentioned in this study we can end up by stating that the higher doses of IMI had potential to induce hepatotoxicity as manifested by biochemical results, antioxidant enzyme assessment and histopathological findings. Using higher doses of commercial products with IMI as an active ingredient can be a potential risk for human health which warrants additional molecular studies to understand the mechanisms underlying the IMI-induced hepatotoxicity.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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