Toxicopathological and Molecular Studies on Imidacloprid andHexaflumuron-induced Hepatorenal Toxicity in Rats.

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Abstract

Pesticides are considered the main source of environmental pollution and causing severe hazardous effects on humans and livestock. Imidacloprid (IC) and hexaflumuron (HFM) are broadly used insecticides for crop protection in the world. Some studies discussed IC toxicity in rats, but the toxicity of HFM doesn't elucidate yet. So that, the current study aimed to investigate the pathogenesis and the mechanistic way of both IC and HFM induced hepatorenal toxicity in rats with comprehensive insight into its molecular mechanism. 21 male Wistar albino rats were divided into 3 groups as the following: group (1), normal saline; group (2), receiving IC; and group (3), receiving HFM. All the following materials were orally administered every day for 28 days. At the end, all rats were euthanized to collect blood and organ samples (liver and kidneys). The results revealed behavioral alterations in walking, body tension, alertness, and head movement as well as a decrease in body weight of rats receiving either IC or HFM. In addition to increasing the levels of MDA with decreasing GHS levels in liver and kidney homogenates. Both liver and kidney tissues showed extensive histopathological alterations associated with increasing the serum levels of ALT, AST, urea, and creatinine as well as reduction in total proteins, albumin, and globulin levels. Furthermore, there was upregulation of m-RNA levels of caspase-3, JNK, and HO-1 genes with strong positive reaction of caspase-3, TNF-α and NF-κB proteins in both liver and kidneys of rats receiving either IC or HFM compared with the control group. We can conclude that both IC and HFM induced oxidative hepatorenal damage via ROS overproduction that activate NF-κB signaling pathways and mitochondrial and JNK dependent apoptosis pathway.

Introduction

Exposure to ecological contamination stays a significant wellspring of wellbeing hazard around the world, particularly in agricultural nations, where destitution, absence of interest in current innovation, and feeble natural enactment join to cause high contamination levels. Among the natural contamination, overexposure to pesticides is considered as one of the causative components of different diseases in human and livestock (Cheng et al. 2011). Pesticides have discovered broad applications in horticultural and veterinary practices worldwide for the most recent forty years (De et al 2014). Pesticides in the climate have the potential for accidental effects on natural life, human and domesticated animals. Humans and animals repeatedly presented to pesticides through water or food (Fisk 2007). Persistent pesticides delivered in one area of the world can be moved through the air to other areas through a continuous cycle of evaporation and deposition (Koirala et al. 2007). Human hazardous effect differs according to the type of pesticides and even though to the degree of weakness.

Imidacloprid, 1[(6-chloro-3-pyridinyl) methyl]-N-nitro-2-imidazolidinimine, is a neonicotinoid insecticide widely used to fight pests of cereals, fruits, and vegetables due to its low soil persistence and high insecticidal activity at a low application rate (Casida and Durkin 2013). Imidacloprid (IC) can exaggerate the toxic properties and adverse effects which may be fatal for human and animal health (Benjamin et al. 2006; Lv et al. 2020). Several studies reported that IC causing severe toxicity in rats and mice including hepatotoxicity, nephrotoxicity, male infertility, and neurological disorders (Bhardwaj et al. 2010; Li et al. 2021). Another example of broadly used insecticides is hexaflumuron (HFM), a Benzoylphenyl urea (BPU) insecticide; it is an insect growth regulator that works by inhibiting a chitin synthesis (Khajepour et al. 2012). Its active
ingredient is categorized as unlikely toxic to human while one recent study about its local formulation showed hepatotoxicity and immunotoxicity in rats but with unclear mechanism of action (Noaishi et al. 2019). Oxidative stress plays an important role in most pesticides-inducing toxicity that leading to free-radicle related cell and DNA damage (John et al. 2001). Oxidative mechanisms have a pivotal role in insecticide-induced tissue damage not only by balancing oxidant-antioxidant status but also by inhibiting neutrophil infiltration and regulating inflammatory mediators (Delgado et al. 2006; Muniz et al. 2008).

Due to the increasing applications of IC and HFM in agricultural and veterinary practices to control insect pests and its likely hazard for consumers by intake of fruits and vegetables with pesticide remains. In addition, the possible mechanism of HFM-induced toxicity in non-target organisms remains to be elucidated. It looked pertinent to investigate the effect of commercial products of these insecticides on rats by means of biochemical parameters, oxidative stress, and histopathological alterations to spot the potential adverse effect of these insecticides on non-target organisms as mammals. Hence, the current study aimed to investigate the possible mechanisms of IC and HFM induced hepato-renal toxicity with comprehensive insight into its molecular mechanism and gene regulating its toxicity in rats.

**Materials And Methods**

**Chemicals**

The study was conducted using the commercial formulations of pesticides that obtained from Kafr El-Zayat Pesticides & Chemicals Company (Kafr El-Zayat, Gharbia, Egypt). The active ingredients of hexaflumuron 10%, the IUPAC name: 1-[3,5-dichloro-4-(1,1,2,2-tetrafluoroethoxy) phenyl]-3-(2,6-difluorobenzoyl) urea with chemical formula C$_{16}$H$_{8}$Cl$_2$F$_6$N$_2$O$_3$. The formulation was supplied as an emulsifiable concentrate (EC). While, the active ingredients of imidacloprid 70%, the IUPAC name: (NE)-N-[1-[(6-chloropyridin-3-yl) methyl] imidazolidine-2-ylidene] nitroamide with chemical formula: C$_9$H$_{10}$ClN$_5$O$_2$. The formulation was supplied as wettable powder. Both pesticides are freshly prepared in deionized water according to the required dose of the active ingredient.

**Animals and animal grouping**

Twenty-one male albino Wistar rats (170±20g) were obtained from the Department of Veterinary Hygiene and Management’s Animal House, Faculty of Veterinary Medicine, Cairo University, Egypt. Animals reared in plastic cages, fed with standard commercial pelleted feed and water was supplied *ad libitum*. They were inspected for health status and acclimatized to the research laboratory environment for two weeks before use. All the procedures and experimental design were permitted by the institutional animal care and use committee (IACUC) of Cairo University.

Rats were randomly divided into 3 groups (n=7) and were given the following materials daily via oral gavage for 28 days. Group (1) received normal saline and kept as a control group. Group (2) received IC at 45 mg active ingredient/kg bwt representing 1/10 LD50. Group (3) received HFM at 11 mg active ingredient/kg bwt representing 1/10 LD50. Doses of pesticides were selected based on their LD50 which was reported to be 450 mg/kg bwt for IC, and 110 mg/kg bwt for HFM formulations (WHO 2010).
Rats in all groups were daily observed for any clinical signs and mortality as well as weighed weekly up to the end of the experimental period.

**Behavioral parameters**

After 28 successful days, four representative behaviors include alteration in walking, body tension, alertness, and head movement were observed and evaluated as the markers of hepatorenal toxicity according to the method described by (Hayase et al. 2000), but with some modifications. These behaviors were scored to determine the degree of behavioral changes and difference between groups and described in a graded scale as the following: 0=normal behavior, 1= light changes, 2= mild changes, 3=moderate changes, and 4=severe changes.

**Sampling**

After the behavioral assessments, rats were anesthetized using Ketamine and Xylazine and blood samples were collected from the orbital sinus, then centrifuged at 3500rpm for 5min to obtain clear serum samples preserved at -20°C till used for biochemical analysis. After that, rats were euthanized by cervical dislocation to collect liver and kidney samples. Part of these samples was preserved at −80°C till used for oxidative stress evaluation and molecular studies while the other part was fixed in 10% neutral buffered formalin to perform histopathological and immunohistochemical examinations.

**Biochemical enzyme markers**

Alanine aminotransferase (ALT), aspartate aminotransaminase (AST), total proteins, albumin, glucose, total cholesterol, high density lipoproteins cholesterol (HDL-C), triglycerides, urea, and creatinine were measured in the collected serum samples using standard kits Marketed by (SPECTRUM-Germany) according to the constructions of the manufacturer. Low density lipoproteins cholesterol (LDL-C) concentration was calculated according to the method of Friedewald et al. (1972). Globulin concentration was calculated from the difference between total proteins and albumin concentrations.

**Oxidative stress evaluations**

It was done by using test kits purchased from Biodiagnostic Co., Egypt for the determination of lipid peroxidation “MDA” level and GSH content in liver and kidney tissue homogenates of different groups according to the instructions of the manufacturer kits (Ohkawa et al. 1979; Koracevic et al. 2001).

**Histopathological examinations**

Formalin fixed liver and kidney tissue specimens were dehydrated using ascending grade of ethanol, purified by Xylene, imbedded in paraffin wax and sliced at 4.5 μm to obtain paraffin embedded tissue sections stained by H&E and examined under light Olympus microscope to determine any pathological alterations (Bancroft 2012).

All the observable pathological parameters were graded using classical semiquantitative scoring system to assess the degree of lesion severity between different groups. Five-pointed ordinal scale used as the
following: (0) none, (1) mild <25%, (2) moderate 25% :50%, (3) severe 50% :75%, and (4) extensive severe >75% tissue damage (Hassanen et al. 2021).

Immunohistochemical studies

Immunohistochemical examination was performed to determine the protein expression of caspase-3 (a marker for apoptosis), Nuclear factor-κ protein (NF-κB) and tumor necrosis factor alfa (TNF-α) as a markers for inflammation in liver and kidney tissue sections using avidin-biotin-peroxidase complex (ABC). Briefly, deparaffinized-tissue sections were incubated with different primary antibodies (Abcam Ltd., USA) then the reagents required for ABC reaction (Vectastain ABC-HRP Kit, Vector Laboratories) were added. Afterward, slides were labeled with peroxidase and colored with DAB-chromogen substrate (Sigma), then examined under light Olympus microscope.

The mean percentage area of various immunostaining expressions in different groups were determined by using Image J software.

Molecular studies

The total RNA was extracting using the RNeasy Mini Kit (Qiagen Cat No./ID: 74104) according to instructions provided. The synthesis of first-strand cDNA was performed using SuperScript Reverse Transcriptase (Thermoscientific) according to the manufacturer's instructions. Quantitative real-time PCR was done using SYBR™ Green PCR Master Mix (Thermoscientific Cat number: 4309155) by the ABI Prism Step One Plus Real-Time PCR System (Applied Biosystems). The assay was performed in duplicates and the ACTB was used as internal standard for calculation of the expression level. The primer sets of the studied genes were shown in Table (1) and he fold change was calculated using $2^{-\Delta\Delta C_{T}}$

Statistical analysis

Data are illustrated as means ± standard deviation of the mean (SD). The recorded results were examined by one-way analysis of variance (ANOVA) and post hoc Duncan's test using the statistical package program (SPSS version 25); P values < 0.05 represent statistical significance. Kruskal Wallis H test was used for comparing the frequency data for nonparametric analysis followed by the Mann-Whitney $U$ test and data were expressed as median.

Results

Clinical signs and body weight of rats:

Rats in different groups didn't show specific clinical signs and mortality all over the experimental period. Regarding body weight, administration of either imidacloprid or hexaflumuron caused a notable reduction in average body weight of rats compared to the control group. Furthermore, hexaflumuron resulted in greater body weight reduction compared to imidacloprid exposed group (Fig. 1).

Behavioral observations
Rats receiving IC showed moderate to severe abnormalities in walking, body tension, drowsiness, and head movement compared to control rats. Furthermore, HFM exposed rats displayed more morbid symptoms than imidacloprid treated rats in which they exhibited a severe reduction in their walking and body tension, as well as they, became lethargic and their head movement was greatly attenuated compared to the rats in the control group (Table 2).

**Biochemical enzyme markers**

Data presented in Table (3) showed that administration of both IC and HFM in rats led to a significant elevation in the entire liver function parameters if compared with the control group. Moreover, HFM exposure showed a significant increase in serum AST and ALT activities in comparison with the imidacloprid group. On the other hand, total proteins, albumin and globulin levels were significantly inhibited in IC and HFM groups when compared to the control group. While there were no significant differences in total proteins, albumin and globulin levels between imidacloprid and hexaflumuron groups.

Concerning kidney function parameters, there was a significant increase in serum urea and creatinine levels in groups receiving either IC or HFM if compared with the control group, but the HFM receiving group showed the highest levels.

In case of glucose and lipid profile, there was a significant elevation in serum glucose levels in both IC and HFM exposed groups in comparison with the control group but, the highest levels were observed in the hexaflumuron group. Serum total cholesterol, triglycerides and LDL concentrations were significantly elevated in IC and HFM groups in comparison with the control group. The highest total cholesterol and LDL Levels were observed in the HFM group, while triglycerides concentration was the same in both treated groups. Regarding serum HDL concentration, there was a significant decline in both IC and HFM exposed groups when compared to the control group, but the lowest levels were detected in the HFM group.

**Oxidative stress evaluations**

The highest MDA levels and lowest GSH levels were observed in the group receiving HFM. Furthermore, IC receiving group showed a significant elevation in MDA levels and decreasing in GSH levels compared with the control group (Fig. 2).

**Histopathological examinations**

Liver tissue sections of the control rat showing normal histological structure (Fig. 3a). On the other hand, liver sections of IC receiving group showed moderate to severe histopathological alterations. There were severe diffuse hepatocellular cytoplasmic vacuolization and individual hepatocellular necrosis (Fig. 3b). Extensive congestion in the central vein and hepatic sinusoids were also recorded with lymphocytic infiltration. Portal triad showed congestion, moderate inflammatory cells infiltrations, and fibroplasia (Fig. 3c). Regarding HFM receiving group, liver sections showed severe diffuse hepatocellular cytoplasmic vacuolization and extensive congestion of the central vein, sinusoids, and portal vein. Hepatocellular coagulative necrosis with either zonal centrilobular or random focal distribution was noticed with or without mononuclear inflammatory cells infiltration (Fig. 3d). In addition, focal to coalescent areas of hemorrhage
were also observed in some sections (Fig. 3e). Portal triad showed severe inflammatory cells infiltration (Fig. 3f). The results of hepatic lesion scoring are illustrated in Fig. 3g and noticed the highest score in all parameters in group receiving HFM. Moreover, IC receiving group showed increase in lesion score in all pathological parameters compared with the control group.

Kidney tissue sections of the control rat showing normal histological structure (Fig. 4a). While those obtained from rat in IC receiving group showed mild to moderate nephrotoxic nephrosis. Renal tubular epithelial cells showed granular and vacuolar swelling. Most glomeruli showed congestion of the capillary tuft with hypercellularity (Fig. 4b). Interstitial tissue showed severe congestion and mild inflammatory cells infiltration. Concerning HFM receiving group, kidney sections showed severe nephrotoxic nephritis and glomerulopathy. Some glomeruli showed atrophy of glomerular tuft with widening of bowmen’s capsule while others showing vacuolation, congestion, and/or hypercellularity (Fig. 4c). Renal tubular epithelium showed severe degeneration and necrosis with intraluminal renal cast and droplets. Interstitial tissue showed severe congestion, hemorrhage, and focal inflammatory cells infiltration (Fig. 4d). The results of renal lesion scoring are illustrated in Fig. 4e and noticed the highest score in all parameters in the group receiving HFM. Moreover, IC receiving group showed increase in lesion score in all pathological parameters compared with control group.

**Immunohistochemical studies**

There were strong positive expressions of caspase-3, NF-κB, and TNF-α in both liver and kidney sections obtained from the group receiving HFM compared with the control group. Moreover, the group receiving IC showed moderate reactions for the above-mentioned immune marker in both liver and kidneys (Figs. 5-7).

**Molecular studies**

Marked increases in the transcript levels of caspase-3, JNK, and HO-1 genes as well as reduction in Keap-1 gene were observed in the groups receiving either IC or HFM compared with the control groups. The highest levels of caspase-1, JNK, and HO-1 genes were recorded in the group receiving HFM compared with the IC group. On the other hand, IC group recorded the lowest level of Keap-1 gene when compared with HFM group (Fig. 8).

**Discussion**

Exposure to environmental pollution remains a major source of health risk worldwide especially in developing countries (Bolognesi and Morasso 2000). Among the environmental pollution, overexposure to pesticides is considered as one of the causative factors of various health problems in human and animals (Cheng et al. 2011). The current study designed to investigate the possible toxic effects of two types of insecticides (IC and HFM) on the liver and kidney of rats through measuring rat’s body weight and behavioral changes, liver and kidney function markers, lipid profile, oxidative stress markers, histopathological examination, caspase-3, NF-κB, and TNF-α immune markers as well as measuring m-RNA levels of caspase-3, JNK, HO-1, and Keap-1 genes to spot the mechanistic way of toxicity of these insecticides in rats.
In the current study, there was a notable decrease in body weight with neurobehavioral alterations of rats after exposure to IC and HFM despite of having free access to food suggesting the toxic effect of both pesticides to animal organs that may interfere with the absorption of some nutrients (Ndonwi et al. 2020). In agreement with our findings, several studies showed that insecticides cause a decrement in body weight (Arfat et al. 2014). In addition, severe hepatotoxicity has been reported to induce hepatencephalopathic neurobehavioral alterations (Hayase et al. 2000).

The results of the present study revealed that IC and HFM induced hepatorenal oxidative stress damage manifested by increased MDA levels and decreased TAC levels associated with the upregulation of HO-1 gene and down-regulation of Keap1 gene levels. These findings agreed with other report showed that lufenuron, belonging to the same hexaflumuron-family, was able to induce oxidative stress damage in the liver of rats (Basal et al. 2020). Also, chronic exposure to IC alters inflammation and oxidative stress markers in the liver and central nervous system of rats (Duzguner and Erdogan 2012). Increasing MDA levels suggest free O$_2$ overproduction that initiates DNA damage, protein degradation, lipid peroxidation and tissue damage particularly liver (organ of detoxication) and kidneys (organ of excretion) (Timoumi et al. 2019). HO-1 is a stress-induced isoform located in the endoplasmic reticulum, mitochondria, cell nucleus, and plasma membrane of several cell types mainly liver and kidneys (Hopper et al. 2018). Its upregulation occurred in many conditions associated with oxidative stress, chemical toxicity, and inflammatory reactions (Ferrándiz ML 2008). Keap1 has been shown to interact with Nrf2, a master regulator of the antioxidant response, which is important for the amelioration of oxidative stress (Wang et al. 2008; Deshmukh et al. 2017). Several studies confirmed that Keap-1 or Nrf2 down-regulation increased mitochondrial ROS production and induced the process of apoptosis and inflammation (Shibata et al. 2008).

The results of oxidative stress evaluations reflected on the histopathological picture of liver and kidneys of rat receiving IC and HFM that showing severe pathological alterations related to ROS overproduction. Several studies reported that ROS overproduction increasing cell and mitochondrial membrane permeability and alters Na/K/ATPase pump causing ionic imbalance (Khalaf et al. 2020). This mechanism explained the observed hepatocellular and renal tubular epithelial cells degeneration in the present study. Furthermore, oxidative stress causing mitochondrial dysfunction and opining mitochondrial transition pores that increase the cytosolic Ca levels which activate several enzymes leading to protein degradation, lipid peroxidation and DNA damage (Mansour and Mossa 2010). Those causing further membrane damage and cell death via necrosis and apoptosis pathway. Apoptosis is a programmed cell death that initiated several factors including ROS production (Kandemir et al. 2017). Caspase-3 has been known as a marker of apoptosis in mammalian cells and initiates the apoptotic cascade by activating other caspase enzymes (Eldutar et al. 2017). JNK belongs to the mitogen-activated protein kinase family that plays an important role in the process of cell apoptosis (Vlahopoulos and Zoumpourlis 2004). It is activated by several factors including ROS and proinflammatory cytokines causing apoptosis through a series of intermediate (Oltemans et al. 2003). These data suggest our results about strong caspase-3 protein expressions in both liver and kidney sections of both insecticides receiving groups together with up-regulation of m-RNA levels of different apoptotic markers as caspase-3 and JNK genes.
The observable hepatocellular pathological alterations were confirmed by measuring the hepatocellular integrity through the determination of two biochemical markers, serum ALT and AST. Our results showed a significant elevation in both biochemical hepatic markers indicating a defect in the permeability of cell membrane and cellular necrosis (Manfo et al., 2020). It is reported that IC and HFM exposure led to a significant impairment in the hepatic parameters (Toor et al., 2012). Our results also showed a decline in the total proteins levels in the IC and HFM groups suggesting a reduction in albumin synthesis in response to hepatocellular damage (Mansour and Mossa, 2010). Our finding agreed with Chakroun et al., (2017) that reported a significant decrease of total proteins following imidacloprid exposure. Data illustrated in the current study also showed a significant elevation in both urea and creatinine concentrations upon exposure to either IC or HFM suggesting their nephrotoxic potential. These findings can be explained by the histopathological observations that showed severe nephrotoxic nephrosis, nephritis, and glomerulopathy in the groups receiving either IC or HFM.

The data presented in our study demonstrated a significant elevation in serum glucose levels in both IC and HFM administration indicating a disturbance of carbohydrate metabolism as a result of enhancing liver breakdown of glycogen. Results obtained by Kim et al (2013) support our findings as IC could affect insulin signaling pathways. Additionally, IC and HFM administration led to a significant elevation in total cholesterol, triglycerides and low-density lipoprotein (LDL) cholesterol concentrations, while high density lipoprotein (HDL) cholesterol level was significantly diminished in both interventions in comparison to the control group. Pesticides was reported to induce oxidative stress associated with mitochondrial dysfunction and impairment of glucose and lipid metabolism (Bonvallot et al. 2018). In addition, pesticides exposure alters lipid homeostasis together with the hepatic and adipocytes lipid storage impairment, thus the lipid level in the blood is affected by pesticides that led to disruption of energy balance (He et al. 2020).

Furthermore, the present study showed moderate to severe TNF-α and NF-κB expressions in both liver and kidney sections of all insecticides receiving groups. It is reported that there were correlations between insecticides exposure and changes in cytokine activity. Omurtag et al. (2008) reported that pesticides administration resulted in hepatotoxicity related to proinflammatory cytokine expression (TNF-α) which in turn was increased by oxidative stress. Additionally, several insecticides can trigger ROS production leading to oxidative stress and enhanced activation of the NF-κB pathway (Yang et al. 2009). In addition, it induces significant production of TNF-α and NO in macrophages and thus contributes to inflammatory reactions, cytokine imbalance and immune dysregulation (Dutta et al. 2008). Videla et al. (2004) have proposed that lindane induced oxidative stress in liver triggers DNA binding activity of NF-κB, with a consequent increase in the expression of NF-κB-dependent genes for TNF-α, therein identifying factors that may mediate the hepatotoxic effect of insecticides. In the current study the observed increase of MDA levels in liver and kidneys, correlated with the stimulation of pro-inflammatory cytokine expression suggests that both IC and HFM may mediate its toxicity via activation of NF-κB signaling pathway in the chronic phase of inflammation.

Conclusion
From the results of the current study we can concluded that both IC and HFM insecticides exerts severe hepatorenal oxidative stress damage. IC and HFM induced oxidative stress via several ways including ROS production, antioxidants exhaustion, HO-1 upregulation, and inactivation of Keap-1 gene that inactivate Nrf2 signaling pathway. In addition, both insecticides activate JNK signaling pathway via ROS overproduction causing apoptosis via several cascade activation including upregulation of caspase-3 gene and protein overexpression. Activation of NF-κB signaling pathway is another mechanism for both insecticides induced hepatorenal toxicity in rats. Both IC and HFM stimulate cytokines production including TNF-α via several ways such as ROS overproduction and JNK activation which activate nuclear translocation of NF-κB within hepatic and renal cells initiate the process of inflammatory reactions.

**Declarations**

**Ethics approval and consent to participate**

All Institutional and National Guidelines for the care and use of animals (fisheries) were followed.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data are available on request

**Competing interest**

The authors declare that they have no competing interests

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**Authors’ contributions**

E.I.H conceived the study, designed the experiment, reviewed all the results, drafted the manuscript, and performed the pathological studies. A.M.H performed the oxidative stress evaluations and carried out data analysis, S.M performed the experimental study and measured the animal's weights and behavioral changes. M.A performed the molecular assays. N.H.H performed the biochemical tests and carried out data analysis. All authors read, revised, and approved the final manuscript.

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**Tables**

**Table. 1: The primer sets of the studied genes**

| Sense          | Antisense                  | Amplicon | Accession no |
|----------------|----------------------------|----------|--------------|
| Caspase 3      | GAGCTTGGAACGCGAAGAAA      | TTGCGAGCTGACATTCCAGT | 221          | NM_012922.2  |
| JNK            | GTCATTCTCGGCATGGGCTA      | TGGACGCATCTATCACCAGC | 337          | NM_053829.2  |
| HO-1           | AGCGAAACAAACGAGACACCCA    | ACCTCGTGGAGACGCTTTAC | 166          | NM_012580.2  |
| Keap-1         | ATGTGATGAACGGGGCAGTTCTC  | AAGAACTCCTCCTCCTCCCCGAA | 190         | NM_057152.2  |
| **ACTB**       | CCGCGAGTACAACCTTCTTG      | CAGTTGGTGACAATGCCGTTG | 297          | NM_031144.3  |
Abbreviations: JNK, c-Jun N-terminal kinases; HO-1, Heme oxygenase; Keap-1, Kelch Like ECH Associated Protein 1; ACTB, Beta actin (housekeeping gene).

**Table. 2: The effect of imidacloprid and hexaflumuron on some behavioral changes in rats**

| Group/parameters | Control | Imidacloprid | Hexaflumuron |
|------------------|---------|--------------|--------------|
| Walking          | 0<sup>a</sup> | 3<sup>b</sup> | 4<sup>c</sup> |
| Body tension     | 0<sup>a</sup> | 3<sup>b</sup> | 4<sup>c</sup> |
| Alertness        | 0<sup>a</sup> | 4<sup>b</sup> | 4<sup>b</sup> |
| Head movement    | 0<sup>a</sup> | 3<sup>b</sup> | 3<sup>b</sup> |

Data expressed as Median. (N=5 rats/group), values having different letters in the same row means significantly different at \( p \leq 0.05 \).

Note: 0, normal behavioral patterns; 3, moderate behavioral alterations; 4, severe behavioral alterations

**Table. 3: The effect of Imidacloprid and Hexaflumuron on serum biochemical parameters in rats.**

| Hexaflumuron (HFM) | Imidacloprid (IC) | Control | PARAMETERS                          | GROUPS  |
|-------------------|-------------------|---------|------------------------------------|---------|
| 70.88 ± 4.98<sup>c</sup> | 63.02 ± 4.36<sup>b</sup> | 40.89 ± 2.72<sup>a</sup> | ALT (U/L) | |
| 102.70 ± 4.77<sup>c</sup> | 79.52 ± 5.91<sup>b</sup> | 57.66 ± 4.69<sup>a</sup> | AST (U/L) | |
| 5.82 ± 0.94<sup>b</sup> | 6.50 ± 0.90<sup>b</sup> | 8.60 ± 0.52<sup>a</sup> | TP (g/dl) | |
| 3.43 ± 0.34<sup>b</sup> | 3.56 ± 0.62<sup>b</sup> | 4.52 ± 0.36<sup>a</sup> | Albumin (g/dl) | |
| 3.18 ± 1.01<sup>b</sup> | 3.01 ± 0.37<sup>b</sup> | 4.14 ± 0.38<sup>a</sup> | Globulin (g/dl) | |
| 180.74 ± 5.63<sup>c</sup> | 122 ± 5.43<sup>b</sup> | 83.80 ± 7.19<sup>a</sup> | Glucose (mg/dl) | |
| 137.96 ± 9.23<sup>c</sup> | 116.10 ± 7.13<sup>b</sup> | 74.43 ± 7.74<sup>a</sup> | Total cholesterol (mg/dl) | |
| 17.78 ± 9.18<sup>c</sup> | 31.96± 4.55<sup>b</sup> | 41.25 ± 5.21<sup>a</sup> | HDL-C (mg/dl) | |
| 103.27 ± 8.00<sup>c</sup> | 68.43 ± 10.4<sup>b</sup> | 23.86 ± 5.00<sup>a</sup> | LDL-C (mg/dl) | |
| 84.83 ± 7.42<sup>b</sup> | 78.35 ± 6.72<sup>b</sup> | 46.16 ± 4.40<sup>a</sup> | Triglycerides (mg/dl) | |
| 61.04 ± 3.53<sup>c</sup> | 45.76 ± 3.29<sup>b</sup> | 33.52 ± 3.48<sup>a</sup> | Urea (mg/dl) | |
| 1.97 ± 0.17<sup>c</sup> | 1.40 ± 0.18<sup>b</sup> | 0.78 ± 0.25<sup>a</sup> | Creatinine (mg/dl) |
Data expressed as Mean ± SD. (N=5/group), a; b; c means having different superscript letters in the same row differ significantly at p≤ 0.05.

Abbreviations: AST, aspartate amino transferase; ALT, alanine aminotransferase; TP, total proteins; HDL-C, high density lipoprotein cholesterol; and LDL-C, low density lipoprotein cholesterol.

Figures

Figure 1

Effects of Imidacloprid and Hexaflumuron on body weight (g) of male rats. Values are presented as mean ± SD. (n = 5 rat/ group). Values with different letters are significantly different at P ≤ 0.05.
effects of imidacloprid and hexaflumuron on hepatic and renal MDA level and GSH content of male rats. Values are presented as mean ± SD. (n= 5 rat/ group). Values with different letters are significantly different at P ≤ 0.05. Abbreviations: Control (C), Imidacloprid (IC) and hexaflumuron (HFM), Malondialdehyde (MDA), Reduced Glutathione (GSH).
Figure 3

Photomicrograph of liver tissue sections stained by H&E stain representing, (a) control group with normal histological structure; (b-c) IC receiving group showing, (b) diffuse hepatocellular cytoplasmic vacuolization with severe congestion in central vein (arrow), sinusoids (arrowhead), and portal vein (star). (c) Portal triad showing moderate mononuclear congestion (star) and inflammatory cells infiltration (arrow). (d-f) HFM receiving group showing, (d) large focal area of hepatocellular coagulative necrosis (arrow) infiltrated with mononuclear inflammatory cells. (e) Large coalescent area of hepatic hemorrhage (star). (f) Portal triad
showing severe congestion (star), edema, and mild inflammatory cells infiltration (arrow). (g) Bar chart representing microscopic lesion scoring in liver sections of different groups. Values are presented as median. (n= 5 sections representing 5 rats/group). Values with different letters are significantly different at P ≤ 0.05. Abbreviations: Control (C), Imidacloprid (IC) and hexaflumuron (HFM).

Figure 4

Photomicrograph of kidney tissue sections stained by H&E stain representing, (a) control group with normal histological structure. (b) IC receiving group showing severe congestion in glomerular capillary tuft (arrow),
and interstitial bl vs with mild individual cell necrosis in renal tubular epithelium (arrowhead). (c-d) HFM receiving group showing, (c) glomerular atrophy (arrowhead) with widening of bowman’s capsule. Note: degeneration and necrosis of renal tubular epithelial cells. (d) Severe congestion in glomeruli and renal bl vs (star) with interstitial inflammatory cells infiltration (arrows). (e) Bar chart representing microscopic lesion scoring in kidney sections of different groups. Values are presented as median. (n= 5 sections representing 5 rats/group). Values with different letters are significantly different at P ≤ 0.05. Abbreviations: Control (C), Imidacloprid (IC) and hexaflumuron (HFM),

Figure 5

Photomicrographs representing IHC examinations in liver sections of different groups. (a-c) Control group showing normal mild to negative caspase-3, TNF-α, and NF-KB expressions respectively. (d-f) Imidacloprid receiving group showing strong to moderate reactions for the above-mentioned immune markers. (g-i) Hexaflumuron receiving group showing strong reactions for all examined immune markers.
Figure 6

Photomicrographs representing IHC examinations in kidney sections of different groups. (a-c) Control group showing normal caspase-3, TNF-α, and NF-KB expressions respectively. (d-f) Imidacloprid receiving group showing strong reactions for the above-mentioned immune markers. (g-i) Hexaflumuron receiving group showing strong reactions for all examined immune markers.
Figure 7

Bar charts representing mean percentage area of caspase-3, TNF-α, NF-KB immunopositivity in liver (a), and kidney (b) tissue sections. Values are presented as mean ± SD. (n = 10 low power fields per section, total 5 sections representing 5 rat/group). Values with different letters are significantly different at P ≤ 0.05.
Figure 8

Bar charts representing the transcript levels of caspase-3 (a), JNK (b), HO-1 (c), and Keap-1 (d) genes in liver and kidney tissue homogenates from different groups. Values are presented as mean ± SD. (n= 5 rats/group). Values with different letters are significantly different at P ≤ 0.05. Abbreviations: Control (C), Imidacloprid (IC) and hexaflumuron (HFM), c-Jun N-terminal kinases (JNK), Heme oxygenase (HO-1), Kelch Like ECH Associated Protein-1 (Keap-1).