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Original article

Effects of exposure to imidacloprid contaminated feed on the visceral organs of adult male rabbits (*Oryctolagus cuniculus*)

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ABSTRACT

The best-known and often used systemic, broad-spectrum neonicotinoid pesticide is imidacloprid (IMI). This study was carried out on adult male rabbits (n = 12) to assess the residual effects of exposure to IMI-contaminated diet on the liver, lung, heart, and kidney. Pesticide-exposed rabbits (n = 6) received IMI contaminated green grass (Bildor® 0.5 ml (100 mg)/L water) every alternative day once daily for up to 15 days. The remaining rabbits were fed a standard diet free of pesticides as a control. During routine monitoring of the rabbits throughout the experiment, there were no apparent toxic symptoms identified. On days 16, after deep anesthesia blood and visceral organs were collected. The levels of hepatic serum aspartate transaminase and alanine transaminase were considerably elevated in IMI-exposed rabbits ($p \leq 0.05$). Thin layer chromatography revealed that the residue of IMI was at the detectable level in the liver and stomach. Histopathologically, the liver revealed coagulation necrosis with granulomatous inflammation and congestion in portal areas with dilated and congested central veins. The lungs showed congestion of blood vessels and granulomatous inflammation around the terminal bronchiole. Accumulations of inflammatory cells were observed in the cortico-medullary junction in the kidney. The heart showed necrosis and infiltration of mononuclear cells within the cardiac muscles. The findings of the current study emphasize that IMI-contaminated feed exposure causes toxicity into the cellular level of different visceral organs of adult male rabbits and it may also cause the similar toxic effects of the other mammals specially the occupationally exposed persons.

1. Introduction

Hazardous effect of pesticide is a significant global public health issue. Pesticides cover a wide spectrum of compounds such as insecticides, herbicides, fungicides, rodenticides, molluscicides, nematicides and other chemicals intended to kill or control pests (Aktar et al., 2009; Hassaan and El Nemr, 2020). Due to a lack of proper knowledge, pesticides are commonly abused by the majority of Bangladeshi crop, vegetable, and fruit farmers (Kobir et al., 2020). The worst scenario is now prevailing in the pest management arenas of Bangladesh.

Imidacloprid (IMI) is one of the top used and the most well-known broad-spectrum, systemic, neonicotinoid pesticide used extensively against sucking, boring, and root-feeding insects, representing more than 25% of the world's pesticide market (Craddock et al., 2019; Goulson, 2013; Ihara and Matsuda, 2018). It is also utilized in numerous veterinary medications to treat pets for fleas (Thompson et al., 2020). It works primarily on pests' nervous systems via nicotinic acetylcholine receptors, which results in a favorable toxicological profile in humans (Mundhe et al., 2017). IMI is found in a variety of commercial insecticides and using data from animal research, it is categorized as being moderately harmful (Class-II WHO; toxicity category-II EPA) (Mohamed et al., 2009). These chemicals can be acquired through food, cutaneous absorp-

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tion, inhalational absorption, with oral consumption causing the most serious poisoning (Tomizawa & Casida, 2005).

Pesticide compounds (insecticides) can cause oxidative stress, which alters antioxidant and free radical-scavenging enzyme systems and produces free radicals (Duzguner and Erdogan, 2010; Wang et al., 2018). The liver's function is hampered by oxidative damage induced by free radicals in the liver, which leads to abnormalities in body homeostasis (Zama et al., 2007). IMI has an impact on multiple systems in the body of chicken, including the liver, lungs, kidneys, spleen, gut, pancreas, heart etc. where congestion and hemorrhages are evident (Komal et al., 2016). In male mice, IMI has been demonstrated to cause oxidative stress (El-Gendy et al., 2010); as well as being hepatotoxic and gonadotoxic (Arfat et al., 2014; Mohamed et al., 2009; Yang et al., 2020). The spermatogenic and interstitial or Leydig cell populations in adult male rabbits are toxicologically affected by IMI (Kobir et al., 2022).

Risks to human health differ based on the variety of pesticides used and the level of susceptibility (Arfat et al., 2014). Toxicological studies in animals are frequently extended to human toxicity, however their applicability is still poorly defined. Chemical pesticides are considering threat to human health, environment, biodiversity, soil and water health.

So, the purpose of the present experiment was to assess the effects of IMI-contaminated feed exposure in adult male rabbits by analyzing serum biochemistry and gross and microscopic changes of different visceral organs.

2. Material and methods

2.1. Ethic al approval

The Animal Welfare and Experimentation Ethics Committee, Bangladesh Agricultural University, Mymensingh, Bangladesh (AWEEC/BAU/19/41) developed criteria for the care and use of animals, and the current study and all experimental protocols were established and carried out in accordance with those standards.

2.2. Chemical preparation

Imidacloprid (IMI, Bildor®, a product marketed by Corbel International Ltd., Tejgaon, Dhaka – 1215, Bangladesh) was purchased from the registered pesticide dealer (Mymensingh City Corporation Market, Mymensingh Sador, Bangladesh) and dealt with caution and the required safeguards. The pesticide IMI was properly diluted in fresh tap water 0.5 ml (100 mg)/liter according to data sheet to use for pest control in agriculture. Using a Suja Global Hand Sprayer Machine (SEGARTEX, Dhaka-1230, Bangladesh), the appropriately mixed IMI water was applied on the evening to the green grass in the confined field that was fenced. In the following morning, the sprayed green grasses were collected.

2.3. Animals and experimental procedures

For this study, twelve male Netherland dwarf rabbits (*Oryctolagus cuniculus)* aged 11 months and weighing around 2–3 kg each was selected. Individual metal cages were used to keep the animals in the department's experimental animal room, which was kept at 25–30 °C with a 12-hour light–dark cycle. The rabbits were given a typical diet of green grass and wheat bran, as well as unlimited access to drinking water. After acclimatization for one-week, in the morning of every other day, six rabbits were given IMI exposed green grass (48 h interval) up to 15 days and fresh green grass with wheat bran on evening; provided normal pesticide-free green grass and wheat bran on pesticide-free days. As a control, six rabbits were given wheat bran and pesticide-free green grass. During the study, dietary patterns related to food and water consumption were noted. Every rabbit was regularly observed, and the state of their health was recorded. All of the exposed and control rabbits were sacrificed on day 16 of the pesticide exposure under deep isoflurane anesthesia.

2.4. Serum analysis

Blood was drawn from the abdominal aorta during the necropsy and isolated sera were then tested biochemically for aspartate transaminase (AST), alanine transaminase (ALT), bilirubin, and creatinine. Using commercially available kits (Randox Laboratories, Crumlin, UK) and according to the manufactures instructions, the serum test was analyzed by spectrophotometry.

2.5. Detection of IMI residues by using thin layer chromatography (TLC)

Immediately after collection of the target organs such as stomach, liver, lungs, kidneys from sacrificed rabbits, saline wash was performed to remove dirt and clotted blood and then packed into separate plastic zipper bags, transported to the lab by ice bags, and stored there until being extracted at −20 °C. The experiment was conducted using glass-distilled water and analytical reagent grade. The standard solution of IMI was prepared in ethanol (10 ml) whereas p-Dimethyl-aminobenzaldehyde was prepared by dissolving it (5 g) in concentrated hydrochloric acid, HCl (100 ml). The mobile phase used in this experiment was chloroform-acetone @ 7:3. About 4 gm from each sample (stomach, liver, lungs, and kidney) was divided into small parts, ground, and blended. 10 ml phosphate buffer saline (pH-6.5) was added and stirred for vortexing (Vortex- XHC, Wincom, China). The mixture was centrifuged for 20 min at 6000 rpm after being combined with 2 ml of 30% trichloroacetic acid (TCA) (Hettich D-78532, Germany). Whatman filter paper and funnel were used to collect and filter the supernatant and the same volume of diethyl ether was applied and kept at room temperature for 10 min. After obtaining the bottom layer, diethyl ether supernatant extraction was carried out twice. The final amount of extracts was carefully combined into a screw-top vial and refrigerated for further analysis. The TLC plate was spotted using standard imidacloprid solution (10 µl) and placed in the presaturated TLC chamber at a distance of approximately 10 cm with mobile phase (chloroform-acetone @ 7:3). The plate was taken out of the chamber and allowed to dry at room temperature. The UV detector box at 254 nm showed normal pink color spots on the TLC plate.

2.6. Collection of different vital organs for gross and histopathology

Under deep anesthesia, internal organs such as liver, lungs, kidney, heart were visualized and examined very carefully for gross changes particularly, organs's color, gross texture and any lesion and recorded carefully. After gross examination, samples of the liver, lungs, kidney, and heart were obtained and immediately fixed using a standard procedure in 10% neutral buffered formalin (NBF). Next, after being dehydrated with ascending grades of alcohol, paraffin was used to embed the NBF-fixed tissues, which were then sectioned at a thickness of 6 µm using a sliding microtome (Euromax®, Japan). For histopathological examination, Hematoxylin and eosin (H and E) staining was performed on the deparaffinized sections. A light microscope was used to examine the histopathological changes induced by IMI (LABOMED, Labo America Inc., CA 94538).

2.7. Statistical evaluation

Using SPSS, version 18.0 software (IBM Corp., NY, USA), all of the collected data were analyzed and statistically evaluated applying the 'Student t-test'. Statistical significance was defined as $p \leq 0.05$ and the values were presented as mean ± standard deviation (SD).

2.8. Processing of photographs and images

With a digital camera (DS-Fi1, Nikon, Tokyo, Japan) or a virtual slide scanner (VS-120, Olympus, Tokyo, Japan) mounted on a microscope, high- and low-power photomicrographs were captured. In Adobe Photoshop 7.0 J and Adobe Illustrator 10.0 J, the contrast, brightness, and sharpness of the pictures, as well as the layout and lettering, were adjusted.

3. Results

3.1. Effect of IMI on rabbit's body weight

Rabbits exposed to IMI were regularly monitored during the exposure period, but no toxic symptoms were noticed. When compared to control rabbits, the mean body weight of IMI-exposed rabbits did not differ substantially (data not shown).

3.2. Effect of IMI on serum biochemistry

Hepatic serum enzyme aspartate transaminase (AST) and alanine transaminase (ALT) levels were substantially higher in rabbits exposed to IMI compared to control rabbits ($p \leq 0.05$) (Fig. 1A-B). There was no significant change found in bilirubin and creatinine level in IMI-exposed rabbit group (Fig. 1C-D).

3.3. Detection of IMI residue in tissues by thin layer chromatography (TLC)

The thin layer chromatographic plate analysis revealed that the residue of IMI was deposited at a detectable level in tissue of liver and stomach (Fig. 2A) and was undetectable in kidney and lungs of IMI-exposed rabbits (Fig. 2B). The pink spot was found at 0.52 in UV detection box at 254 nm wave length on the TLC plate.

3.4. Effect of IMI on gross morphology of vital organs

Grossly, no change was found in collected visceral organs (liver, lungs, kidney, and heart) of control and IMI treated rabbits. The size, shape, color and texture of organs of IMI-treated rabbits were normal and similar to the control rabbits.

3.5. Histopathological changes of vital organs of IMI exposed rabbits

3.5.1. Liver

The liver of control rabbits displayed a regular, normal histological structure devoid of any abnormalities (Fig. 3A). In brief, the

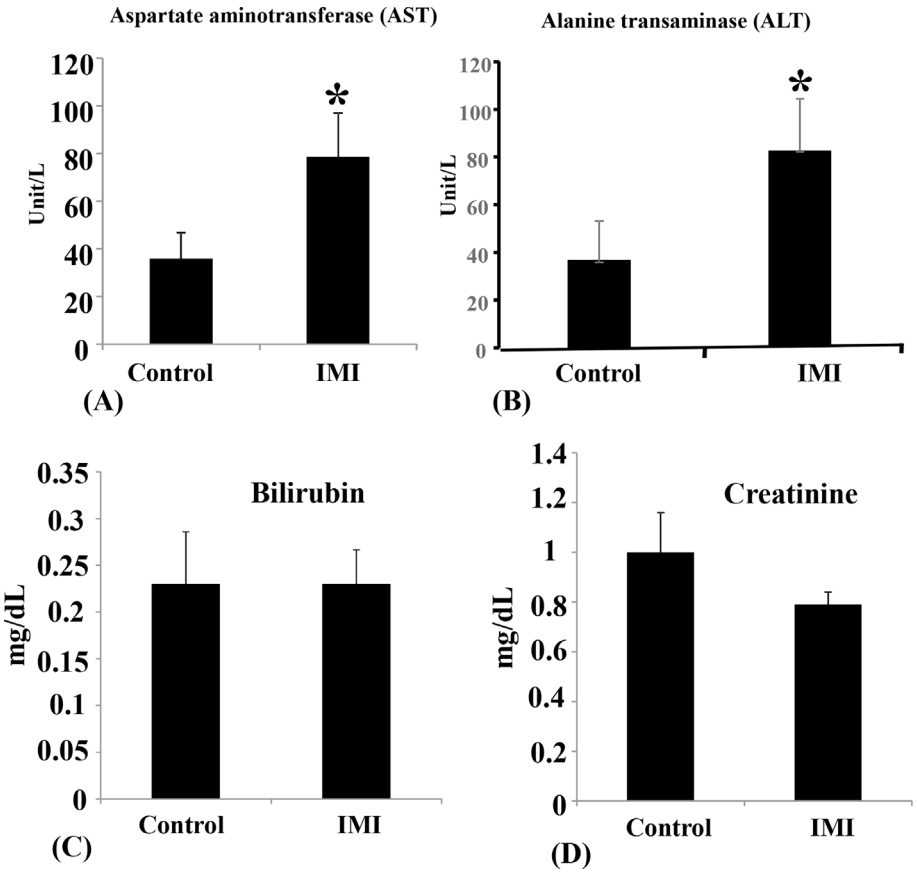

Fig. 1. A-B: Dynamics of hepatic enzymes, AST (A) and ALT (B). The value of AST and ALT were increased substantially in the imidacloprid exposed group compared to the control. C-D: Dynamics of bilirubin and serum creatinine. No significant change in bilirubin and creatinine level. Student *t*-test, *the p-values of ≤ 0.05 were considered significant.

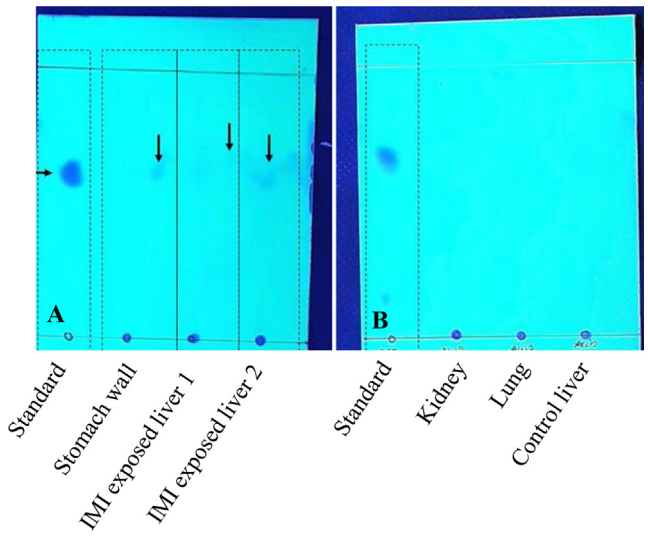

Fig. 2. Chromatographic plate showing the presence of residue of imidacloprid (black arrows) in tissues of liver and stomach of imidacloprid (IMI) exposed rabbits (A) and not detected in lungs and kidney of IMI exposed rabbit and liver of control rabbit (B).

parenchymal hepatocytes were located around the central veins (considered as perivenular areas) (Fig. 3A) and around the portal canals (considered as periportal areas). The portal canal was referred to enlarged regions of the interlobular connective tissue between the liver lobules that contain a branch of the portal vein, hepatic artery, bile duct, and lymph vessel (Fig. 3A). IMI treated rabbit revealed clear histopathological alterations in liver parenchyma (Fig. 3B-D). Particularly, coagulation necrosis with infiltration of inflammatory cells were present in periportal areas (Fig. 3B-D). Dilatation of sinusoids and congested portal veins were seen (Fig. 3B). Dilatation and congestion of central vein were also seen (Fig. 3D). Presence of RBC within necrosed areas indicated hemorrhage within liver parenchyma (Fig. 3E).

3.5.2. Lungs

Numerous alveoli, alveolar duct, alveolar sac, bronchioles and part of bronchus constituted the histoarchitecture of lungs of control rabbits (Fig. 4A). In the IMI treated rabbit, granulomatous inflammation and accumulation of inflammatory cells were seen around the terminal bronchiole close to the blood vessels (Fig. 4-B-C). Marked congestion of blood vessels was also found (Fig. 4D).

3.5.3. Kidney

In the kidney of IMI-exposed rabbits, granulomatous inflammation was found near the cortico-medullary junction (Fig. 5A-B). Presence of congestion and inflammatory cells in between kidney tubules was also observed (Fig. 5C).

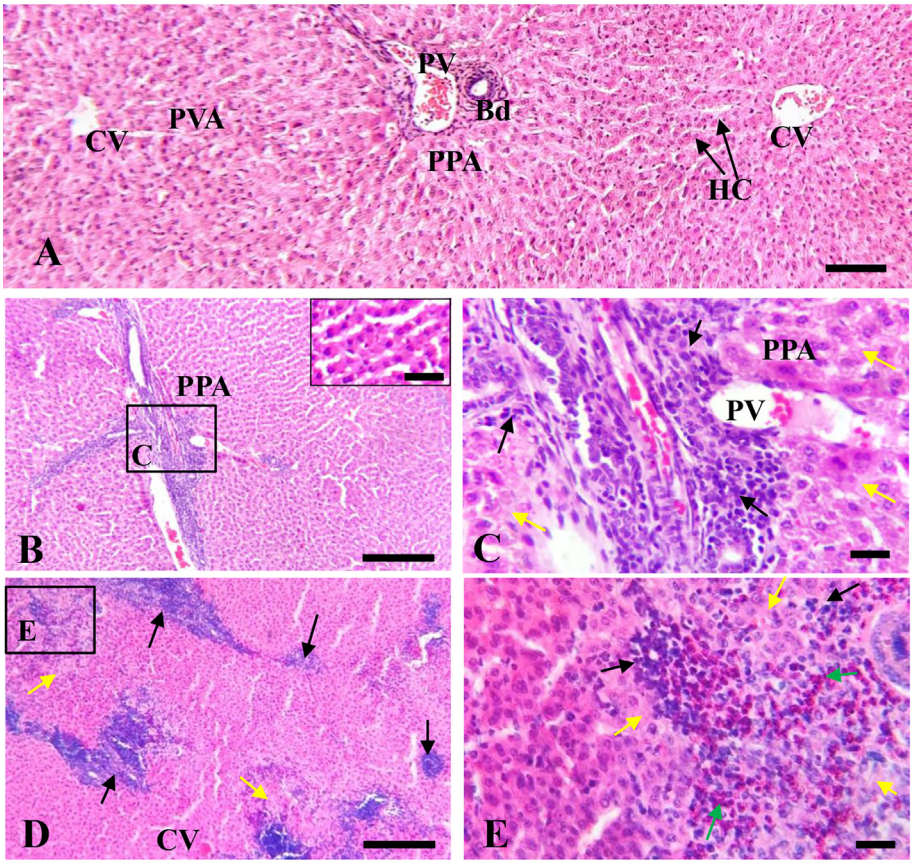

Fig. 3. Histopathological observation of liver of control and IMI-exposed rabbits. A: Normal histoarchitecture of the liver of control rabbit. B: Congested portal veins and coagulation necrosis with infiltration of inflammatory cells was present in portal areas and periportal areas. Inset showing dilatation of sinusoids in periportal areas in higher magnification. C: Showing the coagulative necrosis (yellow arrows) and inflammatory cells (black arrows) in higher magnification. D: Marked infiltration of inflammatory cells in portal areas and presence of coagulative necrosis (yellow arrows) in liver parenchyma with congested central vein. E: Showing the inflammatory cells (black arrows) and coagulative necrosis (yellow arrows) in higher magnification. Presence of RBC (green arrows) indicating hemorrhage within liver parenchyma. CV- Central Vein, PVA- Perivenular area, PPA- Periportal area, PV- Portal Vein, HC– Hepatic cord, Bd- Bile duct, H & E stain. Bars: A, B, D = 400 μm; C, E = 100 μm.

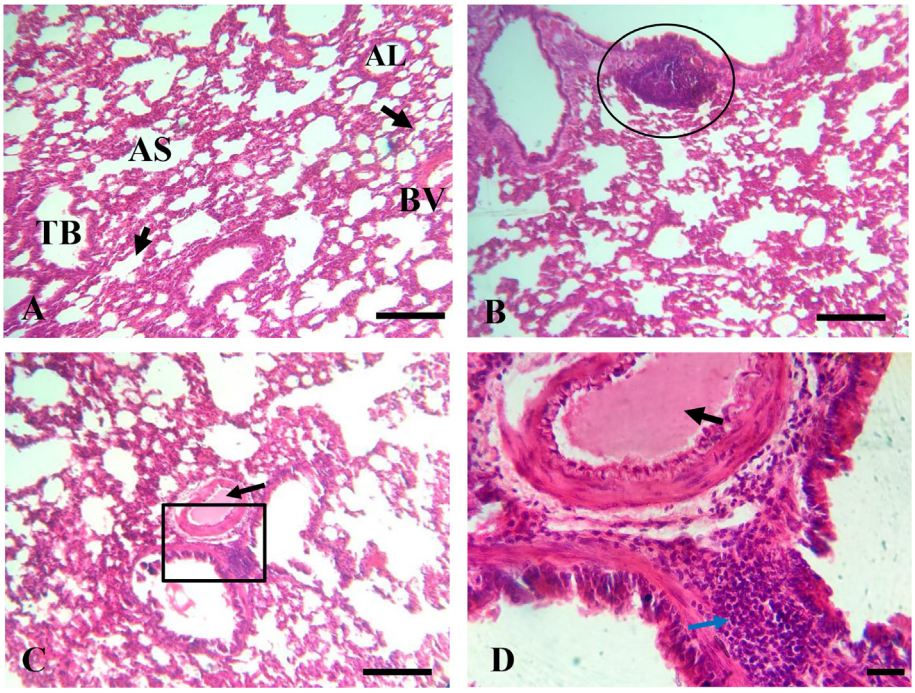

Fig. 4. A: Histology of the lungs of control rabbits. AL- alveoli, AS- alveolar sac, Arrows- alveolar duct, TB- terminal bronchiole, BV- blood vessel. B-C: Lungs of IMI-exposed rabbits showing granulomatous inflammation (encircled area) around the terminal bronchiole close to the blood vessel which was congested (black arrows). D: Granulomatous inflammation (blue arrow) with accumulation of inflammatory cells around the terminal bronchiole. Black arrow indicated congestion of blood vessels. H & E stain. Bars: A-C = 400 μm; D = 100 μm.

3.5.4. Heart

The heart of IMI-exposed rabbits showed marked infiltration of mononuclear cells in myocardium (Fig. 5D). The inflammatory cells were pleomorphic and heterogeneously arranged alongside the necrosed cardiac muscles (Fig. 5E-F).

4. Discussion

Average body weight and gross morphology of visceral organs of IMI (0.5 ml (100 mg IMI)/liter, sprayed on grass) exposed rabbits were not changed in the present study. At 15 mg IMI/kg/day (oral gavage) exposure, albino mice reported a decrease in body weight (Arfat et al., 2014). The variation in results could be brought about by dose, formulation, or species differences. Interestingly, IMI-exposed rabbits had considerably higher values of the hepatic serum enzymes AST and ALT than the control rabbit which indicates liver injury. Similarly, IMI caused a significant increase of AST and ALT in albino mice at 15 mg/kg/day (Arfat et al., 2014) and in *Labeo rohita* (Qadir and Iqbal, 2016). Repeated oral administration of IMI in wister rats resulted in a considerable increase in AST and ALT values as well as antioxidant enzyme activities that cause oxidation-induced hepatotoxicity (Mehmood et al., 2017, Mahajan et al., 2018, and Hassan et al., 2019), these reports are similar to the present study.

The value of bilirubin and creatinine in IMI-exposed rabbits showed no significant difference in comparison with control rabbit in the current study but it is reported that treatment with IMI increased the bilirubin level in albino mice (Arfat et al., 2014) and creatinine and urea level in rats (Hassan et al., 2019). A research conducted on *C. idella* and *C. auratus* fish revealed that creatinine was significantly lower in IMI-exposed groups of both species as compared to the control group (Ilahi et al., 2018) which is not consistent with the present study.

The residue of IMI deposited at a detectable level in tissue of liver and stomach and undetectable in kidney and lungs of IMI-exposed rabbits in TLC in the present study. The pink spot was found at RF 0.52 in UV detection box at 254 nm wave length on the TLC plate. A pink spot was observed at RF 0.55 by Chandegaonkar et al., 2009 in TLC for detection of IMI.

The histopathology of livers of IMI-treated rabbits revealed coagulation necrosis of hepatocytes with infiltration of inflammatory cells in *peri*-portal areas. Dilatation of sinusoids and congested portal veins were also seen. Similar lesions were reported by Kammon et al. (2010) in liver of IMI intoxicated chickens. In male albino rats, IMI resulted in congested central vein, homogeneous cytoplasm in the hepatocytes, leukocytic infiltration, and fibroblasts surrounding the bile duct and central vein (Mohany et al., 2012) but in the present study no fibrotic lesion was found in perivenular and periportal areas in IMI-exposed rabbits. The liver of female albino rat showed that IMI treatment resulted in dilations of central vein Toor et al., 2013 which concurs with the present investigation.

Mehmood et al. (2017) stated that liver treated with long time exposure (31 days) of IMI revealed degenerative changes in hepatocytes along with bile duct hyperplasia. Hyperplasia of bile duct was not found in the present investigation may be due to short time exposure. According to Soujanya et al. (2013), considerable dilation and congestion of the central vein were observed in male rats, which were found in the present study.

Lungs of IMI treated rabbits showed granulomatous inflammation and accumulation of inflammatory cells around the terminal bronchiole close to the blood vessels. Marked congestion of blood vessels was also found. Pandit et al. (2016) reported that IMI mixed in corn oil taken orally for 30 days produced peribronchial and perivascular infiltration of lymphocytes and enlarged perivascular space in the lungs of male albino rats, which supports the findings in lungs of IMI-exposed rabbits in the present study. Peribronchiolar lymphocyte infiltration was also described by Komal et al. (2016) in IMI treated broilers. Wankhede et al. (2017) noted cellular oedema, bronchial epithelium disruption, and hemorrhages in

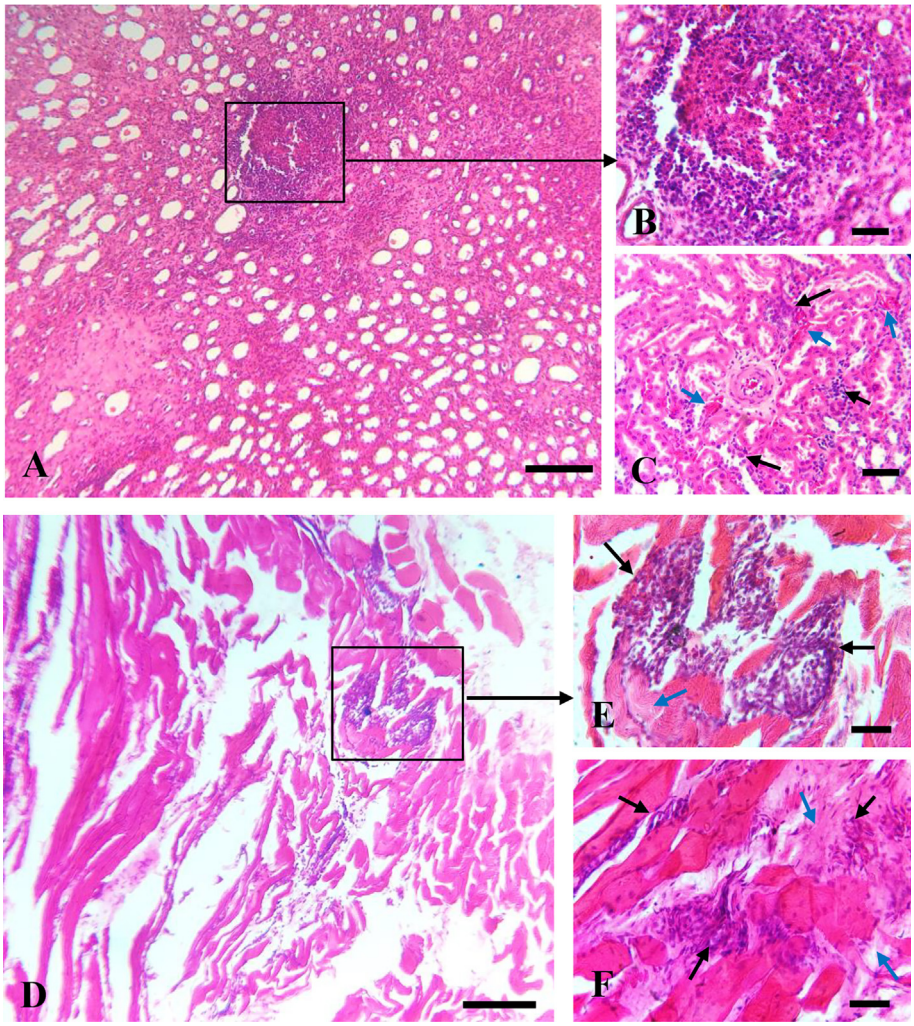

Fig. 5. Histopathological observation of kidney and heart of IMI-exposed rabbits. A: Granulomatous inflammation was present in cortico-medullary junction of kidney. B: Showing the granulomatous inflammation in higher magnification. C: Presence of congestion and inflammatory cells in between kidney tubules. D: Marked infiltration of mononuclear cells in between cardiac muscles. E-F: Showing the heterogenously arranged, pleomorphic inflammatory cells (black arrows) in higher magnification along with necrosed cardiac muscles (blue arrows). H & E stain. Bars: A, D = 400 μm; B, C, E, F = 100 μm.

the lungs of two weeks old Japanese quails treated with IMI. Premlata et al. (2004) observed that IMI causes focal interstitial pneumonia with alveolar wall thickened and aggregation of lymphoid follicles surrounding the bronchioles in the lungs of rat which is partially consistent with the current study.

In the kidney, granulomatous inflammation of mononuclear cells was found near the cortico-medullary junction in IMI-exposed rabbits in the present study. Similarly, Qadir & Iqbal (2016) reported that kidney of *L. rohita* revealed necrosis, wide Bowman's space, renal tubular lumen enlargement, and inflammation. Presence of hemorrhages and inflammatory cells in between kidney tubules indicated interstitial nephritis in the present study which is also described by Komal et al. (2017) in broiler chickens. It was reported by Wankhede et al. (2017) that kidneys of two weeks old Japanese quails treated with IMI showed interstitial haemorrhages and vacuolar degenerative changes.

Necrosis of cardiac muscle fibers with marked infiltration of mononuclear cells was found in the present study. Myocarditis was found in heart of IMI treated broiler chickens (Komal et al., 2017) similar to current investigation. Degenerative changes in myofibers of heart were also described in two weeks old Japanese quails treated with IMI (Wankhede et al., 2017).

5. Conclusions

The present study revealed that acute IMI exposure causes significant increase in serum AST and ALT level that are important critical enzymes in biological processes and consequently they are considered specific indicators of the liver lesion. The liver is a primary target organ for the toxic effect of xenobiotics, therefore it can be used as an index of toxicity of various toxicants. IMI exposure causes hazardous effects on various visceral organs (liver, lungs, kidney, and heart) of adult male rabbits. Due to ubiquitous use of pesticide, the residue of these pesticides may enter into food chain and may affect the public health. So, it's a burning issue to address the need of cautious use of pesticides as they cause toxic effects on the health of human and other mammals and also affects the biodiversity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contribution

The experiment was designed by MRK and MP. LA, MRK and MAK conducted the experiment. Recording, analysis of data, and interpretation of results done by LA, MRK, and MAK. LA, MAK, and MRK wrote the draft. MN, NHS and MP made significant revisions to the text. All authors read and approved the final manuscript.

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