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Effects of imidacloprid contaminated feed exposure on the spleen, lymph node, and mucosa-associated lymphoid tissues of adult male rabbits (*Oryctolagus cuniculus*)

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Abstract

Pesticide is one of the top chemical substances that pose a serious risk to public health. Imidacloprid (IMI) is a widely used broad-spectrum insecticide over the past decade. Here, oral IMI-contaminated green grass (at the dose rate of 100 mg/liter and sprayed) was fed to adult male rabbits (n=6), every alternate day for up to 90 days. The control rabbits (n=6) were fed pesticide-free green grass, wheat bran, and water ad libitum. For gross and histopathology, spleen, lymph nodes, and mucosa-associated lymphoid tissues were collected by ventromedial opening. No evident gross changes were found during the collection of the organs. Histopathologically, lymph nodes showed atrophy and degenerative lymphoid follicles. The cortex and medulla of the lymph nodes were less differentiated. The size and number of the lymphoid follicles in the cortex were also reduced in IMI-exposed rabbits. The spleen exhibited atrophic changes in the white pulps. The white pulp revealed degenerative changes with the depletion of the lymphoid tissues. As for the red pulp, it showed an irregular and depleted mesh network of reticular fibers in IMIexposed rabbits compared to the control. The trachea and esophagus of IMI-exposed rabbits showed infiltration of the mononuclear cells in the propria-submucosa and tunica adventitia, respectively. The infiltration of mononuclear cells population was loaded in the core of the villi, tunica mucosa, and propria-submucosa of the duodenum, jejunum, and ileum of the IMI-exposed rabbits compared to the control. The present study findings suggest that IMI has toxic effects on the lymphoid organs and tissues of adult rabbits.

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Introduction

Imidacloprid (IMI) is one of the top-used broad-spectrum systemic insecticides (1). IMI works primarily on the nervous systems of pests via nicotinic acetylcholine receptors and kills the insects by paralysis (2). It is considered a moderately hazardous chemical (Class-II, WHO; toxicity category-II, EPA) based on animal studies (3), which can be absorbed through ingestion, cutaneous absorption, or inhalation (4). In response to restriction on the usage of organophosphorus insecticides, particularly

chlorpyrifos and diazinon, Bayer Pharmaceuticals launched IMI in 1991. Since then, it has been registered in more than 120 countries to protect the crop and vegetables from insect attacks (5,6). The indiscriminate use of IMI throughout the world, particularly in developing countries like Bangladesh, has been a serious concern for public health (5-9). Due to its unregulated usage and persistent nature in the environment, including water bodies, IMI has been incorporated into our food chain, consequently, posing a serious threat to the occupationally exposed person (5,8,9). Pesticides and their active toxic metabolites induce functional impairment in

various vital organs when exposed repeatedly (10). IMI induces oxidative stress in rats, which was detected using different stress marker enzymes profile (11). IMI affects multiple systems of the body of mammals, such as the blood vascular system in mice and rabbits (12-14), particularly causing a significant increase in total leukocyte count (TLC) in male albino rats (14). Among the differential leukocyte count, the percentage of lymphocytes was significantly increased in rabbits exposed to IMI for 15 alternate days at the dose rate of 100 mg/L (7). It also causes hepatotoxicity, characterized by coagulation necrosis with infiltration of huge numbers of inflammatory cells in the periportal areas, nephrotoxicity with the features of inflammations in the corticomedullary junction, neurotoxicity, and gonadaltoxicity in rats (15-18). Depending on the duration of the exposure, IMI causes detrimental effects in the testicular tissues. In particular, the spermatogenic and Leydig cell populations reduced significantly with morphologically abnormal spermatozoa in IMI-exposed adult male rabbits for 15 alternative days at the dose rate of 100 mg/L (5). IMI affects the heart, characterized by pericarditis and myocarditis, the lungs accompanied by severe congestion along with hyperplasia of the bronchus-associated lymphoid tissue (BALT), inflammation in the intestines and pancreas of broiler chickens exposed to IMI orally for 14 days (19). The domestic chicken showed neurobehavioral alterations including muscle tremors, ataxia, and a depressed tendency after being exposed to IMI orally for 7 consecutive days at the dose rates of 0, 0.03, 0.34, 3.42, 10.25, and 15.5 mg/kg/day (20). The total immunoglobulin, especially IgG, increased significantly in IMI-exposed male albino rats. A significant decrease in phagocytic activity, chemokinesis, and chemotaxis was observed in rats exposed to IMI for 4 weeks at the dose rate of 0.21 mg/kg body weight. Histopathologically, the spleen showed lymphocytic depletion with increased fibroblasts and bundles and lymphocytic depletion with pyknotic nuclei in the thymus in rats (21). The cell-mediated immune response was suppressed with prominent histological alterations in the spleen of IMI-exposed mice for 28 days (22). Unfortunately, the effects of IMI on the immune system and mucosaassociated lymphoid tissues (MALT) through contaminated feed exposure have not been reported or established in adult male rabbits.

The study aims to assess the residual effects of IMI on the immune system at the cellular level, particularly in the lymph nodes, spleen, trachea, esophagus, duodenum, jejunum, and ileum of adult male rabbits.

Materials and methods

Chemical preparation

Imidacloprid (Bildor®, marketed by Corbel International Ltd., Tejgaon, Dhaka-1215, Bangladesh) was collected from the authorized pesticide dealer (Mymensingh City

Corporation Marker, Mymensingh Sador, Bangladesh) and handled carefully with appropriate precautions. The insecticide imidacloprid (IMI) 0.5 ml (100 mg)/liter was properly mixed with fresh tap water according to a data sheet used for pest control in agriculture. The properly mixed IMI water was sprayed on green grass in a fenced, restricted field using the Suja Global Hand Sprayer Machine (SEGARTEX, Dhaka-1230, Bangladesh) in the evening. The sprayed green grasses were collected the next morning.

Animals and experimental procedures

Twelve male Netherland dwarf rabbits (Oryctolagus cuniculus) aged 11 months, with an average body weight of approximately 1.4 to 1.6 kg each, were selected for the study. Animals were housed in individual metal cages in the departmental experimental animal room, maintained at 25°-30°C with a 12-hour light-dark cycle. The animals were fed a standard diet (green grass and wheat bran) and supplied with tap water ad libitum. After acclimatization for one week, six rabbits received IMI-exposed green grass at one time in the morning for every alternative day up to 90 days and fresh green grass with wheat bran on evenings and on pesticide-free days. Six rabbits as a control received normal pesticide-free green grass and wheat bran. Dietary habits concerning water and food consumption were recorded during the study. All rabbits were observed regularly, and their health condition was recorded as well. At the end of the imidacloprid exposure, all exposed and control rabbits were sacrificed on day 91 under deep chloroform anesthesia (using chloroform anesthesia (EMSURE®, EMD Millipore Corporation, Germany).

This study was undertaken humanely with sufficient care for the animals and all the experimental protocols were approved by the "Animal Welfare and Experimentation Ethics Committee", Bangladesh Agricultural University, Mymensingh, Bangladesh (AWEEC/BAU/19-41).

Collection of lymph node, spleen, and mucosa-associated lymphoid tissues for histopathology

The axillary lymph nodes, spleen, trachea, esophagus, duodenum, jejunum, and ileum were collected and immediately fixed in 10% neutral buffered formalin (NBF) by the standard method. Then, NBF-fixed tissues were dehydrated with ascending graded-alcohols, then cleared, and infiltrated with xylene, and embedded in paraffin. The embedded tissues were then sectioned at 6 μ m in thickness using a sliding microtome. The sectioned tissues were deparaffinized using xylene, rehydrated with descending grades of alcohol, and stained with hematoxylin and eosin (H and E) for histopathological examination. Imidaclopridinduced histopathological changes were studied using a light microscope.

Photograph and image processing

Photomicrographs at high- and low-power magnification were taken with a digital camera (DS-Fi1, Nikon, Tokyo, Japan) or a virtual slide scanner (VS-120, Olympus, Tokyo, Japan) mounted on a microscope. Adjustment of photographs for contrast, brightness and sharpness, layout, and lettering were performed in Adobe Photoshop 7.0J.

Results

Gross Changes

Grossly, no change was found in collected organs (lymph nodes, spleen, trachea, esophagus, duodenum, ileum) of control and IMI-exposed rabbits. The size, shape, color and texture of organs of IMI-exposed rabbits were normal and similar to the control rabbits.

Histopathological examination Lymph Node

Histopathological observation of lymph nodes from control rabbits revealed normal organization of the capsule, trabeculae, cortex, and medulla (Figures 1A-C). The IMI-exposed rabbits showed a lack of well demarcated cortex and medulla (Figures 1D, E). Degenerated, and atrophied lymphatic nodules were seen (Figures 1D, E). Severe depletion of the lymphoid tissues was seen in the cortex containing lymphatic nodules (Figure 1F). The number of the lymphatic nodules was also reduced (Figures 1D, E). Numerous small vacuoles were found in both the cortical and medullary regions (Figures 1D, E). Higher magnification of lymphatic nodules showed the degenerative changes of lymphoid tissues in the medulla (Figure 1F). The germinal or reactive center was not so prominent (Figure 1F).

Spleen

Histopathological observation of the spleen of control rabbits revealed a normal organization of capsule, trabeculae, white pulp, and red pulp (Figure 2A). The IMI-exposed spleen of rabbits revealed atrophic changes in the white pulps (Figure 2B). The area of white pulp was regressed. The higher magnification of the white pulp showed degenerative changes with the depletion of the lymphoid tissues (Figure 2B). The subcapsular sinuses were extended compared to the control rabbits. The splenic cords in the red pulp were loosely arranged with congested areas (Figure 2B).

Trachea and esophagus

The trachea of IMI-exposed rabbits showed infiltration of mononuclear cells in the propria-submucosa layer (Figures 3A-B), while the esophagus revealed granulomatous inflammation in the tunica adventitia (Figures 3C-D)

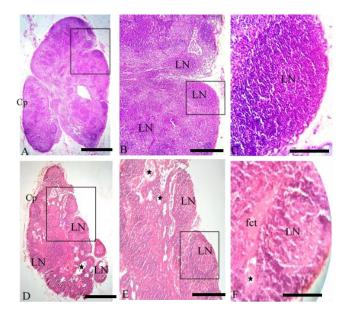


Figure 1: Histopathology of the lymph node. A-C: Normal histo-architecture of lymph nodes of control rabbits. D: Not well-differentiated cortical, medullary regions with degenerated and atrophied (D-F) LN are seen in the IMI-exposed group. Severe depletion of the lymphoid tissues is seen in the cortex containing LN. The number of the LN is also reduced. Numerous small vacuoles were found in both the cortical and medullary regions (asterisks). In higher magnification of LN showing the generative changes of lymphoid tissues and fibrous connective tissues in the medulla (F). Capsule: Cp, Lymphatic nodule = LN, C = cortex, M= medulla, fct= fibrous connective tissue. H & E stain. Bars: A, D = 400μm; B, E = 200 μm, C, F= 100μm.

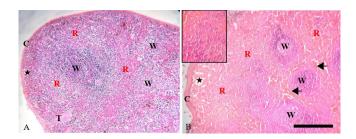


Figure 2: Histopathology of the spleen. A: Normal histoarchitecture of spleen of control rabbits. B: In the IMI-exposed group, the spleen reveals atrophic changes in the white pulps. The white pulp is degenerated and its size is decreased. The higher magnification of the white pulp (Inset) showed degenerative change with the depletion of the lymphoid tissues. The subcapsular sinuses were extended compared to the control rabbits (asterisk). The splenic cords in the red pulp were loosely arranged with hemorrhages (arrows). H & E stain. Capsule (C), Trabeculae (T), Red pulp (R) White pulp (W), Asterisk indicates subcapsular sinus. Bars: $A-B=200~\mu m$; Inset = $100~\mu m$.

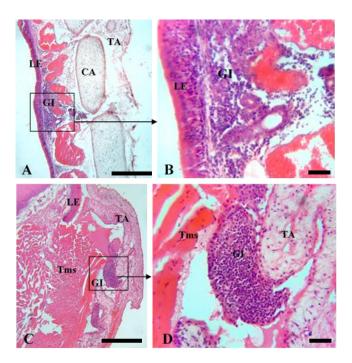


Figure 3: Histopathological observation of trachea and esophagus of IMI-exposed rabbits. A: Infiltration of mononuclear cells in propria-submucosa of trachea was seen. B: Showing the granulomatous infiltration of mononuclear cells in higher magnification. C: Granulomatous inflammation in tunica adventitia of esophagus was found. GI- Granulomatous inflammation, LE- Lamina epithelia, SM- Submucosa, Tms- Tunica muscularis, TA- Tunica adventitia, CA-Cartilage. H & E stain. Bars: A, C = 200 μ m; B, D = 100 μ m.

Duodenum and jejunum

The small intestines of IMI-exposed rabbits revealed a little change in the tunica mucosa. Aggregation of mononuclear cells was found in the tunica mucosa of the duodenum (Figures 4A-B), jejunum (Figures 4C-D) and ileum (Figures 4A-D).

Ileum

In the ileum, the lymphoid tissue, particularly lymphocytes, were located in the lamina epithelia, lamina propria, and submucosa in control rabbits (Figures 5A-B). The frequency of the lymphocytic infiltration is more in IMI-contaminated feed exposed rabbits compared to the control rabbits, and organized lymphatic nodules have been found in the submucosa, which is extended towards the mucosa of the ileum (Figures 5C-D). The lymphocytes population in the core of the villi has also been increased (Figures 5C-D).

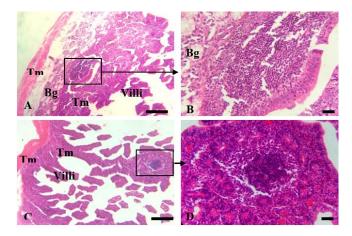


Figure 4: Histopathological observation of the duodenum and jejunum of IMI-exposed rabbits. A-B: Aggregation of mononuclear cells in the tunica mucosa of the duodenum is seen. C-D: Accumulation of mononuclear cells in the tunica mucosa of the jejunum is seen. Bg- Brunner's gland, Tm-Tunica mucosa, Tms- Tunica muscularis. H & E stain. Bars: A, C = $200 \, \mu m$; B, D = $100 \, \mu m$.

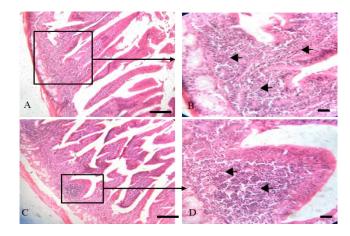


Figure 5: Histopathological observation of ileum of control and IMI-exposed rabbits. A-B: Normal distribution of lymphocytes in the lamina epithelia, lamina propria and submucosa of ileum of control rabbits (arrows). C-D: The frequency of lymphocyte infiltration is higher in IMI-exposed rabbits compared to control, and aggregated lymphatic nodules show a huge number of lymphocytes in the submucosa of ileum of IMI-exposed rabbits (arrows). H & E stain. Bars: A, C = 200 μ m; B, D = 100 μ m.

Discussion

Exposure to pesticide and its toxicity in occupationallyexposed persons is a prime concern in the present day. Immunosuppression due to prolonged exposure to pesticide leads to a great risk of cancer (23). To maintain the integrity of the body, organs of the immune system and associated cells work together against pathogens and xenobiotics. Among all the organs responsible for immunity, secondary lymphoid organs, such as lymph nodes, spleen, tonsils, and mucosa-associated lymphatic tissues encounter foreign bodies, which are distributed at the entry point throughout the body in adults (24). In the present study, the IMI-induced alterations in the lymph nodes histologically revealed a lack of proper differentiation of the parenchyma into cortex and medulla with no visible germinal center. The lymphatic nodules were degenerated and atrophied. The lymphoid tissues were depleted severely, and the size and number of lymphatic nodules were reduced.

Numerous small vacuoles were found in both the cortical and medullary regions. No relevant study or reason responsible for such degenerative changes in the lymph nodes has been found. However, several study findings have been reported and established that when toxic chemicals and other xenobiotics enter the body repeatedly, they cause similar degenerative changes in the lymph nodes of mammals, which is similar to the findings of the present study (25-31).

The spleen is the major secondary lymphatic organ, which is involved in filtering blood and triggering an immune response against bloodborne antigens and pollutants. The ability to filter blood is enhanced by a reticular fiber network filled with reticular cells and macrophages. In adults, the spleen served as an organ of lymphopoiesis (32).

In the present study, IMI affects the spleen and causes structural alterations. The white pulp in the spleen of IMI-exposed rabbits revealed atrophy, and depletion of the lymphoid tissues with decreased size of lymphoid follicles. The subcapsular sinuses were extended, and the splenic cord in red pulp was loosely arranged. This derangement of the splenic cords resulted in the loss of integrity of the reticular mesh network, which hampers the filtration of blood in the spleen. Similar changes were reported in albino male rats (21,33) and white leghorn cockerels (34).

The trachea of IMI-exposed rabbits showed infiltration of huge numbers of mononuclear cells in propria-submucosa in the present study, but no such lesion was described by any authors. The esophagus of IMI-exposed rabbit revealed granulomatous inflammation in the tunica adventitia. The sublethal concentration of IMI caused histological alterations in the midgut epithelium, and the cytotoxic features were irregular border epithelium, cytoplasmic vacuolation observed in predatory bugs (35).

Aggregation of mononuclear cells in the tunica mucosa of duodenum, jejunum, and ileum was found in the present study. Aggregated and scattered lymphocytes usually inhabit the small intestine for a normally functioning immune system. The populations of this lymphocytic infiltration significantly increased in the duodenum, jejunum, and ileum in the IMI-exposed rabbits compared to the control. Similar results were reported in association with the desquamation

of villi of the epithelium and necrotic infiltration of huge inflammatory cells in intestine of IMI-exposed rats (36) and Japanese quails (37).

Conclusion

The results of the present study reveal that chronic exposure to IMI-contaminated feed (Bildor @ 100mg/L, for 90 days) induces immunotoxicity in adult male rabbits. Histopathologically, the lymph node and spleen of IMIexposed rabbits showed severe degenerative changes, including a smaller number of lymphoid follicles with decreased size in the lymph nodes and depleted white pulp and splenic cords in the red pulp of the spleen. The mononuclear cells infiltration in the propria-submucosa of the trachea, granulomatous inflammation in the tunica adventitia of the esophagus, and huge number of inflammatory cells infiltration in the propria-submucosa and core of villi of the duodenum, jejunum, and ileum were identified. These results indicated that IMI causes toxicity in all the organs involved to protect and maintain the body's immunity, directly or indirectly.

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Conflicts of interest

There is no conflict of interest among the authors.

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تأثير الأعلاف الملوثة بإيميداكلوبريد على الطحال والعقدة الليمفاوية والأنسجة اللمفاوية المرتبطة بالغشاء المخاطي في ذكور الأرانب الذكور البالغة

ألامجير كبير'، نازمول حسن صديقي'، مرشدة نصرين'، لطيفة أكتر'، مونمون بيرفين'، ضياء الحق'، محمد ربيع الكريم'

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الخلاصة

المبيدات هي واحدة من أهم المواد الكيميائية التي تشكل خطر اجسيما على الصحة العامة. (IMI) Imidacloprid هو مبيد حشري واسع الطيف يستخدم على نطاق واسع على مدى العقد الماضي. هنا ، تم اعطاء العشب الأخضر الملوث بـ IMI عن طريق الفم (بمعدل جرعة ١٠٠ ملغم / I الأخرانب الذكور البالغين (نI) ، كل يوم بديل لمدة تصل إلى ٩٠ للأرانب الذكور البالغين (نI) ، كل يوم بديل لمدة تصل إلى ٩٠

يوما. تم تغذية الأرانب في مجموعة السيطرة (ن = ٦) بالعشب الأخضر الخالي من المبيدات ونخالة القمح والماء حسب الحاجة. لغرض الفحص العياني والنسيجي، تم جمع الطحال والغدد الليمفاوية والأنسجة اللمفاوية المرتبطة بالغشاء المخاطي عن طريق الفتحة البطنية. لم يتم العثور على تغييرات جسيمة واضحة أثناء جمع الأعضاء. من الناحية النسيجية، وجد ضمور العقد الليمفاوية وتنكس الحويصلات اللمفاوية. كانت قشرة ونخاع الغدد الليمفاوية أقل تمايزا. كما لوحظ انخفاض حجم وعدد الحويصلات اللمفاوية في القشرة في الأرانب المعرضة لـ IMI. أظهر الطحال تغيرات ضامرة في اللب الأبيض. كشف اللب الأبيض عن تغيرات تنكسية مع نضوب الأنسجة اللمفاوية. أما بالنسبة لللب الأحمر، فقد أظهر شبكة شبكية غير منتظمة ومستنفدة من الألياف الشبكية في الأرانب المعرضة لـ IMI مقارنة بالسيطرة. أظهرت القصبة الهوائية والمريء للأرانب المعرضة لـ IMI ارتشاح للخلايا أحادية النواة في IMI ارتشاح للخلايا و tunica adventitia، على التوالى. إرتشاح مجاميع الخلايا أحادية النواة تركز في وسط الزغابات، والغشاء المخاطى المغلالة، والـ propriasubmucosa للاثني عشر، الصائم، واللفائفي للأرانب المعرضة للـ IMI مقارنة بالسيطرة. تشير نتائج الدراسة الحالية إلى أن الـ IMI له تأثيرات سامة على الأعضاء والأنسجة اللمفاوية للأرانب البالغة.