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Immunotoxic effects of imidacloprid following 28 days of oral exposure in BALB/c mice

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ABSTRACT

Imidacloprid, a neonicotinoid insecticide has been in use worldwide for several years in agriculture and veterinary medicine. It is possible that residue of this compound may be recycled in the food chain and thus information regarding effects from potential exposure to it is warranted. The objective of the present study was to evaluate immunotoxic effects of imidacloprid in female BALB/c mice. Imidacloprid was administered orally daily at 10, 5, or 2.5 mg/kg over 28 days. Specific parameters of humoral and cellular immune response including hemagglutinating antibody (HA) titer to sheep red blood cells (SRBC; T-dependent antigen), delayed type hypersensitivity (DTH) response to SRBC, and T-lymphocyte proliferation in response to phytohemagglutinin (PHA) were evaluated. The results showed that imidacloprid at high dose, specifically suppressed cell-mediated immune response as was evident from decreased DTH response and decreased stimulation index of T-lymphocytes to PHA. At this dose, there were also prominent histopathological alterations in spleen and liver. Histopathological analysis of footpad sections of mice revealed dose-related suppression of DTH response. Imidacloprid at low dose of 2.5 mg/kg/day did not produce any significant alterations in cellular and humoral immune response and it seemed to be an appropriate dose for assessment of 'no observable adverse effects level' for immunotoxicity in BALB/c mice. The results also indicated that imidacloprid has immunosuppressive effects at doses >5 mg/kg, which could potentially be attributed to direct cytotoxic effects of IMD against T cells (particularly T_H cells) and that long-term exposure could be detrimental to the immune system.

1. Introduction

Pesticides are the largest group of chemicals that are used widely in modern agricultural practices with the aim of preventing, destroying, repelling or mitigating any pest. Imidacloprid (IMD) belongs to a relatively newer group of insecticides, the neonicotinoids. The neonicotinoids are major class of insecticides developed in the past three decades. Neonicotinoids are primarily used as plant systemic

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insecticides. IMD was introduced in 1991 as the first chloronicotinyl insecticide (syn. neonicotinoid) and has been highlighted because of its extremely high intrinsic insecticidal potency, low mammalian toxicity, broad insecticidal spectra, excellent systemic properties and plant compatibility (Mencke and Jeschke, 2002). It is most commonly used on rice, cereals, maize, potatoes, vegetables, sugar beets, fruits, cotton, hops and turf and is especially systemic when used as a seed or soil treatment. It has been the largest selling insecticide worldwide for agricultural use and as a veterinary medicinal remedy to control ectoparasitic insects in the last decade.

Exposure of animals to residual concentration of pesticides can lead to immunosuppression either directly or with the participation of stress mechanisms/the neuroendocrine system (Kacmar et al., 1999). In recent years, tremendous use of IMD in agriculture may have added to its soil persistence and soil storage (Sarkar et al., 2001) and ground water contamination (Gervais et al., 2010). Additionally, it may enter water bodies from spray drift or accidental spills, leading to local point-source contamination. In water sediment system, IMD is degraded by microbes into guanidine compound. The time to disappearance of one-half of the residues (DT_{50}) of IMD was 30–162 days (Gervais et al., 2010). IMD was detected in a range of fresh and processed fruits and vegetables (Gervais et al., 2010). Fernández-Alba et al., 2000 also reported that degradation products of IMD could make it to be frequently recovered in fruits and vegetables. It is possible that extensive use of IMD over a decade may have resulted in residues of this insecticide to be recycled in the food chain. However, it was hard to trace a study having comprehensive information on levels of imidacloprid residues in food chain vis-à-vis its toxic effects on humans and/or animals.

Despite original belief that imidacloprid has low mammalian toxicity, there is increasing evidence that it may cause heart, kidney, and other organ damages along with gastrointestinal irritation, neurological symptoms and even death when ingested along with alcohol (Yeh et al., 2010). It has been reported that, 90 days oral administration of IMD at 20 mg/kg/day produces pathomorphological changes and hormonal imbalance in female rats (Kapoor et al., 2011). Previous studies with IMD have shown genotoxic effects in rats (Karabay and Oguz, 2005) and cultured human lymphocytes (Demsia et al., 2007). In albino rats, IMD induced immunological effects (at single dose tested, i.e., 0.21 mg/kg/day for 28 days) were successfully ameliorated with daily supplementation of thymoquinone, an anti-oxidant (Mohany et al., 2012). Generally, immunotoxicity experiments performed in inbred animals lead to better assessment of immune system functions; since, intra-group and intergroup variations in the results is nullified owing to the identical genotype of inbred animals. Thus, the present investigation was undertaken to explore the impact of 28 days exposure of IMD on the humoral and cell-mediated immune responses of inbred BALB/c mice.

2. Materials and methods

2.1. Experimental animals

Female BALB/c mice (4–6-week-old) were obtained from National Institute of Pharmaceutical Education and Research (Mohali, India). Mice were housed in polystyrene cages (eight/cage) with ad libitum access to standard pellet feed (Ashirwad Industries Ltd., Chandigarh, India) and filtered tap water. The room was maintained under a 12/12 h light–dark cycle, an ambient temperature of 20–25 °C, and a relative humidity of 45 (±15)%. All mice were housed for 1 week for acclimatization before initiation of any experiment. All animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of the University.

2.2. Chemicals

Technical grade imidacloprid (>98% purity) was provided by Indofil Chemicals Company (Mumbai, India). This was suspended in carboxymethyl cellulose, purchased from CDH (New Delhi, India). Dexamethasone, cyclophosphamide, Freund's complete adjuvant, phytohemagglutinin-P (PHA), phenazine methosulphate (PMS), XTT dye, and Tris (hydroxymethyl)-amino-methane were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) powder (with high glucose, L-glutamine, and pyridoxine hydrochloride, but lacking sodium pyruvate/bicarbonate), antibiotic–antimycotic solution, and fetal bovine serum were each procured from Gibco (Paisley, UK). Dulbecco's phosphate-buffered saline (DPBS; without calcium or magnesium) and hematoxylin and eosin (H&E) stains were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai).

2.3. Maximum tolerated dose

The maximum tolerated dose (MTD) of imidacloprid (IMD) by oral route was determined in a pilot dose range study, based on the method of Moser and Padilla (1998). For this pilot study, an apparent LD_{50} of 168 mg/kg for technical grade IMD in female mice (Solecki, 2001) was considered. Here, small groups of animals (n = 2–3/dose) were administered a single dose of IMD (by oral gavage) and observations were taken at various times thereafter. A range of doses was used initially including at least one lethal dose, to determine the doses that produced overt signs of toxicity, but no lethality (MTD).

To verify the MTD, additional groups (n = 2–3/dose) were administered that dose and were tested to define more closely the time course of effects. While conducting the experiments for MTD determination, the effects of various doses of IMD on gross observable behavioral activity profiles were also noted without manipulating or disturbing the mice. Observations were made for individual mice for up to 24 h. The MTD was the dose that did not cause mortality or reduce body weight by >10%, but still induced these types of toxicities.

2.4. Doses and exposure schedules

The selection of three test doses of IMD was based on the MTD for technical grade IMD, (determined in preliminary studies to be 50 mg/kg body weight in female BALB/c mice [single dosing]). Based on this initial value, three MTD-based test (toxic) doses were proposed for use: (i) 10 mg/kg (20% of MTD; high); (ii) 5 mg/kg (10% of MTD; medium); and (iii) 2.5 mg/kg (5% of MTD; low).

Animals were divided into five groups (6–8 mice/group); three groups treated with three different IMD doses, one positive control group (received dexamethasone [DXM] or cyclophosphamide [CYP], depending on experiment), and one negative control group (carboxymethyl cellulose, vehicle control). IMD suspension of 1.0, 0.5 and 0.25 mg/ml final concentrations was prepared in 0.5% (w/v aq.) carboxymethyl cellulose vehicle. Mice in the three test groups were then administered (by oral gavage) 10, 5, or 2.5 mg IMD/kg in appropriate volumes. Each dose was prepared to accommodate changes in body weight by adjusting the gavage volume between 120 to 210 μl. Animals in the test groups were administered IMD daily for 28 days. The negative control mice received carboxymethyl cellulose (0.5% solution in distilled water) vehicle in 150 μl oral deliveries daily for 28 days. Positive control mice were administered CYP (50 mg/kg, orally) or DXM (2 mg/kg, orally) daily for 5 days, using fresh solutions of DXM (0.2 mg/ml) and CYP (5 mg/ml) prepared in sterile normal saline. Each IMD suspension in carboxymethyl cellulose was also prepared fresh daily and thoroughly vortexed before administering orally. All dosing were performed between 11:00 and 13:00 each day as far as possible; body weights were recorded daily prior to the time of dosing.

Antigen, i.e., sheep red blood cells (SRBC), was injected in mice as a single injection 6–8 h after completion of a day's IMD dosing (specific days indicated below) in the experiments examining hemagglutination antibody titers and DTH response.

2.5. Non-functional assays

2.5.1. Peripheral blood cell analyses

At the end of exposure period, i.e., day 29 for IMD-treated and negative control groups and day 5 for positive control groups, 0.5 ml peripheral blood samples were collected from the orbital sinus of each mouse prior to euthanization. Slide smears were immediately prepared and total leukocytes then estimated in the remaining volume using a hematological auto-analyzer (Abacus Hematology Analyzer, Diatron, Lenexa, KS). Leukocyte differential counts (e.g., lymphocytes, neutrophils, monocytes) were determined by examining a total of 200 WBC in blood smears after Leishman's staining; duplicate slides were analyzed each time and results for each cell type expressed in terms of percent of all cells counted.

2.5.2. Body and organ weights and splenic cellularity

Mice from each group were weighed daily just before dosing and at the time of autopsy to record their mean body weights. At the end of the experimental trial (i.e., after 28 days), mice were euthanized by chloroform over-anesthetization. The spleen, liver, kidney, and lungs were then excised, lightly blotted on tissue paper, and weighed; all data were expressed as relative organ weight. For determination of splenic cellularity, spleen single-cell suspensions were made as noted below, red blood cells lysed, and splenocytes counted in a hemocytometer.

2.5.3. Histopathology

The spleen, liver, kidney and lung tissues were placed in 10% buffered formalin. Thereafter, paraffin-embedded sections of these tissues were cut (5–6 μm thickness) and stained with H&E.

2.6. Functional assays

2.6.1. Preparation of spleen cell suspension

On day 29, each mouse was euthanized and its spleen removed aseptically and placed in a petri dish containing cold DMEM medium. The organ was teased apart to generate a single cell suspension; the resulting material was centrifuged at $800 \times g$ for 5 min at 4 °C. The supernatant was discarded and the pellet suspended in 0.75% NH_4Cl (in Tris buffer, pH 7.2) to lyse any erythrocytes present. After storage for 5–7 min on ice, the cells were washed twice with DMEM and centrifuged again at $800 \times g$ for 5 min at 4 °C. Cell viability was then determined by a trypan blue dye exclusion test. With each sample, the final splenocyte concentration was adjusted to 2×10^6 live cells/ml in complete DMEM medium containing 10% fetal bovine serum and 1% antibiotic–antimycotic solution.

2.6.2. Hemagglutinating antibody (HA) titer

Fresh blood from healthy sheep (collected in sterile Alsever's solution) was washed (centrifuged for $800 \times g$ for 10 min at 4 °C) three times with sterile DPBS. The pelleted sheep red blood cells (SRBC) were then diluted to 1.5×10^9 cells/ml with DPBS for immunization. For evaluation of HA titer, separate sets of treated/control mice were immunized by an intraperitoneal (IP) injection of 0.3 ml of the SRBC suspension (4.5×10^8 cells/mouse) 7 days before completion (i.e., on day 21 of regimen) of the treatment period (Elsabbagh and El-Tawil, 2001).

At the end of the experimental period (day 29 for IMD treated and negative control groups/day 5 for CYP-treated mice group), sera were prepared from peripheral blood samples from each immunized mouse and de-complemented (56 °C, 30 min). To prevent non-specific agglutination, a 1% (v/v) SRBC suspension was prepared in DPBS containing 1% (w/v) bovine serum albumin. The microtiter HA technique was then employed to determine serum antibody titer. Serial 2-fold dilution of each serum sample were made in 96-well U-bottom microtiter plates; an equal volume of 1% SRBC suspension was then added to each well and the plate was incubated for 2 h at room temperature. The reciprocal of the highest dilution yielding hemagglutination was taken as the antibody titer. Serum samples of mice from all five groups were tested again for confirmation of HA titer.

2.6.3. Delayed-type hypersensitivity (DTH) response

DTH response (using SRBC as antigen) was assessed as described in Hassan et al. (2004), with some modifications. On day 18 of the exposure period (or day 2 for DXM control group), mice were sensitized by a subcutaneous (SC) injection into their back with 50 μl of SRBC (10^8 cells) suspended in Freund's complete adjuvant (FCA). After 10 days (i.e., on day 28), these sensitized mice were challenged (under light ketamine [100 mg/kg body weight, IP] anesthesia) by injecting 50 μl of SRBC (10^8 cells) into their right hind footpad. Swelling in the right hind footpad was measured using a pressure sensitive micrometer screw gauge (Mitutoyo, Kawasaki, Japan) 24 and 48 h post-challenge.

After the final measurement, the mice were euthanized and footpad sections prepared for histopathological examination to evaluate cellular changes in DTH response. In brief, the right footpad was isolated from each host, immediately placed in 10% buffered formalin, and then processed for histopathology in a manner similar to other organs (see above).

2.6.4. *Lymphocyte proliferation assay*

Lymphocyte proliferation assay was performed by the method of Roehm et al. (1991), with some modifications. Briefly, the assay was performed in flat-bottom 96-well tissue culture plates (Greiner Bio-one, Frickenhausen, Germany). Triplicate cultures of each splenocyte sample isolated above were prepared, with and without presence of mitogen (phytohemagglutinin [PHA]). Specifically, splenocytes were added at 200 μl/well (4×10^5 cells) and then 20 μl of a 25-μg PHA/ml solution (0.5 μg/well of culture) or vehicle was added. The plate was then transferred to a humidified CO_2 (7% CO_2) incubator maintained at 37 °C, and incubated for 72 h. After the incubation, the number of proliferating cells was determined with tetrazolium salt XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) dye combined with PMS (phenazine methosulphate). The XTT solution (1 mg/ml) was made fresh each time by dissolving XTT in warm (40 °C) media and filtered (0.2 μm filter; Millipore, Billerica, MA) prior to use. PMS was made up as a 10 mM (3.06 mg/ml) solution in DMEM and stored at 4 °C for 3–5 days maximum. PMS was added to the XTT (at 2.5 μl PMS/ml XTT solution) immediately before use. For the assay itself, 50 μl XTT/PMS solution was added to each well, the plates were gently shaken (to mix contents), and incubation allowed to continue an additional 3–4 h. The absorbance (450 nm) in each well was then determined using a UVmax kinetic microplate reader (with reference wavelength of 650 nm) (Tecan Group Ltd., Männedorf, Switzerland).

2.7. *Statistical analysis*

Data were presented as mean (±SE), tested for normality (Shapiro–Wilks W-test) and homogeneity (Bartlett's test for unequal variances) and, if needed, appropriate transformations were made. Statistical analysis was performed using a Kruskal–Wallis H test in Statext-v13 software (www.statext.com).

3. Results

3.1. *Maximum tolerated dose*

The MTD of technical grade IMD in BALB/c mice was found to be 50 mg/kg by the oral (gavage) route (data not shown). The following symptoms were noted in mice while evaluating the MTD: toxic symptoms started 10–15 min after IMD administration and were dose-dependent in onset and severity. Overt signs were prominent mouth smacking/chewing 8–10 min after IMD administration (and continued for 45–60 min); head tremors started after 12–15 min (lasting for 2 s–1 min) and peak tremors were noted at 20–22 min of administration. Whole body tremors were sometimes noted; body posture was also abnormal, with the hind limbs fully extended away from the body. Mice that died presented with prominent whole body (mainly head and neck) convulsions for 5–20 min, difficulty in respiration, and a complete prostrate position just prior to death.

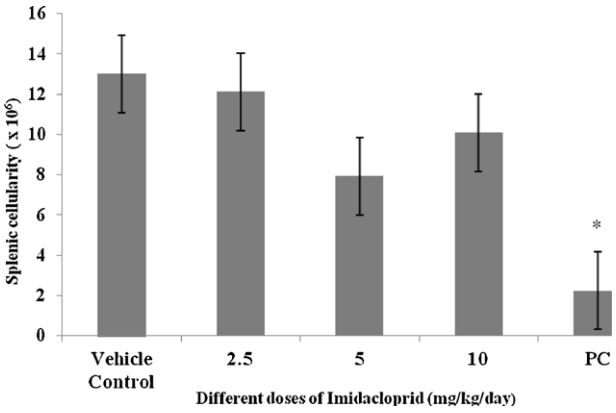

Fig. 1 – Splenic cellularity of BALB/c mice following oral IMD exposure for 28 days. Values are presented as mean ± SE of 5 mice/group. PC: positive control: (CYP – 50 mg/kg/day by gavage for 5 days). *Value significantly different from vehicle control at $p \leq 0.05$.

3.2. *Hematology*

All animals survived the experimental period of 28 days and no pesticide related death was observed. Only in the high IMD dose group (10 mg/kg) total leukocyte count (TLC) and percent lymphocytes were lower than those in the vehicle control group (Table 1). Differential leukocyte counts (DLC) of the three IMD-treated dose groups also did not vary significantly from those of the vehicle control. In contrast, lymphocyte and neutrophil percentages of DXM-treated mice were, respectively, significantly lower and higher than those of vehicle control counter-parts. Platelet count in mice in the high dose group was significantly lower compared to vehicle control; the other doses had no significant effect on this endpoint. Other blood parameters such as total erythrocyte count (TEC), hemoglobin (Hb) count, and packed cell volume (PCV) did not reveal any significant differences (relative to vehicle control values) arising from IMD or DXM treatments.

3.3. *Body and organ weights and splenic cellularity*

Apart from the finding that the final body weights of mice treated with the high IMD dose slightly decreased (≈8%), there were no significant differences in body weight gain among any of the various dosage groups (Table 1). Repeated exposure to IMD led to decreased spleen weight with all three test doses (significantly so in DXM-treated hosts); the decrease in splenic index was greatest for the high dose and, expectedly, only significant with the DXM mice. There was a reduction (albeit not significantly so) in splenic cellularity at the medium dose and a significant reduction in the CYP-treated mice (Fig. 1). This outcome with the 5 mg/kg dose is unclear as the differences

Table 1 – Effect of imidacloprid on organ weight and blood cell counts in BALB/c mice dosed orally for 28 days.

Parameters	Vehicle control	[a]IMD 10 mg/kg	IMD 5 mg/kg	IMD 2.5 mg/kg	Positive control
Body weight (g)					
Before dosing	[b]15.30 ± 0.94	18.60 ± 0.53	17.40 ± 0.83	17.20 ± 1.03	19.40 ± 0.84
After 28 days	15.60 ± 1.15	17.10 ± 0.68	17.90 ± 0.86	16.10 ± 0.75	17.80 ± 0.73 ([c]C)
Spleen weight (g)	0.101 ± 0.008	0.078 ± 0.002	0.081 ± 0.007	0.079 ± 0.005	*0.072 ± 0.005 ([c]D)
Splenic index (%)	0.63 ± 0.06	0.50 ± 0.03	0.52 ± 0.04	0.54 ± 0.03	*0.41 ± 0.03 (D)
[a]TLC (10^3/mm^3)	6.49 ± 0.27	5.10 ± 0.40	6.69 ± 0.55	6.60 ± 0.58	6.91 ± 0.63 (D)
Lymphocyte (%)	74.00 ± 1.08	72.00 ± 1.58	70.20 ± 0.58	73.50 ± 2.72	*67.75 ± 0.63 (D)
Neutrophils (%)	23.5 ± 1.19	25.5 ± 1.71	26.8 ± 0.80	23.75 ± 2.39	*29.75 ± 0.48 (D)
Monocytes (%)	2.50 ± 0.65	2.50 ± 0.29	3.00 ± 0.45	2.75 ± 0.48	2.50 ± 0.29 (D)
Platelets ($\times 10^5$/mm^3)	2.99 ± 0.28	*1.98 ± 0.05	2.79 ± 0.46	2.30 ± 0.14	3.58 ± 0.40 (D)
[a]TEC (10^6/mm^3)	4.22 ± 0.40	2.82 ± 0.41	4.36 ± 0.47	3.80 ± 0.77	3.98 ± 0.33 (D)
[a]Hb Count (gm %)	12.23 ± 0.81	11.45 ± 0.39	12.70 ± 0.76	13.28 ± 1.21	12.23 ± 1.38 (D)
[a]PCV (%)	31.98 ± 1.13	30.48 ± 0.88	30.12 ± 0.78	32.30 ± 0.72	31.15 ± 1.91 (D)

[a] Abbreviations: IMD, imidacloprid; TLC, total leukocyte count; TEC, total erythrocyte count; Hb, hemoglobin count; and PCV, packed cell volume.
[b] Data shown as mean ± SE; $n = 5$ mice/group, except for body weight and spleen weight ($n = 6$).
[c] C: cyclophosphamide (50 mg/kg) and D: dexamethasone (2 mg/kg) by gavage for five consecutive days.
* Value significantly different from vehicle control at $p < 0.05$.

from the other IMD groups were not significant and did not follow any firm dose-trend. The indices of the other organs; *viz*. liver, lung, and kidney of IMD treated mice groups did not differ significantly from those of the vehicle control mice (Table 2).

3.4. *Hemagglutination antibody (HA) titer*

While high and low doses of IMD had no significant effects on serum anti-SRBC agglutinin titer, the medium dose (5 mg/kg) caused a very significant ($p \leq 0.01$) decrease in the titer. As expected, the CYP treatment led to a near-complete abrogation of response (Table 3).

3.5. *Delayed-type hypersensitivity (DTH) response*

DTH response to SRBC was characterized by intense local inflammatory reaction with erythema, edema, vesiculation, and swelling in the vehicle control mice. In IMD-treated mice, the intensities of these symptoms/inflammatory reactions were inversely related to the dose of IMD administered, i.e., at high dose, only mild inflammatory reaction with mild edema and erythema was noted. Mice in the low dose group showed reactions nearly similar to those of the vehicle control. Once again, as expected, the DXM-treated mice group showed only very mild inflammatory reactions, very mild edema, and negligible swelling (Fig. 2).

The DTH response (i.e., percent increase in paw thickness at a given timepoint) decreased non-significantly in the medium IMD dose mice after 24 h (Table 3); in contrast, high IMD dose and DXM-treated mice displayed significantly suppressed responses compared to those of the vehicle control. Interestingly, at 48 h post-challenge, DTH responses were suppressed significantly in the medium and high IMD dose group and even more so (by ≈20% more) than at 24 h in the DXM-treated mice.

3.6. *Lymphocyte proliferation assay*

Stimulation indices, used as measure of lymphocyte proliferation were not significantly impacted by the low and the medium dose regimens (Table 3). In contrast, the high dose regimen led to a significantly lower value. Mice in the CYP positive control group also showed significant suppression in their lymphoproliferative responses.

Table 2 – Effect of imidacloprid on relative organ weight in BALB/c mice dosed orally for 28 days.

Groups	Relative organ weight {organ weight (g)/body weight (g) × 100} Organs			
	Spleen	Liver	Kidney	Lung
CMC – 0.5%	0.627 ± 0.058[a]	4.13 ± 0.153	0.61 ± 0.042	0.933 ± 0.022
Low dose – 2.5 mg/kg	0.537 ± 0.032	4.36 ± 0.202	0.58 ± 0.031	0.924 ± 0.071
Medium dose – 5 mg/kg	0.516 ± 0.036	4.24 ± 0.195	0.54 ± 0.018	0.878 ± 0.022
High dose – 10 mg/kg	0.498 ± 0.032	3.74 ± 0.215	0.54 ± 0.032	0.813 ± 0.045
Dexamethasone (positive control)[b]	0.413 ± 0.032*	4.07 ± 0.382	0.52 ± 0.024	0.808 ± 0.044

[a] Data shown as mean ± SE; $n = 6$ mice/group.
[b] Dexamethasone – 2 mg/kg orally for 5 consecutive days.
* Value significantly different from vehicle control at $p \leq 0.05$.

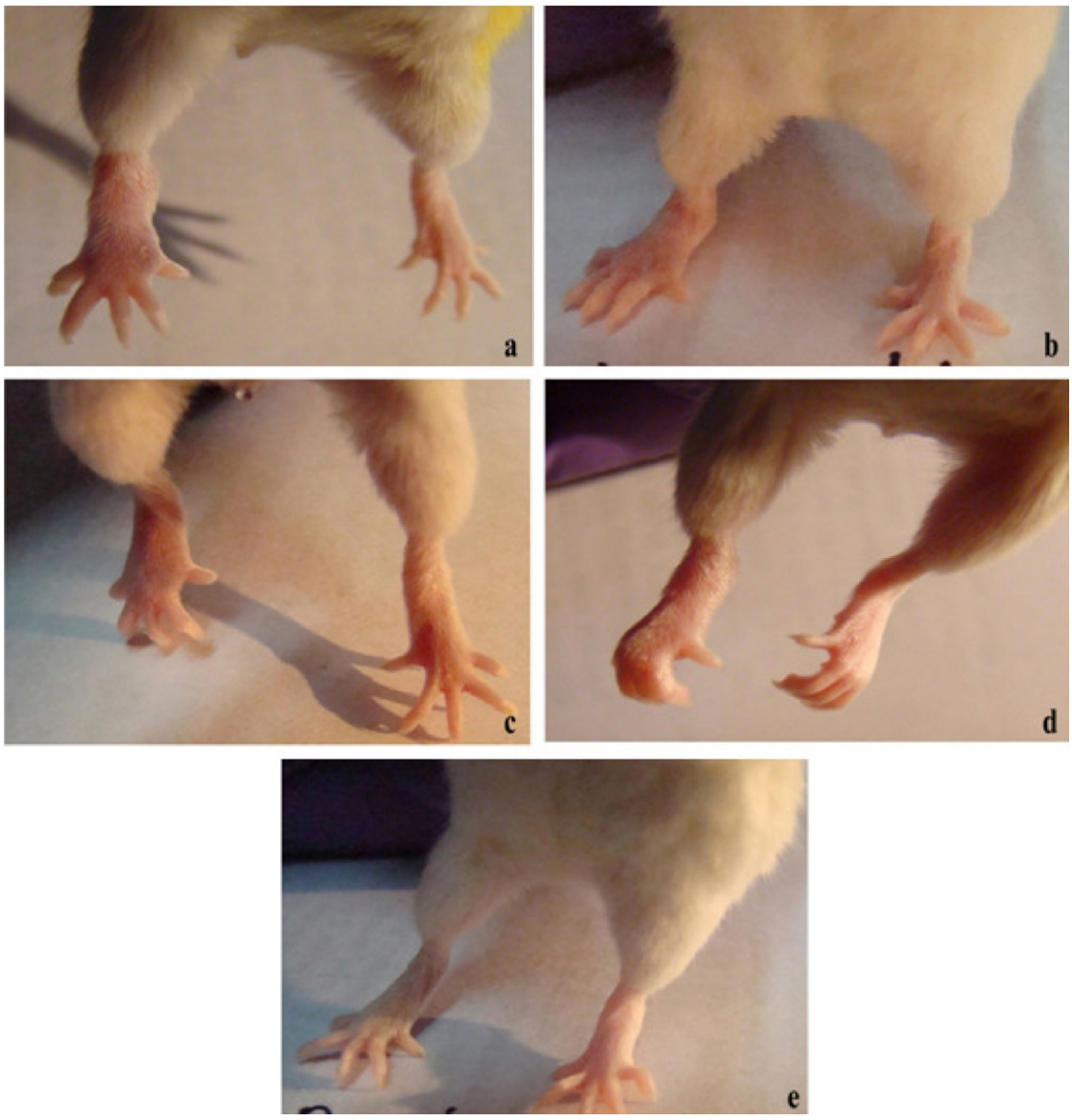

Fig. 2 – DTH reaction (after 48 h) in right hind footpad of BALB/c mice following oral IMD exposure for 28 days; gross symptoms. (a) Vehicle control – footpad showing intense inflammatory reaction characterized by erythema, edema, vesiculation, swelling. (b) High dose (10 mg/kg daily, 28 days) – mild inflammatory reaction showing mild edema, erythema and mild swelling. (c) Medium dose (5 mg/kg daily, 28 days) – comparatively mild-to-modeate inflammatory reaction, moderate edema and swelling. (d) Low dose (2.5 mg/kg daily, 28 days) – moderate-to-normal inflammatory reaction, edema and swelling, and erythema. (e) DXM-treated [positive control] mice (2 mg/kg daily, 5 days) – footpad showing very mild inflammatory reaction, very mild edema, and negligible swelling.

3.7. *Histopathology*

Histopathological examination of organs did not reveal any significant changes in the kidneys and lungs except for some instances of mild-to-moderate congestion (data not shown). In contrast, the spleen and liver tissues of mice in the test groups did reveal some significant pathological alterations (Fig. 3; shows results for vehicle, high dose, and DXM hosts only). There was a seeming dose-related depletion of lymphocytes in the splenic white pulp, with spleen of mice in the high dose group showing moderate-to-severe lymphocyte depletion and an increased presence of neutrophils and reticuloendothelial cells, along with congestion.

Footpad sections from vehicle control mice (48 h after challenge with antigen) revealed an intense local inflammatory reaction characterized by the presence of a large number of mono-nuclear cells (i.e., macrophages and lymphocytes) and a few neutrophils/polynuclear cells in the dermis (Fig. 4). Footpad sections of mice from the high dose group and DXM-treated group revealed very mild inflammatory reaction with very few macrophages/lymphocytes in the dermis, suggesting marked suppression of the DTH

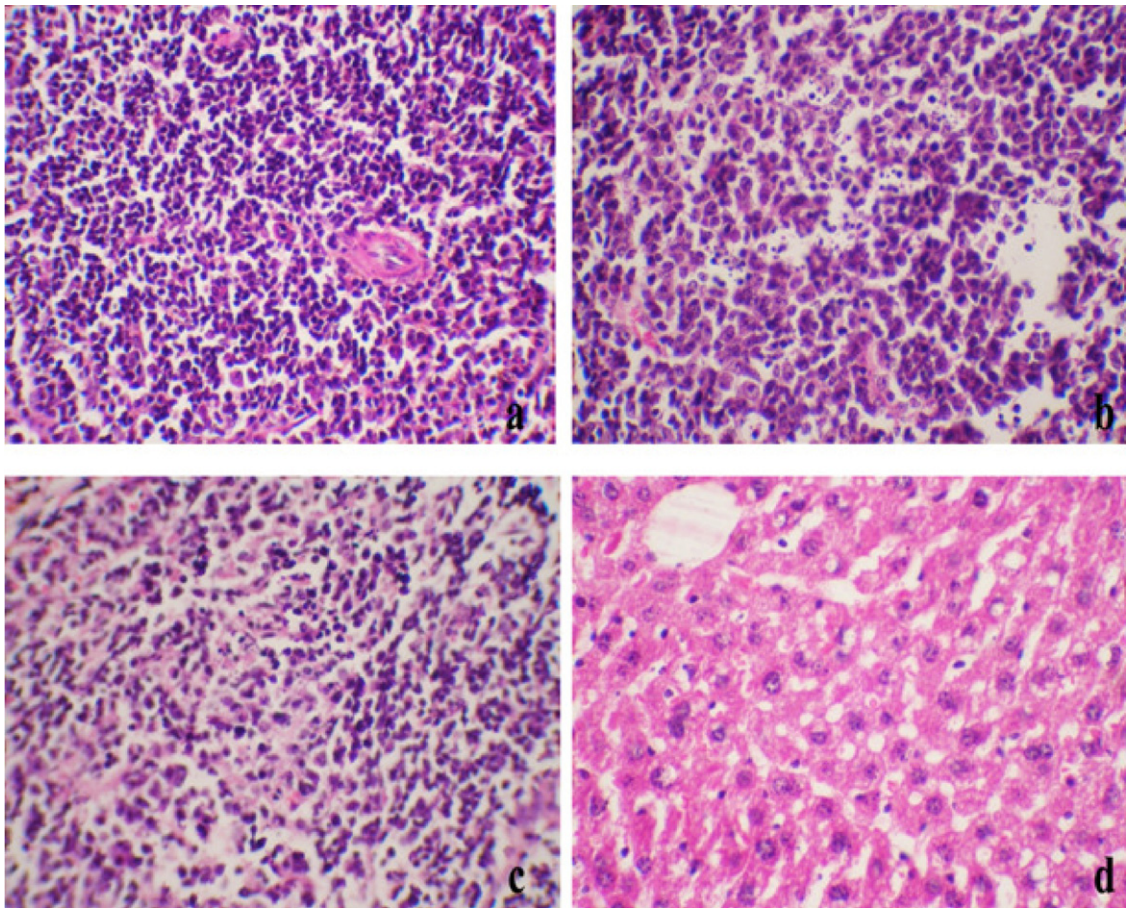

Fig. 3 – Histopathology of spleen and liver section of BALB/c mice. Image shown is a representative image from each group. (a) Vehicle control – normal histological architecture of spleen showing white pulp. (b) IMD-treated high dose group (10 mg/kg/day daily, 28 days) – moderate-to-severe depletion of lymphocytes and mild congestion in white pulp. (c) DXM-treated (2 mg/kg daily, 5 days) positive control – severe depletion of lymphocytes and congestion in white pulp. (d) IMD-treated high dose group showing moderate fatty degeneration in the liver. H&E staining; magnification 400×.

response. Footpad sections of mice from the medium dose exhibited moderate-to-good inflammatory reaction, and cells such as macrophages and lymphocytes were present in the dermis, but in less number/concentration than that of the control, indicating moderate suppression of DTH response. Comparable to vehicle control mice, food pad sections of mice from low dose revealed intense inflammatory reaction with presence of plenty of lymphocytes and macrophages thus, showed little or negligible suppression of DTH response (Table 4 and Fig. 4).

Table 3 – Effect of imidacloprid on cellular and humoral immune responses in BALB/c mice dosed orally for 28 days.

Treatment group	DTH response (%)[a]		Stimulation index[b]	Log_2 antibody titer [$n = 6$ mice/group]
	24 h later	48 h later		
Vehicle control	57.81 ± 7.68[c]	53.15 ± 4.36	1.253 ± 0.061	4.33 ± 0.21
Low dose – 2.5 mg/kg	42.51 ± 3.46	40.96 ± 2.48	1.153 ± 0.048	3.83 ± 0.17
Medium dose – 5 mg/kg	38.89 ± 3.49	36.63 ± 1.64**	1.158 ± 0.030	3.17 ± 0.17**
High dose – 10 mg/kg	35.49 ± 1.93*	34.36 ± 1.38**	1.102 ± 0.034*	4.00 ± 0.00
Positive control	30.33 ± 3.11 (D)[d,*]	24.56 ± 1.31 (D)**	1.026 ± 0.012 (C)[d,*]	0.26 ± 0.15 (C)**

[a] DTH: delayed-type hypersensitivity (percent of increase in paw thickness at given timepoints).
[b] For T-cell proliferation response to PHA, $n = 6$ mice/group except for CPY control ($n = 5$).
[c] Data shown as mean ± SE; $n = 6$ mice/group.
[d] C: cyclophosphamide (50 mg/kg) and D: dexamethasone (2 mg/kg) by gavage for five consecutive days.
* Value significantly different from vehicle control at $p \leq 0.05$.
** $p \leq 0.01$.

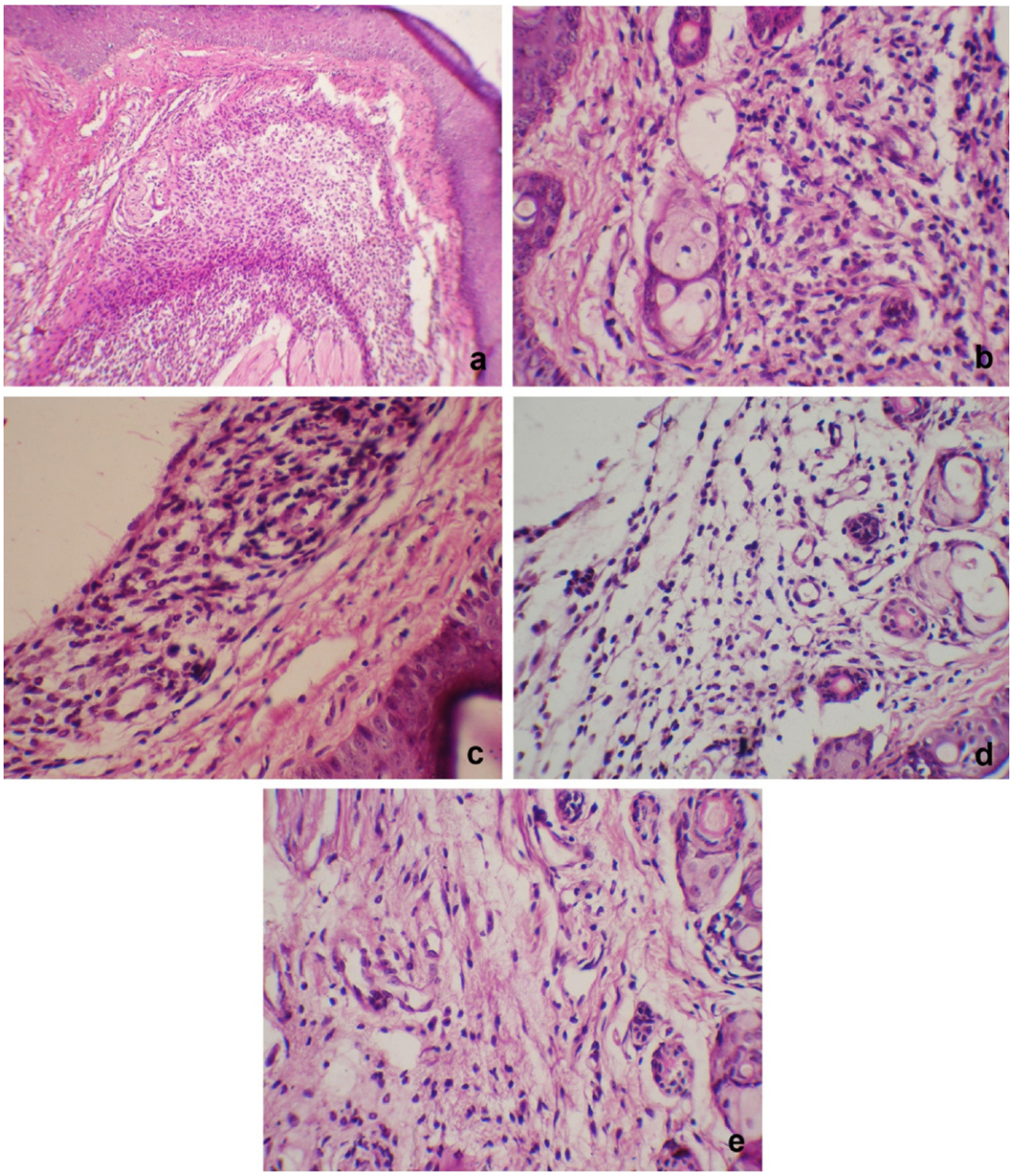

Fig. 4 – Histopathology of right hind foot pad of BALB/c mice tested for DTH reaction (after 48 h). Image shown is a representative image from each group. Footpad section from: (a) vehicle control – intense inflammatory reaction characterized by large number of lymphocytes in dermis (H&E, magnification 100×). (b) vehicle control – large numbers of inflammatory cells (such as lymphocytes and macrophages) are evident in the epidermis and dermis (H&E, magnification 400×). (c) IMD-treated low dose group (2.5 mg/kg daily, 28 days) – moderate to intense/severe inflammatory reaction with a presence of large number of lymphocytes and macrophages in the dermis and epidermis (H&E, magnification 400×). (d) IMD-treated medium dose group (5 mg/kg daily, 28 days) – comparatively mode-rate inflammatory reaction with a presence of a moderate number of lymphocytes in the dermis (H&E, magnification 400×). (e) IMD-treated high dose group (10 mg/kg daily, 28 days) – very mild inflammatory reaction with a presence of few lymphocytes in the dermis (H&E, magnification 400×).

4. Discussion

The immunosuppressive effects of pesticides may also be associated with an increased cancer risk; as, an increase in the number of cases has recently been observed among agricultural workers (Sathiakumar et al., 2011). Toxicological studies of imidacloprid are limited and acceptable daily intake (ADI) was earlier reported as 0.006 mg/kg/day based on mostly

Table 4 – Histopathological alterations in footpad sections of mice examined for DTH response (48 h post-SRBC antigen challenge) after 28 days repeated oral exposure to imidacloprid.

Groups	Inflammatory reaction (in general)	Type of cells present in dermis/epidermis			DTH response/ reaction
		Macrophages	Lymphocytes	Neutrophils	
Vehicle control	Intense	+++[a]	+++	+	No suppression
Low dose (IMD – 2.5 mg/kg)	Moderate to intense	++	+++	–	Little or no suppression
Medium dose (IMD – 5 mg/kg)	Moderate	+	++	–	Mild-to-moderate suppression
High dose (IMD – 10 mg/kg)	Mild	+	++	–	Moderate-to-high suppression
Dexamethasone (positive control)[b]	Very mild	+	+	–	Marked suppression

[a] +++: high in number; ++: mild-to-moderate in number; +: few in number.
[b] Dexamethasone – 2 mg/kg orally for 5 consecutive days.

unpublished reports (Solecki, 2001; California Environmental Protection Agency, 2006). The residue study of imidacloprid in dairy cows conducted by Heukamp (1992) and Klein (1992) revealed detectable levels of IMD in milk. Craig et al. (2005) found transferable residue of imidacloprid on dog's coat and suggested that repeated chronic exposure may pose health risks to veterinarians, veterinary technologists, dog caretakers, and owners. Thus, exposure to IMD residues in food as well as occupational exposure (Demsia et al., 2007) can have human health implications too.

Immunomodulatory effects of various xenobiotic classes, including polycyclic aromatic hydrocarbons, halogenated aromatic hydrocarbons, heavy metals, organochlorine organophosphorous, carbamates and other majority of pesticides, have been relatively well characterized (Blakley et al., 1999; Luebke et al., 2007), but not those of the neonicotinoid insecticides. Along with recent development in the area of immunotoxicology in the last decade, agrochemical compounds (such as insecticides) have been presented as important candidates for the testing of immunotoxic potential in order to determine 'no observable adverse effect levels' (NOAEL) (Thomas, 1998; Vohr and Ruhl-Fehlert, 2001). Despite overt use of IMD in agriculture and veterinary medicine, there is little information about its immunotoxicity and its NOAEL in mice. Very recently, a developmental immunotoxicity study in Wistar rats has shown age-related, dose-dependent developmental immunotoxic effects of IMD and clearly demonstrated developmental immunotoxicity as one of the potential risk associated with IMD exposure at high doses (Gawade et al., 2013). Specifically, other studies have shown IMD to be immunosuppressive in the rat (Gatne et al., 2006) and the White leghorn cockerel (Siddiqui et al., 2007) models. Available literature in these animal models shows IMD to be immunotoxic, but mechanisms involved therein have not been demonstrated. Efforts have been made in the present investigation (as far as possible) to evaluate parameters described by Jong and Loveren (2007), who defined parameters indicative of direct immunotoxicity into non-functional and functional assays (themselves originally derived from WHO IPCS monographs (IPCS, 1996)).

This paper is the first report to establish an oral (by gavage) MTD for technical-grade IMD in BALB/c mice. In this study, the high dose of IMD (10 mg/kg/day) decreased both body and spleen weights (albeit that the ratio of spleen:body weights remained unchanged) (see Table 1) and splenic cellularity. Significant reduction in the platelet count was observed in the mice exposed to high IMD dose and could lead to clotting disturbances. These findings are complimentary to the findings of Gawade et al. (2013). Similar reduction in platelet count and disturbed blood clotting was also reported in a sub-chronic IMD toxicity study in female Wistar rats (Eiben and Rinke, 1989). Results of the present study clearly demonstrated immunomodulatory effects of IMD in mice at doses of 10 and 5 mg/kg/day following 28 days of exposure (see Tables 1 and 2). Decreased percent lymphocytes and total leukocyte count in IMD treated high dose group indicated a risk to lymphopaenia and immunomodulation (Gawade et al., 2013). This may eventually have an immunosuppressive effect, through the adverse effects on the normal functioning of bone marrow, stress or other varied factors responsible for normal leukocyte balance. Significant reduction in hemagglutination antibody titer was observed at the medium dose (5 mg/kg); however, at the low dose, reduction was insignificant. Parallel to these findings, progressive and proportional decrease in hemagglutination antibody titers in Sprague Dawley rats treated with different doses of IMD have been reported (Gatne et al., 2006). Recent study of developmental immunotoxicity of IMD in rats has also supported our findings of decrease in hemagglutination antibody titer (Gawade et al., 2013).

In the current study, the post-IMD treatment lymphoproliferative responses to mitogen PHA were evaluated using XTT dye. This is the first time, combination of tetrazolium salt XTT dye and mitogen PHA, instead of MTT dye and Con A/PHA was used for the evaluation of murine T-cell response, since XTT/PHA method is more convenient and reliable in general. This included the fact that there was no need to solubilize any formazan crystals (using dimethyl sulfoxide), thereby avoiding an additional series of steps/handling of the cultures as required in the MTT/PHA or MTT/ConA methods.

The stimulation indices derived in the lymphocyte proliferation test (XTT/PHA studies), were significantly decreased in the high IMD dose and CYP-treated mice. IMD has recently been shown to have genotoxic effects on lymphocytes from human, rats, and other animals (Demsia et al., 2007; Costa et al., 2009). Therefore, IMD here could have inhibited T-cell proliferation via direct genotoxicity and (if based on those other studies) gave rise to apoptosis. To date, the precise molecular mechanism of IMD action against T-cell activity has not yet been fully defined. Nevertheless, the results of T-lymphoproliferation assay obtained here demonstrated that

IMD could cause inhibition of T-cell activity. T-helper (T_H) cells are involved in the generation of B-cell responses (T and B-cell co-operation for antibody synthesis) leading to production of antibodies against T-dependent antigens such as SRBC. Hence, the noted suppression of HA titer (antibody response) observed with IMD treatments here could be attributed to the impairment of T_H cell activity alone (either in numbers or ability to proliferate) or in conjunction with some as-yet undefined effects of the insecticide on intrinsic B-cell functions. The latter possibility is currently under investigation in our laboratories.

Effects of subacute oral exposure to IMD on T-cell function was further assessed through measurement of DTH response which was significantly suppressed after 48 h in the high and the medium IMD dose groups. Furthermore, histopathological evaluation of footpad sections of mice tested for DTH reaction revealed significant pathological or cellular alterations (Table 4 and Fig. 4). Comparative histological evaluation of the food pad sections from vehicle control and IMD-treated mice confirmed the gross symptoms of DTH reaction and the decreases in paw thickness seen with the high and medium IMD doses. Despite our best efforts with literature search, we could not find an immunotoxicity study with IMD or other insecticides, wherein cellular changes in an organ tested for DTH reaction have been described. Such histological studies to corroborate the gross findings of DTH reaction are of upmost importance while evaluating pesticides for immunotoxicity.

Significant reduction in DTH reaction to SRBC, a T-cell-dependent antigen, often is indicative of reductions in cell-mediated immunity. Alterations in the magnitude of DTH reaction, symptomatically and/or at a histologic level, are usually indicative of an impairment of T_H1 effector cells. T_H1 effector cells (also termed T_{DTH} cells) are responsible for the DTH reaction. Specifically, following interaction with a specific antigen, the T_H1 cells produce cytokines that invoke mononuclear cell infiltration, mononuclear cell interaction, and increased vascular permeability in the vicinity of stimulus (Luster et al., 1982). Histopathological findings in the present study indicating reductions in mononuclear cell involvement at the injection site and a generalized lower inflammatory response could be explained by an IMD-induced effect on T_H1 cells in particular and/or their capacity to invoke the three physiologic outcomes noted above (by still unknown mechanisms).

Histopathological alterations in the spleen of mice exposed to high IMD dose are indicative of past/ongoing tissue destruction and injury reflecting IMD induced death of lymphocytes. Gatne et al. (2006) also showed depopulation of lymphocytes, mild fibrous tissue proliferation, and disintegration of white pulp in the spleen of IMD-treated rats, with severity of the lesions being maximal at the highest dose level (160 mg/kg body weight) tested. Additionally, these results are in accordance with the histopathological lesions observed in spleen of rats exposed to 0.21 mg/kg of IMD (Mohany et al., 2012). Although there are no reports as to how IMD induced the death of splenocytes, it has been reported that parathion (an organophosphate pesticide) induces apoptosis in murine germ cells (Bustos-Obregon et al., 2001). Hence, it would be interesting to investigate further the effect of IMD on splenic changes.

Lastly, in this study, the liver of mice exposed to the high IMD dose evidenced congestion and fatty degeneration. Such outcomes are highly suggestive of mild-to-moderate hepatotoxic effects for this insecticide. The hepatotoxic effects noted here are in agreement with the findings of EL-Gendy et al., 2010 who reported, increase in lipid peroxidation (LPO) as well as in activities of anti-oxidant enzymes such as catalase, SOD, GSHPX, and GSH-T in the liver of Swiss albino mice 24 h after a single oral dose (≈15 mg/kg body weight) of IMD. Complementary to our findings, mild focal necrosis of the liver and hepatocellular damage has also been reported following subchronic IMD exposure in rats (Bhardwaj et al., 2010).

In conclusion, our results have indicated a direct immunotoxic effect of IMD in inbred BALB/c mice. It was clear from the present study that, subacute (28 days) oral IMD exposure suppressed immune responses, with prominent inhibition of T-cell-mediated response being noted at the high (i.e., 10 mg/kg) dose tested. Since the low IMD dose (2.5 mg/kg/day) did not significantly alter normal function of the mouse immune system, this seems to be on its face an appropriate dose for the establishment of a 'no observable adverse effect level' (NOAEL) for immunotoxicity in female BALB/c mice. For more than 30 years, it has been clearly defined that immunosuppressive effects of environmental agents often reflect functional defects in immunocompetent cells and/or a depletion of responding immune system cell types (Faith et al., 1980). As such we believe that, when all of the results here are taken together, the suppression of cellular immune responses by IMD in the present investigation could potentially be attributed to direct cytotoxic effects of IMD against T cells (particularly T_H cells). The present study clearly demonstrates immunotoxicity as one of the potential risks associated with chronic exposure to IMD at high doses possibly leading to immunocomprised state in humans and caution should be taken to avoid direct or indirect exposure to IMD through residues and by occupational means. At the same time, frequent assessment of pesticide residues and further studies are warranted to better characterize these toxicities/mechanisms therein observed here.

Conflict of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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REFERENCES

Bhardwaj, S., Srivastava, M.K., Kapoor, U., Srivastava, L.P., 2010. A 90-day oral toxicity of imidacloprid in female rats: morphological, biochemical, and histopathological evaluations. Food Chem. Toxicol. 48, 1185–1190.

Blakley, B., Brousseau, P., Fournier, M., Voccia, I., 1999. Immunotoxicity of pesticides: a review. Toxicol. Ind. Health 15, 119–132.
Bustos-Obregon, E., Diaz, O., Sobarzo, C., 2001. Parathion induces mouse germ cells apoptosis. Ital. J. Anat. Embryol. 106, 199–204.
California Environmental Protection Agency, 2006. Imidacloprid, Risk Characterization Document Dietary and Drinking Water Exposure 2006, Department of Pesticide Regulation, February 9, pp. 1–195.
Costa, C., Silvari, V., Melchini, A., Catania, S., Heffron, J.J., Trovato, A., de Pasquale, R., 2009. Genotoxicity of imidacloprid in relation to metabolic activation and composition of the commercial product. Mutat. Res. 672, 40–44.
Craig, M.S., Gupta, R.C., Candery, T.D., Britton, D.A., 2005. Human exposure to imidacloprid from dogs treated with advantage. Toxicol. Mech. Methods 15, 287–291.
Demsia, G., Vlastos, D., Goumenou, M., Matthopoulos, D.P., 2007. Assessment of the genotoxicity of imidacloprid and metalaxyl in cultured human lymphocytes and rat bone marrow. Mutat. Res. 634, 32–39.
Eiben, R., Rinke, M., 1989. NTN 33893. Sub-chronic Toxicity Study on Wistar Rats (administration in the feed for 96 days). Unpublished Report #18187, submitted to WHO by Bayer AG, Mannheim, Germany. INCHEM Toxicological Evaluations: Imidacloprid, International Programme on Chemical Safety, World Health Organization, Geneva, Switzerland.
Elsabbagh, H.S., El-Tawil, O.S., 2001. Immunotoxicity of cupravit and pervicur fungicides in mice. Pharmacol. Res. 43, 71–76.
EL-Gendy, A.K., Aly, N.M., Mahmoud, F.H., Kenawy, A., El-Sebae, A.K., 2010. The role of vitamin C as antioxidant in protection of oxidative stress induced by imidaclo-prid. Food Chem. Toxicol. 48, 215–221.
Faith, R.E., Luster, M.I., Vos, J.G., 1980. Effect of immunocompetence by chemicals of environmental concern. Rev. Biochem. Toxicol. 2, 173–212.
Fernández-Alba, A.R., Tejedor, A., Agüera, A., Contreras, M., Garrido, J., 2000. Determination of imidacloprid and benzimidazole residues in fruits and vegetables by liquid chromatography–mass spectrometry after ethyl acetate multiresidue extraction. J. AOAC Int. 83, 748–755.
Gatne, M.M., Ramesh Bhoir, P.S., Deore, M.D., 2006. Immunotoxicity studies of imidacloprid in rats. Toxicol. Int. 13, 82–84.
Gawade, L., Dadarkar, S.S., Husain, R., Gatne, M., 2013. A detailed study of developmental immunotoxicity of imidacloprid in Wistar rats. Food Chem. Toxicol. 51, 61–70.
Gervais, J.A., Luukinen, B., Buhl, K., Ston, D., 2010. Imidacloprid Technical Fact Sheet. National Pesticide Information Center, Oregon State University Extension Services. http://npic.orst.edu/factsheets/imidacloprid pdf (accessed 01.06.12).
Hassan, Z.M., Ostad, S.N., Minaee, B., Narenjkar, J., Azizi, E., Neishabouri, E.Z., 2004. Evaluation of immunotoxicity induced by propoxure in C57BL/6 mice. Int. Immunopharmacol. 4, 1223–1230.
Heukamp, U., 1992. NTN 33893: Cattle Feeding Study: Lab Project Number: P 67315000. Bayer AG. MRID 42556139, p. 318.
IPCS (International Programme on Chemical Safety), 1996. Environmental Health Criteria 180. WHO, Geneva.
Jong, W.H.D., Loveren, H.V., 2007. Screening of xenobiotics for direct immunotoxicity in an animal study. Methods 41, 3–8.
Kacmar, P., Pistl, J., Mikula, I., 1999. Immunotoxicology and veterinary medicine. Acta. Vet. Brno. 68, 57–79.
Kapoor, U., Srivastava, M.K., Srivastava, L.P., 2011. Toxicological impact of technical imidacloprid on ovarian morphology hormones and antioxidant enzymes in female rats. Food Chem. Toxicol. 49, 3086–3089.
Karabay, N.U., Oguz, M.G., 2005. Cytogenetic andgenotoxic effects of the insecticides imidacloprid and methamidophos. Genet. Mol. Res. 4, 653–662.
Klein, O., 1992. Imidacloprid: [Methylene-carbon 14]: Absorption, Distribution, Excretion, and Metabolism in the Liver and Kidney of a Lactating Goat: Lab Project Number: M 184 0528-8. Bayer AG, MRID 42556115, p. 147.
Luebke, R., House, R., Kimber, I., 2007. Immunotoxicology and Immunopharmacology: Target Organ Toxicology Series, third ed. CRC Press, Boca Raton, FL.
Luster, M.I., Dean, J.H., Moore, J.A., 1982. Evaluation of immune functions in toxicology. In: Hayes, A.W. (Ed.), Principle and Methods of Toxicology. Raven Press, New York, pp. 561–586.
Mencke, N., Jeschke, P., 2002. Therapy and prevention of parasitic insects in veterinary medicine using imidacloprid. Curr. Top. Med. Chem. 2, 701–715.
Mohany, M., El-Feki, M., Refaat, I., Garraud, O., Badr, G., 2012. Thymoquinone ameliorates the immunological and histological changes induced by exposure to imidacloprid insecticide. J. Toxicol. Sci. 37, 1–11.
Moser, V.C., Padilla, S., 1998. Age and gender related differences in the time-course of behavioral and biochemical effects produced by oral chloripyrifos in rats. Toxicol. Appl. Pharmacol. 149, 107–119.
Roehm, N.W., Rodgers, G.H., Hatfield, S.M., Glasebrook, A.L., 1991. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J. Immunol. Methods 142, 257–265.
Sarkar, M.A., Roy, S., Kole, R., Chowdhury, A., 2001. Persistence and metabolism of imidacloprid in different soils of West Bengal. Pest Manag. Sci. 57, 598–602.
Sathiakumar, N., MacLennan, P.A., Jack Mandel Delzell, E., 2011. A review of epidemiologic studies of triazine herbicides and cancer. Crit. Rev. Toxicol. 41 (1), 1–34.
Siddiqui, A., Choudhary, M., Goriya, H.V., Bhavsar, S.V., Thaker, A.M., 2007. Evaluation of immunotoxic effect of short-term administration of quinalphos and imidacloprid in white leghorn cockerels. Toxicol. Int. 14, 15–19.
Solecki, R., 2001. Pesticide residues in food. Toxicological evaluations – imidacloprid. Joint FAO/WHO Meeting on Pesticide Residues, JMPR. http://www.inchem.org/documents/jmpr/jmpmono/2001pr07.htm/ (accessed 01.06.12).
Thomas, P.T., 1998. Immunotoxicology hazard identification and risk assessment. Nutr. Rev. 56, 1–6.
Vohr, H.W., Ruhl-Fehlert, C., 2001. Industry experience in the identification of the immunotoxic potential of agrochemicals. Sci. Total Environ. 270, 123–133.
Yeh, I.J., Lin, T.J., Hwang, D.Y., 2010. Acute multiple organ failure with imidacloprid and alcohol ingestion. Am. J. Emerg. Med. 28, 255.e1–e3.