



A detailed study of developmental immunotoxicity of imidacloprid in Wistar rats

Lalita Gawade^a, Shruta S. Dadarkar^{b,*}, Raghbir Husain^b, Madhumanjiri Gatne^a

^a Department of Pharmacology and Toxicology, Bombay Veterinary College, Mumbai 400012, Maharashtra, India

^b Department of Toxicology, Piramal Life Sciences Limited, Mumbai 400063, Maharashtra, India

ARTICLE INFO

Article history:

Received 10 April 2012

Accepted 11 September 2012

Available online 18 September 2012

Keywords:

Developmental immunotoxicity

in utero

Imidacloprid

ABSTRACT

Human exposure to imidacloprid is likely to occur during its use as an acaricide or an ectoparasiticide. Accordingly, the developmental immunotoxic potential of imidacloprid was investigated. Oral exposure was initiated in timed pregnant female Wistar rats on gestation day 6 (GD 6) till GD 21. On GD 20, half of the gravid dams were sacrificed, and *in utero* fetal development was assessed. In the other half of the dams, administration was continued till weaning on postnatal day 21 (PND 21) and maternal toxicity was investigated. A subgroup of weaned pups was sacrificed to assess immunotoxicity parameters. The other half of the pups were exposed to imidacloprid till PND 42, and immunotoxicity was assessed. The findings revealed post-implantation loss in the highest dose group, indicating the risk of abortion. Soft tissue abnormalities and skeletal alterations were observed in the highest dose group. Humoral immunity was assessed by estimating hemagglutination titer and immunoglobulin production. Cell mediated immunity was assessed by Delayed Type Hypersensitivity, whereas, non-specific immunity was assessed by phagocytic index, and other phenotypic parameters. These data revealed that imidacloprid caused age-dependent adverse effects on the developing immunity which was aggravated when exposure continued throughout development, leading to a compromised immune system.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

The perinatal development of the immune system proceeds through a well-defined sequence of organ and cellular events involving specialized microenvironments, during cell selection and apoptosis, and establishment of immunologic memory (West, 2002). Many of these events are restricted to *in utero* development or, if they occur postnatally, have a different impact after birth. As a result, it is not surprising that early life, developmentally timed, immune maturational events are exquisitely sensitive to environmental disruption (Luebke et al., 2006). Depending upon the nature of the environmental disruptor and the timing of the exposure, different adverse outcomes can result (Dietert and Piepenbrink, 2006). In fact, even exposure to the same xenobiotic during different periods of prenatal development can result in different postnatal immunotoxic changes. Limited information is available regarding the possibility of inhibited postnatal immune capacity in humans as a consequence of developmental immunotoxicant exposure. However, the available animal data suggest the potential for altered postnatal immune function in humans exposed to immunotoxicants (e.g., environmental chemicals and therapeutic agents) during fetal and/or early postnatal life (Holladay and Smialowicz, 2000).

With the advent of increasing knowledge on toxicity induced by chemicals, various tests have been added to the existing toxicity study panel that are considered mandatory by regulatory authorities to assess risk associated with use of these chemicals. Accordingly, the protocol for immunotoxicity was designed and added as a part of the US EPA guidelines (OPPTS 870-7800) in August 1998. In recent years developmental immunotoxicity has gained increasing recognition and indicates probable risk of ailments during adulthood (Dietert, 2009). The exposure of animals to residual concentration of pesticides can lead to immunosuppression, either directly, or through participation of stress mechanisms and the neuroendocrine system. Immunosuppression leads to a change in life-span, increased susceptibility to infectious diseases and decreased immune response to foreign antigens. Therefore, there is a necessity to explore the effects of pesticides on the immune system.

Imidacloprid, 1[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine, a chloronicotyl has been commonly used as an insecticide for crop protection worldwide over the last decade. Although, some work on the immunotoxic potential of imidacloprid in rats has been done (Gatne et al., 2006), there is need to further explore the developmental immunotoxic effects of imidacloprid in rats. Human exposure to imidacloprid, especially pregnant women is relatively possible due to its extensive use as an insecticide, with low soil persistence and high insecticidal activity at low application rates. Toxicological studies of imidacloprid

* Corresponding author. Tel.: +91 22 30818530; fax: +91 22 30818411.

E-mail address: shruta.dadarkar@piramal.com (S.S. Dadarkar).

are limited and the acceptable daily intake was reported as 0.006 mg/kg per day based on a majority of unpublished reports (California Dept. of Pesticide Regulation, 2000). The no observed effect level (NOEL) for maternal effects of imidacloprid is designated at 10 mg/kg per day (Bhardwaj et al., 2010), and the no observed adverse effect level (NOAEL) for developmental effects is 30 mg/kg per day (Becker et al., 1988) for female rats. However, it has been established that on repeat administration at 20 mg/kg per day, imidacloprid produces pathomorphological changes and hormonal imbalance in female rats (Kapoor et al., 2011). The availability of such information regarding the effect of imidacloprid on the female reproductive system, indicates the necessity to explore a two-generation study, to assess the outcome of imidacloprid exposure to gravid females and their subsequent progeny.

In this study, we explored the effects of imidacloprid when administered to dams from gestation day six (GD 6) through parturition, followed by the entire period of lactation during which the pups were exposed indirectly to imidacloprid through milk, subsequently, the pups were directly exposed to imidacloprid till puberty. Thus, the current study was undertaken to evaluate the toxic potential of imidacloprid on the immune system at various developmental stages.

2. Materials and methods

2.1. Experimental design

Nulliparous female Wistar rats (6–8 weeks of age, weighing 160–180 g) were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in individually ventilated cages in a temperature/humidity-controlled room, with access to water and food *ad libitum*. All animal experiments were double blinded (i.e. animal dosing and parameter evaluations were done by different personnel) and handled in accordance with the guidelines of "Committee for the Purpose of Control and Supervision of Experiments on Animals". All animal experiments were approved by Institutional Animal Ethics Committee of Piramal Life Sciences Limited. Imidacloprid was made into a paste with 2–3 drops of Tween-80 and suspended in 0.5% carboxymethylcellulose (CMC; Sigma Aldrich; St. Louis, MO) in order to administer it orally (p.o.) at a volume of 10 ml/kg body weight. The selected dosage regimen for the study, depicted in Fig. 1, was derived from prior literature (Meister, 1994), and by conducting Maximum Tolerated Dose (MTD) studies in dams and weanlings (for sake of brevity, data not shown).

2.2. Assessment of *in utero* developmental toxicity

Oral imidacloprid administration was initiated in dams from GD 6 and was continued throughout gestation till GD 21. On GD 20, a subgroup of 7 gravid dams from each group were sacrificed and assessed for *in utero* exposure (Husain et al., 1992) through effect of imidacloprid on the development of fetuses. In the remaining dams imidacloprid administration was continued through parturition till pups were weaned on postnatal day 21 (PND 21). All nursing pups were exposed to imidacloprid through lactation. On PND 21 hemato-biochemical status of dams from each group was assessed, followed by euthanasia. Half of the weaned pups were sacrificed on PND 21, to assess immunotoxicity. The pups were randomized based on body weight and care was taken to avoid including any runts. An even number of pups from each dam were selected at every stage. The remaining half of the pups were exposed orally to imidacloprid till PND 42 and at the end of this period immunotoxicity parameters were assessed.

2.2.1. Maternal exposure

Throughout the experimental period, the pregnant dams were clinically observed at least once a day or more frequently if signs of toxicity were evident. At the time of sacrifice or unscheduled death during the study, the dams and fetuses

were examined macroscopically for any abnormalities or pathological changes in the organs of the reproductive system. Gross necropsy of pregnant rats and fetal evaluation for both soft tissue and skeletal changes in the fetuses was carried out according to Christian, 2001. During gestation, females were weighed daily from day of conception to day of parturition and the group average of daily body weights were calculated for each group. On PND 20, 7 dams from each group were sacrificed and organs viz; liver, kidney, uterus (after removal of fetuses, Christian et al., 2003), and gravid uterus with fetuses were collected and weighed to derive percent organ weight factor of live body weight. Gross pathological examination of the abdominal viscera of each dam was performed. Liver, kidney and spleen were excised for histological assessment and subjected to routine histological processing. Finally, the uterus and ovaries were removed. The implantation sites were counted through the translucent uterine wall and both ovaries were removed and carefully examined for presence of corpus luteum. The uterine horns were cut along the anti-mesometrial (greater) curvature and macroscopically examined for presence of live or dead fetuses, which were numbered subsequently from the ovarian end of each horn.

2.2.2. Fetal assessment by caesarean section

Uterine horns were excised through a midline abdominal incision and the following macroscopic observations were made: (i) The number of fetuses in uterus, (ii) Number of live and dead fetuses, runts, still births, body weight and gross external alterations, (iii) Soft tissue alteration in fetuses: Half of the fetuses from each dam sacrificed on GD 20 were selected randomly and examined immediately by dissection for evidence of soft tissue alterations (Christian, 2001), and (iv) Skeletal alteration in fetuses: Remaining half of the fetuses were examined for skeletal abnormalities using double-staining (Alizarin red S plus Alcian blue) method (Christian, 2001) with some modifications. Images were captured using a Stemi-2000 stereomicroscope (Carl Zeiss, Germany).

2.3. Assessment of developmental immunotoxicity on PND 21 and PND 42

Throughout lactation both the dams and nursing pups were clinically observed at least once a day, and more frequently in case of signs of toxicity. At the time of sacrifice or unscheduled death during the study, animals were examined macroscopically for any pathological changes in the abdominal viscera. During the lactation period, dams were weighed daily from PND 1 to PND 21 (weaning) and the pups were weighed daily from PND 22 to PND 42. From the live weights, the group average daily body weights of dams and pups were calculated for each group.

2.3.1. Assessment of humoral immunity of pups on PND 21 and PND 42 through quantitative hemagglutination test

To detect antibody titers against sheep red blood cell (SRBC) antigen, the quantitative hemagglutination test was carried out (Mediratta et al., 2002). SRBCs were procured from the sheep farm of Bombay Veterinary College, Mumbai and were used as the antigen for the hemagglutination test. The SRBCs were collected in Alsever's solution (Sigma Aldrich), washed in sterile pyrogen free, normal saline, and administered intraperitoneally (0.5×10^9 cells/ml per 100 g body weight) for immunization on PND 7 and on PND 28 to pups. On PND 21 and PND 42, the animals were lightly anesthetized with ether and blood was collected from the retro orbital plexus. The serum was separated and analyzed for hemagglutination titer. Two fold serial dilutions of rat sera were made in microtitre plates using normal saline. To each well 50 μ l of 1% (v/v) SRBC was added in 50 μ l of diluted sera. The plates were then incubated at 37 °C for 1 h and observed for hemagglutination. The reciprocal of the highest dilution giving hemagglutination was taken as the hemagglutination titer.

2.3.2. Assessment of humoral immunity of pups on PND 21 and PND 42 through Immunoglobulin (Ig) estimation

Total serum immunoglobulins were estimated by using the zinc sulfate turbidity test (Ismail and Asad, 2009; McEwan et al., 1970) on PND 21 and PND 42. The turbidity at wavelength 545 nm was expressed as 20 zinc sulfate turbidity (ZST) units. The obtained ZST value was converted to g/ml immunoglobulin using the following formula:

$$\text{Zinc sulfate turbidity (ZST units)} = (\text{O.D. of Z tube} - \text{O.D. of C tube}) \times 10$$

$$\text{Total immunoglobulin (g/ml)} = 0.04 + 0.98 \text{ ZST units.}$$

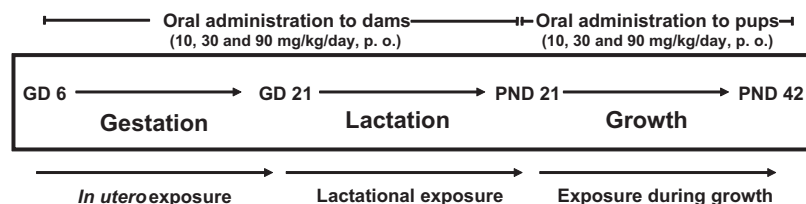


Fig. 1. Schedule of imidacloprid exposure during *in utero* and developmental phases in Wistar rats. Pups were exposed to imidacloprid (10, 30 and 90 mg/kg per day), *in utero* through dams, followed by exposure through lactation, till weaning and subsequently by oral administration to young ones till age of puberty.

2.3.3. Assessment of cell-mediated immunity of pups on PND 21 and PND 42 through Delayed Type Hypersensitivity (DTH) test

The Delayed Type Hypersensitivity test reaction to Dinitrofluorobenzene (DNFB) was assessed as per the procedure described by Horiuchi et al. (1999). The pups were primed twice with DNFB application to the shaved skin of the abdomen. A subsequent application to the surface of the ear elicited an inflammatory reaction which was monitored by measuring the ear thickness. (i) *Sensitization with DNFB*: This parameter was assessed in pups on PND 21 and PND 42. For PND 21 assessment, DNFB was used for sensitization on PND 12 and PND 13 and for PND 42 assessment, DNFB was used for sensitization on PND 33 and PND 34. The sensitizing dose composed of 100 µl of 0.5% DNFB (Sigma Aldrich), dissolved in ethanol (Merck KGaA; Darmstadt, Germany). (ii) *Contact hypersensitivity*: The respective challenge of DNFB for assessing DTH, for PND 21 and PND 42 were applied on PND 20 and PND 41, respectively. The challenge dose containing 10 µl of 0.3% DNFB dissolved in acetone (Sigma Aldrich) and olive oil 4:1 (v/v) was applied to both surfaces of the right ear of sensitized rats; while both surfaces of the left ear (control) were painted with 10 µl acetone and olive oil 4:1 (v/v). The difference in ear thickness (mm) between the left ear painted with vehicle and the right ear painted with DNFB was measured with constant-tension, spring-loaded calipers (POCO 2T; Kröplin Längenmesstechnik, Schlüchtern, Germany), at 0 h (before challenge) and 3, 6, 12, 24 and 48 h after application of the challenge dose.

2.3.4. Assessment of non-specific immunity through in vitro phagocytosis

Phagocytosis is demonstrated in vitro by the blood leukocytes (Hari Babu, 2004). A suspension of 18 h growth of *Staphylococcus aureus* from a nutrient agar slant was prepared in 5 ml of normal saline. Rat blood (0.5 ml by cardiac puncture) was mixed with 0.5 ml of the culture suspension and was incubated at 37 °C for 2 h. The smear was prepared and stained using Leishman's stain (Sigma Aldrich). The phagocytic activity was estimated as percent of cells showing phagocytosis whereas phagocytic index (P.I.) was calculated by considering average number of bacteria per phagocyte (Bukovsky et al., 1998). The phagocytic index was calculated using the following formula: P.I. = (Percentage of neutrophils that contain bacteria × the average number of bacteria per neutrophil) × 100.

2.3.5. Assessment of general toxicity

Hematology was performed on PND 21 and PND 42 with the Beckman Coulter Counter (Brea, CA). The serum was separated on PND 21 and PND 42 and clinical chemistry parameters were quantified using Mindray Serum Biochemistry Auto-analyser BS-400 (Mindray, China). Tissues were fixed in 10% neutral buffered formalin and subjected to routine histological processing. Paraffin embedded sections were stained with Hematoxylin (Sigma Aldrich) and Eosin (Loba Chemie; Mumbai, India) and evaluated for microscopic changes by an investigator blinded to the treatment groups. The liver, thymus and spleen of rats sacrificed on PND 21 and PND 42 were weighed for the estimation of organ weight factor. The organ weight factor was calculated to determine the effect of imidacloprid on overall growth and growth of immune organs of the pups. Percent organ weight factor was calculated as follows:

$$\% \text{ Organ Weight Factor} = \frac{\text{Organ weight}}{\text{Live body weight}} \times 100$$

2.4. Statistical analysis

For analyzing differences among multiple groups, a single factor ANOVA followed by Dunnett's multiple comparison test was used. Data was subjected to Bartlett's test to meet the homogeneity of variance before conducting ANOVA. *P* values <0.05 were considered statistically significant. Unless stated otherwise, all error bars represent standard error of mean.

Table 1

Assessment of *in utero* developmental toxicity parameters in fetuses exposed to imidacloprid through maternal exposure. Number of still births, runts and post implantation losses increased dose-dependently in imidacloprid-treated dams at all the three doses over that of vehicle treated dams. However, soft tissue malformations were absent in all the groups.

Group	Implantation sites	Fetal assessment										
		Total births	Still births	Live births	Runts	No. of males	No. of females	Fetus wt. male (mg)	Fetus wt. female (mg)	Total fetus wt. (mg)	Average fetus wt. (mg)	Post implantation loss (%)
Vehicle	12.29 ± 0.92	12.29 ± 0.92	0.00	12.29 ± 0.92	0.00	6.43 ± 0.61	5.86 ± 0.74	30.53 ± 2.26	28.05 ± 3.08	58.57 ± 2.66	4.89 ± 0.33	0.00
10 mg/kg	13.13 ± 0.55	13.13 ± 0.54	0.00	13.13 ± 0.54	0.25 ± 0.16	6.88 ± 0.58	6.25 ± 0.49	31.34 ± 4.17	27.88 ± 2.43	59.21 ± 5.07	4.57 ± 0.43	0.00
30 mg/kg	14.00 ± 0.58	13.71 ± 0.47	0.14 ± 0.14	13.29 ± 0.61	0.29 ± 0.18	6.29 ± 0.57	7.43 ± 0.81	26.43 ± 3.46	24.20 ± 3.50	50.63 ± 5.61	3.75 ± 0.46	4.88 ± 3.17
90 mg/kg	12.43 ± 2.01	11.71 ± 1.92	1.57 ± 1.57	10.14 ± 2.34	0.43 ± 0.30	5.43 ± 0.65	6.43 ± 1.48	28.09 ± 5.97	22.61 ± 5.57	50.70 ± 10.22	4.73 ± 0.67	16.86 ± 11.69

3. Results

3.1. Assessment of *in utero* developmental toxicity

3.1.1. Assessment during maternal exposure

There were no signs of toxicity observed in pregnant females in any of the experimental groups. Daily gain in body weight of pregnant dams in all groups remained unaffected. In the present study percent organ weight factor of seven female rats from each group sacrificed on PND 20 was determined. The percent organ weight factor decreased in a dose-dependent manner in liver and gravid uterus, whereas it increased in the uterus. A dose-dependent post implantation loss, still births and runts were observed in dams exposed to imidacloprid (Table 1).

3.1.2. Fetal assessment on caesarean section on GD 20

In dams receiving 90 mg/kg imidacloprid, external abnormalities were observed, included one fetus with anasarca, five fetuses with malformation and three were macerated. At 30 mg/kg dose one fetus was dead and one was macerated. Soft tissue malformation did not occur in any treatment groups. Skeletal malformations included an absence of thoracic rib in one fetus, fused ribs in two fetuses, wavy ribs in three fetuses, bifid vertebral centrum in two fetuses and incomplete ossification of phalangeal cartilages in two fetuses of 90 mg/kg dose group (Fig. 2). Only one fetus with fused ribs was found in the 30 mg/kg dose group.

3.2. Assessment of developmental immunotoxicity on PND 21 and PND 42

Dams were monitored for health status during the phase of lactation (PND 1 to PND 21). After parturition, lactating females were closely observed for general health and behavior. There were no signs of toxicity observed in lactating females in any of the groups. Mortality was observed in nursing pups (2 out of 30 pups) only in the highest group. Few incidences of hypoactivity, piloerection, melena and diarrhea were evident in all imidacloprid exposed pups. Daily gain in body weight of pregnant dams in all groups remained unaffected (Fig. 3A). However, it was observed that mean weight from PND 1 to PND 21 was numerically less in imidacloprid exposed groups as compared to the control group (Fig. 3B). From PND 1 to PND 21 the pups were exposed to imidacloprid only through milk (lactational exposure) and thus, only a part of the selected dose was delivered to the pups from PND 1 to PND 21. However, from PND 21 to PND 42 the pups were directly exposed to selected dose levels of imidacloprid through oral gavage. From PND 22 to PND 42 there was no significant difference in the weights of imidacloprid exposed pups compared to the control (Fig. 3C). Percent organ weight factor was derived on PND 21 and PND 42. On PND 21 there was reduction in the percent organ weight factor for spleen and thymus indicating the affiliation of

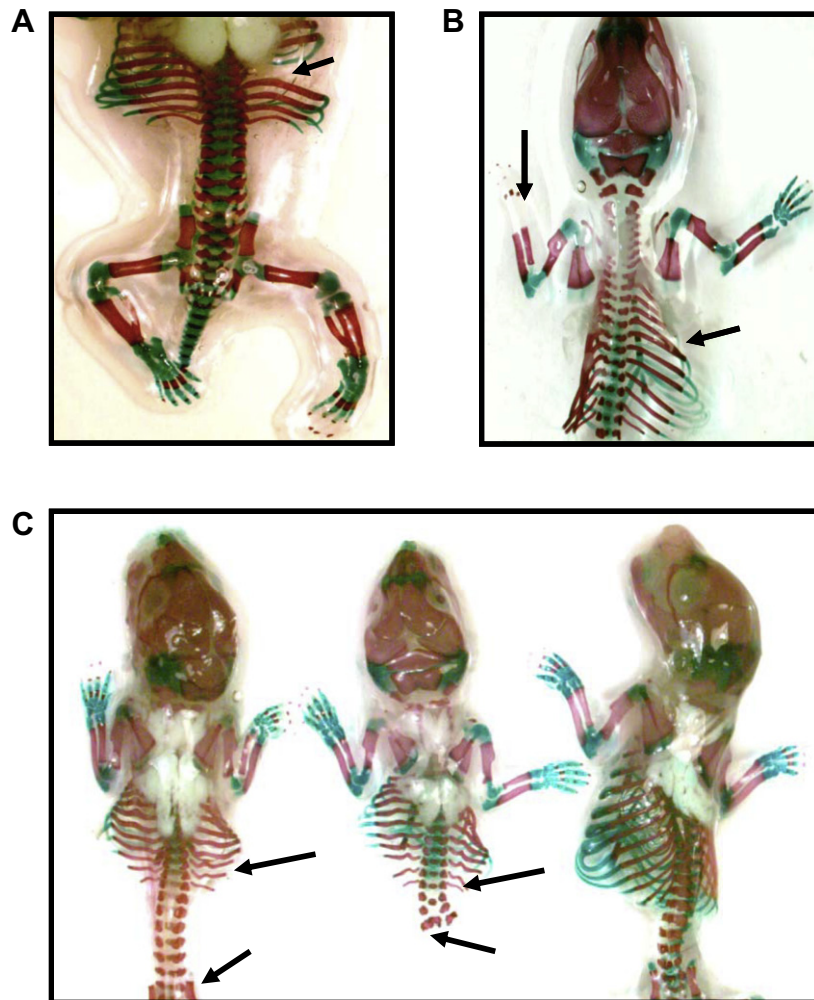


Fig. 2. Skeletal malformations induced in pups of dams exposed to high dose imidacloprid. (A) Absence of rib, (B) incomplete ossified phalangeal cartilage and (C) wavy ribs with bifid vertebral centrum, were observed in pups of dams exposed to 90 mg/kg imidacloprid.

imidacloprid to the immune system. This may also represent a metabolic response to the high dose of imidacloprid. On PND 42, a dose-dependent reduction in percent organ weight factor of spleen and liver was observed, as against that, a dose-dependent increase in percent organ weight factor was evident for thymus, the percent organ weight factor was 0.24, 0.28, 0.28 and 0.33 for thymus, 0.56, 0.29, 0.28 and 0.28 for spleen and 5.97, 4.96, 4.96 and 5.13 for liver of rats receiving vehicle, 10, 30 and 90 mg/kg imidacloprid respectively.

3.3. Assessment of humoral immunity

3.3.1. Hemagglutination titer against SRBC antigens on PND 21 and PND 42

Antibody titer against SRBC antigens measured on PND 21 and PND 42 in the group receiving highest dose (90 mg/kg) was lowest (9.50 ± 1.50 on PND 21 and 21.33 ± 3.37 on PND 42) indicating the effect of imidacloprid administration on antibody response. In the vehicle control group mean hemagglutination titers were 36.00 ± 6.59 on PND 21 and 58.67 ± 5.33 on PND 42. In the other two groups (30 and 10 mg/kg) a dose-dependent decrease in hemagglutination titers were evident with the mean values being 11.00 ± 1.46 , 26.67 ± 3.37 at 30 mg/kg and 17 ± 2.36 , 32.00 ± 7.16 at 10 mg/kg dose on PND 21 and PND 42, respectively. The present study revealed a reduction in the antibody titers against SRBCs in a

dose-dependent manner in rats exposed to imidacloprid on PND 21 and PND 42 (Table 2). In case of treatment groups, the mean antibody titers reduced significantly on PND 21 (9.50 ± 1.50) and very significantly on PND 42 (21.33 ± 3.37) as compared to the vehicle control antibody titers (36.00 ± 6.59 and 58.67 ± 5.33 on PND 21 and PND 42, respectively). The comparison of means of the hemagglutination titer of the respective groups on PND 21 and PND 42 revealed that hemagglutination titers were lower on PND 21 as compared to PND 42 in all groups and the difference was statistically significant only in the control groups indicating that in the treated group, development of humoral immunity was not at par with the control.

3.3.2. Assessment of serum immunoglobulin levels in pups on PND 21 and PND 42

The mean immunoglobulin levels (g/ml) of the group receiving moderate dose (30 mg/kg) were lowest among all the groups (2.28 ± 0.12 on PND 21 and 3.20 ± 0.18 on PND 42) indicating the effect of imidacloprid administration on immunoglobulin levels (Table 2). These levels differed significantly on PND 21 and PND 42 as compared to the vehicle control. The mean immunoglobulin levels were 3.07 ± 0.32 on PND 21 and 5.10 ± 0.23 on PND 42. In the other two groups (90 and 10 mg/kg) decrease in immunoglobulin levels was evident with the mean values being 2.31 ± 0.16 , 3.55 ± 0.34 at 90 mg/kg and 2.44 ± 0.20 , 4.41 ± 0.38 at 10 mg/kg

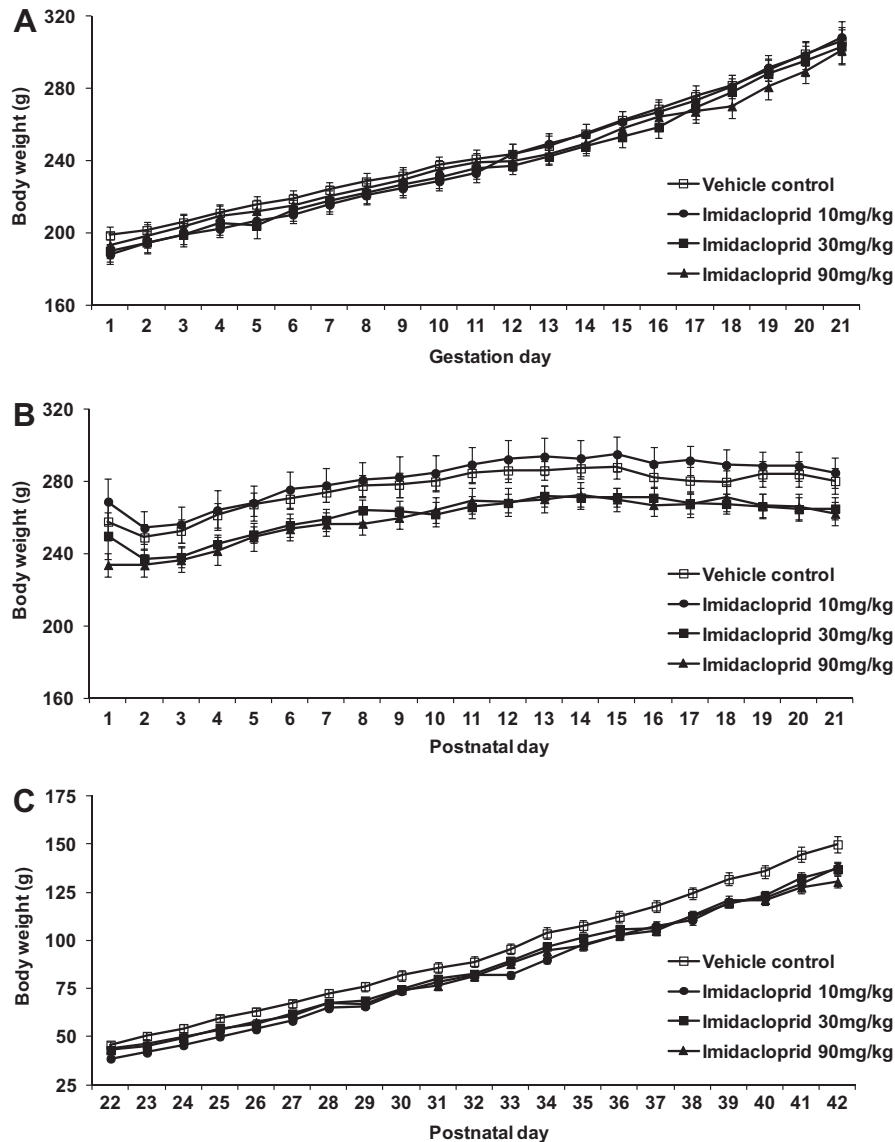


Fig. 3. Daily body weight of animals. (A) Body weight of dams exposed to 10, 30 and 90 mg/kg of imidacloprid from GD 1 to GD 21, (B) body weight of dams exposed to 10, 30 and 90 mg/kg of imidacloprid from PND 1 to PND 21, (C) body weight of pups exposed to imidacloprid from PND 22 to PND 42. All values are averages \pm S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to vehicle exposed dams or pups on the corresponding day.

dose on PND 21 and PND 42, respectively (Table 2). The comparison of the mean immunoglobulin levels of the respective groups on PND 21 and PND 42 revealed that immunoglobulin levels were lower on PND 21 as compared to PND 42 in all groups and the difference was statistically significant (Table 2).

3.3.3. Assessment of cell-mediated immunity through Delayed Type Hypersensitivity test in rats on PND 21 and PND 42

The mean increase in ear thickness (mm) of the ear (right ear) painted with DNFB over that of unpainted ear (left ear) of the group receiving highest dose imidacloprid (90 mg/kg) was lowest among all the groups (0.15 ± 0.007 on PND 21 and 0.24 ± 0.024 on PND 42) indicating the effect of imidacloprid administration on immune response. In the vehicle control group, the mean difference in ear thickness was 0.33 ± 0.013 on PND 21 and 0.43 ± 0.02 on PND 42. In the other two groups a dose-dependent decrease in ear thickness was evident with the mean values being 0.22 ± 0.013 , 0.31 ± 0.02 at 30 mg/kg and 0.31 ± 0.007 , 0.40 ± 0.02 at 10 mg/kg dose on PND 21 and PND 42, respectively. In all the

mid and high dose imidacloprid exposed pups, the difference in ear thickness differed very significantly as compared to control on PND 21 and PND 42 (Table 3). The comparisons of the means of ear thicknesses of the respective groups on PND 21 compared to values on PND 42 revealed that the difference in ear thicknesses were lower on PND 21 as compared to PND 42 in all groups and the difference was statistically significant.

3.3.4. Assessment of non-specific immunity through *in vitro* phagocytosis

Phagocytosis by neutrophils was observed in all the groups on PND 21 and PND 42 (Fig. 4). Mean phagocytic index of the rats receiving highest dose (90 mg/kg) was lowest among all the groups (31.17 ± 1.56 on PND 21 and 29.83 ± 2.34 on PND 42) indicating the effect of imidacloprid administration on non-specific immune response. In the vehicle control group, mean phagocytic index was 57.17 ± 4.69 on PND 21 and 73.33 ± 3.70 on PND 42. In the other two groups treated with imidacloprid, a dose-dependent decrease in phagocytic index was evident with the mean values being

Table 2
Immunotoxicological assessment of pups exposed to imidacloprid. Hemagglutination titer decreased dose-dependently across all the imidacloprid-treated groups compared to vehicle treated group at both, PND 21 as well as PND 42. Immunoglobulin levels dropped at 30 mg/kg on PND 21 and 30 and 90 mg/kg on PND 42. Phagocytic index was lower in all the imidacloprid-treated groups as compared to vehicle control on PND 21 and PND 42. All values are average \pm S.E.M.

Time point	Group	Hemagglutination titer	Immunoglobulin level (g/ml)	Phagocytic index (%)
PND 21	Vehicle	36.00 \pm 6.59	3.07 \pm 0.32	57.17 \pm 4.69
	10 mg/kg	17.00 \pm 2.36*	2.44 \pm 0.20	55.17 \pm 1.90
	30 mg/kg	11.00 \pm 1.46**	2.28 \pm 0.12*	35.33 \pm 2.96**
	90 mg/kg	9.50 \pm 1.50**	2.31 \pm 0.16	31.17 \pm 1.56***
PND 42	Vehicle	58.67 \pm 5.33	5.10 \pm 0.23	73.33 \pm 3.70
	10 mg/kg	32.00 \pm 7.16*	4.41 \pm 0.38	53.33 \pm 3.76**
	30 mg/kg	26.67 \pm 3.37***	3.20 \pm 0.18***	33.00 \pm 1.57***
	90 mg/kg	21.33 \pm 3.37***	3.55 \pm 0.34**	29.83 \pm 2.34***

* $P < 0.05$ compared to vehicle-treated group

** $P < 0.01$ compared to vehicle-treated group

*** $P < 0.001$ compared to vehicle-treated group.

Table 3
Average ear thickness changes induced by DNFB in pups exposed to imidacloprid on PND 21 and PND 42. An increase in ear thickness change was observed in the vehicle control group over time till 48 h indicating an active immune response to Delayed Type Hypersensitivity reaction. However, the difference in ear thickness change between vehicle-treated group and 90 mg/kg dose group of imidacloprid was significantly different on both PND 21 and PND 42. All values are average \pm S.E.M. of 6 rats.

Time point	Group	Ear thickness changes (mm) at different time intervals					
		0 h	3 h	6 h	12 h	24 h	48 h
PND 21	Vehicle	0.02 \pm 0.014	0.05 \pm 0.014	0.09 \pm 0.006	0.12 \pm 0.010	0.17 \pm 0.025	0.33 \pm 0.013
	10 mg/kg	0.00 \pm 0.015	0.09 \pm 0.025	0.14 \pm 0.022	0.16 \pm 0.018	0.21 \pm 0.007	0.31 \pm 0.007
	30 mg/kg	0.01 \pm 0.011	0.04 \pm 0.010	0.06 \pm 0.014*	0.08 \pm 0.007**	0.15 \pm 0.007	0.22 \pm 0.013***
	90 mg/kg	0.00 \pm 0.017	0.05 \pm 0.014	0.05 \pm 0.007***	0.03 \pm 0.015***	0.10 \pm 0.019*	0.15 \pm 0.007***
PND 42	Vehicle	0.01 \pm 0.01	0.28 \pm 0.01	0.32 \pm 0.01	0.34 \pm 0.02	0.34 \pm 0.02	0.43 \pm 0.02
	10 mg/kg	0.00 \pm 0.00	0.09 \pm 0.01***	0.22 \pm 0.02***	0.29 \pm 0.02	0.36 \pm 0.01	0.40 \pm 0.02
	30 mg/kg	0.01 \pm 0.01	0.15 \pm 0.02***	0.23 \pm 0.03*	0.26 \pm 0.02*	0.28 \pm 0.02*	0.31 \pm 0.02***
	90 mg/kg	0.02 \pm 0.01	0.13 \pm 0.02***	0.19 \pm 0.01***	0.18 \pm 0.01***	0.23 \pm 0.02***	0.24 \pm 0.02***

* $P < 0.05$ compared to vehicle-treated group.

** $P < 0.01$ compared to vehicle-treated group.

*** $P < 0.001$ compared to vehicle-treated group.

35.33 \pm 2.96 and 33.00 \pm 1.57 at 30 mg/kg and 55.17 \pm 1.90 and 53.33 \pm 3.76 at 10 mg/kg dose on PND 21 and PND 42 respectively. In case of treatment groups, the phagocytic index was reduced significantly on PND 21 and PND 42 as compared to the vehicle control. The comparison of the mean phagocytic index of the respective groups on PND 42 with PND 21 values revealed that phagocytic indices were lower on PND 42 as compared to PND 21 in all the imidacloprid treated pups.

3.3.5. Assessment of general toxicity on PND 21 and PND 42

In the present study, on PND 21, a decreased platelet count was observed in nursing dams in imidacloprid exposed groups (Table 4). A decrease in percentage of granulocytes was observed (in 30 and 90 mg/kg treated groups) and an increase in hematocrit and monocyte count (in all treatment groups) was observed (Table 4). However, except for hematocrit, these differences were not statistically significant. In pups on PND 21, a trend of decrease in hemoglobin, mean corpuscular hemoglobin concentration and granulocyte percentage was observed (Table 5). The platelet count and percent lymphocytes showed an increase (Table 5). Whereas in pups on PND 42, a significant decrease in total leukocyte count, and a trend of decrease in percent lymphocytes and platelet count was observed (Table 5). Biochemical analysis, in maternal females, on PND 21, revealed an increase in alanine transaminase (ALT), and decrease in serum protein, albumin, globulin and albumin/globulin (A/G) ratio at 30 and 90 mg/kg (Table 6). Similarly, in pups, on PND 21, an increase in aspartate transaminase (AST), serum protein, albumin, A/G ratio and alkaline phosphatase (ALP), was observed at 30 and 90 mg/kg (Table 7). On PND 42, an increase in AST, albumin, A/G ratio and ALP was observed across all imidacloprid-treated groups (Table 7). Pre-defined visceral organs were collected for

histological evaluation after sacrifice on GD 20 from dams, whereas, on PND 21 and PND 42 from pups. The organs collected were thymus, spleen, lymph node, liver, kidney, brain and sex organs. Histological analysis revealed renal casts in kidneys of two dams administered with highest dose (90 mg/kg) on GD 20 and in kidneys of pups on PND 42. On PND 21, brain from one rat exhibited homogenous eosinophilic bodies in cerebrum and testis with pink homogenous exudates in epididymis. None of the other organs exhibited any findings that could be attributed to imidacloprid treatment.

4. Discussion

Available literature on imidacloprid mediated immune system effects has been aptly collated by Mohany et al. (2012). There has also been an attempt to explore the genotoxic potential of imidacloprid (Demsia et al., 2007), as well as the chronic toxicity of imidacloprid in female rats (Bhardwaj et al., 2010; Kapoor et al., 2011). The current study focuses on the *in utero* and concomitant developmental toxic effects of imidacloprid. We also evaluated the effect of imidacloprid treatment on weanlings and young-adults. An approach to developmental immunotoxicity that addresses all critical windows of immune system development was thus evaluated. Considering the different stages of development, the developmental toxicity protocols are designed in which pregnant females are exposed to the test chemicals during organogenesis of the fetus, lactating females are exposed to the test chemicals and weanlings are exposed to test chemicals till they attain maturity. Available literature reveals that most researchers have chosen only one of the above three periods for exposure of animals to

Table 4
Imidacloprid-induced changes in hematological parameters of dams on PND 21. Hematological assessment of dams exposed to imidacloprid (10, 30 and 90 mg/kg) on PND 21. All values are average \pm S.E.M. of 6 rats.

Time point	Group	Hematological parameters of dams										
		WBC ($\times 10^3/\mu\text{l}$)	RBC ($\times 10^6/\mu\text{l}$)	Hb (g/ml)	HCT (%)	MCV (ft)	MCH (pg)	MCHC g/ml	PLT ($\times 10^3/\mu\text{l}$)	LY (%)	MO (%)	GR (%)
PND 21	Vehicle	12.20 \pm 0.85	6.42 \pm 0.23	15.10 \pm 0.33	6.56 \pm 0.10	50.42 \pm 0.14	22.80 \pm 0.42	46.24 \pm 0.71	820.00 \pm 92.67	82.18 \pm 1.94	11.12 \pm 1.44	6.70 \pm 1.55
	10 mg/kg	12.68 \pm 1.22	6.28 \pm 0.50	15.18 \pm 0.35	7.62 \pm 0.18*	50.52 \pm 0.11	30.08 \pm 3.37	45.00 \pm 0.53	525.00 \pm 49.24*	77.38 \pm 1.47	13.42 \pm 0.66	8.68 \pm 1.54
	30 mg/kg	12.38 \pm 0.35	5.97 \pm 0.69	14.78 \pm 0.42	7.04 \pm 0.12*	50.46 \pm 0.11	29.26 \pm 4.57	44.86 \pm 0.46	645.00 \pm 109.53	82.78 \pm 1.04	13.88 \pm 0.82	3.18 \pm 0.23
	90 mg/kg	12.54 \pm 1.72	7.32 \pm 0.36	15.04 \pm 0.23	7.00 \pm 0.09*	50.32 \pm 0.21	19.84 \pm 0.58	43.48 \pm 0.70	790.40 \pm 88.78	80.58 \pm 0.90	14.24 \pm 1.22	5.26 \pm 1.32

* $P < 0.05$ compared to vehicle-treated group.

Table 5
Imidacloprid-induced changes in hematological parameters of pups on PND 21 and PND 42. Hematological assessment of pups exposed to imidacloprid (10, 30 and 90 mg/kg) on PND 21 and PND 42. All values are average \pm S.E.M. of 6 rats.

Time point	Group	Hematological parameters of pups										
		WBC ($\times 10^3/\mu\text{l}$)	RBC ($\times 10^6/\mu\text{l}$)	Hb (g/ml)	HCT (%)	MCV (ft)	MCH (pg)	MCHC g/ml	PLT ($\times 10^3/\mu\text{l}$)	LY (%)	MO (%)	GR (%)
PND 21	Vehicle	4.42 \pm 0.78	3.37 \pm 0.24	12.08 \pm 0.44	3.90 \pm 0.28	57.92 \pm 0.64	36.68 \pm 2.12	63.27 \pm 3.46	410.83 \pm 52.10	76.80 \pm 1.14	12.60 \pm 0.41	10.60 \pm 1.29
	10 mg/kg	5.75 \pm 0.42	3.85 \pm 0.24	12.75 \pm 0.63	4.37 \pm 0.28	56.43 \pm 0.79	33.43 \pm 1.06	59.22 \pm 1.51	599.17 \pm 62.26	83.13 \pm 0.72	10.37 \pm 0.40	6.50 \pm 0.51
	30 mg/kg	5.75 \pm 0.78	3.58 \pm 0.25	9.08 \pm 0.55**	4.05 \pm 0.27	57.05 \pm 1.33	25.92 \pm 1.54**	45.27 \pm 1.71***	585.83 \pm 107.48	76.68 \pm 1.24	12.97 \pm 0.99	10.35 \pm 0.75
	90 mg/kg	4.08 \pm 0.40	3.75 \pm 0.38	10.50 \pm 0.37*	4.55 \pm 0.48	60.45 \pm 0.69*	29.88 \pm 3.73	49.60 \pm 6.58	669.17 \pm 111.46	78.32 \pm 0.80	13.47 \pm 0.36	8.92 \pm 0.92
PND 42	Vehicle	18.20 \pm 2.50	6.39 \pm 0.74	11.93 \pm 0.44	15.70 \pm 0.60	54.92 \pm 0.72	20.83 \pm 0.29	37.98 \pm 0.42	1012.00 \pm 143.87	83.58 \pm 3.29	10.45 \pm 2.03	5.97 \pm 1.42
	10 mg/kg	8.90 \pm 0.49**	6.53 \pm 0.26	13.47 \pm 0.44	17.75 \pm 0.69*	54.42 \pm 0.41	20.65 \pm 0.20	37.95 \pm 0.32	1088.00 \pm 79.11	80.18 \pm 1.28	9.58 \pm 1.05	10.23 \pm 1.97
	30 mg/kg	5.75 \pm 0.78	3.58 \pm 0.25	9.08 \pm 0.55**	4.05 \pm 0.27	57.05 \pm 1.33	25.92 \pm 1.54**	45.27 \pm 1.71***	585.83 \pm 107.48	76.68 \pm 1.24	12.97 \pm 0.99	10.35 \pm 0.75
	90 mg/kg	7.40 \pm 0.98**	6.91 \pm 0.25	13.57 \pm 0.42*	17.58 \pm 0.51*	53.50 \pm 0.33	20.58 \pm 0.25	38.52 \pm 0.32	850.93 \pm 170.32	81.15 \pm 1.53	10.88 \pm 1.02	7.97 \pm 1.29

* $P < 0.05$ compared to vehicle-treated group.

** $P < 0.01$ compared to vehicle-treated group.

*** $P < 0.001$ compared to vehicle-treated group.

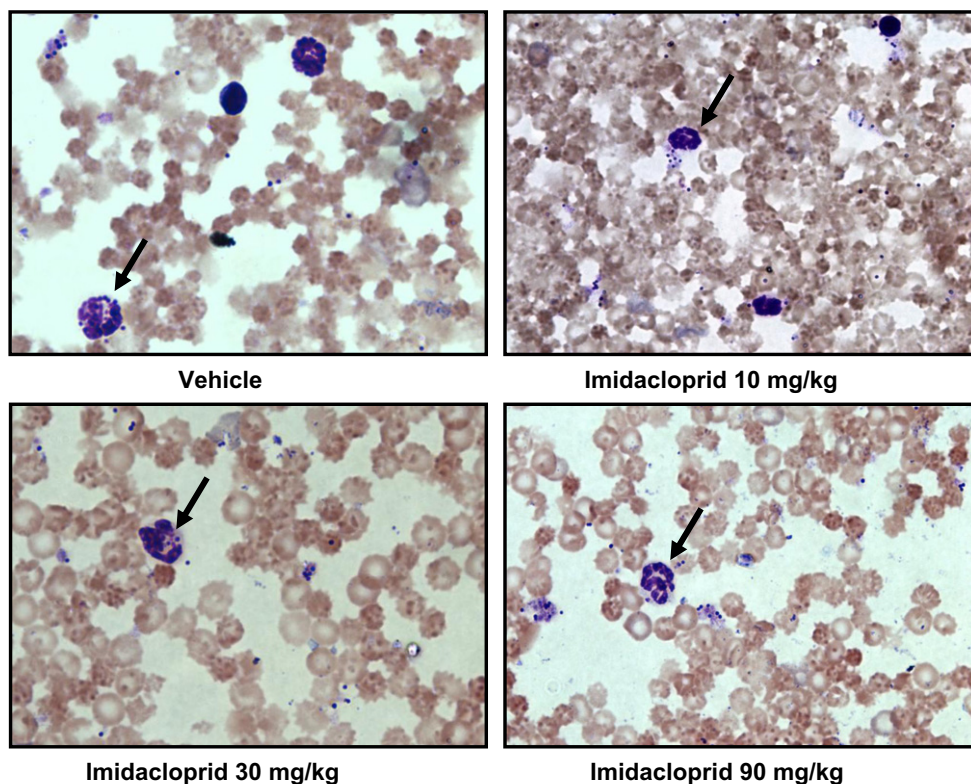


Fig. 4. Phagocytosis shown by neutrophils in imidacloprid exposed rats on PND 42. Microscopic evaluation of rat blood smear after *ex vivo* exposure of imidacloprid treated rat blood to *Staphylococcus aureus* using Leishman's stain at 100X. Arrows represent phagocytic activity by neutrophils.

Table 6
Imidacloprid-induced changes in clinical chemistry parameters of dams on PND 21. Clinical chemistry assessment of dams exposed to imidacloprid (10, 30 and 90 mg/kg) on PND 21. All values are average \pm S.E.M. of 6 rats.

Time point	Group	Clinical chemistry parameters of dams							
		AST (U/l)	ALT (U/l)	Creatinine (g/l)	Total Protein (g/l)	Albumin (g/ml)	Globulin (g/ml)	A/G ratio	ALP (U/l)
PND 21	Vehicle	234.94 \pm 20.69	152.46 \pm 30.93	0.83 \pm 0.03	8.25 \pm 0.31	4.25 \pm 0.21	4.00 \pm 0.23	1.08 \pm 0.08	670.60 \pm 168.70
	10 mg/kg	211.48 \pm 49.75	219.18 \pm 48.51	0.86 \pm 0.12	8.71 \pm 0.22	4.64 \pm 0.25	4.06 \pm 0.11	1.15 \pm 0.08	841.40 \pm 186.85
	30 mg/kg	209.78 \pm 33.51	189.20 \pm 43.01	0.66 \pm 0.11	6.35 \pm 1.10	3.27 \pm 0.74	3.08 \pm 0.41	1.02 \pm 0.18	763.2 \pm 203.29
	90 mg/kg	264.08 \pm 55.14	175.18 \pm 46.84	0.70 \pm 0.14	7.53 \pm 1.44	3.52 \pm 0.80	4.01 \pm 0.68	0.81 \pm 0.15	615.60 \pm 121.32

Table 7
Imidacloprid-induced changes in clinical chemistry parameters of pups on PND 21 and PND 42. Clinical chemistry assessment of pups exposed to imidacloprid (10, 30 and 90 mg/kg) on PND 21 and PND 42. All values are average \pm S.E.M. of 6 rats.

Time point	Group	Clinical chemistry parameters of pups							
		AST (U/l)	ALT (U/l)	Creatinine (g/l)	Total protein (g/l)	Albumin (g/ml)	Globulin (g/ml)	A/G ratio	ALP (U/l)
PND 21	Vehicle	221.32 \pm 20.11	69.38 \pm 5.16	0.57 \pm 0.01	5.62 \pm 0.08	3.21 \pm 0.06	2.41 \pm 0.03	1.33 \pm 0.02	793.33 \pm 66.54
	10 mg/kg	203.42 \pm 19.40	58.93 \pm 2.59	0.53 \pm 0.02	6.02 \pm 0.16	3.60 \pm 0.11	2.43 \pm 0.06	1.48 \pm 0.03	646.67 \pm 30.58
	30 mg/kg	289.43 \pm 14.92	51.80 \pm 5.27	0.59 \pm 0.01	5.90 \pm 0.07	3.48 \pm 0.09	2.42 \pm 0.11	1.46 \pm 0.10	1206.83 \pm 82.16
	90 mg/kg	265.77 \pm 26.75	68.47 \pm 6.96	0.59 \pm 0.01	5.89 \pm 0.13	3.61 \pm 0.13	2.28 \pm 0.07	1.59 \pm 0.08	1265.33 \pm 66.34
PND 42	Vehicle	225.50 \pm 19.24	70.00 \pm 3.03	0.67 \pm 0.03	6.83 \pm 0.19	1.92 \pm 0.08	4.91 \pm 0.18	0.39 \pm 0.02	394.33 \pm 46.04
	10 mg/kg	290.70 \pm 41.82	79.33 \pm 4.56	0.79 \pm 0.04	7.41 \pm 0.33	2.27 \pm 0.19	5.14 \pm 0.18	0.44 \pm 0.03	399.67 \pm 44.50
	30 mg/kg	265.23 \pm 28.23	69.03 \pm 3.22	0.70 \pm 0.03	6.98 \pm 0.18	2.30 \pm 0.14*	4.68 \pm 0.07	0.49 \pm 0.03*	410.33 \pm 75.29
	90 mg/kg	329.15 \pm 43.31*	80.20 \pm 6.42	0.78 \pm 0.08*	7.86 \pm 0.80	2.73 \pm 0.55	5.13 \pm 0.29	0.52 \pm 0.07	469.33 \pm 72.96

* $P < 0.05$ compared to vehicle-treated group.

immunotoxicants (Bhardwaj et al., 2010; Kapoor et al., 2011). There are hardly any studies covering the entire span of all afore-

mentioned stages of mammalian development. This may be due to the fact that all repeat dose toxicity studies demand a delicate

balance between induction of toxicity and the challenge to keep animals alive till the end of the exposure period. Apart from other studies, few deaths have also been reported in humans from imidacloprid ingestion (Proenca et al., 2005). Whereas studies by Becker et al. (1988) cover only exposure during organogenesis. Thus taking into account the available literature and results of pilot dose range finding studies, the three dose levels (10, 30 and 90 mg/kg) for oral imidacloprid administration were selected in the current study.

Further, PND 21–PND 42 are the most critical periods of development in murine life, which could be considered equivalent to human adulthood, and hence the nature of toxicity observed would signal the probable risk associated with direct exposure to the chemical in adulthood. Holsapple et al. (2005) have emphasized on the rationale for PND 42 exposure and stated that most immune functions attain adult status at PND 42. Our comprehensive literature survey failed to adequately trace assessment of imidacloprid toxicity reports during PND 21–PND 42. The repeated exposure to imidacloprid during postnatal period is shown to result in adverse effects on the body weights of the animals. However, an adverse effect on maternal body weight during this period was not evident in our study. It is also known that the effect on pups is indicative of excretion of imidacloprid in milk. The residue study of imidacloprid in dairy cows conducted by previous researchers (Heukamp, 1992; Klein, 1992) revealed detectable levels of imidacloprid in milk. Thus, apart from occupational exposure (Demsia et al., 2007), the issue of direct or indirect exposure of nursing young ones to imidacloprid can have human health implications too. This provided us the hypothesis that lactating dams when administered with imidacloprid, would provide part of the exposure to their nursing pups.

Besides developmental toxicity, the effects of imidacloprid on the immune system have also been reported earlier, though exclusively in adult male rats (Mohany et al., 2012) and in adult female rats (Bhardwaj et al., 2010). Hence, assessment of humoral, cell-mediated, as well as non-specific immunity was explored in the present two-generation design of experiment. In the current study, hemagglutination titers in the control group indicate that in treated groups, development of humoral immunity was not at par with the vehicle control group. Imidacloprid adversely affected humoral immunity during the postnatal and growth stages of the animals. When the test animals were pretreated with imidacloprid, the titers of circulating antibodies exhibited a decline. The mean immunoglobulin levels were reduced significantly on PND 21 and PND 42, in the imidacloprid treated group as compared to the vehicle control. Our results are contrary to the findings of Mohany et al. (2012), who have attributed the increase in circulating immunoglobulins to leukocyte activation. Such differences in observations may arise due to variation in exposure levels of imidacloprid, owing to the use of dissimilar formulations. Imidacloprid adversely affected humoral immunity in postnatal and growing pups. Delayed Type Hypersensitivity was measured on PND 20 and PND 41 in both the imidacloprid and vehicle treated groups. Mean difference in ear swelling in highest treatment group (90 mg/kg) was reduced significantly on PND 21 and PND 42 as compared to the vehicle control on PND 21 as well as on PND 42. Imidacloprid thus revealed an adverse effect on cellular immunity as well, in postnatal period during the growth of pups. It may be noteworthy to mention that DNFB has irritant properties in addition to its sensitizing effects. However, since a challenge only control was not used in this experiment, the inflammatory reaction causing the ear swelling could be due to a combination of the irritant and the sensitizing effect of DNFB.

The mean phagocytic index was reduced significantly on PND 21 and PND 42 as compared to the vehicle control. Thus, at the highest dose, imidacloprid exhibited adverse effects on non-spe-

cific immunity at the perinatal and growing stage in pups. These findings are complimentary to the findings of Mohany et al. (2012), who have recorded a decrease in phagocytic activity of leukocytes. Imidacloprid exposed rats may become more prone to infection since the ability to clear infectious microorganisms decreases, as is evident from the phagocytic activity parameter. However, an *in vivo* phagocytosis experiment to verify this feature of imidacloprid is warranted.

Hematology parameters were estimated in parturient female rats on PND 21. The mean platelet count and percentage of granulocytes decreased whereas an increase in monocyte count and significant increase in hematocrit was observed, other parameters were in the normal range, which complements the findings of Bhardwaj et al. (2010). On PND 21, hemoglobin and mean corpuscular hemoglobin concentration decreased, and platelet count and percent lymphocytes increased. On PND 42, a significant decrease in total leukocyte, percent lymphocytes and platelet count was observed in imidacloprid exposed groups. Decreased percent lymphocytes and total leukocyte count in treated groups correlated with continuous exposure to imidacloprid, which indicated a risk to lymphopaenia and immunomodulation (Loveless et al., 2008). 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.

Biochemical parameters of maternal females were estimated on PND 21 and in pups on PND 21 and PND 42. Imidacloprid-induced elevation of hepatic enzymes is known in literature (Bhardwaj et al., 2010; Kapoor et al., 2011). In the current study, hepatic functioning seems to be modulated in pups, on both PND 21 and PND 42, manifested through an elevated activity of AST and ALP, which also indicates hepatocellular damage. Whereas, alteration in A/G ratio indicates initiation of liver and kidney insufficiency, and results in decreased immunity against bacterial and parasitic infections. On PND 21, in pups, there was reduction in the percent organ weight factor of spleen and thymus, and an increase in that of liver in the mid and high dose groups. The increase in percent organ weight factor of liver may represent a metabolic response to the high dose of imidacloprid. Similarly, on PND 42, in pups, a dose-dependent reduction in the percent organ weight factor of spleen was observed. Reduction in mean body weights and change in weight of spleen, thymus, liver and kidney in relation to body weight are considered as a criteria for assessing immunotoxicity (Loveless et al., 2008). The increase in percent organ weight factor of liver was associated with a concomitant increase in the activity of AST and ALP in the high dose group on PND 21. Similar to the findings of the current study, an elevation of hepatic enzymes on imidacloprid administration have also been reported previously (Mohany et al., 2012). Findings of the tests performed for assessment of humoral immunity (antibody titer against SRBC antigens and immunoglobulin estimation), cell-mediated immunity (Delayed Type Hypersensitivity) and non-specific immunity (percent phagocytic index) revealed that imidacloprid has an age-dependent adverse effect on immunity. This is further to the fact that apart from ovarian toxicity (Kapoor et al., 2011), a clear mechanism involved in reproductive function is unknown. However, at higher doses, imidacloprid is capable of significantly altering endocrine homeostasis, governing female reproductive capability.

Considering the fact that imidacloprid is a 'general use pesticide' as designated by the US-EPA (Demsia et al., 2007), developmental immunotoxicity studies of imidacloprid in rodents covering the entire span of exposure from GD 6 to PND 42 are scarce. The present study was aimed at a thorough evaluation of the risks associated with chronic administration of imidacloprid to pregnant dams, nursing pups and young-adults. As evident from the current study, imidacloprid showed age-related, dose-depen-

dent, developmental immunotoxic effects. Humans who are continuously associated with imidacloprid during the process of its production, transport or uses are the ones most likely to get chronically exposed to it. Considering the results of all the parameters together, the present study clearly demonstrates developmental immunotoxicity as one of the potential risks associated with chronic exposure to imidacloprid at high doses. This suggests that continuous exposure to imidacloprid during development may affect the immune system adversely, making the subject prone to various secondary ailments. Though, there is less possibility of individuals getting chronically exposed to such high doses by oral route, caution should be taken to avoid not only the occupational exposure to imidacloprid, but also exposure to the vulnerable population such as pregnant women and growing children.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We would like to thank Dr. Somesh Sharma, Chairman and Dr. Arun Balakrishnan, Senior Vice President, HTS, Natural Products and External Liaison, Piramal Life Sciences Limited for granting a state-of-the-art facility to undertake this research work.

References

- Becker, H., Vogel, W., Terrier, C., 1988. Embryo toxicity study (including teratogenicity) with NTN 33893 technical in the rat (Research & Consulting Company AG, Report No. R 4582 submitted to WHO by Bayer AG, Mannheim, Germany).
- Bhardwaj, S., Srivastava, M.K., Kapoor, U., Srivastava, L.P., 2010. A 90 days oral toxicity of imidacloprid in female rats: morphological, biochemical and histopathological evaluations. *Food Chem. Toxicol.* 48, 1185–1190.
- Bukovsky, M.A., Kardosova, H., Koscova, Kosfalova, D., 1998. Immunomodulating activity of 4-O-methyl-D-glucuronol-xylan from *Rudbeckia fulgida* var. *sullivantii*. *Biologia* 53, 771–775.
- California Dept. of Pesticide Regulation, 2000. Database entry for Bayer Advanced Lawn Season-Long Grub Control Ready-to-Use, February 13. Available from: <<http://www.cdpr.ca.gov/docs/label/prodman.html>>.
- Christian, M.S., 2001. Test Methods for Assessing Female Reproductive and Developmental Toxicology. Taylor & Francis, Philadelphia.
- Christian, M.S., Schulte, S., Hellwig, J., 2003. Developmental (embryo-fetal toxicity/teratogenicity) toxicity studies of synthetic crystalline lycopene in rats and rabbits. *Food Chem. Toxicol.* 41, 773–783.
- Demsia, G., Vlastos, D., Goumenoub, 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.
- Dietert, R.R., 2009. Developmental immunotoxicity (DIT), postnatal immune dysfunction and childhood leukemia. *Blood Cells Mol. Dis.* 42, 108–112.
- Dietert, R.R., Piepenbrink, M.S., 2006. Perinatal immunotoxicity: why adult exposure assessment fails to predict risk. *Environ. Health Perspect.* 114, 477–483.
- Gatne, M.M., Ramesh, Bhoir, P.S., Deore, M.D., 2006. Immunotoxicity studies of imidacloprid in rats. *Toxicol. Int.* 13, 89–92.
- Hari Babu, Y., 2004. Handbook of Basic Immunological and Serological Techniques. Academia Publishers, New Delhi.
- Heukamp, U., 1992. NTN 33893: Cattle Feeding Study: Lab Project Number: P 67315000. Bayer AG: MRID 42556139, p. 318.
- Horiuchi, H., Ota, M., Kitahara, S., Ohta, T., Kiyoki, M., Komoriya, K., 1999. Allopurinol increases ear swelling and mortality in a Dinitrofluorobenzene-induced contact hypersensitivity mouse model. *Biol. Pharm. Bull.* 22, 810–815.
- Husain, R., Malaviya, M., Seth, P.K., Husain, R., 1992. Differential responses of regional brain polyamines following in utero exposure to synthetic pyrethroid insecticides: a preliminary report. *Bull. Environ. Contam. Toxicol.* 49, 402–409.
- Ismail, S., Asad, M., 2009. Immunomodulatory activity of *Acacia catechu*. *Indian J. Physiol. Pharmacol.* 53, 25–33.
- 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.
- 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.
- Loveless, S.E., Hoban, D., Sykes, G., Frame, S.R., Everds, N.E., 2008. Evaluation of the immune system in rats and mice administered linear ammonium perfluorooctanoate. *Toxicol. Sci.* 105, 86–96.
- Luebke, R.W., Chen, D.H., Dietert, R.R., 2006. The comparative immunotoxicity of five selected compounds following developmental or adult exposure. *J. Toxicol. Environ. Health B Crit. Rev.* 9, 1–26.
- McEwan, A.D., Fisher, E.W., Selman, I.E., Penhale, W.J., 1970. A turbidity test for the estimation of immune globulin levels in neonatal calf serum. *Clin. Chim. Acta* 27, 155–163.
- Mediratta, P.K., Sharma, K.K., Singh, S., 2002. Evaluation of immunomodulatory potential of *Ocimum sanctum* seed oil and its possible mechanism of action. *J. Ethnopharmacol.* 80, 15–20.
- Meister, R.T., 1994. Farm Chemicals Handbook. Meister Publishing Company, Willoughby, OH.
- Holsapple, M.P., Burns-Naas, L.A., Hastings, K.L., Ladics, G.S., Lavin, A.L., Makris, S.L., Yang, Y., Luster, M.I., 2005. A proposed testing framework for Developmental Immunotoxicology (DIT). *Toxicol. Sci.* 83, 18–24.
- 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.
- Proenca, P., Teixeira, H., Castanheira, F., Pinheiro, J., Monsanto, P.V., Marques, E.P., Vieira, D.N., 2005. Two fatal intoxication cases with imidacloprid: LC/MS analysis. *Forensic Sci. Int.* 153, 75–80.
- Holladay, D.H., Smialowicz, R.J., 2000. Development of the murine and human immune system: differential effects of immunotoxicants depend on time of exposure. *Environ. Health Perspect.* 108, 463–473.
- West, L.J., 2002. Defining critical windows in the development of the human immune system. *Hum. Exp. Toxicol.* 21, 499–505.