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A detailed study of developmental immunotoxicity of imidacloprid in Wistar rats

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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.

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

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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 imadacloprid 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 µl of 1% (v/v) SRBC was added in 50 µl 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:

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

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

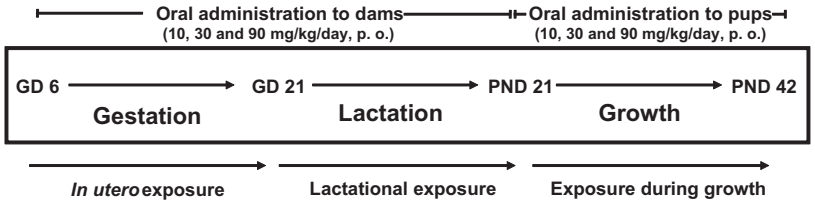

Fig. 1. Schedule of imidacloprid exposure during *in utero* and developmental phases in Wistar rats. Pups were exposed to imidacloprid (10, 30 and 90 mg/kg per day), *in utero* through dams, followed by exposure through lactation, till weaning and subsequently by oral administration to young ones till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