Effect of Imidacloprid on Reproduction of Female Albino Rats in Three Generation Study

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Abstract

In the present study, the effect of oral administration of imidacloprid over three generations on biochemical, histological and physiological alterations in female rats was assessed. Female rats were divided into three groups. Group 1 was control and was given corn oil, group 2 was administered imidacloprid at the rate of 10 mg/kg bw/day, group 3 was administered imidacloprid at the rate of 20 mg/kg bw/day. F0 and F1 generation female albino rats were dissected for this study. Weight of ovary decreased significantly at higher dose of treated female rats of F0 and F1 generation. Histopathology of ovary of group 2 and group 3 revealed different stages of follicles. The level of acid phosphatase (ACP) and alkaline phosphatase (ALP) increased significantly at higher dose in the ovary of females of both the generations. In either generation, non-significant changes were observed in fertility index, live birth index, gestation lengths and sex ratio. Female F1 pups in the 20 mg/kg/day group showed a significant decreased body weight on postnatal day 21 as compared to F0 pups on day 21.

Conclusion: The lower dose of imidacloprid (10 mg/kg/day T1) had no effect on various reproductive parameters of female rats and higher dose (20 mg/kg/day T2) of imidacloprid had some significant effects on feed consumption and reproductive parameters for three generation reproductive study.

Keywords: Imidacloprid; Female albino rats; Fertility index; Sex ratio; Reproductive parameters; Lower dose; Higher dose; Three generations

Introduction

Imidacloprid, a neonicotinoid the newest class of major insecticide has outstanding potency and systemic action for crop protection against piercing and sucking insect pests and also highly effective for control of flea on cats and dogs [1]. Few cases of acute human poisoning have been reported following ingestion of imidacloprid formulations [2,3]. There are some reports that show imidacloprid has an adverse effect on the reproductive tract [4], also this compound has been identified as having teratogenic [5], mutagenic [6] and carcinogenic [7] effects in animals and humans. Many pesticides having endocrine disruptor properties are also known to adversely impair the reproductive competence of males. Imidacloprid may adversely affect reproduction and cause developmental delays as a result of maternal toxicity. The effects of imidacloprid on reproduction and development were examined in a two generation, two-litter study in Wistar rats (30/sex/dose in the parental generation, P1). The dietary doses were 100, 250 and 700 ppm [8]. Maternal toxicity at 700 ppm included decreased body weight gain and food consumption with a marked reduction during lactation. In one study, pregnant rats fed technical grade imidacloprid throughout pregnancy and lactation at doses of 0, 100, 250 and 750 ppm revealed no effects other than significantly reduced food consumption (14% relative to controls) in the mother rats [9]. Imidacloprid was not found to affect reproductive variables or cause birth defects. However, reduced mean body weight and body weight gain relative to controls was observed in the males and females of all generations at the highest dietary concentration tested (700 ppm). At this concentration, parental animals also had reduced body weights, relative to controls, in association with reduced food consumption [10]. In spite of a number of studies on the effects of imidacloprid on reproductive behavior of animals, no information is available on the effect of imidacloprid on the two generation reproduction of rats when females were treated with imidacloprid. Aminotransferases and phosphatases are important and critical enzymes in the liver metabolic activity and are responsible for detoxification processes. So any interference in various enzyme levels lead to biochemical impairment and lesions of the tissue. The liver is the principal target of imidacloprid toxicity, as demonstrated by its elevated serum transaminase, alkaline phosphatase and/or glutamate dehydrogenase activities; and alterations of other clinical parameters. Therefore, this study was designed to investigate the effects of imidacloprid on three generation reproduction of rats (when only females were treated with imidacloprid).

Materials and Methods

Chemical

Commercial product of imidacloprid (Confidor, 17.8%, w/w imidacloprid as active ingredient) used in this study was purchased from the local market in Ludhiana, India.

Animals and experimental design

The study was conducted on sexually mature female albino rats, 3 months of age, weighing 100-150 g obtained from Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana. The animals were housed in groups of two rats per cage. The rats were acclimatized for one week before using them for experimentation. The rats were maintained under controlled conditions of temperature (22 ± 2°C) and humidity (30-70%) with 12 h light and dark cycle. After acclimatization for one week, healthy rats were subjected to this study.

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with 6 female rats constituting each group. Animals were divided into three groups. Group I served as control and given corn oil orally. Group II rats were given 10 mg/kg bw/day dose of imidacloprid. Group III rats were given 20 mg/kg bw/day dose of imidacloprid. The animals were given standard diet containing pelleted food and water ad libitum. The experimental protocol met the National guidelines on the proper care and use of animals in the laboratory research. The Institutional Animal Ethics Committee (IAEC) approved this experimental protocol. The study began with 6 female rats/group (F0 generation), and they were exposed to imidacloprid orally at 10 and 20 mg/kg bw/day. After 8-week administration of imidacloprid, each treated female was mated with a normal male having no dosage group and pregnant females were allowed to deliver and nurse their pups. F0 females were necropsied after weaning of their pups. Administration of imidacloprid was continued throughout the mating, gestation and lactation periods until necropsy. For the second generation, 6 female weanlings in each group of F0 generation were selected as F1 parents on postnatal days 21-25 to equalize the mean body weights among groups as much as possible. The day on which F1 parental animals were selected was designated as day 0 of dosing for the F1 generation. F1 selected rats were given imidacloprid orally dissolved in corn oil mated after 8-week administration. They were allowed to deliver and nurse their F2 pups.

Dosing

Imidacloprid was dissolved in corn oil to obtain the desired test concentrations and given orally to female rats for two generations. Two doses of imidacloprid 10 and 20 mg/kg bw/day were given to females of F0 and F1 generation, and control rats of each generation were given corn oil. F0 females mated with normal males to get F1 generation and F1 females mated with normal rats to get F2 generation. 10 mg/kg/day dose was reported as NOEL (No Observed Effect Level Dose) by Bhardwaj et al. and Kapoor et al. [11,12] and other higher than NOEL was taken in the present study. Administration to F0 parental female animals was started at 3 months of age. Administration to F0 females lasted until necropsy through 10 weeks or more of the pre-mating, mating, gestational, lactational periods and during weaning of the F1 offspring. Administration to F1 parental animals was started from age of 6 weeks old, until necropsy through 8 weeks or more of the pre-mating, mating, gestational, lactational periods and during weaning of the F2 offspring.

Parental data (F0 and F1)

Clinical observations: Throughout the study, all F0 and F1 parental female rats were observed at least twice daily and cages were inspected daily for evidence of ill health or reaction to the treatment. F0 females underwent physical examinations beginning on the day treatment commenced, and weekly until mating. After mating, the physical examinations occurred on days 1, 7, 14, 21 and 28. The same schedule of examinations was used for the selected F1 rats. F0 females were weighed on the day treatment commenced, at weekly intervals until mating was detected, on days 0, 6, 13 and 20 after mating, on days 0, 4, 7, 14 and 21 of lactation and prior to necropsy. After selection, the F1 parental rats were given imidacloprid orally and kept until day 25 after mating. The selected F1 generation was allocated to its specific treatment group when they were 6 weeks old.

Vaginal smears were obtained from the female rats every day in the morning to examine the estrous cycle during four weeks before mating; starting from 6 weeks of age for the F0 parents and from 11 weeks old for the F1 parents, and the mean days of estrous cycle were calculated. Cases with estrous cycle other than 4 to 6 days were regarded as abnormal. F0 and F1 parental females were anaesthetized by chloroform. The following organs were weighed in all the F0 and F1 females: the liver, ovaries and uterus.

Biochemical analysis

After sacrficication, the tissue sample of liver was homogenized in the phosphate buffer saline. The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) were estimated by the method of Reitman and Frankel as described by Bergmeyer and acid phosphatase (ACP), alkaline phosphatase (ALP) was estimated by method of Bessey et al.

Processing of tissues for histopathology

All F1 and F2 rats were weighed and sacrificed by chloroform anesthesia. For histopathological studies, ovaries of treated and control rats were fixed in 10% formalin. After routine processing and dehydration of each tissue, paraffin sections were cut at 5 um and stained with hematoxylin-eosin for microscopic examination. Serial sections of ovary were studied for various observations like total number of follicles, number of normal and atretic follicles, oocyte and nucleus as described by Kaur and Guraya.

Offspring evaluation (F1 and F2)

All pups derived from F0 and F1 parents (F1 and F2 litters, respectively) were examined as soon as possible on the day of birth to determine the number and sex of pups, the number of live born and stillborn members of each litter and gross abnormalities. All individual offspring’s were examined approximately 24 h after birth (day 1) and daily thereafter for any evidence of ill health. Litter size and mortality were recorded daily from days 1 to 21. The sex ratios of each litter were recorded. Individual body weights were recorded on days 0,4,7,14,21. The dams were removed and offspring weaned on day 21 of age, and the selection of the F1 generation was made on day 25. For the selected F1 females, sexual maturation was assessed daily from day 25 of age until vaginal opening occurred. Females that littered and reared offspring to
weaning were euthanized on day 28 post-partum (after weaning). For all F0 and the selected F1 adult females, detailed necropsies involving full macroscopic examinations weighing of the liver, ovary and uterus was carried out.

Reproductive performance of F0 and F1 rats: The reproductive parameters from this study were expressed in terms of indices, weights and ratios that considered all stages from conception to weaning [13]. These parameters were defined below:

Fertility index (%) = (Number of females delivering /number of females cohabited) × 100

Live birth index (%) = (Number of live pups at day 0/number of pups born) × 100

4-day survival index (%) = (Number of live pups on day 4/ number of pups alive on day 0) × 100

21-day (weaning) survival index (%) = (Number of pups alive on day 21/ number of pups alive on day 4) × 100

Litter size = Number of pups/number of pregnant females

Body weight of pups = The body weight of pups were recorded on days 0, 4, 7, 14 and 21

Statistical analysis

All values were presented as the mean ± standard error of means (S.E.M). Comparisons were made between control and treated groups using “Analysis of Variance (ANOVA)” as a statgraphics statistical package. The body weight of parental animals, food consumption, length of estrous cycle, organ weight was evaluated by student t test for pairwise comparisons between control and individual treatment groups.

Results

Data for parental animals

Clinical signs and body weight: The most consistent finding was aggression/hyperactivity which was observed throughout the study in both F0 and F1 females of imidacloprid treated groups. Some females of the low and high dose groups had vaginal discharges. Mean estrous cycle days were significantly reduced in higher dose (T2) treated group (Table 2).

Histology

Histologically, sections of ovary of control rats showed different stages of follicular development viz. primary, secondary, tertiary, early

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Litter size=Number of pups/number of pregnant females

Body weights of pups=The body weight of pups were recorded on days 0, 4, 7, 14 and 21

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Histology

Histologically, sections of ovary of control rats showed different stages of follicular development viz. primary, secondary, tertiary, early
Values are Mean ± SE of 6 animals in each group
**Significantly different from control at P<0.01
*Significantly different from control at P<0.05

**Table 2: Effect of imidacloprid on body weight of female albino rats after pairing of F0 and F1 generation as compared to control.**

<table>
<thead>
<tr>
<th>Traits</th>
<th>F0</th>
<th>F1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>T1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mating</td>
<td>141.6 ± 3.06</td>
<td>152.5 ± 3.81</td>
</tr>
<tr>
<td>Gestation</td>
<td>152.5 ± 4.95</td>
<td>166.6 ± 4.21</td>
</tr>
<tr>
<td>After delivery</td>
<td>146.6 ± 1.67</td>
<td>155.3 ± 3.65</td>
</tr>
<tr>
<td>During lactation</td>
<td>165.8 ± 2.38</td>
<td>173.3 ± 8.33</td>
</tr>
</tbody>
</table>

Values represent the mean ± SE of 6 animals in each group
*Significantly different from control at P<0.05
*Significantly different from control at P<0.01

**Table 3: Relative organ weights for F0 and F1 females.**

<table>
<thead>
<tr>
<th>Traits</th>
<th>F0</th>
<th>F1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of females examined</td>
<td>No. of females examined</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>129.16 ± 3.982</td>
<td>141.7 ± 3.333</td>
</tr>
<tr>
<td>T1</td>
<td>140.8 ± 4.013</td>
<td>150.8 ± 3.515</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovary (g)</td>
<td>0.030 ± 0.002</td>
<td>0.029 ± 0.001</td>
</tr>
<tr>
<td>T1</td>
<td>0.037 ± 0.001</td>
<td>0.038 ± 0.004</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterus (g)</td>
<td>0.135 ± 0.021</td>
<td>0.172 ± 0.036</td>
</tr>
<tr>
<td>T1</td>
<td>0.206 ± 0.043</td>
<td>0.206 ± 0.043</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent the mean ± SE of 6 animals in each group
*Significantly different from control at P<0.05
*Significantly different from control at P<0.01

**Discussion**

The present three generation reproductive study was performed to provide general information concerning the effects of imidacloprid on the performance of female reproductive system, and on the growth and development of the offspring. Mean estrous cycle days were significantly reduced in T2 treated group of F0 females (4.06 ± 0.03, P<0.01) as compared to control (5 ± 0.21). Studies of Borgeest et al. [14] experienced a significant increase in the percentage of days in estrous phase compared with control and methoxychlor treated mice. It is obvious that monitoring of body weight provides information on health level of animals which can also be important interpretation of reproductive effects [15]. Body weight of females increased significantly during gestation and lactation period of F0 and F1 generation.

The relative ovarian weight was significantly reduced in higher dose (T2) group in F0 and F1 generation. Reduction in body weight at 20 mg/kg/day dose level as observed in the present study may be correlated with decreased sex organ weight (ovary weight) in both the generations, which reflects the effect of imidacloprid on the reproductive system. Organophosphates like methyl parathion, dimethoate and monocrotophos given to female albino rats have also resulted in significant decrease in the ovarian weights [16].

Food consumption of females before pairing for the F0 and F1 generation decreased significantly in treated females of both the generations. The decrease in feed consumption is correlated with decrease in body weight gain in all the treated groups [17] in the two generations. There was significant increase in food consumption of females during gestation and lactation in F0 and F1 generation. Significant increase in food consumption was observed on 13-21 days of gestation in both the generations. During the period of gestation and lactation metabolism increases by 82.5% on average and the female assimilates additionally 304 kcal (including 60.5 kcal for gestation and 243.5 kcal for lactation) [18]. Lactation makes considerably greater demands on the mother’s body than pregnancy does in species where the young are helpless at birth and depend on mother's milk for a comparatively long time. The requirements for the production of milk are met partly from within, by the mobilization of the mother's body tissues, and partly from without, by an increased food intake [19]. Similar results were found by Margaret et al. [20] that at different intervals throughout the gestation and lactation periods, increased food consumption was observed in F0 generation females of the mid- and high-dose groups of stanol esters,
Values represent the mean ± SE of 6 animals in each group

<table>
<thead>
<tr>
<th>Parameters (µmole/g)</th>
<th>Dosage/mg/kg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>0 (Control) 10 20</td>
</tr>
<tr>
<td>AST</td>
<td>53.54 ± 5.71 63.73 ± 9.59</td>
</tr>
<tr>
<td>AKP</td>
<td>61.08 ± 4.28 70.30 ± 3.35</td>
</tr>
<tr>
<td>ACP</td>
<td>63.06 ± 2.50 69.00 ± 8.94</td>
</tr>
</tbody>
</table>

Values represent the mean ± SE of 6 animals in each group

Significantly different from control at P<0.01

Table 8: Liver biochemical data of female rats of F2 generation orally administered imidacloprid.

Food consumption are expected as a result of the animals’ attempt to compensate for the reduced caloric value of the test diet compared to controls. Cripps and Williams [21] measured feed consumption during lactation in Sprague-Dawley rats and found an approximately 3- to 5-fold increase in daily feed consumption between postnatal day 1 and postnatal day 21. Similar increased food consumption during lactation in rats was reported by Shirley [22] and Arnold et al. [23].

There was significant increase in ALT and AST activity in the liver. Bhardwaj et al. [11] also observed that oral administration of imidacloprid in female rats at the rate of 5, 10 and 20 mg/kg bw/day
has no adverse effects on either generation. Studies on imidacloprid have indicated 10 mg/kg/day as No Observed Effect Level (NOEL) as evidenced by various biochemical, hematological, neurobehavioral and oxidative stress parameters and produced significant changes at high dose levels (20 mg/kg/day) [11,12]. There have been no studies reported in the literature concerning the effects of imidacloprid on multigenerational reproduction after oral exposure to imidacloprid in female rats [32]. In our multigenerational experiment, the results indicated only minimal effects upon reproductive performance of rats. Imidacloprid exposure caused reduction in fertility index of both the generations. Suter et al. [8] also reported a mild effect or no effect on reproductive performance after exposure to imidacloprid.

### Conflict of Interest Statement
The authors declare that there are no conflicts of interest.

### Acknowledgements
We are thankful to Department of Zoology, PAU Ludhiana for providing facilities for conducting research.

### References

<table>
<thead>
<tr>
<th>Live pup weight (g)</th>
<th>Control</th>
<th>T 1</th>
<th>T 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 0 parents/F1 pups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>3.90 ± 0.59</td>
<td>4.13 ± 0.24</td>
<td>6.08 ± 1.32*</td>
</tr>
<tr>
<td>Day 4</td>
<td>9.06 ± 0.59</td>
<td>9.05 ± 1.00</td>
<td>10.5 ± 2.27</td>
</tr>
<tr>
<td>Day 10</td>
<td>16.38 ± 0.50</td>
<td>16.01 ± 0.95</td>
<td>18.94 ± 4.01</td>
</tr>
<tr>
<td>Day 14</td>
<td>23.9 ± 1.19</td>
<td>22.83 ± 1.35</td>
<td>25.29 ± 5.38</td>
</tr>
<tr>
<td>Day 21</td>
<td>31.95 ± 6.78</td>
<td>30.44 ± 1.48</td>
<td>29.24 ± 1.48</td>
</tr>
<tr>
<td>F 1 parents/F2 pups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>2.73 ± 0.22</td>
<td>2.64 ± 0.19</td>
<td>2.81 ± 3.05</td>
</tr>
<tr>
<td>Day 4</td>
<td>6.38 ± 0.42</td>
<td>6.31 ± 0.10</td>
<td>6.62 ± 0.43</td>
</tr>
<tr>
<td>Day 7</td>
<td>13.09 ± 0.73</td>
<td>12.1 ± 0.93</td>
<td>12.67 ± 0.94</td>
</tr>
<tr>
<td>Day 14</td>
<td>17.29 ± 0.74</td>
<td>18.30 ± 0.86</td>
<td>17.60 ± 1.41</td>
</tr>
<tr>
<td>Day 21</td>
<td>29.93 ± 0.86</td>
<td>23.08 ± 2.46</td>
<td>20.74 ± 2.20*</td>
</tr>
</tbody>
</table>

Values represent the mean ± SE of 6 animals in each group.

*Significantly different from control at P<0.01

### Table 10: Effect of imidacloprid on the body weight gain of F1 and F2 pups.

<table>
<thead>
<tr>
<th>Hormones</th>
<th>F1</th>
<th>Control</th>
<th>T 1</th>
<th>T 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone (ng/ml)</td>
<td>19.8 ± 7.29</td>
<td>19.4 ± 7.46</td>
<td>7.3 ± 1.82*</td>
<td></td>
</tr>
<tr>
<td>Estrogen (pg/ml)</td>
<td>253.3 ± 47.23</td>
<td>256.6 ± 34.02</td>
<td>256.6 ± 28.00*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>10.6 ± 6.90</td>
<td>8.6 ± 2.71</td>
<td>8.5 ± 2.44*</td>
<td></td>
</tr>
<tr>
<td>Estrogen (pg/ml)</td>
<td>201.6 ± 11.66</td>
<td>218.3 ± 19.73</td>
<td>235.0 ± 16.48*</td>
<td></td>
</tr>
</tbody>
</table>

### Table 11: Effect of imidacloprid on hormones of F1 and F2 generation.

for 90 days resulted in elevation of serum ALT, AST, glucose, Blood Urea Nitrogen (BUN). It has been suggested that an increase in alkaline phosphatase (ALP) level occurs due to the damage of the cells of liver, kidney, small intestine, and bone resulting in the liberation of this enzyme in the blood systems [26].

The body weight of F0 pups increased significantly on day 0, and at day 21 body weight of F1 pups was significantly lowered in higher dose group as compared to control. The body weight of F1 pups at day 21 in high dose group was significantly lowered compared to control. Svetlana [25] reported a decrease in body weight gain of offspring’s up to 13% compared to control until weaning at postnatal day 21. Similar results were found by Leslie et al. that body weight gain of offspring was significantly decreased from days 21-25 for Han Wistar rat females receiving 7500 ppm and 25,000 ppm rebaudioside A. Pup body weight and weight gains were reduced throughout lactation, with statistically identified lower weights on post natal day 21 in all generations at 100 mg/kg/day [27].

Oral administration of cypermethrin to female rats has resulted in significant decrease in plasma progesterone levels [28]. Earlier studies of Mani et al. also observed significant reduction in testicular enzyme 17ß-hydroxysteroid dehydrogenase, responsible for testosterone biosynthesis in male rats exposed to fenevalerate which may ultimately be leading to net decrease in testosterone concentration in group of rats [29].

Two doses of imidacloprid to female rats over two successive generations resulted in some effects on weight gain, food consumption, fertility index and showed no effects on gestation index, weaning index, sex ratio, live birth index of F1 and F2 pups [30,31]. Higher dose of imidacloprid (20 mg/kg bw/day) in both the generations showed a reduction in their body weight and food consumption and some effects on reproduction. Lower dose of imidacloprid (10 mg/kg bw/day) showed non-significant effects. Thus 10 mg/kg/day dose of imidacloprid which has been reported as NOEL (No Observed Effect Level) dose


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