International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 08-09-2024, Accepted: 09-10-2024, Published: 24-10-2024
Volume 9, Issue 10, 2024, Page No. 175-180

Impact of imidacloprid on the mammalian endocrine system: The case of the wistar strain of rattus norvegecus

Siapo Yao Martin[1], Kablan Kassi Jean-Jacques[1*], Ano Ehui Joachim[2], Abeto Claude Joice Dominique[1], Diby Yao Kan Séraphin[3], Tahiri Annick[1]

[1]Department of Biosciences, Biology and Health Laboratory, University Félix Houphouët- Boigny, Côte d'Ivoire
[2] Department of Science and Techniques, Laboratory of Biological Sciences, University Alassane Ouattara, Côte d'Ivoire
[3] Department of Biosciences, Natural Environment Laboratory and Biodiversity Conservation, University Félix Houphouët-Boigny, Côte d'Ivoire

Abstract
Pesticides are chemical substances used to protect plants and their products. They are intended to curb undesirable organisms. However, they also have a toxic impact on non-target organisms, particularly humans. To verify the impact of imidacloprid, a product used in agriculture, we evaluated the pesticide imidacloprid on the endocrine system of female wistar rats. For the treatment, doses of 2.25, 4.5 and 9 mg/kg body weight were administered to female rats for 28 days. With regard to treatment, female rats treated with the 9 mg/kg body weight dose showed a decrease in body weight compared with control female rats. In terms of vital organ and reproductive mass, there was no significant variation between treated and control female rats. However, histopathology showed lesions and dysfunction of the liver, kidneys and ovaries. Imidacloprid adiminitration caused no disturbance in urea, creatinine, ALAT, ASAT or estrogen production. Imidacloprid does not cause any apparent endocrine disruption, but in the long term could have an impact on reproduction and on the general state of health of human beings.

Keywords: Pesticides, CAO-NET 30 SC, imidacloprid, endocrine system, female rat

Introduction
Pesticides are substances used in agriculture to curb the effect of organisms deemed undesirable and harmful (Latif *et al*., 2011) [7]. Pesticides are an almost indispensable method in most agricultural practices, whatever the country's level of development. However, they are now at the heart of a health and environmental problem, contaminating flora and fauna through their massive use (Bonde *et al*., 2008) [4]. Indeed, because of their toxicity, pesticides represent a real danger to humans if they are not used under appropriate conditions (Lee *et al*., 2007) [8]. As a result, the chemical industry is always on the lookout for molecules that are equally effective against target organisms and less harmful to non-target organisms. However, epidemiological studies have shown a correlation between the professional use of neonicotinoids and the appearance of certain pathologies in the populations concerned. Neonicotinoids would therefore be considered endocrine disruptors (Saadi *et al*., 2014) [10]. Endocrine disruptors refer to natural or synthetic substances capable of interfering with the endocrine system (WHO, 2002) [13].
With the aim of contributing to our knowledge of the extent of damage that can be caused by neonicotinoids, this study's general objective is to assess the toxicity of imidacloprid, a neonicotinoid widely used in Ivory Coast in food and cocoa crops (IMI), on the endocrine system of female rats (wistar strain).

Material
Animal material
The animal material consisted of female rats (Rattus norvegicus) of the wistar strain aged 4-6 weeks from the vivarium of the Ecole Normale Superieure d'Abidjan.

Chemicals used
The chemical used was the insecticide CAO-NET 30SC with the active ingredient imidacloprid. This insecticide was supplied by AF-CHEM-SOFACO of Côte d'Ivoire. The active ingredient imidacloprid was chosen due to its high use in cocoa plantations in Côte d'Ivoire (Siapo *et al*., 2018) [11].

Methods
Constitution of animal batches
The different treatment batches are made up of female rats aged 4-6 weeks, so 4 batches of 5 female rats are used. The animals are acclimatized 7 days before the experiment and fasted the day before pesticide administration.

Dose preparation
Treatment doses are obtained from the 30g/l stock solution, based on the LD50 of the product supplied by AF-CHEM-SOFACO. These doses are diluted according to the manufacturer's recommendations, i.e. 250 ml/10 liters of distilled water. The LD50 of imidacloprid is 450 mg/kg body weight. Three doses are formulated from the stock solution: 2.25, 4.5 and 9 mg/kg body weight.

- Control batch 1 receives distilled water at a rate of 1ml/100g body weight (bw).
- Batch 2 receives pesticide spray at 2.25 mg/kg body weight.
- batch 3 receives pesticide spray at 4.5 mg/kg body weight
- batch 4 receives pesticide spray at 9 mg/kg body weight

Animal treatment
Female rats were treated in accordance with OECD 407 guidelines. Rats were dosed daily for 28 days by tube-feeding with water for controls and pesticide slurry for

batches 2, 3 and 4. The animals were weighed every other day and their behavior observed daily. After 28 days of treatment, the animals were sacrificed after ether anesthesia. Blood from each animal was collected in dry tubes for chemical and hormonal (estrogen) parameters. Vital organs (heart, kidneys, liver and lung) and reproductive organs (ovaries, uterine horns) were removed, weighed and preserved in 10% formalin for histopathological studies.

Impact of imidacloprid on female rat body mass

The change in body mass of female rats was determined according to the following formula:

$$\text{variation in body weight (\%)} = \frac{\textit{final mass-initial mass}}{\textit{initial mass}} \times 100$$

Relative mass of vital and reproductive organs

Once the organs have been harvested, they are rinsed in NaCl and wrung out on paper towels to remove all traces of water, then weighed. The relative mass of each organ is determined according to the formula below:

$$\text{Relative mass (g /100g wb)} = \frac{\textit{mass of organs}}{\textit{body weight}} \times 100$$

Biochemical analysis

Urea, creatinine, AST and ALT a are determined from blood collected in dry tubes. Blood samples are centrifuged at 4,000 rpm for around 10 minutes in a centrifuge (LC-04 PLUS). Assays are performed on a Hycel Lisa 300 automated analyzer.

Hormone assay (estrogen)

This test is based on the competition principle and is performed using the solid-phase enzyme-linked immunosorbent assay (ELISA). The fixed amount of enzyme-conjugated antigen competes for antibody binding sites. After incubation, the wells are washed to stop the competition reaction.

Histopathology

The aim of organ histology is to reveal any toxic effects of imidacloprid under the microscope. This technique makes it possible to obtain thin sections of the various organs: the liver and kidney for the vital organs, and the ovaries and uterine horns for the reproductive organs. To achieve this, several successive stages are required.

- **Fixation organs**
  The aim of organ fixation is to maintain the cells in a state close to life. Fixation hardens the organ, enabling it to hold its various tissue formations in place. In this way, the cells are protected against bacterial attack and retraction. The organs were immersed in 10% formalin for 48 hours at room temperature.

- **Déshydratation**
  Directly removed from the formalin, the organs were placed individually in cassettes and then dehydrated in four successive alcohol baths of increasing degrees (80°; 90°; 96° and 100°) for 1, 2, 2 and 2 hours respectively.

- **Éclaircissement**
  The cassettes containing the organs were clarified in three successive toluene baths, each lasting two hours. This clarification removed all traces of alcohol from the organs and prepared them for impregnation.

- **Imprégnation**
  For two and three hours respectively, the organs are impregnated in two liquid kerosene baths. This operation was carried out in an oven (MEMMERT, Germany) at 60°C.

- **Inclusion in kerosene**
  Using a mold, kerosene embedding is carried out in ambient air. To do this, the cassettes were opened by loosening the closure, removing the organ parts and placing them in the mold. The mold is then covered by the cassette, into which the liquid kerosene is poured until it fills. After cooling, and to facilitate demolding, the blocks formed were hardened in the freezer.

- **Coupe of blocks using a microtome**
  The 3µm-thick sections were cut using a LEICA microtome model (RM 2125 RTS).

- **Organ dewaxing**
  The sections were placed in a 40°C water bath, then mounted on slides. The slides are then placed in an oven at 60°C for 30 min to be dewaxed. Organ sections spread out on slides are dewaxed again in three successive 15 min toluene baths.

- **Réhydratation**
  Rehydration is carried out in three successive alcohol baths of decreasing strength (100°, 90° and 80°C) for 5 min each. The sections are then rinsed with distilled water.

- **Coloration organ sections**
  Sections are first introduced into a hematoxylin bath for 3 min. After rinsing with distilled water, the sections are immersed in 3% eosin for 5 min.

- **Déshydratation**
  After staining, the sections are dehydrated again in three increasing baths of alcohol (75°,95°and 100°) for five minutes each.

- **Montage and observation of sections**
  A few drops of EUKITT inclusion are placed on the organ sections, which are then covered with a coverslip. A tri-ocular electron microscope (Olympus CX31, Philippines) with a camera (AmScope, MD130) connected to a computer (HP Elitebook Folio 1040, China) running videomet software was used for observations. Magnifications (GX40 and GX100) were used to assess any organ tissue anomalies.

Statistical analysis

Statistical analysis was carried out using Graph Pad prisme software. The analysis of variance (ANOVA) applied to the results obtained enabled us to assess the effects of the different treatments at a significance level of 5 %.

Results

Effect of imidacloprid on body mass in female rats

For 28 days, gavage administration of distilled water and imidacloprid enabled us to check the variation in body mass of female rats. Animals were dosed at 2.25, 4.5 and 9 mg/kg body weight. The results showed no significant difference between the body mass of control rats (distilled water) and rats treated with 2.25 and 4.5 mg/kg body mass ($p>0.05$). On the other hand, there was a significant decrease in the body mass of rats treated at the highest dose of 9 mg/kg body mass of the order of 45.83 % ($p<0.05$) (Fig 1).

Effect of imidacloprid on the mass of vital and reproductive organs

Statistical analysis showed no significant difference between control rats and rats treated with imidacloprid at doses of 2.25; 4.5 and 9 mg/kg body weight ($p>0.05$) for the mass of vital organs (heart, liver and kidneys) and reproductive organs (adrenal glands, uterine horn and ovaries) (Table I).

Effect of imidacloprid on biochemical parameters

Statistical analysis of plasma urea, creatinine, Alanine Amino Transferase (ALAT) and Aspartate Amino Transferase (ASAT) of control rats and those treated with imidacloprid at doses of 2.25; 4.5 and 9 mg/kg body weight showed no significant difference ($p>0.05$) (Table II).

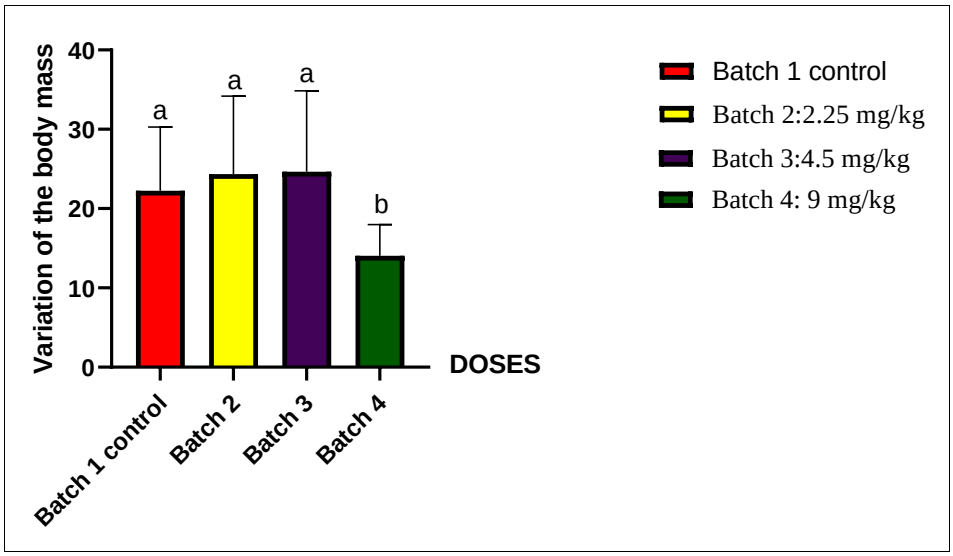

Fig 1 : Effect of imidacloprid on body weight in female rats
(Histograms with the same letters are not significantly different) (ANOVA, $p<0.05$)

Table 1: Effect of imidacloprid on the mass of vital and reproductive organs

Organs	Control (Distilled Water)	Batch 2 (2.25 mg/kg)	Batch 3 (4.5 mg/kg)	Batch 4 (9 mg/kg)	P-value
Heart	0.576 ± 0.057	0.669 ± 0.041	0.638 ± 0.041	0.611 ± 0.023	P=0.416
Liver	4.519 ± 0.386	4.531 ± 0.075	4.671 ± 0.257	4.199 ± 0.395	P=0.740
Kidneys	0.822 ± 0.049	0.786 ± 0.172	949 ± .026	0.902 ± 0.038	P=0.609
Adrenal glands	0.056 ± 0.003	0.064 ± 0.006	0.050 ± 0.009	0.041 ± 0.007	P=0.196
Ovaries	0.096 ± 0.015	0.096 ± 0.003	0.100 ± 0.010	0.083 ± 0.014	P=0.775
Uterine horn	0.328 ± 0.027	0.278 ± 0.020	0.283 ± 0.025	0.383 ± 0.104	P=0.556

(ANOVA, $p>0.05$)

Table 2: Effect of imidacloprid on biochemical parameters**.**

Parameters	Control (distilled water)	Batch 2 (2.25 mg /kg)	Batch 3 (4.5mg/kg)	Batch 4 (9mg/kg)	P value
Urea	0.540 ± 0.015	0.533 ± 0.063	0.546 ± 0.078	0.523 ± 0.016	P=0.99
Creatinine	5.000 ± 1.000	5.000 ± 0.000	5.667 ± 0.3333	4.333 ± 0.333	P=0.82
ALAT	43.67 ± 2.333	41.33 ± 4.410	48.00 ± 7.937	39.33 ± 2.667	P=0.91
ASAT	196.7 ± 20.17	220.0 ± 17.04	198.0 ± 7.371	224.3 ± 19.85	P=0.76

(ANOVA, p>.05); ALAT: Alaninie Amino Transferase; ASAT: Aspartate Amino Transferase

Effect of imidacloprid on vital organ histology (kidneys and liver)

Histological studies of the kidneys showed, in controls, the presence of Bowman's capsules, the glomerular chamber, convoluted tubules and well-differentiated glomeruli. However, in female rats treated with 2.25 and 4.5 mg/kg body weight, there was a mismatch between convoluted tubules and glomeruli, incipient necrosis of the convoluted tubules, alteration of the glomeruli and incipient shrinkage of their size. In female rats treated with 9 mg/kg body weight, observations showed advanced necrosis of the convoluted tubules and glomeruli, shrinkage of glomerular size, disintegration of the glomerular camber and absence of Bowman's capsules (Fig 2).

In the liver, control rats showed intact hepatic parenchyma, normal steatosis, blood vessel and hepatic trabeculae. However, all female rats treated at doses of 2.25, 4.5 and 9 mg/kg body weight showed dilatation of the central vein and sinusoidal capillaries, necrosis, swelling of the liver parenchyma and numerous steatoses (Fig 3).

Effects of imidacloprid on histology of reproductive organs

Histology of the ovaries after daily treatment for 28 days with imidacloprid at doses of 2.25; 4.5; and 9 mg/kg body weight showed in control female rats the presence of follicles in the secondary and tertiary stages, well differentiated by theca and corona radiata. The follicles progressed normally to the De Graaf stage. On the other hand, in female rats treated with 2.25, 4.5 and 9 mg/kg body weight, atresic follicles were observed. Cross-section of the ovary shows undifferentiated follicles, the corona radiata is unordered and the cumulus oophorus is atresic. This is most intensified in female rats treated with the highest dose of 9 mg/kg bw (Fig 4).

As regards the uterine horn, no abnormalities were observed in female rats treated at doses of 2.25, 4.5 and 9 mg/kg bw compared with control female rats (Fig 5).

Effect of imidacloprid on estradiol levels

In rats treated with 2.25, 4.5 and 9 mg/kg body weight, analysis of estradiol levels showed no significant difference between treated and control rats (ANOVA, $P < 0.05$) (Fig 6).

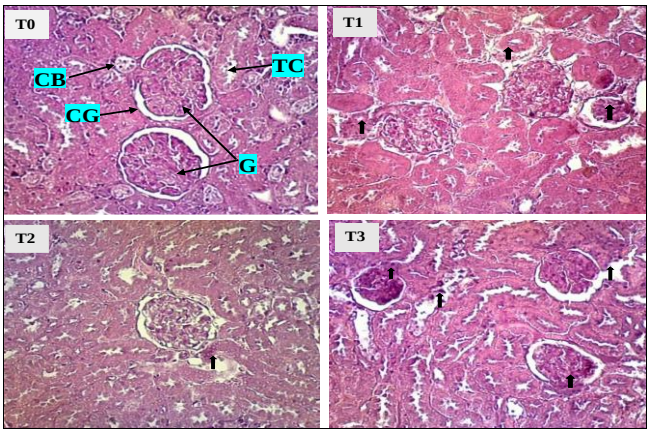

Fig 2: Histological section of the kidney

T0: Control (distilled water); CB: Bowman's capsule; CG: Glomerular chamber; G: Glomerulus; T1: Treated 2.25 mg/kg bw; T2: Treated 4.5 mg/kg bw; T3: Treated 9 mg/kg bw; TC: Contoured tube; Arrows: Anomaly and necrotic tissue; Gx100; staining; Haematoxylin-eosin

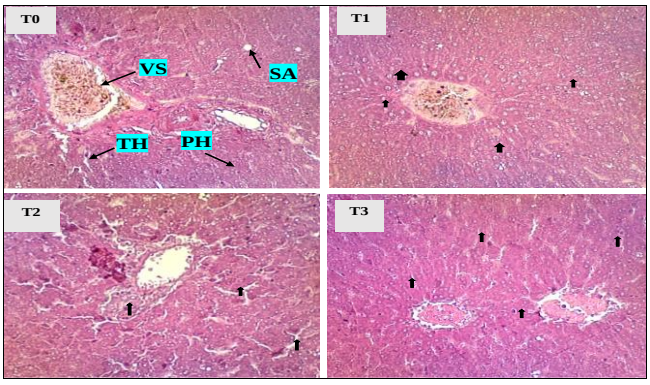

Fig 3: Histological section of the liver

T0: Control; VS: blood vessel; SA: normal steatosis; PH: liver parenchyma; TH: liver trabeculae; T1: Treated 2.25 mg/kg bw; T2: Treated 4.5 mg/kg bw; T3: Treated 9 mg/kg bw; CT: Contoured tube; Arrow: abnormal tissue, high number of steatosis and liver trabeculae, Gx100; staining; Haematoxylin- eosin

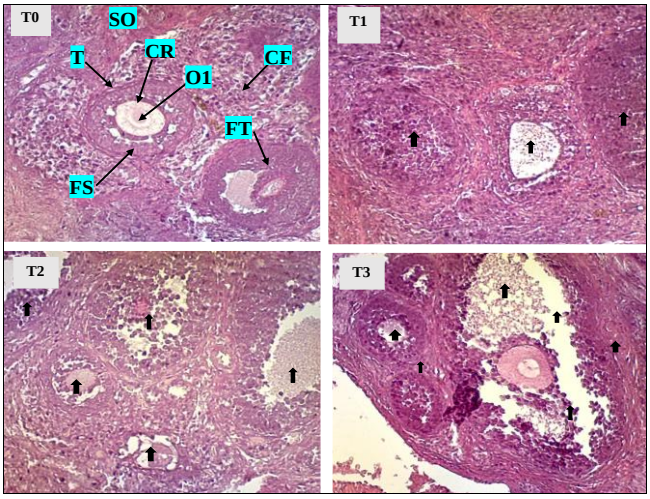

Fig 4: Histological section of the ovary

T0: Control; CR: corona radiata; CF: follicular cell; FT: tertiary follicle; FS: secondary follicle; O1: oocyte 1; SO: ovarian stroma; T: theca; T1: Treated 2.25mg/kg bw; T2: Treated 4.5mg/kg bw; T3: Treated 9mg/kg bw; Arrow: abnormality, tissue necrosis Gx100; staining; Haematoxylin-eosin

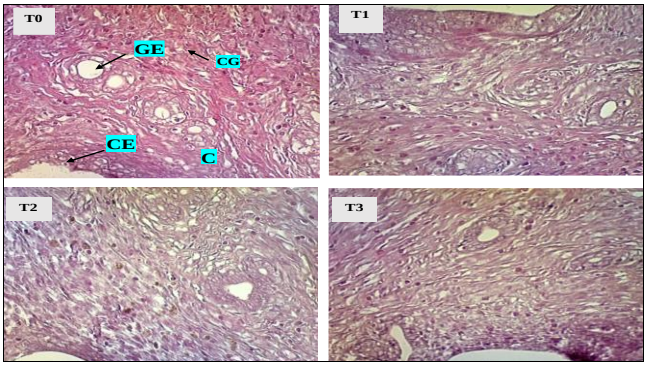

Fig 5: Histological section of the uterine horn

T0: Control; C: chorion; EC: epithelial cell; GC: glandular cell; EG: endometrial gland; T1: Treated 2.25 mg/kg bw; T2: Treated 4.5 mg/kg bw; T3: Treated 9 mg/kg bw, Gx100; staining; Haematoxylin-eosin

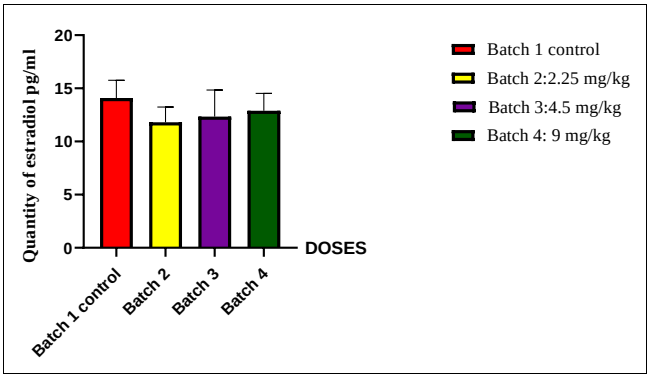

Fig 6: Effect of imidacloprid on estradiol levels (ANOVA, $p>0.05$)

Discussion

Oral administration of imidacloprid, a synthetic pesticide, to female rats for 28 days at doses of 2.25 and 4 mg/kg body weight showed no significant difference in body weight between treated and control animals. On the other hand, the dose of 9 mg/kg body weight showed a significant reduction in the body mass of treated animals. This result shows that as imidacloprid concentration increases, feed intake decreases. Our results are in line with the work of Bhardwaj *et al* (2010) [3]. In their work, they observed a reduction in body mass in female rats treated orally with 20 mg/kg/d imidacloprid for 90 days. These authors consider that this regression could be due to the low food consumption of the treated female rats.

For the doses used in this study, imidacloprid showed no significant variation in the mass of vital organs (kidneys, liver) and reproductive organs (ovaries, uterine horn). These results show that the doses of imidacloprid tested do not affect the relative mass of the vital and genital organs of rats. The results of this study are contrary to those of Vohra and Khera (2014) [12], who found that as oral intake of imidacloprid increased, the ovaries of female rats became smaller, but uterine size increased. Vohra and Khera (2014) [12] also showed that liver and kidney mass decreased with increasing imidacloprid concentration at doses of 10 and 20 mg/kg body weight. This difference could be due to the duration of treatment. Our treatment lasted 28 days with 2.25, 4.5 and 9 mg/kg body weight imidacloprid, whereas that of Vohra and Khera (2014) [12] lasted 60 days with 10 and 20 mg/kg body weight imidacloprid.

Biochemical assays of renal constituents showed no significant difference in urea and creatinine in all female rats treated with imidacloprid for 28 days compared with the control. In contrast, with another pesticide from the neonicotinoid family, acetamiprid, Bellabiod and Kheris (2017) [2] observed a significant decrease in urea and no change in creatinine in male mice treated with 1/3 LD50 (66 mg/kg body weight) of acetamiprid for 30 days. Acetamiprid being more toxic (LD50= 198 mg/kg bw) than imidacloprid (LD50 = 450mg/kg bw), this would justify their results. This difference in results could be explained by the nature of the active ingredient used.

Analysis of liver substances, ALAT and ASAT at doses 1/200, 1/100 and 1/50 of the LD50 of imidacloprid, showed no significant difference in all imidacloprid-treated rats compared with controls. The results obtained in the present study are consistent with those observed by Harmandeep *et al*. (2012) [6]. These authors administered 9 mg/kg bw imidacloprid to female albino rats for 28 days by oral intubation and observed that ASAT and ALAT activity varied non-significantly in the plasma of rats treated with 9 mg/kg (1/50 LD50) body weight.

In the present study, histopathological tests revealed abnormalities in vital organs (kidneys, liver) and reproductive organs (ovaries, uterine horn). Kidney histology showed alterations and degeneration of tubular epithelium, necrosis of convoluted tubules and altered glomeruli in rats treated at doses of 2.25; 4.5 and 9 mg/kg body weight compared with control rats. Our results are similar to those of Bellabiod and Kheris (2017) [2] who worked on the acetamiprid family identical to imidacloprid. These authors studied mice treated with high doses, i.e. 1/3DL50 of acetamiprid, and observed histopathological effects on the kidneys, reporting degeneration of the tubular epithelium with structural changes in the glomeruli. These authors state that these alterations appear to be accentuated as a function of the dose administered and the duration of experimentation.

Histological sections of the liver revealed numerous degenerative changes resulting in dilatation of the central vein and sinusoids between hepatocytes and necrosis of some liver tissues. Our results are similar to those of Bhardwaj *et al*. (2010) [3], who reported mild focal liver necrosis and hepatocellular damage following subchronic exposure to imidacloprid in female rats. The results of work by Arfat *et al*. (2014) [1] also indicate that imidacloprid induced hepatotoxicological effects in mice at a dose of 15 mg/kg/day, when exposed for a period of 15 days. Their work highlighted congestion and fatty degeneration of the liver, when mice were exposed to high levels of imidacloprid.

Histology of the ovaries of imidacloprid-treated rats after 28 days showed atresia of the secondary and tertiary follicles. Oocytes I underwent necrosis in all treated female rats, with alteration of the various follicle cells. Our results are similar to those of Nabiuni *et al*. (2015) [9] on pregnant laboratory wistar rats. These authors demonstrated the impact of imidacloprid on reproduction. Indeed, these authors' study was conducted for 20 days with the administration of 10 mg/kg body weight of imidacloprid by ingestion with food. The results of the experiment by Nabiuni *et al*. (2015) [9] showed that the treatment of rats with imidacloprid affected not only the number of ovarian follicles but also the diameter of these follicles compared with controls, thereby altering the reproductive process.

Hormonal analysis revealed non-significant levels of estradiol secretion in treated rats compared with the control. These results differ from the work of Hafez *et al*. (2016) [5], which showed a highly significant decrease in testosterone and estradiol levels in imidacloprid-treated rats compared with control rats. Rats received doses of 45 and 90 mg/kg body weight of imidacloprid for 5 days. Hafez *et al*. (2016) [5] report that the decrease in testosterone levels was more pronounced in rats given the highest dose of imidacloprid, i.e. 90 mg/kg body weight; thus, the higher the dose of imidacloprid, the greater its effect on testosterone levels. This difference in results could be due to the doses used in our trials, which were lower than those of Hafez *et al*. (2016) [5].

Conclusion

Exposure of female rats to imidacloprid at doses of 2.25, 4.5 and 9 mg/kg body weight revealed its effect on several parameters. For 28 days. Female rats treated with the highest dose of 9 mg/kg body weight of imidacloprid showed a loss of body weight.

Imidacloprid at these three doses did not alter the relative mass of the vital and reproductive organs, nor the biochemical parameters of the kidneys and liver, but it did affect the histological structure of the kidneys, liver and ovaries.

With regard to estrogen production, the study carried out showed that imidacloprid does not disrupt the endocrine function of the ovary due to the low dose used.

In this study, the doses of imidacloprid used did not appear to be an apparent endocrine disruptor, but could in the long term prove disruptive, given its impact on the ovaries and therefore on reproduction in women.

Acknowledgement
The authors would like to thank the teaching staff of the Pedagogical Research Unit Endocrinology and Reproductive Biology of University Félix Houphouët Boigny, for providing us with the necessary equipment. We are also grateful to the Ecole Normale Supérieure for making the vivarium available to us.

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