

# Insecticide imidacloprid induces morphological and DNA damage through oxidative toxicity on the reproductive organs of developing male rats

Ramazan Bal<sup>1</sup>, Mustafa Naziroğlu<sup>2\*</sup>, Gaffari Türk<sup>3</sup>, Ökkes Yılmaz<sup>4</sup>, Tuncay Kuloğlu<sup>5</sup>, Ebru Etem<sup>6</sup> and Gıyasettin Baydas<sup>7</sup>

<sup>1</sup>Faculty of Medicine, Department of Physiology, Firat University, Elazığ, Turkey

<sup>2</sup>Faculty of Medicine, Department of Biophysics, Suleyman Demirel University, Isparta, Turkey

<sup>3</sup>Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Firat University, Elazığ, Turkey

<sup>4</sup>Faculty of Science, Department of Biology, Firat University, Elazığ, Turkey

<sup>5</sup>Faculty of Medicine, Department of Histology and Embryology, Firat University, Elazığ, Turkey

<sup>6</sup>Faculty of Medicine, Department of Medical Biology, Firat University, Elazığ, Turkey

<sup>7</sup>Department of Physiology, Bingöl University, Bingöl, Turkey

We investigated whether treatment with imidacloprid would induce morphological changes, DNA fragmentation, antioxidant imbalance and apoptosis in the reproductive system of developing male rats. Twenty-four male rats were included in this 90-day study, starting at 7 days of age. The rats were divided into four groups. The first group was used as control. The second, third and fourth groups received oral 0.5-, 2- and 8-mg/kg imidacloprid, respectively. Serum, sperm and testis samples were collected from all groups at the end of the experimental period. The weights of the epididymis, vesicula seminalis, epididymal sperm concentration, body weight gain, testosterone and reduced glutathione values were lower in the imidacloprid-treated groups than that in the controls. All treated groups had increased lipid peroxidation, fatty acid concentrations and higher rates of abnormal sperm. Apoptosis and fragmentation of seminal DNA were higher in rats treated at the two higher doses of imidacloprid. These results show that this compound has a negative effect on sperm and testis of rats. Copyright © 2012 John Wiley & Sons, Ltd.

KEY WORDS—imidacloprid; oxidative stress; apoptosis; testis; fatty acids

## INTRODUCTION

Reactive oxygen species (ROS) are mainly composed of superoxide anion, hydrogen peroxide and singlet oxygen. The oxygen-derived species can attack DNA, producing a distinctive pattern of DNA alterations.<sup>1</sup> ROS play an important role in the pathogenesis of male reproductive system because the existence of polyunsaturated fatty acids in the testes makes this organ more sensitive to oxidative damage.<sup>2,3</sup>

There are numerous studies indicating that organophosphate-induced ROS caused reproductive tissue damage and that it has an important role in the pathophysiology of testis, probably via inhibition several enzymes involved in DNA synthesis and pathology of membrane polyunsaturated fatty acids,<sup>4,5</sup> decreasing activity of glutathione (GSH), GSH peroxidase<sup>6,7</sup> and antioxidant vitamins.<sup>5,8,9</sup>

Many studies have reported adverse effects of various environmental toxicants on reproductive systems of

humans and experimental animals.<sup>10</sup> Imidacloprid [1-(6-chloro-3-pyridylmethyl)-2-nitroimino-imidazolidine (IMI)] has gained great attention as a synthetic insecticide that acts in a similar manner as nicotine and is now widely used for the control of pests on crops and fleas on domestic animals.<sup>11</sup>

Many pesticides having endocrine disruptor properties are also known to adversely impair the reproductive competence of males. Endocrine disruptors in the animal/human body and their adverse effects are related with their ability to interfere with sex steroid action, which finally causes adverse effects to the adult reproductive system.<sup>4</sup> IMI is metabolized by human cytochrome P<sub>450</sub> isozymes through two pathways: (i) imidazolidine hydroxylation and desaturation, resulting in 5-hydroxyIMI and an olefin, respectively, and (ii) nitroimine reduction and cleavage, yielding the nitrosoimine, guanidine and urea derivatives.<sup>12</sup>

Testicular toxicity of insecticides is an important side effect on account of infertility, although there is no report of infertility in IMI toxicity. Extensive investigations were conducted to evaluate cellular and molecular aspects of gonadal damage in animals during spermatogenesis following chemical exposure to insecticides.<sup>7,13</sup>

\*Correspondence to: Mustafa Naziroğlu, Faculty of Medicine, Department of Biophysics, Suleyman Demirel University, Dekanlik Binasi, TR-32260, Isparta, Turkey. E-mail: mnaziroglu@med.sdu.edu.tr

The exposure to insecticides such as carbofuran caused cellular-chromosomal alterations leading to oligozoospermia in men.<sup>14</sup> In view of the evident cytotoxic nature of insecticides, the study of cellular proliferation using rat as animal model became imperative to explore the possible mechanism and dose of drug-dependent cytotoxicity.<sup>15</sup>

Although there are potential sources of toxic hazards from IMI, such as the global increase in its use and its persistence in crops, vegetables and fruits and physical contact in pets, there are few investigations on the toxic effects of chronic IMI exposure in human and experimental animal models.<sup>16,17</sup> To our knowledge, the effects of IMI exposure on reproductive functions of male rats have not been previously reported.

Consequently, the primary objective of the present study was to determine the possible effects of IMI exposure on the reproductive organ of male rats during early stages of postnatal development.

## MATERIALS AND METHODS

### *Animals and experimental design*

The Animal Use Committee of Firat University (Elazig, Turkey) approved all of the experimental protocols, which complied with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1985).

Twenty-four healthy adult male Wistar albino rats, aged 7 days, were obtained from and maintained at the Experimental Research Centre, Firat University. The animals were housed in polycarbonate cages in a room with a 12-h day–12-h night cycle at a temperature of  $24 \pm 3^\circ\text{C}$  and humidity of 45% to 65%. During the whole experimental period, the animals were fed a standard commercial diet (Elazig Food Company, Elazig, Turkey) and allowed to drink water *ad libitum*.

### *Subchronic 90-day oral toxicity study*

The animals were randomly divided into four groups with six animals in each group.

Group 1 Control, receiving corn oil as vehicle by gastric gavage in the same amount as that used for dissolving IMI in the other groups starting at postnatal day 7 for 3 months.

Group 2 Received 0.5 mg/kg body weight (BW) IMI dissolved in corn oil, daily by gastric gavage during the 3-month experimental period.

Group 3 Received IMI at a dose of 2 mg/kg BW as previously described.

Group 4 Received IMI at a dose of 8 mg/kg BW, as previously described.

The treatment doses were chosen to be lower than the reported no observable effect level, which is 5–10 mg/kg BW/day in male rats.<sup>18</sup>

### *Sample collection and homogenate preparation*

After the experimental period, the animals (at age 97 days old) were decapitated, and their testis, epididymis, seminal vesicles and ventral prostate were removed, cleared of adhering connective tissue and weighed. Blood samples were also collected right after decapitation.

The right testicles were fixed with Bouin's fluid and the left testicles frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  until use for lipid peroxidation (LP), GSH, fatty acids and DNA analyses analysis. The serum was separated and also stored at  $-70^\circ\text{C}$  until use for the estimation of the testosterone levels using the appropriate kits (Boehringer Mannheim, Germany).

### *Localization of apoptotic cells in the testis*

Apoptotic cell death of spermatogenic cells was established by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay by the ApopTag Peroxidase In-Situ Apoptosis Detection Kit (Chemicon, Temecula, CA). Briefly, the fixed testicular tissue was embedded in paraffin and cut into 4- $\mu\text{m}$ -thick sections. The paraffin was removed by immersing the sections in xylene, after which they were dehydrated through graded alcohol and washed in PBS. The sections were treated with 0.05% proteinase K for 5 min followed by treatment with 3% hydrogen peroxide for 5 min to inhibit endogenous peroxidase. After washing in PBS, the sections were then incubated with the TUNEL reaction mixture containing terminal TdT enzyme and digoxigenin-11-dUTP at  $37^\circ\text{C}$  for 1 h in a humidified chamber and then stop/wash buffer was applied for 30 min at  $37^\circ\text{C}$ . The sections were visualized with diaminobenzidine substrate, counterstained with Mayor's hematoxylin, dehydrated in graded alcohol and cleared. Negative controls were performed using distilled water in the place of the TdT enzyme.

### *Sperm analyses*

*Epididymal sperm concentration.* The epididymal sperm concentration was determined with a hemocytometer using a modified procedure.<sup>19</sup> The right epididymis was finely minced with anatomical scissors within 1 ml of isotonic saline in a Petri dish and then completely disintegrated with tweezers and allowed to incubate at room temperature for 4 h to accomplish the migration of all spermatozoa from epididymal tissue to fluid. After incubation, the epididymal tissue–fluid mixture was strained to separate the homogenate from tissue particles. The homogenate fluid containing all epididymal spermatozoa was drawn into an erythrocytes pipette up to the 0.5 mark of the capillary tube. A solution containing 0.6 M sodium bicarbonate, 1% formalin and 0.025% eosin was pulled into the bulb up to line 101 of the pipette, producing a 1:200 diluted sperm suspension. Then, approximately 10  $\mu\text{l}$  of this suspension was transferred to the two counting chambers of an improved Neubauer counter (Deep 1/10 mm, LABART, Darmstadt, Germany) and allowed to stand for 5 min. The spermatozoa

in both chambers were counted with the help of light microscope at 200× magnification.

**Sperm motility.** Freshly isolated left epididymal tissue was used for the analysis of sperm motility.<sup>19</sup> The percentage sperm motility was evaluated using a light microscope with heated stage. For this process, a slide was placed on a light microscope warmed up to 37°C, and then several small drops of Tris buffer solution [0.3 M Tris (hydroxymethyl) aminomethane, 0.027 M glucose, 0.1 M citric acid] were placed on the slide, mixed and covered for observation at 400× magnification. Motility estimates were performed from three different fields in each sample. The mean of the three successive estimations was used as the final motility score.

**Sperm morphology.** To determine the percentage of morphologically abnormal spermatozoa, the slides were stained with a mixture of 1.67% eosin and 10% nigrosin in 0.1 M sodium citrate for viewing under a light microscope at 400× magnification. A total of 300 spermatozoa were examined on each slide [1800 cells in each group ( $n=6$ )], and the head, tail and total abnormality rates of spermatozoa were expressed as percentage.<sup>19</sup>

#### *Determination of lipid level in testis*

Lipids were extracted from the left testis with hexane-isopropanol (3:2 v/v) by the method of Hara, Radin.<sup>20</sup> The samples (1 g) were homogenized with 10 ml of mixture of hexane-isopropanol. Subsequently, the fatty acids were converted into methyl esters with 2% sulfuric acid (v/v) in methanol and extracted with 5 ml n-hexane for chromatographic analysis in a Shimadzu GC-17A gas chromatograph equipped with a 25-m, 0.25-mm i.d. Permabond fused silica capillary column (Machery-Nagel, Germany) and a flame ionization detector. The oven temperature was programmed from 145°C to 215°C, in 4°C/min intervals. The injector and flame ionization detector temperatures were 240°C and 280°C, respectively. The nitrogen carrier gas flow was 1 ml/min. The methyl esters of fatty acids were identified by comparison with authentic standard mixtures analysed under the same conditions. The values were expressed as micrograms per gram of tissue.

#### *Serum testosterone*

A DRG Elisa kit (ELISA EIA-1559, 96 Wells kit, DRG Instruments, GmbH, Marburg, Germany) was used for the determination of serum testosterone following to the analytical protocol supplied by the kit manufacturer.

#### *Analysis of DNA fragmentation in testis*

The fragmentation of DNA in left testis was determined by a modification of a previously described procedure.<sup>21</sup> Semen was homogenized in lysis buffer containing 50 mM of Tris-HCl (pH 8.0), 10 mM of ethylenediaminetetraacetic acid, 0.5% (w/v) of sodium dodecyl sulfate, 1% of Triton X-100, 0.25 mg/ml of

RNase A and 100 µg/ml of proteinase K (final concentration, 2.5 µg/µl) and incubated for 1 h at 65°C. After centrifugation at 12 000g, 4°C for 20 min, the supernatant was extracted with phenol and chloroform and DNA precipitated by 100% ethanol, washed with 70% ethanol. DNA was resuspended in Tris-ethylenediaminetetraacetic acid buffer for electrophoretic analysis in 2% agarose gel. The gel was stained with ethidium bromide and visualized under UV light.

#### *Analysis of LP [malondialdehyde (MDA)] and reduced GSH levels in testis of rats*

The concentration of thiobarbituric acid-reactive substances in the tissue samples was estimated by the method of de las Heras.<sup>22</sup> In brief, 1 ml of tissue homogenate (supernatant; Tris-HCl buffer, pH 7.5) was mixed with 2 ml of a 1:1:1 mixture of TBA/TCA/HCl (0.37% thiobarbituric acid, 0.25 N HCl and 15% TCA) placed in a water bath for 60 min, allowed to cool and centrifuged at room temperature for 10 min. The MDA complex was analysed using the high-performance liquid chromatography equipment (Shimadzu, Kyoto Japan). The values were expressed as micrograms per gram of tissue.

Reduced GSH was determined by the method of Ellman<sup>23</sup> as described in our study.<sup>24</sup> Briefly, 1 ml of tissue homogenate was treated with 1 ml of metaphosphoric acid (Sigma, St. Louis, MO), and the mixtures were centrifuged at 5000 rpm to collect the supernatant. After deproteinization, the supernatant was allowed to react with 1 ml of Ellman's agent (30 mM 5, 5'-dithiobis-nitrobenzoic acid in 100 ml of 0.1% sodium citrate). The absorbance of the yellow product was read at 412 nm in a spectrophotometer. The values were expressed as micrograms per gram of tissue.

#### *Statistical analysis*

One-way ANOVA and *post hoc* Tukey HSD test were used to determine differences between groups for all parameters. The results are presented as mean ± SEM. Values were considered statistically significant if  $P < 0.05$ . The SPSS/PC program (Version 10.0; SPSS, Chicago, IL) was used for the statistical analysis.

## RESULTS

#### *Effect of IMI on final BW and serum testosterone*

Table 1 shows the mean values of BW gain and serum testosterone levels at the end of the experimental period. These parameters were significantly lower in the three IMI-treated groups than that in the controls ( $P < 0.001$ ).

#### *Reproductive organ weights*

The absolute organ weights of testis, epididymis, right cauda epididymis, prostate and seminal vesicles of controls and IMI-treated rats are shown in Figure 1. The

Table 1. Effect of IMI on final BW gain and serum testosterone levels in rats (mean  $\pm$  SEM)

Parameters	Control (n=6)	IMI-0.5 (n=6)	IMI-2 (n=6)	IMI-8 (n=6)
BW (g)	274.3 $\pm$ 3.0	251.8 $\pm$ 3.3 <sup>a</sup>	235.4 $\pm$ 5.7 <sup>b</sup>	222.5 $\pm$ 8.8 <sup>b</sup>
Testosterone (ng/dl)	97.6 $\pm$ 6.7	72.3 $\pm$ 7.9 <sup>b</sup>	66.1 $\pm$ 9.5 <sup>b</sup>	45.8 $\pm$ 9.2 <sup>b</sup>

<sup>a</sup> $P < 0.01$ . <sup>b</sup> $P < 0.001$  versus control.

weights of epididymis ( $P < 0.05$ ), right cauda epididymis ( $P < 0.05$ ) and seminal vesicles ( $P < 0.001$ ) of IMI-05, IMI-2 and IMI-8 group were significantly lower than that in the controls.

#### Epididymal sperm characteristics

The epididymal sperm characteristics of control and IMI-administered rats are presented in Table 2. There was no significant difference in sperm motility, epididymal sperm concentration and abnormal sperm rate in the three groups. However, epididymal sperm concentration ( $P < 0.05$ ) and abnormal sperm rate (head, tail and total values) ( $P < 0.001$ ) were significantly higher in IMI-8 group than that in control group.

#### Evaluation of TUNEL staining

Apoptosis levels in testis of control and IMI-treated rats, demonstrated by TUNEL staining, are shown in Figure 2. TUNEL-positive cells had the typical morphological features of apoptosis, including chromatin condensation, cytoplasmic budding and apoptotic bodies. To estimate the apoptotic index, TUNEL-positive cells in seminiferous tubules (100 per animal) in 20 randomly chosen fields were counted. The apoptotic index was calculated as the percentage of cells with TUNEL positivity. No or few TUNEL-positive cells were observed along the basement of seminiferous tubules (Figure 2A). Treatment with IMI

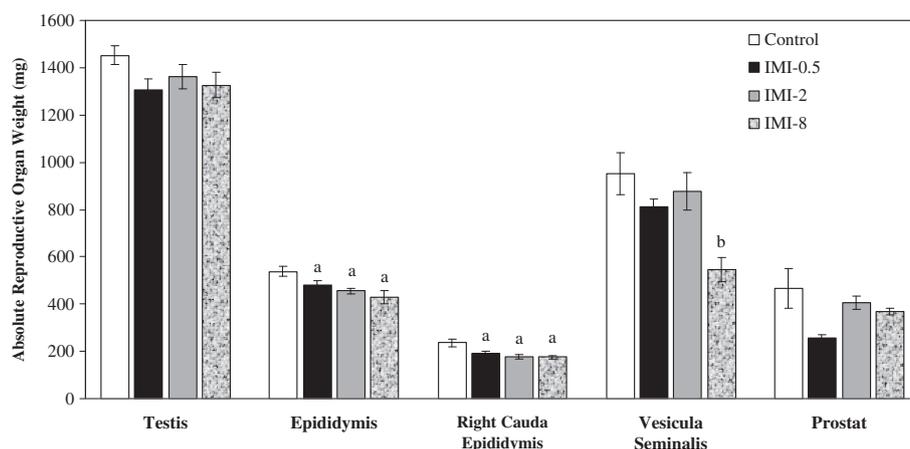
resulted in apoptotic death of germ cells at the seminiferous tubules (Figures 2D–2F). The apoptotic indexes were increased in the testis of IMI groups in a dose-dependent manner: 1.35  $\pm$  0.67% (0.5 mg/kg), 3.60  $\pm$  1.38% (2 mg/kg) and 5.26  $\pm$  1.68% (8 mg/kg), respectively, relative to control (0.31  $\pm$  0.2%).

#### Analysis of DNA fragmentation

The fragmentation of DNA is shown in Figure 3. Apoptotic cells usually contain fragmented DNA, which can be visualized by gel electrophoresis. Such fragmentations were used as a criterion for apoptosis. The DNA isolated from the sperm of rats exposed to IMI at doses of 8 mg/kg BW and showed degradation into oligonucleotide fragments, forming a clear laddering pattern of apoptosis when separated by electrophoresis (Figure 3). The DNA fragmentation was less obvious in the semen of rats of IMI-2, whereas there was no fragmentation in the seminal DNA of rats from IMI-0.5.

#### Biochemical parameters

The levels of total cholesterol, testicular fatty acid compositions (palmitic, palmitoleic, stearic, oleic, linoleic, arachidonic and docosapentaenoic acids) and cholesterol are shown in Table 3.



<sup>a</sup> $p < 0.05$  and <sup>b</sup> $p < 0.01$  versus control.

Figure 1. Effects of IMI on absolute weights of reproductive organs including testis, epididymis, right cauda epididymis, vesicular seminalis and prostate in rats (mean  $\pm$  SEM and  $n = 6$ )

IMIDACLOPRID AND TESTIS

Table 2. Effect of IMI on epididymal sperm characteristics in rats (mean  $\pm$  SEM and  $n = 6$ )

Groups	Sperm motility (%)	Epididymal sperm concentration (million/cauda epididymis)	Abnormal		Sperm rate	%
			Head	Tail	Total	
Control	74.6 $\pm$ 4.3	101.6 $\pm$ 10.3	2.0 $\pm$ 0.8	2.7 $\pm$ 0.0	4.7 $\pm$ 1.1	
IMI-0.5	70.0 $\pm$ 4.1	102.0 $\pm$ 5.0	2.0 $\pm$ 0.7	2.8 $\pm$ 0.5	4.8 $\pm$ 0.5	
IMI-2	65.0 $\pm$ 2.9	86.3 $\pm$ 8.2	2.3 $\pm$ 0.3	2.7 $\pm$ 0.9	5.0 $\pm$ 1.2	
IMI-8	61.1 $\pm$ 2.2	61.0 $\pm$ 4.4 <sup>a</sup>	6.0 $\pm$ 1.2 <sup>b</sup>	9.0 $\pm$ 1.2 <sup>b</sup>	15.0 $\pm$ 1.4 <sup>b</sup>	

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.001$  versus control group.

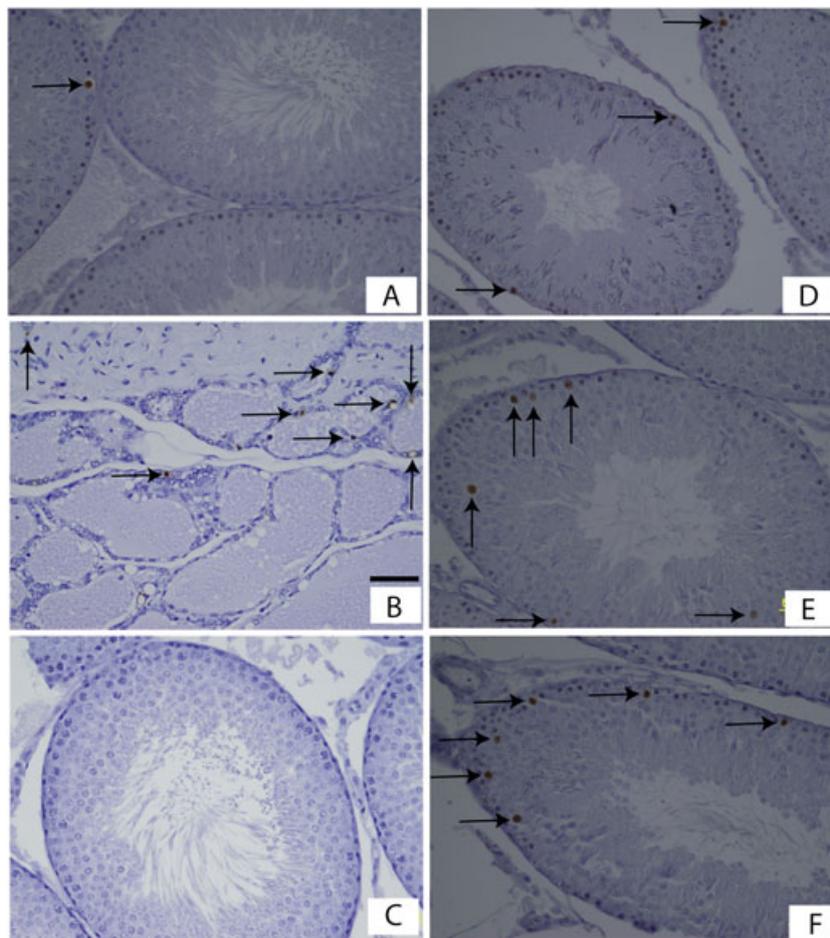


Figure 2. Representative photomicrographs of TUNEL staining in the testes of (A) control, (D) IMI-0.5, (E) IMI-2 and (F) IMI-8 groups. (C) Negative staining control is also illustrated to ensure the staining methods are working well. Note that there were no detectable signals in the negative control. (B) Positive control: TUNEL-stained cells in breast tissue where continuous apoptosis takes place. Arrows indicate candidate apoptotic cells. Calibration bar: 50  $\mu$ m

The serum total cholesterol levels were significantly higher in the IMI-2 ( $P < 0.05$ ) and IMI-8 ( $P < 0.01$ ) groups than that in the controls.

Treatment with IMI resulted in significant ( $P < 0.001$ ) increases in palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2 n6), dihomo- $\gamma$ -linolenic acid (20:3 n6), arachidonic acid (20:4 n6), docosapentaenoic acid (22:5 n6) and total lipid values

in a dose-dependent manner except oleic and linoleic acids ( $P < 0.001$ ).

*LP (MDA) and reduced GSH levels*

The effects of IMI-8 on testis MDA and GSH are shown in Figure 4. The analyses of MDA and GSH were possible only in the IMI-8 group because most of the samples from the

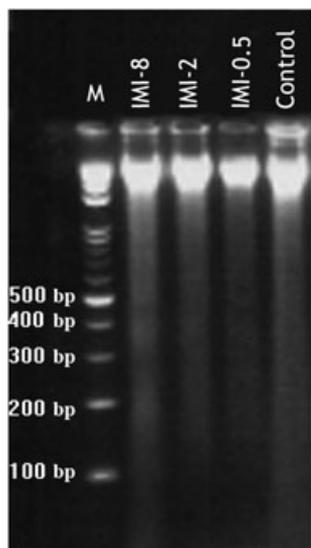


Figure 3. Effects of CTD exposure at doses of 0.5, 2 and 8 mg/kg BW. CTD exposure on DNA fragmentation in infant male rats at particularly 8 mg/kg BW dose induces the cleavage of DNA into oligonucleosome-length fragments, a characteristic of apoptosis. Marker (M) = molecular weight standards; IMI-0.5 = 0.5 mg/kg BW; IMI-2 = 2 mg/kg BW; IMI-8 = 8 mg/kg BW

0.5- and 2-mg/kg groups were used other analysis. The levels of MDA ( $P < 0.05$ ) were significantly higher and those of GSH significantly ( $P < 0.001$ ) lower in the rats treated with 8 mg/kg IMI than that in the controls.

## DISCUSSION

This study is the first to show that exposure of developing male rats to sublethal dosages of IMI, a neonicotinoid pesticide, affects the reproductive organ of male rats by decreasing the mass of accessory sex organs, testosterone level, sperm concentration, by increasing the rate of abnormal sperm morphology, by changing the lipid composition of testicular tissue, by fragmenting seminal DNA and by increasing apoptosis of spermatogenic cells.

We observed a direct relationship between the dose of IMI and the degree of sperm deterioration. At the highest dose of IMI, the epididymal sperm concentration was significantly

decreased accompanied by significantly higher counts of abnormal sperm. The decrease in epididymal sperm concentration might be due either to the inhibition of testosterone biosynthesis as evident by decreased testosterone levels or to the apoptosis in spermatogenic cells, as demonstrated by increased apoptotic index/seminal DNA fragmentation. It might also be due to suppression of the luteinizing and follicle-stimulating hormones (FSH and LH).<sup>4</sup>

Sperm motility can be used as an important functional parameter to predict sperm-fertilizing capacity.<sup>25</sup> A decrease in sperm motility may be due to low levels of ATP.<sup>26</sup> Toxic effects including reduced sperm motility and concentration with increased abnormal sperm morphology are in agreement with the effects of many other pesticides.<sup>27</sup> We noted that the administration of IMI reduces sperm motility, but the extent of the reduction was not significant.

Leydig cell development begins on day 14 postpartum, and the differentiation of adult Leydig cells is finalized by day 56 in the testis of rats.<sup>28</sup> Testosterone is responsible for the development of male secondary sex characteristics and hormonal imprinting of the liver, prostate and hypothalamus in the prepubertal period and supports spermatogenesis, sperm maturation and sexual function in the adult.<sup>29</sup> For these reasons, the impairment of testosterone biosynthesis may consequently result in reproductive dysfunction.

The decrease of testosterone levels induced by IMI exposure may indicate that IMI has a direct inhibitory effect on testosterone production from Leydig cells. The administration of IMI may disrupt the biosynthesis of testosterone in Leydig cells because of decreased LH secretion by the pituitary and/or from decreases in steroidogenic enzyme gene transcriptional activity.

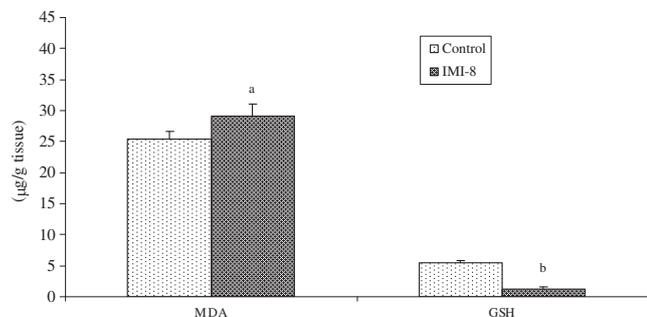
In fact, IMI acts as a nicotine acetylcholine receptor agonist and somehow likely to interfere with the release of gonadotropin-release hormone from hypothalamus and/or with release of LH/FSH from the pituitary, resulting in the reduction of sperm production in the testes, as suggested by Ngoula *et al.*<sup>30</sup> for pirimiphos-methyl, an organophosphorous pesticide in rats.

Furthermore, Akingbemi *et al.*<sup>28</sup> demonstrated that the cytochrome P450 enzymes, which catalyse the first reaction in the testosterone biosynthetic pathway, that is, the conversion of cholesterol to pregnenolone, were down-regulated by a biologically active metabolite of methoxychlor.

Table 3. Effect of IMI on fatty acid concentrations (mg/100 ml) in testis of rats (mean  $\pm$  SEM)

Parameters	Control ( $n=6$ )	IMI-0.5 ( $n=6$ )	IMI-2 ( $n=6$ )	IMI-8 ( $n=6$ )
Palmitic acid (16:0)	3047.8 $\pm$ 130.3	6435.1 $\pm$ 611.4 <sup>a</sup>	6418.4 $\pm$ 736.3 <sup>a</sup>	6436.7 $\pm$ 783.6 <sup>a</sup>
Palmitoleic acid (16:1)	89.5 $\pm$ 35.2	340.2 $\pm$ 58.5 <sup>a</sup>	342.6 $\pm$ 7.1 <sup>a</sup>	410.9 $\pm$ 59.2 <sup>a</sup>
Stearic acid (18:0)	564.3 $\pm$ 104.9	1303.2 $\pm$ 206.9 <sup>a</sup>	1607.4 $\pm$ 165.8 <sup>a</sup>	2130.9 $\pm$ 136.8 <sup>a</sup>
Oleic Acid (18:1)	1044.9 $\pm$ 70.0	1871.1 $\pm$ 323.5 <sup>a</sup>	1923.0 $\pm$ 63.3 <sup>a</sup>	2216.2 $\pm$ 406.6 <sup>a</sup>
Linoleic acid (18:2 n6)	398.4 $\pm$ 63.4	894.2 $\pm$ 237.1 <sup>a</sup>	919.8 $\pm$ 79.1 <sup>a</sup>	939.5 $\pm$ 236.7 <sup>a</sup>
Dihomo- $\gamma$ -linolenic acid (20:3 n6)	78.0 $\pm$ 11.5	176.5 $\pm$ 15.4 <sup>a</sup>	192.8 $\pm$ 5.1 <sup>a</sup>	203.4 $\pm$ 32.9 <sup>a</sup>
Arachidonic acid (20:4 n6)	1642.7 $\pm$ 70.7	2731.2 $\pm$ 249.1 <sup>a</sup>	2851.7 $\pm$ 25.9 <sup>a</sup>	2964.2 $\pm$ 52.1 <sup>a</sup>
Docosapentaenoic acid (22:5 n6)	1644.0 $\pm$ 104.8	2972.4 $\pm$ 258.1 <sup>a</sup>	3229.5 $\pm$ 58.4 <sup>a</sup>	3548.1 $\pm$ 349.9 <sup>a</sup>
Total lipid	8771.3 $\pm$ 362.1	17590.4 $\pm$ 121.1 <sup>a</sup>	17786.3 $\pm$ 59.7 <sup>a</sup>	19956.3 $\pm$ 249.6 <sup>a</sup>

<sup>a</sup> $P < 0.001$  versus control.



<sup>a</sup>p<0.05 and <sup>b</sup>p<0.001 versus control.

Figure 4. Effects of IMI-8 on testis LP (MDA) and reduced GSH in testis (mean  $\pm$  SEM and  $n = 6$ )

During the postnatal development of testis, the number of Leydig cells increases, and they acquire steroidogenic capacity, which are androgen dependent.<sup>29</sup> The disruption of testosterone biosynthesis by IMI consequently may reduce the number of Leydig cells per testis and/or decrease the steroidogenic capacity of individual Leydig cells.

In the present study, MDA and fatty acid concentrations in testis were higher at 8 mg/kg dose than that in control. However, the exposure of developing rat to IMI induces depletion in antioxidant defense systems, as indicated with decreased GSH in testis, because the generation of excessive free radicals/ROS in tissues and subcellular compartments is scavenged by the antioxidant defense system.<sup>31–33</sup>

This may consequently lead to disruption in functional integrity of membrane structures of the mitochondria and other cytoplasmic organelles through peroxidation of phospholipids, proteins and nucleotides in testis.<sup>5,34</sup> The subcellular membranes, rich in unsaturated fatty acids, contain low levels of antioxidants and are highly susceptible to LP. The mitochondrial and microsomal membranes consequently may undergo permeability changes following enhanced LP and GSH depletion.<sup>1</sup>

Male reproductive organs are particularly susceptible to the deleterious effects of ROS and LP, resulting in impaired fertility,<sup>2</sup> as evidenced by the increased apoptosis of germ cells in testis and seminal DNA fragmentation in the present study.

Thus, the adverse effect of IMI on reproduction of male rats appears to be due to the induction of oxidative stress in testis. Apoptosis in the testis occur so much that 75% of germ cells die by spontaneous apoptosis,<sup>17</sup> leading to abnormal spermatogenesis.<sup>3</sup> Consistently, increased apoptotic index in the testicular tissue found in the present study was well correlated with decreased sperm count and increased sperm abnormalities.

In conclusion, we observed that the three doses of IMI are capable of altering significant reproductive functions such as hormonal balance (the oxidant/antioxidant balance) and alters DNA along with marked morphological changes in testis of rats. The current study may be primarily used to understand mechanism of IMI induced testis toxicity.

## CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

## ACKNOWLEDGEMENTS

RB formulated the present hypothesis. MN was responsible for writing the report. GT, ÖY, TK and EE were responsible for analysis of the data. GB made critical revision on the manuscript. There is no financial support and conflict interest in the current study. The authors thank Dr Manuel Flores-Arce, Department of Chemical and Biochemical Engineering, Tijuana Institute of Technology, Tijuana, Mexico, for his scientific comments on the manuscript.

## REFERENCES

- Kovacic P, Somanathan R. Unifying mechanism for eye toxicity, Electron transfer, reactive oxygen species, antioxidant benefits, cell Signaling and cell Membranes. *Cell Membr Free Radic Res* 2008; **2**: 56–69.
- Mathur PP, D'Cruz SC. The effect of environmental contaminants on testicular function. *Asian J Androl* 2011; **13**(4): 585–591.
- Turner TT, Lysiak JJ. Oxidative stress: a common factor in testicular dysfunction. *J Androl* 2008; **29**(5): 488–498.
- Kapoor U, Srivastava MK, Srivastava LP. Toxicological impact of technical imidacloprid on ovarian morphology, hormones and antioxidant enzymes in female rats. *Food Chem Toxicol* 2011; **49**(12): 3086–3089.
- Nagda G, Bhatt DK. Alleviation of lindane induced toxicity in testis of Swiss mice (*Mus musculus*) by combined treatment with vitamin C, vitamin E and alpha-lipoic acid. *Indian J Exp Biol* 2011; **49**(3): 191–199.
- El-Gendy KS, Aly NM, Mahmoud FH, Kenawy A, El-Sebae AK. The role of vitamin C as antioxidant in protection of oxidative stress induced by imidacloprid. *Food Chem Toxicol* 2010; **48**(1): 215–221.
- Mandal TK, Das NS. Testicular gametogenic and steroidogenic activities in chlorpyrifos insecticide-treated rats: a correlation study with testicular oxidative stress and role of antioxidant enzyme defence systems in Sprague–Dawley rats. *Andrologia* 2012; **44**(2): 102–115.
- Uzun FG, Kalender S, Durak D, Demir F, Kalender Y. Malathion-induced testicular toxicity in male rats and the protective effect of vitamins C and E. *Food Chem Toxicol* 2009; **47**(8): 1903–1908.
- Yousef MI, Vitamin E. modulates reproductive toxicity of pyrethroid lambda-cyhalothrin in male rabbits. *Food Chem Toxicol* 2010; **48**(5): 1152–1159.
- Pflieger-Bruss S, Schuppe HC, Schill WB. The male reproductive system and its susceptibility to endocrine disrupting chemicals. *Andrologia* 2004; **36**(6): 337–345.
- Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol Sci* 2001; **22**(11): 573–580.
- Schulz-Jander DA, Casida JE. Imidacloprid insecticide metabolism: human cytochrome P450 isozymes differ in selectivity for imidazolidine oxidation versus nitroimine reduction. *Toxicol Lett* 2002; **132**(1): 65–70.
- Latchoumycandane C, Chitra C, Mathur P. Induction of oxidative stress in rat epididymal sperm after exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Arch Toxicol* 2002; **76**(2): 113–118.
- Gallegos-Avila G, Ancer-Rodriguez J, Niderhauser-Garcia A, Ortega-Martinez M, Jaramillo-Rangel G. Multinucleation of spermatozoa and spermatids in infertile men chronically exposed to carbofuran. *Reprod Toxicol* 2010; **29**(4): 458–460.
- Cupp AS, Uzumcu M, Suzuki H, Dirks K, Phillips B, Skinner MK. Effect of transient embryonic in vivo exposure to the endocrine disruptor methoxychlor on embryonic and postnatal testis development. *J Androl* 2003; **24**(5): 736–745.

16. Abou-Donia MB, Goldstein LB, Bullman S, *et al.* Imidacloprid induces neurobehavioral deficits and increases expression of glial fibrillary acidic protein in the motor cortex and hippocampus in offspring rats following in utero exposure. *J Toxicol Environ Health A* 2008; **71**(2): 119–130.
17. Karabay NU, Oguz MG. Cytogenetic and genotoxic effects of the insecticides, imidacloprid and methamidophos. *Genet Mol Res* 2005; **4**(4): 653–662.
18. Kapoor U, Srivastava MK, Bhardwaj S, Srivastava LP. Effect of imidacloprid on antioxidant enzymes and lipid peroxidation in female rats to derive its No Observed Effect Level (NOEL). *J Toxicol Sci* 2010; **35**(4): 577–581.
19. Turk G, Atessahin A, Sonmez M, Ceribasi AO, Yuce A. Improvement of cisplatin-induced injuries to sperm quality, the oxidant-antioxidant system, and the histologic structure of the rat testis by ellagic acid. *Fertil Steril* 2008; **89**(5 Suppl): 1474–1481.
20. Hara A, Radin NS. Lipid extraction of tissues with a low-toxicity solvent. *Anal Biochem* 1978; **90**(1): 420–426.
21. Wang JY, Shum AY, Ho YJ, Wang JY. Oxidative neurotoxicity in rat cerebral cortex neurons: synergistic effects of H<sub>2</sub>O<sub>2</sub> and NO on apoptosis involving activation of p38 mitogen-activated protein kinase and caspase-3. *J Neurosci Res* 2003; **72**(4): 508–519.
22. de las Heras A. Comparison of methods for determining malondialdehyde in dry sausage by HPLC and the classic TBA test. *Eur Food Res Technol* 2003; **217**: 180–184.
23. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; **82**(1): 70–77.
24. Naziroğlu M, Kilinç F, Uğuz AC, *et al.* Vitamin C and E modulates blood lipid peroxidation and antioxidant levels in maximal exercising basketball players. *Cell Biochem Funct* 2010; **28**: 300–305.
25. Aitken RJ, Best FS, Warner P, Templeton A. A prospective study of the relationship between semen quality and fertility in cases of unexplained infertility. *J Androl* 1984; **5**(4): 297–303.
26. Bai JP, Shi YL. Inhibition of Ca<sup>2+</sup> channels in mouse spermatogenic cells by male antifertility compounds from *Tripterygium wilfordii* Hook. f. *Contraception* 2002; **65**(6): 441–445.
27. Dalsenter PR, de Araujo SL, de Assis HC, Andrade AJ, Dallegre E. Pre and postnatal exposure to endosulfan in Wistar rats. *Hum Exp Toxicol* 2003; **22**(4): 171–175.
28. Akingbemi BT, Ge RS, Klinefelter GR, Gunsalus GL, Hardy MP. A metabolite of methoxychlor, 2,2-bis(p-hydroxyphenyl)-1,1, 1-trichloroethane, reduces testosterone biosynthesis in rat leydig cells through suppression of steady-state messenger ribonucleic acid levels of the cholesterol side-chain cleavage enzyme. *Biol Reprod* 2000; **62**(3): 571–578.
29. Ewing LL, Keeney DS. Leydig cells: structure and function. In *Cell and Molecular Biology of the Testis*, Desjardins C, Ewing LL (eds.). Oxford University Press: New York, 1993; 137–165.
30. Ngoula F, Watcho P, Dongmo MC, Kenfack A, Kamtchoung P, Tchoumboue J. Effects of pirimiphos-methyl (an organophosphate insecticide) on the fertility of adult male rats. *Afr Health Sci* 2007; **7**(1): 3–9.
31. Naziroğlu M, Kutluhan S, Uguz AC, Celik O, Bal R, Butterworth PJ. Topiramate and vitamin E modulate the electroencephalographic records, brain microsomal and blood antioxidant redox system in pentylentetrazol-induced seizure of rats. *J Membr Biol* 2009; **229**(3): 131–140.
32. Naziroğlu M, Uguz AC, Kocak A, Bal R. Acetaminophen at different doses protects brain microsomal Ca<sup>2+</sup>–ATPase and the antioxidant redox system in rats. *J Membr Biol* 2009; **231**(2–3): 57–64.
33. Naziroğlu M. Role of selenium on calcium signaling and oxidative stress-induced molecular pathways in epilepsy. *Neurochem Res* 2009; **34**(12): 2181–2191.
34. Caliskan AM, Naziroglu M, Uguz AC, *et al.* Acamprostate modulates alcohol-induced hippocampal NMDA receptors and brain microsomal Ca<sup>2+</sup>–ATPase but induces oxidative stress in rat. *J Membr Biol* 2010; **237**(1): 51–58.