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Assessment of imidacloprid toxicity on reproductive organ system of adult male rats

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In the current study it was aimed to investigate the toxicity of low doses of imidacloprid (IMI) on the reproductive organ systems of adult male rats. The treatment groups received 0.5 (IMI-0.5), 2 (IMI-2) or 8 mg IMI/kg body weight by oral gavage (IMI-8) for three months. The deterioration in sperm motility in IMI-8 group and epididymal sperm concentration in IMI-2 and IMI-8 groups and abnormality in sperm morphology in IMI-8 were significant. The levels of testosterone (T) and GSH decreased significantly in group IMI-8 compared to the control group. Upon treatment with IMI, apoptotic index increased significantly only in germ cells of the seminiferous tubules of IMI-8 group when compared to control. Fragmentation was striking in the seminal DNA from the IMI-8 group, but it was much less obvious in the IMI-2 one. IMI exposure resulted in elevation of all fatty acids analyzed, but the increases were significant only in stearic, oleic, linoleic and arachidonic acids. The ratios of 20:4/20:3 and 20:4/18:2 were decreased and 16:1n-9/16:0 ratio was increased. In conclusion, the present animal experiments revealed that the treatment with IMI at NOAEL dose-levels caused deterioration in sperm parameters, decreased T level, increased apoptosis of germ cells, seminal DNA fragmentation, the depletion of antioxidants and change in disturbance of fatty acid composition. All these changes indicate the suppression of testicular function.

Keywords: Imidacloprid, neonicotinoids, apoptosis, testis, sperm characteristics, fatty acid composition.

Introduction

Over the past few decades, a lot of evidence has surfaced accentuating the adverse effects of environmental toxicants on male reproduction.[1] Toxicity of pesticides for non-target organisms is of worldwide concern. Imidacloprid [1-(6-chloro-3-pyridylmethyl)-2-nitroimino-imidazolidine] (IMI) belongs to a major new class of synthetic insecticides, called neonicotinoids and is used widely to control insect pests on crops and fleas on domestic animals. Imidacloprid acts as a potent agonist on insect nicotinic acetylcholine receptors (nAChRs), specifically at the α-subunits of the nicotinic receptor, like nicotine.[2] The receptors nAChRs are ligand-gated ion channels and are involved in synaptic transmission in the central nervous system (CNS).[3] Neonicotinoids including IMI are more toxic to insects and less toxic to mammals, thus providing an excellent example of selective toxicity.[2] The favorable selective toxicity of IMI on insects as opposed to mammals is attributed to differences in their binding affinity or potency in the nicotinic acetylcholine receptor.[2,4,5] There is evidence that the interaction of imidacloprid with the α-subunit of the nicotinic AChRs contributes to its partial agonist actions and its selectivity for insect nicotinic AChRs.[6]

Animal studies confirm the relatively low toxicity in vertebrate animals when compared to insects.[4,5,7,8] It is moderately toxic and its acute oral LD50 are 450 mg/kg for rats and 150 mg/kg for mice.[9] In male rats, the NOEL dose of IMI is 14 mg/kgBW/day. Patch clamp experiments

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performed on the neurons of cochlear nucleus slices, whose ionic channels are characterized,[10–16] demonstrated that mammalian nAChRs have been found far less sensitive to IMI by a factor of approximately 100[5] than those on insect neurons.[8,17] This is one of the main reasons for the success of this insecticide.

However, there are some publications reporting that IMI may have deleterious effects on people exposed,[18,19] however reported clinical toxicity in humans is rare.[20] Experimental studies suggest that chronic exposure to IMI may induce neurobehavioral deficits in offspring rats following in utero exposure,[18] genotoxic and mutagenic alterations.[19] Therefore, it appears that this agent may not be as innocuous as reported. Reports of deleterious effects of IMI in veterinarians, veterinary technologists, dog caretakers, and owners and also of human poisoning have highlighted the need to examine the interaction of such compounds with mammalian nAChRs.[20]

Despite that both animals and humans are intensely exposed to neonicotinoid pesticides, there are very little data available on the chronic toxicity of imidacloprid to non-target organisms, particularly about the effects of IMI on mammalian reproductive functions. Therefore, in the current study it was aimed to investigate the toxicity of low doses of IMI on the reproductive organ system of adult male rats, evaluating testicular biochemistry, histology, sperm dynamics and T level in treated rats.

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Materials and methods

Animals and experimental design

The experimental protocols were approved by the local Animal Use Committees of Firat University (Elazig, Turkey). Animal care and experimental protocols 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 8-9 weeks and weight in the range of 180-210 g, were obtained and maintained from Firat University Experimental Research Centre (Elazig, Turkey). The animals were housed in polycarbonate cages in a room with a 12 h day-night cycle, temperature of 24 ± 3°C, humidity of 45 % to 65 %. During the whole experimental period, animals were fed with a balanced commercial diet (Elazig Food Company, Elazig, Turkey) *ad libitum* and fresh distilled drinking water was given *ad libitum*.

Animals and subchronic 90-day oral toxicity study

The animals were randomly divided into four groups with 6 animals in each group. The first group was taken as (i) *control* and the other groups were treated as (ii) *IMI-0.5 group:* rats received IMI daily for a period of 3 months at a dose of 0.5 mg/kg body weight (BW) by gavage; (iii) *IMI-2 group*: rats received IMI daily for a period of 3 months at a dose of 2 mg/kg BW by gavage; and (iv) *IMI-8 group:* rats received IMI daily for a period of 3 months at a dose of 8 mg/kg BW by gavage.

In the present study, a maximum dose of 8 mg/kg for IMI was selected based on the reported reproductive NOAEL for rats.[21]

Sample collection and homogenate preparation

After the animals were decapitated at the age of 97 days old, the blood was collected and testis, epididymis, seminal vesicles and ventral prostate were removed, cleared of adhering connective tissue and weighed. The right testicles were fixed with Bouin's fluid. The left testicles were frozen in liquid nitrogen and stored at –70°C until use for MDA, GSH, fatty acids, α-tocopherol analysis. Serum was separated and also stored at –70°C until use to estimate some biochemical parameters using the appropriate kits (Boehringer Mannheim, Germany).

Localization of apoptotic cells in the testis

The localization of apoptotic cell death in the spermatogenic cells was defined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay with theApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA). Briefly, the fixed testicular tissue was embedded in paraffin, and sectioned at 4 μm. The paraffin sections were deparaffinized in xylene, dehydrated through graded alcohol, and washed in PBS. The sections were treated with 0.05 % proteinase K for 5 min, which was followed by treatment with 3 % hydrogen peroxide for 5 min to inhibit endogenous peroxidase. After washing in PBS, sections were then incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) enzyme and digoxigenin-11-dUTP at 37°C for 1 h in humidified chamber at 37°C for 1 h, and then stop/wash buffer was applied for 30 min at 37°C. Sections were visualized with diaminobenzidine (DAB) substrate. Sections were counterstained with Mayor's hematoxylin, dehydrated in graded alcohol, and cleared. To control for nonspecific incorporation of nucleotides or for nonspecific binding of enzymeconjugate, negative control staining was performed without active TdT but including proteinase K digestion. Equilibration Buffer was substituted for the volume of TdT ENZYME reagent. Also positive control staining was performed in the normal female rodent mammary gland tissue where continous apoptosis takes place.

Sperm analysis

Epididymal sperm concentration. The epididymal sperm concentration was determined with a hemocytometer

using a modified method.[22] The right epididymis was finely minced by anatomical scissors within 1 mL of isotonic saline in a Petri dish. It was completely squashed by a tweezers for 2 min, and then allowed to incubate at room temperature for 4 h to provide the migration of all spermatozoa from epididymal tissue to fluid. After incubation, the epididymal tissue-fluid mixture was filtered via strainer to separate the supernatant from tissue particles. The supernatant fluid containing all epididymal spermatozoa was drawn into the capillary tube up to 0.5 lines of the pipette designed for counting red blood cells. The solution containing 0.595 M sodium bicarbonate, 1 % formalin and 0.025 % eosin was pulled into the bulb up to 101 lines of the pipette. This gave a dilution rate of 1:200 in this solution. Approximately 10 μl of the diluted sperm suspension was transferred to both counting chamber of Improved Neubauer (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 200x magnification.

Sperm motility. Freshly isolated left epididymal tissue was used for the analysis of sperm motility. The percentage sperm motility was evaluated using a light microscope with heated stage. For this process, a slide was placed on a light microscope with a heated stage warmed up to 37°C, and then several droplets of Tris buffer solution [0.3 M Tris (hydroxymethyl) aminomethane, 0.027 M glucose, 0.1 M citric acid] were dropped on the slide and a very small droplet of fluid obtained from left cauda epididymis with a pipette was added to the Tris buffer solution and mixed by a cover-slip. The percentage of sperm motility was evaluated visually at 400x 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 stained with eosin-nigrosin (1.67 % eosin, 10 % nigrosin and 0.1 M sodium citrate) were viewed under a light microscope at 400x magnification. A total of 300 spermatozoa were examined on each slide (3000 cells in each group), and the head, tail and total abnormality rates of spermatozoa were expressed as percentage.[22]

Determination of MDA-TBA level

The concentration of TBARS in the tissues samples was estimated by the method of Niehaus and Samuelsson.[23] In brief, 1 mL of tissue homogenate (supernatant; Tris-HCl buffer, pH 7.5) was mixed with 2 mL of (1:l:l ratio) TBA-TCA- HC1 reagent (0.37 % thiobarbituric acid, 0.25 N HCI, and 15 % TCA) placed in water bath for 60 min, cooled, and centrifuged at room temperature for 10 min. Thiobarbituric acid-reactive substances (TBARS) were determined by reading the fluorescence detector set at λ (excitation) = 515 nm and λ (emission) = 543 nm. TBARS calculated from a calibration curve using 1, 1, 3, 3-tetraethoxypropane as the standard. The MDA-TBA complex was analyzed using the high performance liquid chromatography (HPLC) equipment. The equipment consisted of a pump (LC-10 ADVP), a Fluorescence detector (RF–10 $_{XL}$), a column oven (CTO-10AS*VP*), an autosampler (SIL-10ADVP) a degasser unit (DGU-14A) and a computer system with class VP software (Shimadzu, Kyoto Japan). Inertsil ODS-3 column (15×4.6 mm, 5 μm) was used as the HPLC column. The column was eluted isocratically at 20 ∞ C with a 5 mM sodium phosphate buffer (pH = 7.0) and acetonitrile (85:15, v/v) at a rate of 1 mL / min.[24] The values were expressed as mmol/ g tissues.

Determination of GSH level in tissue samples

Reduced glutathione (GSH) was determined by the method of Ellman.[25] Briefly, 1 mL tissue homogenate was treated with 1 mL of 5 metaphosphoric acid (Sigma, St. Louis, MO), the mixtures were centrifuged in 5000 rpm and the supernatant was taken. After deproteinization, the supernatant was allowed to react with 1 mL of Ellman's magent (30 mM 5, 5'-dithiobisnitro benzoic acid in 100 mL of 0.1 % sodium citrate). The absorbance of the yellow product was read at 412 nm in spectophotometer. Pure GSH was used as standard for establishing the calibration curve.[26]

Lipid extraction

Lipid extraction of tissue samples were extracted with hexane-isopropanol (3:2 v/v) by the method of Hara and Radin.[27] A tissue sample measuring 1 g was homogenized with 10 mL hexane-isopropanol mixture. Fatty acids in the lipid extracts were converted into methyl esters including 2 % sulphuric acid (v/v) in methanol.[28] The fatty acid methyl esters were extracted with 5 mL n-hexane. Analysis of fatty acid methyl ester was performed in a Shimadzu GC-17A instrument gas chromatograph equipped with a flame ionization detector (FID) and a 25 m, 0.25 mm i.d. permabond fused-silica capillary column (Machery – Nagel, Germay). The oven temperature was programmed between 145 – 215 ° C, 4 ° C / min. Injector and FID temperatures were 240 and 280°C, respectively. The rate of nitrogen carrier gas was at 1 mL / min. The methyl esters of fatty acids were identified by comparison with authentic external standard mixtures analyzed under the same conditions. *Class GC 10* software version 2.01 was used to process the data. The results were expressed as μg / g tissue.

Saponification and extraction

Alpha-tocopherol and cholesterol were extracted from the lipid extracts by the method of Sánchez-Machado et al.[29] with minor modifications. Five milliliters n-hexane/

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isopropyl alcohol mixture was treated with 5 mL of KOH solution (0.5 M in methanol), which was immediately vortexed for 20 s. The tubes were placed in a water bath at 80°C for 15 min. Then after cooling in iced water, 1 mL of distilled water and 5 mL of hexane was added, and the mixture was rapidly vortexed for 1 min, then centrifuged for 5 min at 5000 rpm. The supernatant phase was transferred to another test tube and dried under nitrogen. The residue was redissolved in 1 mL of the HPLC mobile phase (68:28:4 (v/v/v) methanol:acetonitrile:water). Finally, an aliquot of 20 μL was injected into the HPLC column. Before injection, the extracts were maintained at −20°C away from light.

Chromatographic conditions

Chromatographic analysis was performed using an analytical scale (15 cm× 0.45 cm I.D) Supelco LC 18 DB column with a particle size 5 μm (Sigma, USA). HPLC conditions were as follows: mobile phase 60:38:2 (v/v/v): acetonitrile/methanol/water; a flow rate of 1 mL / min; column temperature 30°C. The detection was operated using two channels of a diode-array spectrophotometer, and 202 nm for α-tocopherol and cholesterol. Alpha-tocopherol, and cholesterol were identified by retention and spectral data.[30]

Serum testosterone

The plasma testosterone (T) level was measured by ELISA method using DRG Elisa kit (ELISA EIA-1559, 96 Wells kit, DRG Instruments, GmbH, Marburg, Germany) according to the standard protocol supplied by the kit manufacturer.

Analysis of DNA fragmentation

DNA fragmentation was determined by a modification of a previously described procedure.[31] Semen was homogenized in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.5 % (w/v) SDS, 1 % Triton X-100, 0.25 mg/mL RNAse A and 100 μg/mL proteinase K (final concentration 2.5 μg/μl) and incubated for 1 h at 65°C. After centrifugation at 12,000 g at 4°C for 20 min, the supernatant was extracted with phenol and chloroform and DNA was precipitated by 100 % ethanol, and then washed with 70 % ethanol. DNA was resuspended in Tris–EDTA buffer and analyzed by electrophoresis in 2 % agarose gel. The gel was stained with ethidium bromide and visualized under UV light.

Chemicals

All chemicals were purchased from Sigma.

Statistical analysis

One-way analysis of variance (ANOVA) and *post hoc* Tukey-HSD test were used to determine differences between groups in all parameters except serum T level, whose statistical comparison was performed using the nonparametric Mann-Whitney U test. Results are presented as mean ± S.E.M. 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 body weight gain

The effects of IMI at doses of 0.5, 2 and 8 mg/kg body weight (BW) on body weight gain (final body weight minus initial body weight) of male rats were demonstrated in Table 1. Body weight gains of IMI-2 and IMI-8 groups were significantly less than that of control group ($P > 0.001$).

Reproductive organ weights

Absolute and relative weights of reproductive organs including testis, epididymis, right cauda epididymis, seminal vesicles and prostate of control and IMI groups are shown in Figure 1 as bar graph. The relative organ weights were estimated by dividing the absolute reproductive organ weights to body weight.

While absolute weights of epididymis ($P < 0.01$), right cauda epididymis ($P < 0.01$) and seminal vesicles ($P < 0.05$) of IMI-2 and IMI-8 groups and absolute weights of epididymis in IMI-0.5, IMI-2 and IMI-8 groups were significantly less than those of control, relative weights of epididymis, right cauda epididymis and seminal vesicles of only IMI-8 groups were significantly less than those of control group ($P < 0.05$).

Epididymal sperm characteristics

Epididymal sperm characteristics of control and IMI- administered rats are presented in Table 2. There were no significant differences in sperm motility, epididymal sperm

Table 1. Effect of IMI on body weight gain.

	Initial body weight (g)	*Final body weight (g)*	*Body weight gain (g)*
Control	167.8 ± 3.4^{a}	306.8 ± 4.7	139.1 ± 6.2^{a}
IMI-0.5	165.0 ± 2.1^{a}	303.0 ± 5.0	138.0 ± 3.5^{a}
IMI-2	161.2 ± 3.6^{a}	271.5 ± 5.7	$110.3 \pm 6.8^{b\#}$
IMI-8	166.8 ± 3.9^{a}	267.0 ± 4.6	$100.2 \pm 4.8^{b\#}$

The mean differences between the values bearing different superscript letters within the same raw are statistically significant ($P < 0.05$). (#: $P < 0.001$ compared with control).

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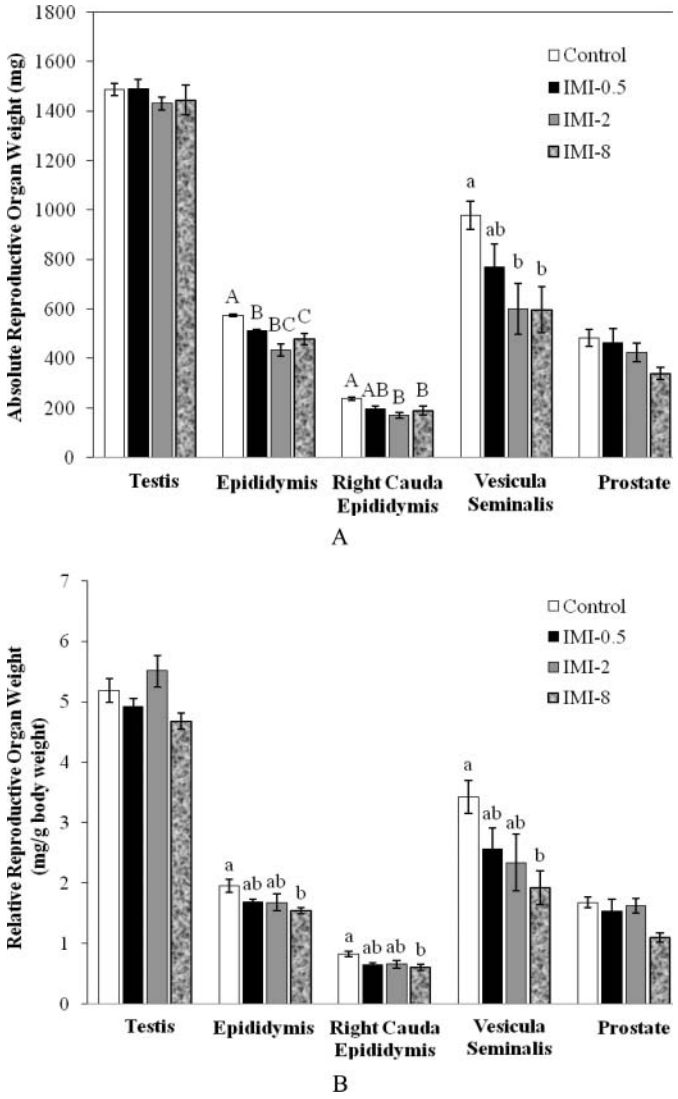

Fig. 1. Absolute (A) and relative (B) weights of reproductive organs including testis, epididymis, right cauda epididymis, vesicular seminalis and prostate (Mean ± SEM). The mean differences between the values bearing different letters for the same organs are statistically significant (a and b: $P < 0.05$; A, B and C: $P < 0.01$).

concentration and abnormal sperm rate between control and 0.5 IMI groups. However, administration of IMI at 2 mg/kg BW/day resulted in significant decreases in epididymal sperm concentration ($P < 0.01$). Furthermore, administration of IMI at 8 mg/kg BW/day resulted in significant decreases in sperm motility ($P < 0.05$) and epididymal sperm concentration ($P < 0.01$) and a significant increase ($P < 0.05$) in the rate of abnormal sperm rate when compared to control.

Evaluation of TUNEL staining

Apoptosis in the spermatogenetic cells of testis of control and IMI-treated rats, demonstrated by TUNEL staining, are shown in Figure 2. TUNEL-positive cells had the typical appearance for apoptosis, including chromatin condensation, cytoplasmic budding and apoptotic bodies. In order 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 TUNEL positive cells. TUNEL-positive cells were occasionally observed in the testis of control rats and therefore, the apoptotic index was very low in control group (0.40 ± 0.25 %) (Fig. 2A). Yet, the number of positive cells was increased in the testis of IMI groups, it reach statistical significance in only IMI-8 group compared to control (Fig. 2F). The apoptotic indexes were 0.85 ± 0.55 %, 1.33 ± 0.88 % and 3.66 ± 1.23 % in IMI-0.5, IMI-2 and IMI-8 respectively.

Analysis of DNA fragmentation

The cells going through apoptosis often include fragmental DNA, which can be visualized by DNA-agarose gel electrophoresis. For that reason, we have employed DNA fragmentation as the criterion for apoptosis. DNA isolated from the sperm of rats exposed to IMI at a dose of 8 mg/kg body weight (BW) for 3 months showed a striking degradation into oligonucleotide fragments forming a clear laddering pattern of apoptosis when separated by 2 % agarose

Table 2. Mean ± SEM values of sperm parameters.

	Parameters				
			Abnormal sperm rate (%)		
Groups	*Sperm motility (%)*	*Epididymal sperm concentration (million/cauda epididymis)*	*Head*	*Tail*	*Total*
Control	72.7 ± 3.1^{a}	93.4 ± 4.8^{A}	4.0 ± 1.1^{a}	4.6 ± 0.5	8.6 ± 0.7
IMI-0.5	73.3 ± 3.3^{a}	76.2 ± 3.3^{A}	8.3 ± 2.1^{ab}	7.0 ± 2.5	15.3 ± 3.0
IMI-2	65.0 ± 5.0^{ab}	46.3 ± 11.4^{B}	7.0 ± 1.0^{ab}	9.0 ± 3.1	16.0 ± 2.9
IMI-8	50.0 ± 7.1^{b}	46.8 ± 7.1^{B}	14.2 ± 4.1^{b}	8.0 ± 1.3	22.2 ± 5.1

The mean differences between the values bearing different superscript letters within the same column are statistically significant (a and b: $P < 0.05$; A and B: $P < 0.01$).

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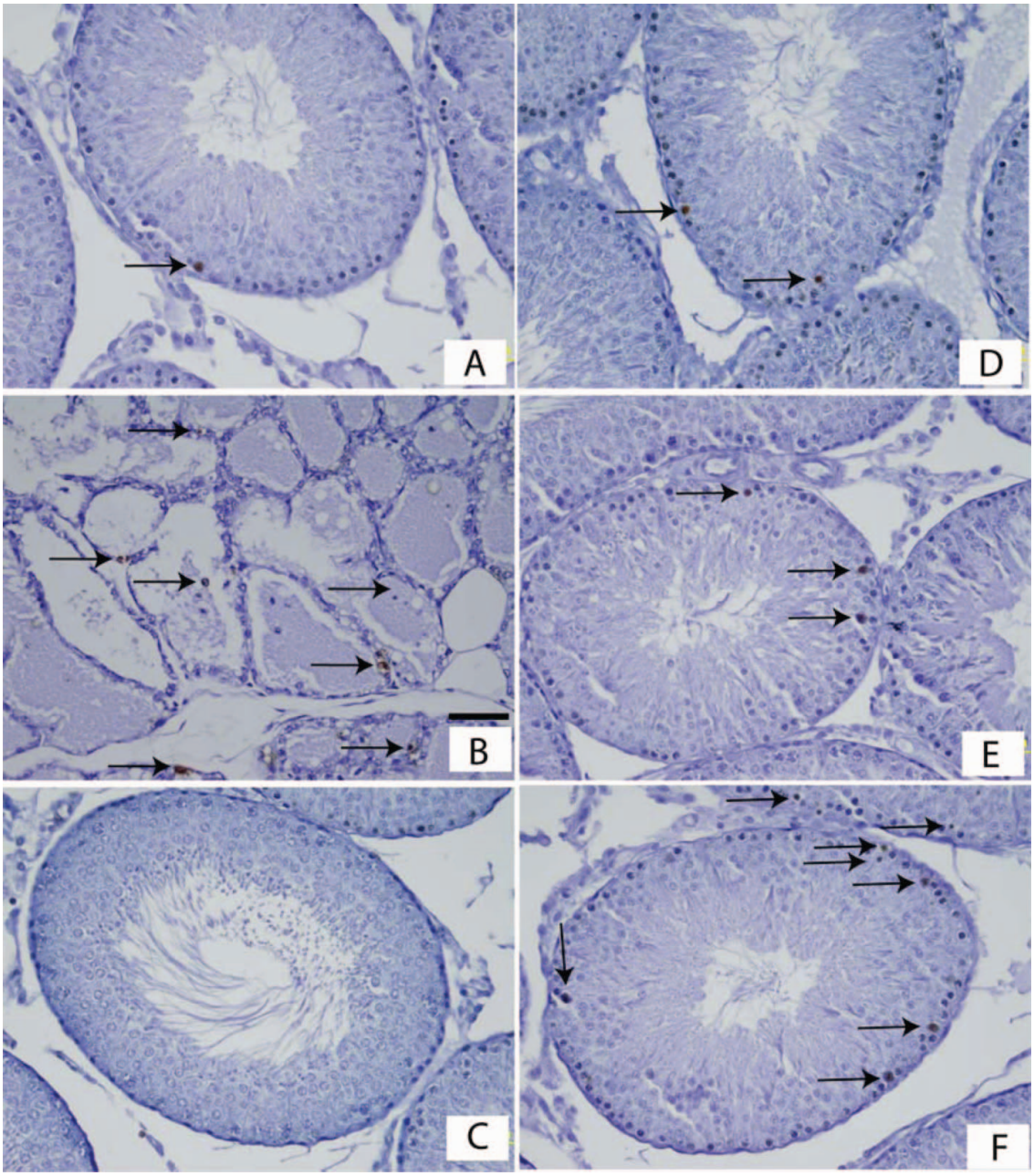

Fig. 2. Representative photomicrographs of TUNEL-staining in the testes of control (A), IMI-0.5 (D), IMI-2 (E) and IMI-8 groups (F). C: Negative control staining is also illustrated to ensure the staining method is working well. Note that there was no detectable signal in the negative control. B: Positive control: TUNEL-stained cells in breast tissue where continuous apoptosis takes place. Arrows indicate candidate apoptotic cells. Calibration bar: 50 μm (color figure available online).

gel electrophoresis (Fig. 3). However, the fragmentation was less obvious in the semen DNA of rats from IMI-2. Whereas, there was no fragmentation in the semen DNA of rats from IMI-0.5.

Biochemical parameters

The effects of IMI administration on the levels of T in serum, the levels of lipid peroxidation (TBARS) and antioxidant (GSH), some fatty acids (palmitic acid, palmitoleic acid, stearik acid, oleic acid, linoleic acid, arachidonic acid and docosapentaenoic acid), cholesterol, α-tocopherol and α-tocopherol acetate in testicular tissue are presented in Table 3.

There was no significant difference in serum T level between control, IMI-0.5 and IMI-2 groups. Serum level of T in IMI-8 group were significantly lower than that in control group ($P < 0.05$) (Table 3).

Table 3. Levels of serum glucose and testosterone and testicular tissue thiobarbituric acid reactive substances (TBARs), glutathione (GSH), cholesterol, α-tocopherol, α-tocopherol acetate and some fatty acids including palmitic, palmitoleic, stearik, oleic, linoleic, arachidonic and docosapentaenoic acids (Mean ± SEM).

	Control (n = 6)	*IMI-0.5 (n = 6)*	*IMI-2 (n = 6)*	*IMI-8 (n = 6)*
Testosterone (ng/dl)	$121,7 \pm 6,9^{a}$	$104,4 \pm 38,2^{ab}$	$88,9 \pm 34,1^{ab}$	$46,9 \pm 4,5^{b}$
TBARS (nmol/g)	$27,8 \pm 1,4^{a}$			$22,6 \pm 1,9^{a}$
GSH (μg/g)	$554,0 \pm 1,4^{a}$			$367,1 \pm 0,7^{b\#}$
Palmitic acid (16:0)(μg)	$3188,8 \pm 231,5^{a}$	$4575,0 \pm 530,9^{a}$	$4466,5 \pm 483,1^{a}$	$4826,1 \pm 656,4^{a}$
Palmitoleic acid (16:1)(μg)	$131,3 \pm 37,5^{a}$	$268,7 \pm 38,6^{a}$	$331,7 \pm 134,2^{a}$	$337,8 \pm 38,9^{a}$
Stearic acid (18:0)(μg)	$677,0 \pm 120,8^{a}$	$918,7 \pm 111,4^{a}$	$692,94 \pm 9,4^{ab}$	$1400,4 \pm 117,7^{b*}$
Oleic Acid (18:1)(μg)	$1127,6 \pm 97,8^{a}$	$1578,9 \pm 99,4^{ab}$	$1829,0 \pm 282,3^{b}$	$1846,5 \pm 205,8^{b}$
Linoleic acid (18:2 n6)(μg)	$417,6 \pm 57,2^{a}$	$752,0 \pm 157,5^{ab}$	$932,0 \pm 196,9^{b}$	$934,3 \pm 117,7^{b}$
Dihomo-γ-linolenic acid (20:3 n6) (μg)	$97,8 \pm 13,9^{a}$	$155,4 \pm 19,5^{a}$	$176,3 \pm 37,7^{a}$	$188,2 \pm 22,1^{a}$
Arachidonic acid (20:4 n6) (μg)	$1671,2 \pm 86,9^{a}$	$2284,4 \pm 188,2^{ab}$	$2267,5 \pm 145,7^{ab}$	$2577,5 \pm 311,9^{b}$
Docosapentaenoic acid (22:5 n6) (μg)	$1805,8 \pm 189,6^{a}$	$2647,7 \pm 251,6^{a}$	$2727,0 \pm 270,9^{a}$	$3072,3 \pm 378,2^{a}$
Total lipid(μg)	$9158,0 \pm 628,4^{a}$	$14381,1 \pm 171,2^{a}$	$14099,7 \pm 212,1^{a}$	$14621,4 \pm 193,3^{a}$
α-tocopherol (μg)	$131,2 \pm 11,4^{a}$			$169,7 \pm 17,8^{a}$
Testis cholesterol(μg)	$5033,6 \pm 322,7^{a}$			$5832,3 \pm 635,4^{a}$

The mean differences between the values bearing different superscript letters within the same raw are statistically significant ($P < 0.05$). (*: $P < 0.05$ vs. control; †: $P < 0.01$ compared with control; #: < 0.001 compared with control).

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Oxidative stress in testicular tissue was studied by estimating tissue peroxidation by the thiobarbituric acid (TBA) test. It is a widely used test for tissue oxidative stress because it can detect many peroxidation products and intermediates, although its specificity is low. Administration of IMI to rats at 8 mg/kg BW/day caused the level of GSH to decrease significantly compared to that of control group ($P < 0.001$) (Table 3). Whereas, IMI exposure did not cause any significant changes in the level of MDA and α-tocopherol.

Levels of all fatty acids increased in all IMI-administered groups. However, the increases were significant only in the levels of stearic, oleic, linoleic and arachidonic acids in IMI-8 group and the levels of oleic, and linoleic acids in IMI-2 group when compared to control group ($P < 0.05$) (Table 3). As can be seen in Table 3, the administration of IMI at 0.5 mg/kg BW did not make any significant changes in fatty acids.

In the testis of rats treated with IMI at 0.5, 2 and 8 mg/kgBW/day, decreases in 20:4/20:3 ratio (∼15, ∼13 and ∼14 respectively) (∼17); in 20:4/18:2 ratio (∼3.0, ∼2.4 and ∼2.7 respectively) (∼4.0) and increases in 16:1n-9/16:0 ratio (∼0.06, ∼0.07 and ∼0.07 respectively) (∼0.04) were observed when compared to control.

Discussion

In the current study, the matured animals were treated with IMI by daily oral gavage at doses comparable (0.5, 2 and 8 mg/kgBW/day) to the reported no observed adverse effect level (NOAEL) for 90 consecutive days. Our study revealed that ingestion of IMI led to decreased weights of reproductive organs, decreased epididymal sperm concentration and sperm motility along with increased sperm abnormalities, decreased level of serum T, decreased GSH level, disturbances in fatty acid composition, increase in apoptotic index in testicular tissue and fragmentation in the seminal DNA. To our knowledge, this is the first report on male reproductive toxicity of IMI.

The reduction in the weights of epididymis and accessory sex organs could possibly be explained by the significantly low T level measured in the rats treated with IMI, since the epididymis and accessory sex organs require a continuous androgenic stimulation for their normal growth and functions.[32] Underdeveloped epididymis and accessory sex organs were also reported for some other insecticides.[33, 34]

The current study showed deterioration in sperm motility of the rats treated with IMI at the highest dose. A decrease in sperm motility may seriously reduce fertilizing ability,[35] which is related to low level of ATP content.[36] For the normal forward movement of spermatozoa, full ATP pool is required and a slight deprivation of ATP leads to reduction in motility, which may cause infertility.[35] Therefore sperm motility can be hindered by altered enzymatic activities of oxidative phosphorolytic process, which is required for ATP production.[35] Akbarsha et al. suggested that cytoplasmic droplet is targeted by cytotoxic drugs and chemicals including insecticides and the impairment of sperm motility induced by insecticides might also be associated with the retention of the cytoplasmic droplet.[37]

Exposure of rats to NOAEL dose-levels of IMI, particularly 2 and 8 mg/kgBW/day, led to decline by nearly half in epididymal sperm concentration. The increased IMI dosage was correlated with the deteriorations in sperm properties. The decreased epidydimal sperm concentration and increased abnormal sperm morphology are common observations in the experimental studies with many other pesticides.[38, 39] Sperm count and sperm abnormality are

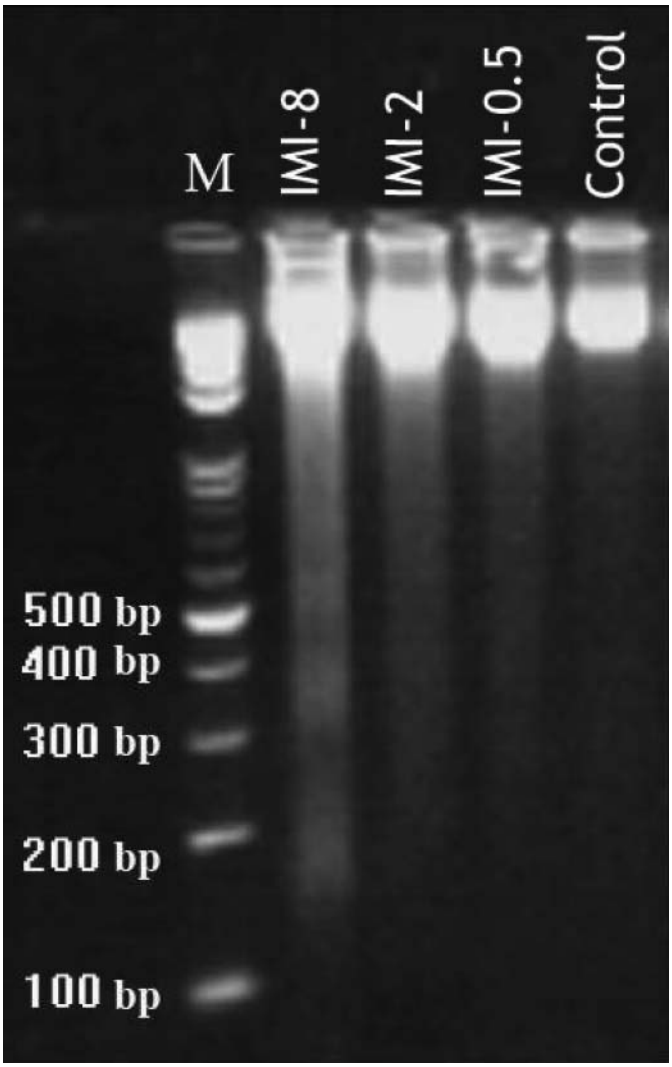

Fig. 3. Effects of IMI exposure at doses of 0.5, 2 and 8 mg/kg body weight (BW). IMI exposure on DNA fragmentation in adult male rats at any of these doses did not induce the cleavage of DNA into oligonucleosome-length fragments, a characteristic of apoptosis. Marker (M) = Mol weight standards; IMI 0.5 = 0.5 mg/kg body weight (BW); IMI-2 = 2 mg/kg body weight (BW); IMI-8 = 8 mg/kg body weight (BW).

the prominent factors affecting fertility.[40] Suppressions of gonadotrophins might be an underlying mechanism for the decreased sperm density.[41] Also, some toxicants may have direct effects on sertoli cell function, which is involved in the control of spermiation. The decrease in epididymal sperm concentration might also be because of either the inhibition of T biosynthesis as indicated by decreased T level or to apoptosis in spermatogenetic cells as indicated by increased number of TUNEL-positive cells in the current study.

Our results showed that serum T in the animals treated with IMI was reduced in a dose-dependent fashion. The reduction of T biosynthesis by IMI can be accounted for by decreased number of Leydig cells per testis and/or the inhibition of T biosynthesis by individual Leydig cells or the increased elimination of androgen. Mahgoub and, El-Medany[42] suggested that the changes in T-level in animal exposed to methomyl orally for two months may be due to a direct toxic effect of the insecticide or possibly through an alteration in the neuroendocrine environment.

Khokha et al. suggested that an increase in testicular lipid peroxidation leads to a decrease in the production of testicular androgens, since high levels of corticosterone produced during oxidative stress may result in the decline in T biosynthesis because this steroid is able to induce Leydig cell apoptosis.[43] It is also possible that the production of T is negatively affected by the prostaglandin F2a (PGF2a) level, which is synthesized from its precursor arachidonic acid by the inducible enzyme cyclooxygenase-2 (COX-2). PGF2a activates its own biosynthetic pathway by induction of COX-2 via protein kinase C in an autoamplification cascade.[44] Therefore, pesticides induce oxidative stress (OS) and disturb the conversion of arachidonic acid into PGF2a via COX-2. In addition, it has been shown that COX-2 and PGF2a are both inhibitors of Leydig cell steroidogenesis.[44]

The reduction in GSH level due to IMI treatment in the current study could reflect the adverse effect of IMI on the antioxidant system in testis. The decrease in GSH level could be the consequence of its increased rate of consumption within the lipophilic compartment of the interstitial cells. Similarly, generation of free radicals and activation of the antioxidant defense system in testicular tissue have been reported after exposure to toxic chemicals.[45,46] Free radicals are considered to play an important role in toxicity of pesticides and environmental chemicals.[47] The increase in ROS leads to damages to membranous structures of the mitochondrial and other cytoplasmic organelles through peroxidation of phospholipids, proteins and nucleotides. Spermatozoa and Leydig cells are richer in polyunsaturated fatty acids (PUFAs) with a prevalence of linoleic C18:2 n6, arachidonic C20:4 n6 and docosapentaenoic acids C22:5 n6[48] and are particularly susceptible to oxidative damage.[48] The mitochondrial and microsomal membranes consequently may undergo permeability changes following enhanced lipid peroxidation and glutathione depletion.[49]

Since MDA is a breakdown product of polyunsaturated fatty acids (PUFA), a measure of the level of MDA can be used as an indicator of lipid peroxidation.[50, 51, 52, 53] However, despite the significant decrease in GSH, the level of MDA did not appear to increase. This could be accounted for by that even though IMI treatment led to an increase in ROS level, this may be overcompensated for by the induced production of antioxidant and other enzymes.[54]

Treatment with IMI resulted in increased apoptosis of germ cells in the seminiferous tubules. The increased IMI dosage was correlated with the increased apoptotic index and the presence of fragmentation in the seminal DNA. There is an agreement that oxidative stress is associated

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with programmed cell death and thus wide range of pathological conditions including infertility.[31,51,55,56,57] This is consistent with the present study, since the level of GSH in the testicular tissue of rats treated with IMI decreased significantly. The low T level might also contribute to the increased level of apoptosis in the testis of animals treated with IMI, since it is the main hormone functioning for ensuring normal spermatogenesis and for inhibition of germ cell apoptosis.[58] Lu et al. suggested that saturated free fatty acids such as arachidonic acid and stearic acid induce apoptosis of the Leyding cells by ceramide production.[59] Consistently, in the current study, these saturated free fatty acids increased significantly. Therefore, the increase in arachidonic acid and stearic acid might partly explain the increased apoptosis in the germinal cell of IMI groups.

The fatty acid profile of testicular tissue of control rats is comparable to the one reported previously.[60] IMI exposure resulted in elevation of all fatty acids. Considering that IMI acts as agonist of nAChR, the effects of IMI on fatty acid composition might be inducted through nAChR. A similar disturbing effect on fatty acid profile were observed with nicotine administration in the brain.[61] Although high concentration of PUFA are needed for normal spermatogenesis and the androgenic activity of Leydig cells,[62] excessive PUFAs was shown to increase oxidative stress, as demonstrated in the heart.[63] Gutteridge et al. suggested that oxidative stress is correlated with the increased capacity for PUFA synthesis.[64] Excessive fatty acids, particularly arachidonic acid, can be cytotoxic and thereby induces apoptosis.[65] This might possibly be the reason for the increased apoptotic index induced by IMI exposure in the current study. Furthermore, excessive PUFA are suggested to have deleterious effects on T production.[66, 67]

In the testis of IMI administered rat, the decrease 20:4/20:3 and 20:4/18:2 ratios in our study indirectly indicate decreases activities of Δ5 desaturase and Δ6 desaturase and the increases in 16:1n-9/16:0 ratio indicates an increased activity of Δ9 desaturase. Similar drops in Δ5 and Δ6 desaturase activities in cultured Sertoli cells after T administration[68] and an increase in Δ9 desaturase activity in cultured rat hepatoma cells was reported.[34] Taken together, IMI appear to modify the fatty acid profile of testicular tissue by modulating the activity of fatty acid desaturases in the testicular tissue.

In conclusion, the present animal experiment revealed that treatment with IMI at NOAEL dose-levels caused deterioration in sperm parameters, decreased T level, increased apoptosis of germ cells, fragmentation of seminal DNA, the depletion of antioxidants and change in disturbance of fatty acid composition. All these changes indicate the suppression of testicular function. Therefore, we should be aware that IMI exposure may be toxic to the reproductive system and necessary precautions must be taken to minimize the harmful side effects of IMI to human and also animals aiming to avoid environmental pollution.

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