Drug and Chemical Toxicology

http://informahealthcare.com/dct
ISSN: 0148-0545 (print), 1525-6014 (electronic)

Drug Chem Toxicol, 2013; 36(4): 421–429
 DOI: 10.3109/01480545.2013.776575

informa healthcare

RESEARCH ARTICLE

Effects of the neonicotinoid insecticide, clothianidin, on the reproductive organ system in adult male rats

Ramazan Bal[1], Gaffari Türk[2], Mehmet Tuzcu[3], Ökkes Yılmaz[3], Tuncay Kuloğlu[4], Gıyasettin Baydaş[5], Mustafa Naziroğlu[6], Zabit Yener[7], Ebru Etem[8], and Zeynep Tuzcu[9]

[1]Department of Physiology, Faculty of Medicine, [2]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, [3]Department of Biology, Faculty of Science, [4]Department of Histology and Embryology, Faculty of Medicine, Firat University, Elazig, Turkey, [5]Bingol University Rectorate, Bingol University, Bingol, Turkey, [6]Department of Biophysics, Faculty of Medicine, Suleyman Demirel University, Isparta, Turkey, [7]Department of Pathology, Faculty of Veterinary Medicine, Yuzuncu Yil University, Van, Turkey, [8]Department of Medical Biology, Faculty of Medicine, Firat University, Elazig, Turkey, and [9]Department of Biology, Faculty of Science, Bingol University, Bingol, Turkey

Abstract

Clothianidin (CTD) is a novel, broad-spectrum insecticide. In the current study, it was aimed to study the effect of subchronic exposure to low doses of CTD (2, 8 and 24 mg/kg body weight/day) on the reproductive system in adult rats. CTD treatment did not significantly change serum testosterone level or sperm parameters (e.g. concentration, motility and morphology), but caused significant decreases in weights of epididymis, right cauda epididymis and seminal vesicles. CTD treatment did not cause sperm DNA fragmentation and did not change the apoptotic index in the seminiferous tubules and levels of α-tocopherol and glutathione, but increased the level of thiobarbituric acid-reactive substances and cholesterol levels significantly at all doses. CTD exposure caused significant elevations in palmitic, linoleic and arachidonic acids in testis in all CTD-exposed groups. There was a drop in 20:4/18:2 (arachidonic acid/linoleic acid) ratio and an increase in 18:1*n-9*/18:0 (oleic acid/stearic acid) ratios in all CTD groups, in comparison to the control group. In conclusion, CTD had little detectable detrimental effects on the reproductive system of male rats over the measured parameters.

Keywords

Apoptosis, clothianidin, DNA fragmentation, fatty acid composition, lipid peroxidation, sperm characteristics

History

Received 5 April 2012
Revised 9 July 2012
Accepted 14 December 2012
Published online 25 March 2013

Introduction

Clothianidin [(*E*)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine] (CTD) is a novel, highly effective, broad-spectrum systemic and contact insecticide that functions as a neonicotinoid (Tomizawa & Casida, 2005). CTD, as well as other neonicotinoids, acts as an agonist in the postsynaptic nicotinic acetylcholine receptors (nAChRs) at much lower concentrations in insects than in mammals, resulting in dysfunction of the nervous system, immobilization, or death (Tomizawa et al., 2000). Neonicotinoids show higher affinity for insect nAChRs, accounting, at least in part, for their selective toxicity to insects over vertebrates and are considered far less toxic to mammals, when compared to invertebrates (Tomizawa & Casida, 2005; Tomizawa et al., 2000). Electrophysiological studies performed in the neurons of cochlear nucleus neurons, whose ionic channels are characterized (Oertel et al., 2011), show that sensitivity of mammalian nAChRs to neonicotinoids are far less, compared to insect nAChRs (Bal et al., 2010). However, because some nAChR subunits are expressed in human testis (α5 and β4) and prostate (α5; Flora et al., 2000), and also in mouse testis and sperm (α7; Bray et al., 2005), any drug aimed at the nicotinic system may have multiple effects outside the central nervous system in mammals.

Free radicals are highly reactive molecules with one or more unpaired electron(s) that include reactive oxygen species (ROS) and reactive nitrogen species. They are commonly found in testis and seminal plasma, and they physiologically control sperm maturation, capacitation, and hyperactivation, the acrosome reaction, and sperm-oocyte fusion. Pathologically, when free radicals are generated excessively, they can induce lipid peroxidation (LPO), DNA damage, apoptosis, and antioxidant depletion. Antioxidants are compounds [e.g. glutathione (GSH), α-tocopherol, selenium and ascorbic acid] or enzymes (glutathione-peroxidase, superoxide dismutase and catalase) acting as free radical scavengers that help to keep free radicals at homeostatic levels to maintain physiologic function and prevent pathological effects resulting from the development of oxidative stress (OS), which is an imbalance between ROS and scavenging properties of antioxidants. The sperm plasma membrane is largely composed of polyunsaturated fatty acids (PUFAs), which are susceptible to oxidative damage resulting

Address for correspondence: Gaffari Türk, Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Fırat University, 23119 Elazığ, Turkey. Fax: +90 424 238 81 73. E-mail: gturk@firat.edu.tr

from the existence of double bonds. When the LPO cascade proceeds in sperm, a major part of the PUFAs is destroyed. LPO affects membrane structure and function, such as fluidity, ion gradients, receptor transduction, transport processes and membrane enzymes. As a result, functions, which are necessary for fertilization, are impaired (Kothari et al., 2010). In addition, cholesterol is involved in steroidogenesis in testes; however, an increased level of cholesterol in testes is attributed to decreased androgen concentration, which results in impaired spermatogenesis (Yamamoto et al., 1999).

Pharmacokinetics studies indicate that CTD is rapidly distributed into all tissues and organs within 2 hours after a single oral administration at the low dose (5 mg/kg), and its excretion and metabolism starts immediately after absorption. The concentration of CTD in tissues and organs declines rapidly, and therefore it does not accumulate in tissues and organs, including the testis (Yokota et al., 2003). There are very limited reports related to effects of the neonicotinoids, especially CTD, on male reproduction. For example; Tanaka (2012) has reported that CTD administration through diet to female mice during the gestation period has no effect on selected reproductive parameters, including litter size, litter weight, or sex ratio at birth. However, in our previous study (Bal et al., 2012a), we demonstrated that CTD exposure at the no observed adverse effect level (NOAEL) dose (32 mg/kg) daily for 90 days caused significant decreases in reproductive organ weights, sperm concentration, testicular GSH level, and significant increases in some testicular fatty acid composition, cholesterol level, apoptotic germ cells, and sperm DNA fragmentation in developing male rats. In our other study (Bal et al., 2012b), we also found that the nicotinoid, imidacloprid, when administered at NOAEL dose levels, leads to testicular dysfunction, including deteriorated sperm quality, decreased testosterone level, increased apoptotic germ cells, increased sperm DNA fragmentation, and disturbed oxidant/antioxidant balance and fatty acid composition in adult male rats. However, there is no information about the effects of CTD at or below NOAEL doses on adult mammalian reproductive functions, despite their widespread use. Therefore, in the present study, the aim was to study the testicular toxicity of CDT at low doses [2, 8 and 24 mg/kg body weight (b.w.)/day] in adult male rats after daily oral administration for 90 consecutive days.

Methods

Animals and experimental design

Experimental protocols were approved by the local animal use committees of Firat University (Elazig, Turkey). Animal care and experimental protocols complied with the National Institutes of Health (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 (8–9 weeks old) were obtained from and maintained in the Firat University Experimental Research Center (Elazig, Turkey). Animals were housed in polycarbonate cages in a room with a 12-hour day/night cycle, a temperature of $24 \pm 3\,^{\circ}C$, and humidity of 45–65%. 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

Animals were randomly divided into four groups with 6 animals in each group. CTD (DANTOTSU®) was obtained from the Sumitomo Chemical Co. Ltd. (Tokyo, Japan), which was dissolved in water easily. Different doses of CTD used in this study were separately dissolved in 1 mL of distilled water. A 1 mL/kg b.w. dose volume was administered to rats. The first group was taken as the control, which received only distilled water (not containing CTD) once-daily for 3 months by oral gavage. Treatment groups received CTD at a dose of 2 mg/kg b.w. (group CTD-2), 8 mg/kg b.w. (group CTD-8) and 24 mg/kg b.w. (group CTD-24) once-daily for 3 months by oral gavage.

The maximum treatment dose (24 mg/kg b.w.) in the present study was chosen to be less than the reported NOAEL on reproduction and fertility in male rats (27.9–32.0 mg/kg/day) for 90-day treatment (Federal Register, 2003). The dose was adjusted daily according to body-weight changes of individual animals. To see the maximum effect of any drug on sperm quality, at least 55–59 days are necessary because the spermatogenic cycle, including spermatocytogenesis, meiosis, and spermiogenesis, is 48–52 days (Türk et al., 2010) and epididymal transit of sperm is approximately 1 week (Kempinas et al., 1998) in rats. The treatment period in the present study was therefore set at 90 days for maximum effect.

Sample collection and homogenate preparation

After animals were decapitated under ether anesthesia at the end of the third month, blood was collected and testis, epididymis, seminal vesicles, and ventral prostate were removed, cleared of adhering connective tissue, and weighed. Right testicles were fixed with Bouin's fluid. Left testicles were frozen in liquid nitrogen and stored at $-70\,^{\circ}C$ until use for thiobarbituric acid reactive substances (TBARS), GSH, fatty acids, cholesterol and α-tocopherol analyses. Serum was separated and also stored at $-70\,^{\circ}C$ until use to estimate some biochemical parameters using the appropriate kits (Boehringer, Mannheim, Germany).

Localization of apoptotic cells in the testis

Localization of apoptotic cell death in spermatogenic cells was defined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA). Briefly, fixed testicular tissue was embedded in paraffin and sectioned at 4 μm. Paraffin sections were deparaffinized in xylene, dehydrated through graded alcohol, and washed in phosphate-buffered saline (PBS). Sections were treated with 0.05% proteinase K for 5 minutes, which was followed by treatment with 3% hydrogen peroxide for 5 minutes 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 hour in a humidified chamber, and then stop/wash buffer was applied for 30 minutes at 37 °C. Sections were visualized with diaminobenzidine (DAB) substrate. Negative controls were performed using distilled water in place of the TdT enzyme. Sections were counterstained with Mayer's hematoxylin, dehydrated in graded alcohol, and cleared. To estimate the apoptotic index (AI), TUNEL-positive cells in seminiferous tubules (100 per animal) in 20 randomly chosen fields were counted. The AI was calculated as the percentage of cells with TUNEL positivity.

Sperm analyses

All sperm analyses were performed by using the methods described by Türk et al. (2008). Epididymal sperm concentration in the right cauda epididymal tissue was determined with a hemocytometer. Freshly isolated left cauda epididymal tissue was used for the analysis of sperm motility. Percentage sperm motility was evaluated using a light microscope a with heated stage. To determine the percentage of morphologically abnormal spermatozoa, slides stained with eosin-nigrosin (1.67% eosin, 10% nigrosin and 0.1 M of sodium citrate) were prepared. Slides were then viewed under a light microscope at 400× magnification. A total of 300 spermatozoa were examined on each slide (1800 cells in each group), and the head, tail, and total abnormality rates of spermatozoa were expressed as a percentage. All sperm analyzes were made in a blinded fashion.

Determination of TBARS and GSH level in tissue samples

Concentration of TBARS in tissue samples was estimated by the method of Niehaus & Samuelsson (1968). In brief, 1 mL of tissue homogenate (supernatant; Tris-HCl buffer, pH 7.5) was mixed with 2 mL of (1:l:1 ratio) thiobarbituric acid (TBA)/trichloroacetic acid (TCA)/HC1 reagent (0.37% TBA, 0.25 N of HCI and 15% TCA) and placed in a water bath for 60 minutes, cooled, and centrifuged at room temperature for 10 minutes. TBARS were determined by reading the fluorescence detector set at λ (excitation) = 515 nm and λ (emission) = 543 nm. TBARS were calculated from a calibration curve using 1,1,3,3-tetraethoxypropane as the standard. The TBARs level was analyzed using the high-performance liquid chromatography (HPLC) equipment. The equipment consisted of a pump (LC-10ADvp), a fluorescence detector (RF-10XL), a column oven (CTO-10ASvp), an autosampler (SIL-10ADvp) a degasser unit (DGU-14A), and a computer system with class VP software (Shimadzu, Kyoto, Japan). An 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 (de las Heras et al., 2003). Values are expressed as nmol/g tissue.

Reduced GSH was determined by the method of Ellman (1959). Briefly 1 mL of tissue homogenate was treated with 1 mL of 5% metaphosphoric acid (Sigma, St. Louis, MO); the mixture was centrifuged at 5000 rpm, and the supernatant was taken. After deproteinization, the supernatant was allowed to react with 1 mL of Ellman's reagent [30 mM of 5,5′-dithiobis(2-nitrobenzoic acid) in 100 mL of 0.1% sodium citrate]. Absorbance of the yellow product was read at 412 nm in the spectrophotometer. Pure GSH was used as the standard for establishing the calibration curve (Akerboom & Sies, 1981).

Lipid extraction

Lipids in tissue samples were extracted with hexane-isopropanol (3:2, v/v) by the method of Hara & Radin (1978). One gram of tissue sample was homogenized with a 10-mL hexane-isopropanol mixture. Fatty acids in lipid extracts were converted into methyl esters, including 2% sulphuric acid (v/v), in methanol (Christie, 1992). Fatty acid methyl esters were extracted with 5 mL of *n*-hexane. Analysis of fatty acid methyl esters was performed in a Shimadzu GC-17A instrument gas chromatograph (Kyoto, Japan) equipped with a flame ionization detector (FID) and a (25 m, 0.25 mm i.d.) Permabond fused-silica capillary column (Machery-Nagel GmbH & Co. KG, Düren, Germany). The oven temperature was programmed between 145 and 215 °C, 4 °C/min. Injector and FID temperatures were 240 and 280 °C, respectively. The nitrogen carrier gas flow rate was 1 mL/min. 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; Shimadzu) was used to process the data. Results are expressed as µg/g tissue.

Saponification and extraction

Alpha-tocopherol and cholesterol were extracted from lipid extracts by the method of Sanchez-Machado et al. (2004), with minor modifications. Five milliliters of *n*-hexane/isopropyl alcohol mixture were treated with 5 mL of KOH solution (0.5 M in methanol), which was then immediately vortexed for 20 seconds. Tubes were placed in a water bath at 80 °C for 15 minutes. After cooling in iced water, 1 mL of distilled water and 5 mL of hexane were added, and the mixture was rapidly vortexed for 1 minute, then centrifuged for 5 minutes at 5000 rpm. The supernatant phase was transferred to another test tube and dried under nitrogen. Residue was redissolved in 1 mL of the HPLC mobile phase (methanol/acetonitrile/water; 68:28:4, v/v/v). Finally, an aliquot of 20 µL was injected into the HPLC column. Before injection, extracts were maintained at −20 °C away from light.

Chromatographic conditions

Chromatographic analysis was performed using an analytical scale (15 × 0.45 cm i.d.) Supelco LC 18 DB column with a particle size of 5 µm (Sigma-Aldrich). HPLC conditions were as follows: mobile phase of acetonitrile/methanol/water (60:38:2, v/v/v); flow rate of 1 mL/min; and column temperature at 30 °C. 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 (Lopez-Cervantes et al., 2006).

Serum testosterone

Serum testosterone level was measured by the enzyme-linked immunosorbent assay (ELISA) method using a DRG ELISA testosterone kit (ELISA EIA-1559, 96 Wells kit; DRG Instruments GmbH, Marburg, Germany), according to the standard protocol supplied by the kit manufacturer. Sensitivity of the kit was 0.083–16.000 ng/mL, and the intra-assay coefficient of variation of the kit was 4.16%.

Analysis of sperm DNA fragmentation

Sperm DNA fragmentation was determined by a modification of a previously described procedure (Wang et al., 2003). The right epididymal tissue-fluid mixture was filtered by nylon mesh to separate the supernatant from tissue particles and stored at −20 °C until use. Supernatant fluid containing all epididymal spermatozoa was then thawed at room temperature and homogenized in lysis buffer containing 50 mM of Tris-HCl (pH 8.0), 10 mM of ethylenediaminetetraacetic acid (EDTA), 0.5% (w/v) sodium dodecyl sulfate, 1% 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 hour at 65 °C. After centrifugation at $12\,000 \times g$ at 4 °C for 20 minutes, the supernatant was extracted with phenol and chloroform, and DNA was precipitated by 100% ethanol, 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.

Statistical analysis

One-way analysis of variance and Tukey's post-hoc honestly significant difference test were used to determine differences between groups in all parameters. Results are presented as mean ± standard error of the mean (SEM). Values were considered statistically significant if $p < 0.05$. The SPSS/PC program (version 15.0; SPSS, Inc., Chicago, IL) was used for the statistical analysis.

Results

Body-weight gain

Table 1 demonstrates the effects of CTD at the doses of 2, 8 and 24 mg/kg b.w. on body-weight gain (final body weight minus initial body weight) of male rats. CTD administration did not have any significant effect on body-weight gain.

Reproductive organ weights

Table 2 shows absolute and relative organ weights of testis, epididymis, right cauda epididymis, seminal vesicles, and prostate of control and CTD groups. Absolute weights of seminal vesicles of the CTD-2 group, epididymis and right cauda epididymis of the CTD-8 group, and epididymis of the CTD-24 group were significantly less than those of control ($p < 0.05$). Similarly, significant decreases were observed in the relative weights of epididymis (in all treatment groups), right cauda epididymis (in only the CTD-8 group) and seminal vesicles (in both the CTD-2 and CTD-8 groups) ($p < 0.05$).

Table 1. Effect of CTD on body-weight gain.

Groups	Initial body weight (g)	Final body weight (g)	Body-weight gain (g)
Control	166.5 ± 2.2	311.5 ± 4.8	145.0 ± 4.7
CTD-2	168.5 ± 2.4	317.0 ± 5.9	147.3 ± 6.6
CTD-8	161.3 ± 3.1	296.2 ± 4.6	134.8 ± 5.9
CTD-24	164.2 ± 1.7	302.3 ± 11.2	138.2 ± 12.2

Data are expressed as mean ± SEM.

Epididymal sperm characteristics

Epididymal sperm characteristics of control and CTD-administered rats are presented in Table 3. Although all CTD treatments caused a numerical decrease in sperm motility and concentration, and a numerical increase in abnormal sperm rate, these differences did not reach statistical significance.

Apoptotic cells in the testis

Apoptosis in the testis from control and CTD groups, demonstrated by TUNEL staining, are shown in Figure 1. TUNEL-positive cells were rarely observed in the testis of control rats (Figure 1A). The number of TUNEL-positive cells appeared to increase in the testis of CTD groups (Figure 1D–F) as a dose-dependent manner. But, increases in the AI were not significant in the CTD-2, CTD-8 and CTD-24 groups (0.43 ± 0.21%, 0.57 ± 0.29% and 1.16 ± 0.73%, respectively), compared to control (0.23 ± 0.23%).

Sperm DNA fragmentation

Apoptotic cells often contain fragmental DNA, which can be visualized by DNA agarose gel electrophoresis. Therefore, we have used DNA fragmentation as the criterion for apoptosis. DNA isolated from the spermatozoa of rats exposed to CTD at doses of 2, 8 and 24 mg/kg b.w. for 3 months showed no sign of degradation into oligonucleotide fragments, forming a clear laddering pattern of apoptosis when separated by 2% agarose gel electrophoresis (Figure 2).

Biochemical parameters

Levels of serum testosterone, testicular tissue LPO (TBARS), and antioxidant substances (GSH and α-tocopherol), fatty acid composition (palmitic, palmitoleic, stearic, oleic, linoleic, dihomo-γ-linolenic, arachidonic, and docosapentaenoic acids), and cholesterol are presented in Table 4. There were no significant differences in serum testosterone level between control and CTD groups. The level of LPO, measured as TBARS level, increased significantly ($p < 0.01$) in all treatment groups, but the level of GSH did not change significantly when compared to the control group.

Administration of CTD had limited effects on fatty acid composition of testicular tissue. CTD administration at 2, 8 and 24 mg/kg b.w. resulted in increases in palmitic ($p < 0.05$), linoleic ($p < 0.05$ and $p < 0.01$) and arachidonic acid ($p < 0.05$) levels. In addition, in the testis of rats treated with CTD at 2, 8 and 24 mg/kg b.w., decreases in 20:4/18:2 ratio (∼4.1, ∼3.6 and ∼3.4, respectively), when compared to control (∼5), and increases in 18:1*n-9*/18:0 ratio (∼1.8,

~1.7 and ~2.2, respectively), when compared to control (~1.6), were also noted. CTD administration at doses of 2, 8 and 24 mg/kg b.w. caused a significant increase in cholesterol level, in comparison to the control group ($p < 0.01$). All CTD treatments did not significantly change α-tocopherol levels.

Discussion

To our knowledge, this is the first report about the effects of the recently marketed neonicotinoid insecticide, CTD, on the reproductive organ system of adult male rats. The results presented in the current study demonstrate that ingestion of CTD for 3 months induced some adverse effects, to limited extents, on the male reproductive system in rats. However, several findings, such as changes in reproductive organ weights, testicular tissue fatty acid composition, and cholesterol, were found to be significant in this study.

The statistically significant and also insignificant decreases in absolute and relative weights of epididymis and seminal vesicles were determined in a non-dose-dependent manner after CTD administration in the current study. Reductions in reproductive organ weights were reported for CTD-treated rats, exposed during the developmental stage (Bal et al., 2012a), and imidacloprid (another neonicotinoid) in adult male rats (Bal et al., 2012b; Najafi et al., 2010). Reductions in organ weights are usually explained by decreased levels of serum testosterone, because testosterone is the major regulator of normal growth of these organs (Mallick et al., 2007). In the current study, the level of testosterone tended to decrease, but the decrease did not reach to a significant level.

It is known that increased levels of ROS can be detrimental to testicular function. To overcome this, the testis is equipped with a very potent antioxidant system that protects it from the damaging effects of ROS. The enzymatic and nonenzymatic antioxidants help the testis by counteracting any oxidative effect (Aitken & Roman, 2008; Kothari et al., 2010). Significant increase in TBARS level and nonsignificant change in GSH level observed in all CTD groups in the present study, for which these results are inconsistent with the findings (Bal et al., 2012a,b) of our previous studies (nonsignificant increase in TBARS level and significant decrease in GSH level after CTD and imidacloprid exposure), shows that CTD causes an imbalance in oxidant/antioxidant status and LPO by enhancing ROS generation in testicular tissue of adult male rats.

Spermatozoa and Leydig cells in mammals are rich in PUFAs and are more susceptible to oxidative damage, resulting in impairment of sperm. ROS can attack the unsaturated bonds of lipids of the sperm membrane and destroys the structure of lipid matrix in the membranes of spermatozoa and is associated with rapid loss of intracellular adenosine triphosphate, leading to axonemal damage, decreased sperm viability and increased mid-piece morphological defects, and completely inhibits spermatogenesis in extreme cases (Aitken & Roman, 2008; Kothari et al., 2010). In addition, there are nAChR subunits, which, together with distinct regulatory elements, may contribute to different functional and developmental requirements of non-neuronal organs and cells, including testis, prostate, and sperm (Bray et al., 2005; Flora et al., 2000). It has been reported (Bray et al., 2005) that sperm nAChRs play a central role in the control of motility associated with calcium influx mechanisms, and mice deficient in the nAChR subunit, α7, produce impaired sperm motility. Li et al. (2011) have also reported that imidacloprid, but not CTD, strongly inhibits the α4 and β2 subunits of nAChRs activated by acetylcholine in human embryonic kidney cells. Therefore, any drug-affected nicotinic system may cause disturbed function of testicular tissue

Table 2. Absolute and relative weights of reproductive organs, including testis, epididymis, right cauda epididymis, vesicula seminalis and prostate.

Parameters	Absolute weight (mg)					Relative weight (mg/g b.w.)				
Groups	Testis	Epididymis	Right cauda epididymis	Seminal vesicles	Ventral prostate	Testis	Epididymis	Right cauda epididymis	Seminal vesicles	Ventral prostate
Control	1483.3 ± 19.5	570.8 ± 6.1[a]	233.3 ± 6.7[a]	991.7 ± 48.5[a]	508.3 ± 36.6	5.13 ± 0.17	1.97 ± 0.07[a]	0.80 ± 0.04[a]	3.43 ± 0.21[a]	1.74 ± 0.10
CTD-2	1473.3 ± 49.8	511.7 ± 12.0[a,b]	201.7 ± 12.0[a,b]	638.6 ± 67.3[b]	393.3 ± 28.3	4.60 ± 0.10	1.60 ± 0.03[b]	0.62 ± 0.04[ab]	2.02 ± 0.23[b]	1.24 ± 0.11
CTD-8	1380.5 ± 77.6	464.0 ± 42.6[b]	160.0 ± 26.7[b]	643.3 ± 115.9[a,b]	430.4 ± 69.9	4.65 ± 0.28	1.56 ± 0.16[b]	0.53 ± 0.09[b]	2.16 ± 0.39[b]	1.45 ± 0.23
CTD-24	1336.3 ± 80.1	463.8 ± 29.9[b]	177.5 ± 15.5[ab]	752.5 ± 151.1[a,b]	357.5 ± 37.9	4.48 ± 0.04	1.55 ± 0.03[b]	0.59 ± 0.03[a,b]	2.48 ± 0.37[a,b]	1.18 ± 0.08

Mean differences between values bearing different superscript letters within the same row are statistically significant ($p < 0.05$). Data are expressed as mean ± SEM.

Table 3. Effect of CTD on sperm parameters.

Groups	Sperm motility (%)	Epididymal sperm concentration (million/cauda epididymis)	Abnormal sperm rate (%)		
			Head	Tail	Total
Control	70.6 ± 3.3	90.8 ± 4.7	4.7 ± 1.1	4.5 ± 1.1	9.2 ± 0.8
CTD-2	53.3 ± 4.2	76.2 ± 5.3	5.5 ± 0.8	6.2 ± 2.1	11.7 ± 1.8
CTD-8	64.0 ± 8.1	61.2 ± 17.3	13.5 ± 6.7	8.3 ± 1.5	21.8 ± 8.0
CTD-24	50.0 ± 7.1	57.3 ± 6.6	15.8 ± 3.3	6.2 ± 0.8	22.0 ± 3.1

Data are expressed as mean ± SEM.

and sperm. Although sperm motility and epididymal sperm concentration decreased and the rate of abnormal sperm increased after administration of different CTD doses, they were not statistically significant in this study. In our previous studies (Bal et al., 2012a,b), we observed that developing rats exposed to CTD at the NOAEL dose had significantly decreased sperm concentration and significantly increased abnormal sperm rate, and mature rats exposed to imidacloprid had significantly decreased sperm concentration and motility as well as significantly increased abnormal sperm rate. The decrease in epididymal sperm concentration and sperm motility as well as the increase in abnormal sperm rate, though not significant, appeared to be explained by increased LPO, as evidenced by the increase in TBARS level after CTD administration in this study. In addition to OS hypothesis, a direct effect of CTD on sperm nAChR subunits may be responsible for the nonsignificant disturbed sperm quality parameters observed in this study.

The AI of the testis of control rats was comparable to previous reports (Bal et al., 2012a,b). There were increases in the number of TUNEL-positive cells, but not significantly, in the germinal epithelium of the testis of rats exposed to CTD for 90 consecutive days in a dose-dependent manner. It has been reported (Ateşşahin et al., 2010; Sönmez et al., 2011) that an increase in apoptotic germ cells is shown after exposure of different environmental pollutants associated with increased LPO. Bal et al. (2012a) have reported that only 32 mg/kg of NOAEL dose of CTD, but not lower than the NOAEL dose (2 and 8 mg/kg), caused a significant increase in AI in the testis of developing rats. Similarly, only the NOAEL, but not lower than the NOAEL, dose imidacloprid administration has been reported to increase the testicular AI in adult rats (Bal et al., 2012b). Our findings are in agreement with the above-mentioned reports. The finding that no noticeable DNA fragmentation from spermatozoa of rats exposed to CTD was observed is not surprising, because the

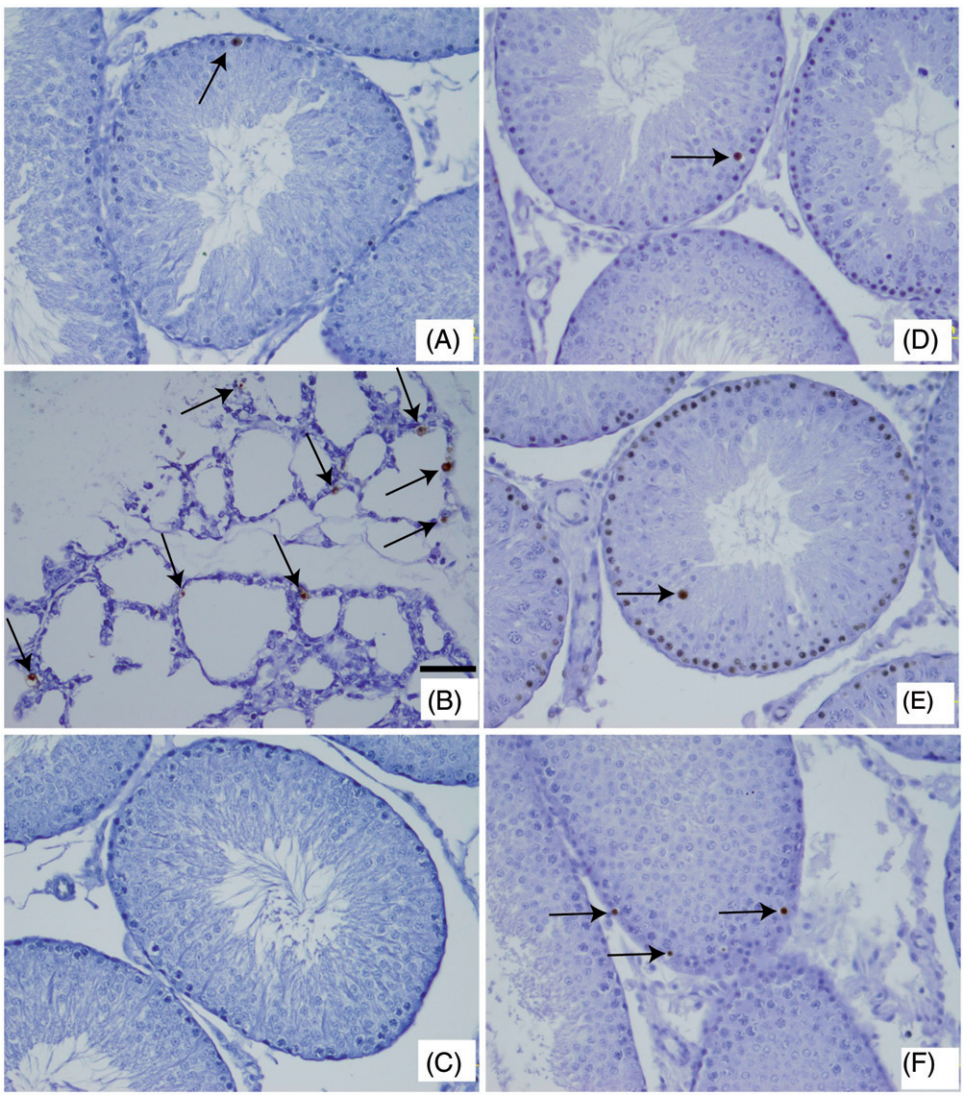

Figure 1. Representative photomicrographs of TUNEL staining in testes of control (A), CTD-2 (D), CTD-8 (E) and CTD-24 groups (F). Arrows indicate candidate apoptotic cells. TUNEL-positive spermatogenic cells had the typical morphological features of apoptosis, including chromatin condensation, cytoplasmic budding and apoptotic bodies. (C) Negative staining control is also illustrated to ensure that the staining method is working well. To control for nonspecific incorporation of nucleotides or for nonspecific binding of enzyme conjugate, negative control staining was performed without active TdT, but including proteinase K digestion. Note that there were no detectable signals in the negative control. (B) Positive control: TUNEL-stained cells in the normal female rodent mammary gland tissue where continuous apoptosis takes place. Note that there are plenty of TUNEL-positive cells. Calibration bar: 50 μm.

increase in the number of TUNEL-positive cells in the germinal epithelium of the testis of rats exposed to CTD was insignificant. Underlying mechanisms of the insignificant increase in apoptosis in a dose-dependent manner could also be the increased LPO, as evidenced by a significant increase in TBARS level in this study, or the direct effect of CTD on testicular nAChRs.

Administration of CTD resulted in an elevation of all fatty acids analyzed, but the increase was significant only in linoleic, arachidonic, and palmitic acids in the testis of rats in the present study. This finding is compatible with the results of our previous studies that both CTD (Bal et al., 2012a) and imidacloprid (Bal et al., 2012b) at or below NOAEL dose levels caused significant increases in some fatty acid compositions. Arachidonic acid is found to be cytotoxic *in vitro*, and arachidonic acid-induced apoptosis has been characterized in various cell models (Cao et al., 2000). In addition, increased levels of saturated free fatty acids, such as palmitic acid, also potentiates apoptosis of Leydig cells by ceramide production (Lu et al., 2003). It is well known that fatty acids in the membranes of the organelles in the testis are largely constituted by PUFAs with a prevalence of linoleic (C18:2*n6*), arachidonic (C20:4*n6*) and docosapentaenoic acids (C22:5*n6*) in the testis (Davis & Coniglio, 1966), which are needed for normal spermatogenesis and androgenic activity (Kimura, 1986; Solano et al., 1988). However, excessive PUFAs make the microsomes and mitochondria of rat testis susceptible to LPO degradation and associated free radical effects (Rice-Evans & Burdon, 1993) and may increase OS, as demonstrated in the heart (Diniz et al., 2004). Therefore, increases in α-tocopherol in the testis of CTD-treated rats, though not significant, may be indicative of a compensation mechanism for increased OS for reducing the susceptibility of tissue PUFA to LPO. Although the reason for the increase observed in fatty acids after CTD treatment is exactly unknown, their nonutilization, which leads to disturbed spermatogenesis and thereby decreased sperm concentration, may be responsible for the increased levels of PUFAs.

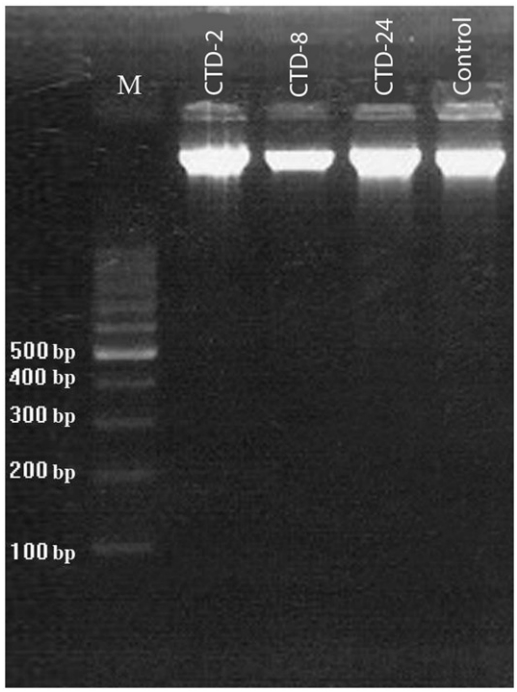

Figure 2. Effects of CTD exposure at doses of 2, 8 and 24 mg/kg b.w. CTD exposure on DNA fragmentation in adult male rats at any of these doses did not induce the cleavage of DNA into oligonucleosome-length fragments, a characteristic of apoptosis. Marker (M), Mol weight standards; Cont, control; CTD-2, 2 mg/kg b.w.; CTD-8, 8 mg/kg b.w.; CTD-24, 24 mg/kg b.w.

In the testis of rats treated with CTD, the decrease in the *n-6* fatty acid (20:4/18:2) ratios in our study indirectly shows a decreased activity of Δ6 desaturase, and the increases in 18:1*n-9*/18:0 ratios indicate an increased activity of Δ9 desaturase. This finding is compatible with the results of our previous studies (Bal et al., 2012a,b). Similarly, in cultured Sertoli cells, treatment with testosterone caused a drop in Δ5 and Δ6 desaturase activities (Hurtado de Catalfo & de Gomez Dumm, 2005). Marra & de Alaniz (1989) demonstrated an increase of Δ9 desaturase activity produced by testosterone in rat hepatoma cells in culture and in livers microsomal fraction of rats. With the presented data, it is fair to suggest that CTD

Table 4. Effect of CTD on levels of serum testosterone and testicular tissue TBARS, GSH, cholesterol, α-tocopherol and some fatty acids.

	Control	CTD-2	CTD-8	CTD-24
Testosterone (ng/mL)	1.2 ± 0.2	1.0 ± 0.6	1.1 ± 0.5	0.9 ± 0.2
TBARS (nmol/g tissue)	6.7 ± 0.2[a]	8.9 ± 0.4[b,†]	8.4 ± 0.4[b,†]	8.8 ± 0.3[b,†]
GSH (μg/g tissue)	543.3 ± 23.2	572.7 ± 3.3	522.3 ± 28.5	501.8 ± 33.5
Palmitic acid (16:0) (μg/g tissue)	3329.9 ± 154.7[a]	5257.0 ± 460.8[b,*]	5396.5 ± 265.2[c,*]	5488.2 ± 754.1[d,*]
Palmitoleic acid (16:1) (μg/g tissue)	133.4 ± 35.5	144.3 ± 35.5	162.3 ± 26.5	138.0 ± 57.8
Stearic acid (18:0) (μg/g tissue)	704.9 ± 94.6	871.5 ± 35.9	982.7 ± 122.8	767.0 ± 68.3
Oleic acid (18:1) (μg/g tissue)	1122.8 ± 99.6	1574.2 ± 86.5	1666.2 ± 218.3	1697.6 ± 142.0
Linoleic acid (18:2*n6*) (μg/g tissue)	348.7 ± 15.9[a]	618.3 ± 65.2[b,*]	743.3 ± 110.9[b,†]	682.3 ± 49.1[b,*]
Dihomo-γ-linolenic acid (20:3*n6*) (μg/g tissue)	99.6 ± 16.2	157.6 ± 15.9	165.0 ± 19.7	136.0 ± 33.3
Arachidonic acid (20:4*n6*) (μg/g tissue)	1741.8 ± 46.9[a]	2533.2 ± 92.6[b,*]	2686.7 ± 81.5[b,*]	2349.0 ± 66.5[b,*]
Docosapentaenoic acid (22:5*n6*) (μg/g tissue)	1869.1 ± 148.3	2904.3 ± 223.4	3095.1 ± 389.7	2771.7 ± 178.8
Total lipid (μg/g tissue)	9514.7 ± 442.8	15 118.5 ± 1274.8	16 258.6 ± 2024.4	13 906.5 ± 1161.8
Cholesterol (mg/g tissue)	0.61 ± 0.01[a]	0.77 ± 0.03[b,†]	0.84 ± 0.03[b,†]	0.86 ± 0.02[b,†]
α-tocopherol (μg/g tissue)	33.4 ± 6.9	34.9 ± 0.7	42.3 ± 2.1	40.3 ± 2.4

Fatty acids are identified by number of carbon atoms in the chain is given first; value following the colon represents number or double bonds (0 means saturated fatty acid); number following n indicates the position of the last double bond counting the double bond from the terminal methyl group. Mean differences between values bearing different superscripted letters within the same row are statistically significant ($^{*}p < 0.05$ versus control and $^{\dagger}p < 0.01$, compared to control). Data are expressed as mean ± SEM.

modifies PUFA biosynthesis, modulating Δ6 and Δ9 desaturase activity in testicular tissue.

After treatment of male rats for 90 days with various doses of CTD, cholesterol level was significantly increased in the testis. Cholesterol is the main precursor for steroidogenesis and is produced mostly in the liver from low-density lipoprotein and high-density lipoprotein. Increase of cholesterol level in testicular tissue may result from its nonutilization for the production of testosterone, as indicated by the insignificant decrease in testosterone level in this study, as was also observed by Bal et al. (2012a). In addition, Yamamoto et al. (1999) have reported that high testicular cholesterol concentration has a detrimental effect on Leydig and Sertoli cell secretory function, spermatogenesis, the epididymal sperm maturation process (decreased sperm motility and concentration), and overall sperm fertilizing capacity. One of the reasons for the insignificant decrease in sperm motility and concentration and insignificant increase in abnormal sperm rate after exposure of different doses of CTD in the present study may be because of the CTD-induced increased testicular cholesterol concentration that negatively affects spermatogenesis.

Conclusion

In conclusion, exposure to CTD appeared to have detectable effects on the reproductive organs, including decreased weights of epididymis and seminal vesicles and disturbances in fatty acid composition, cholesterol, and LPO of testicular tissue. However, its effects on sperm quality, testicular apoptosis, and sperm DNA fragmentation were not notable. Therefore, it is concluded that exposure of rats to CTD at below NOAEL dose levels for 90 consecutive days are weakly toxic to reproductive organ systems.

Declaration of interest

The authors report no financial conflicts of interest. The authors alone are responsible for the content and writing of this paper.

References

Aitken RJ, Roman SD. (2008). Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longev 1:15–24.

Akerboom TP, Sies H. (1981). Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. Methods Enzymol 77:373–82.

Ateşşahin A, Türk G, Yılmaz S, et al. (2010). Modulatory effects of lycopene and ellagic acid on reproductive dysfunction induced by polychlorinated biphenyl (Aroclor 1254) in male rats. Basic Clin Pharmacol Toxicol 106:479–89.

Bal R, Erdogan S, Theophilidis G, et al. (2010). Assessing the effects of the neonicotinoid insecticide imidacloprid in the cholinergic synapses of the stellate cells of the mouse cochlear nucleus using whole-cell patch-clamp recording. Neurotoxicology 31:113–20.

Bal R, Türk G, Yılmaz Ö, et al. (2012a). Effects of clothianidin exposure on sperm quality, testicular apoptosis, and fatty acid composition in developing male rats. Cell Biol Toxicol 28:187–200.

Bal R, Türk G, Tuzcu M, et al. (2012b). Assessment of imidacloprid toxicity on reproductive organ system of adult male rats. J Environ Sci Health B 47:434–45.

Bray C, Son J-H, Kumar P, Meizel S. (2005). Mice deficient in CHRNA7, a subunit of the nicotinic acetylcholine receptor, produce sperm with impaired motility. Biol Reprod 73:807–14.

Cao Y, Pearman AT, Zimmerman GA, et al. (2000). Intracellular unesterified arachidonic acid signals apoptosis. Proc Natl Acad Sci USA 97:11280–5.

Christie WW. (1992). Gas chromatography and lipids. Glasgow: The Oily Press.

Davis JT, Coniglio JG. (1966). The biosynthesis of docosapentaenoic and other fatty acids by rat testes. J Biol Chem 241:610–12.

de las Heras A, Schoch AM, Fischer A. (2003). Comparison of methods for determining malondialdehyde in dry sausage by HPLC and the classic TBA test. Eur Food Res Technol 217:180–4.

Diniz YS, Cicogna AC, Padovani CR, et al. (2004). Diets rich in saturated and polyunsaturated fatty acids: metabolic shifting and cardiac health. Nutrition 20:230–4.

Ellman GL. (1959). Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–7.

Federal Register. (2003). Clothianidin; pesticide tolerances. Authenticated US Government Information 68:32390–400.

Flora A, Schulz R, Benfante R, et al. (2000). Transcriptional regulation of the human α5 nicotinic receptor subunit gene in neuronal and non-neuronal tissues. Eur J Pharmacol 393:85–95.

Hara A, Radin NS. (1978). Lipid extraction of tissues with a low-toxicity solvent. Anal Biochem 90:420–6.

Hurtado de Catalfo GE, de Gomez Dumm IN. (2005). Influence of testosterone on polyunsaturated fatty acid biosynthesis in Sertoli cells in culture. Cell Biochem Funct 23:175–80.

Kempinas WDG, Suarez JD, Roberts NL, et al. (1998). Rat epididymal sperm quantity, quality, and transit time after guanethidine-induced sympathectomy. Biol Reprod 59:890–6.

Kimura T. (1986). Transduction of ACTH signal from plasma membrane to mitochondria in adrenocortical steroidogenesis. Effects of peptide, phospholipid, and calcium. J Steroid Biochem 25:711–16.

Kothari S, Thompson A, Agarwal A, du Plessis SS. (2010). Free radicals: their beneficial and detrimental effects on sperm function. Ind J Exp Biol 48:425–35.

Li P, Ann J, Akk G. (2011). Activation and modulation of human α4β2 nicotinic acetylcholine receptors by the neonicotinoids clothianidin and imidacloprid. J Neurosci Res 89:1295–301.

Lopez-Cervantes J, Sanchez-Machado DI, Rios-Vazquez NJ. (2006). High-performance liquid chromatography method for the simultaneous quantification of retinol, alpha-tocopherol, and cholesterol in shrimp waste hydrolysate. J Chromatogr A 1105:135–9.

Lu Z-H, Mu Y-M, Wang B-A, et al. (2003). Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell. Biochem Biophys Res Commun 303:1002–7.

Mallick C, Mandal S, Barik B, et al. (2007). Protection of testicular dysfunctions by MTEC, a formulated herbal drug, in streptozotocin induced diabetic rat. Biol Pharm Bull 30:84–90.

Marra CA, de Alaniz MJ. (1989). Influence of testosterone administration on the biosynthesis of unsaturated fatty acids in male and female rats. Lipids 24:1014–19.

Najafi G, Razi M, Hoshyar A, et al. (2010). The effect of chronic exposure with imidacloprid insecticide on fertility in mature male rats. Int J Fertil Steril 4:9–16.

Niehaus WGJ, Samuelsson B. (1968). Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem 6:126–30.

Oertel D, Wright S, Cao XJ, et al. (2011). The multiple functions of T stellate/multipolar/chopper cells in the ventral cochlear nucleus. Hear Res 276:61–9.

Rice-Evans C, Burdon R. (1993). Free radical-lipid interactions and their pathological consequences. Prog Lipid Res 32:71–110.

Sanchez-Machado DI, Lopez-Hernandez J, Paseiro-Losada P, Lopez-Cervantes J. (2004). An HPLC method for the quantification of sterols in edible seaweeds. Biomed Chromatogr 18:183–90.

Solano AR, Dada L, Podesta EJ. (1988). Lipoxygenase products as common intermediates in cyclic AMP-dependent and -independent adrenal steroidogenesis in rats. J Mol Endocrinol 1:147–54.

Sönmez M, Türk G, Çeribaşı AO, et al. (2011). Attenuating effect of lycopene and ellagic acid on 2,3,7,8-tetrachlorodibenzo-p-dioxin-

induced spermiotoxicity and testicular apoptosis. Drug Chem Toxicol 34:347–56.

Tanaka T. (2012). Effects of maternal clothianidin exposure on behavioral development in F1 generation mice. Toxicol Indust Health 28:697–707.

Tomizawa M, Casida JE. (2005). Neonicotinoid insecticide toxicology: mechanisms of selective action. Ann Rev Pharmacol Toxicol 45: 247–68.

Tomizawa M, Lee DL, Casida JE. (2000). Neonicotinoid insecticides: molecular features conferring selectivity for insect versus mammalian nicotinic receptors. J Agric Food Chem 48:6016–24.

Türk G, Ateşşahin A, Sönmez M, et al. (2008). 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 89: 1474–81.

Türk G, Çeribaşı AO, Sakin F, et al. (2010). Antiperoxidative and anti-apoptotic effects of lycopene and ellagic acid on cyclophosphamide-induced testicular lipid peroxidation and apoptosis. Reprod Fertil Develop 22:587–96.

Wang JY, Shum AY, Ho YJ, Wang JY. (2003). Oxidative neurotoxicity in rat cerebral cortex neurons: synergistic effects of H2O2 and NO on apoptosis involving activation of p38 mitogen-activated protein kinase and caspase-3. J Neurosci Res 72:508–19.

Yamamoto Y, Shimamoto K, Sofikitis N, Miyagawa I. (1999). Effects of hypercholesterolaemia on Leydig and Sertoli cell secretory function and the overall sperm fertilizing capacity in the rabbit. Hum Reprod 14:1516–21.

Yokota T, Mikata K, Nagasaki H, Ohta K. (2003). Absorption, tissue distribution, excretion, and metabolism of clothianidin in rats. J Agric Food Chem 51:7066–72.