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ORIGINAL RESEARCH

Effects of clothianidin exposure on sperm quality, testicular apoptosis and fatty acid composition in developing male rats

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Abstract Clothianidin (CTD) is one of the latest members of the synthetic organic insecticides, the neonicotinoids. In the present study, it was aimed to investigate if daily oral administration of CTD at low doses for 90 days has any deleterious effects on reproductive functions of developing male rats. Animals were randomly divided into four groups of six rats each, assigned as control rats, or rats treated with 2 (CTD-2), 8 (CTD-8) or 32 (CTD-32) mg CTD/kg body weight by oral gavage. The significant decreases of the absolute weights of right cauda epididymis and seminal vesicles, and body weight were detected in the animals exposed to CTD administration at 32 mg/kgBW/day. Epididymal sperm concentration decreased significantly in CTD-32 group and the abnormal sperm rates increased in CTD-8 and CTD-32 groups when compared to control group. The testosterone level was significantly decreased in CTD-32 group when compared to control group. The administration of all CTD doses resulted in a significant decrease in the level of GSH. The number of TUNEL-positive cells significantly increased in the germinal epithelium of testis of rats exposed to CTD at 32 mg/kgBW/day. In groups CTD-8 and CTD-32, only docosapentaenoic, arachidonic, palmitic and palmitoleic acids were significantly elevated when compared to control. The ratios of 20:4/18:2 and 18:1*n*−9/18:0 were decreased when rats exposed to CTD. Sperm DNA fragmentation was observed in CTD-32 group, but not CTD-2 and CTD-8. It is concluded that low doses of CTD exposure during critical stages of sexual

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maturation had moderate detrimental effects on reproductive organ system and more severe effects are likely to be observed at higher dose levels. In addition, the reproductive system may be more sensitive to exposure of CTD even earlier in development (prenatal and early postnatal), and therefore it could be expected that more severe effects could also be observed at the NOAEL dose levels, if dosing had occurred in utero or early postnatal.

Keywords Apoptosis · Clothianidin · Fatty acid composition · Neonicotinoids · Sperm characteristics · Testis

Introduction

A new generation of insecticides, the neonicotinoids, are increasingly getting popular for controlling insect pests on crops and pets (Tomizawa and Casida 2003). Clothianidin [(*E*)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl- 2-nitroguanidine] (CTD) is a novel and broad-spectrum insecticide, which is one of the latest members of the synthetic organic insecticides, the neonicotinoids (Tomizawa and Casida 2005). The nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects are the primary and selective target for neonicotinoid insecticides, including CTD (Tomizawa et al. 2000). The high selectivity of these compounds for insect compared to mammalian nAChRs accounts, at least in part, for the safety and effectiveness of neonicotinoids (Tomizawa and Casida 2003). CTD exhibits higher insecticidal activity than some other neonicotinoids, such as imidacloprid against both sucking insects, and chewing insects (Tomizawa and Casida 2003). CTD has low acute oral toxicity, with an oral LD50 in rats of >5,000 mg/kg body weight. In male rats, the No Observed Adverse Effect Level (NOAEL) dose of CTD has been reported to be 27.9 mg/kgBW/day after 90 day oral dosing study (Federal Register 2003). Therefore CTD as a neonicotinoid is considered far less toxic to mammals when compared to invertebrates (Bal et al. 2010; Tomizawa and Casida 2005). Patch clamp studies carried out in the neurons of ventral cochlear nucleus neurons, whose ionic channels are characterized (Bal and Oertel 2007; Oertel et al. 2011), show that sensitivity of mammalian nAChRs to neonicotinoids are far less compared to insect nAChRs (Bal et al. 2010). However, as the 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.

Nontarget animals and humans are extensively exposed to neonicotinoids including CTD, because their uses are globally increased; they are persistent in crops, vegetables and fruits. Even though governmental agencies and internal organizations are continuously setting maximum residue levels for the control of each pesticide in order to ensure the safety of foodstuffs, pesticide residues in foodstuffs may exceed the limits due to careless use.

Free radicals are highly reactive molecules that include reactive oxygen species (ROS) and reactive nitrogen species. They are normally generated in subcellular compartments of testis, particularly mitochondria; however excessive production of free radicals can lead to tissue injury and cell death and result in antioxidant depletion. Antioxidants are substances [glutathione (GSH), α-tocopherol, selenium, ascorbic acid] or enzymes (glutathione-peroxidase, superoxide dismutase and catalase) present in tissues with the capacity to balance or neutralize these free radicals. However, this balance can easily be broken by chemicals which disrupt the prooxidant–antioxidant balance, leading to cellular dysfunction (Agarwal et al. 2008). Current theories of membrane fusion suggest that membrane fluidity is a prerequisite for normal cell functions and that the fluidity and flexibility of cell membranes are mainly dependent on their lipid constitution (Lenzi et al. 1996). Although cholesterol is involved in steroidogenesis in testes, increased level of cholesterol in testes is attributed to decreased androgen concentration, which resulted in impaired spermatogenesis (Yamamoto et al. 1999). Additionally, the mitochondrial membrane of spermatozoa is more susceptible to lipid peroxidation, as this compartment is rich in polyunsaturated fatty acids and has been shown to contain low amounts of antioxidants (Agarwal et al. 2008). Some neonicotinoid pesticides induce oxidative stress (increase in lipid peroxidation by-products and decrease in enzymatic and non-enzymatic antioxidants) in the serum (Mohany et al. 2011), liver (El-Gendy et al. 2010) and testis (Zhang et al. 2011). It has been reported that neonicotinoid, imidacloprid, has no significant effect on serum cholesterol

levels in calves (Kaur et al. 2006) and female rats (Bhardwaj et al. 2010). El-Sheekh et al. (1994) have reported that the herbicide, atrazine, stimulates fatty acid synthesis.

Pharmacokinetic studies indicate that CTD is rapidly distributed into all tissues and organs within 2 h after single oral administration at the low dose, and its excretion and metabolism immediately start after absorption. The concentration of CTD in the tissues and organs declines rapidly, and therefore it does not accumulate in the tissues and organs including testis (Yokota et al. 2003). Tanaka (2012) have 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, Najafi et al. (2010) have alleged that the neonicotinoid, imidacloprid, administration to rats daily for 60 days leads to significant reproductive disorders including a histological adverse effect on testicular tissue, spermatogenesis, sperm viability, velocity and abnormality.

Besides, there is no information about if exposure to CTD during developmental stages of postnatal life affected normal development of reproductive system, despite their widespread use. Therefore, in the present study, reproductive organ system of developing male rats were evaluated for biochemical, structural alterations in testicular tissue after daily administration of low doses of CTD to developing rats by oral gavage.

Material 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 male Wistar albino pups at the age of 7 days were obtained and maintained from Firat University Experimental Research Centre (Elazig, Turkey). The pups of the six different dams were used in this study. For randomization, a pup from each dam was randomly selected for each group. Thus, four different pups of each dam received four different doses of CTD (0, 2, 8 and 32 mg/kg). They were kept with their dams until they were 30 days old. 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, Elazıg, Turkey) ad libitum and fresh distilled drinking water was given ad libitum. CTD (DANTOTSU®) was obtained from Sumitomo Chemical Co. Ltd. (Japan), which was dissolved in water easily. Different doses of CTD used in this study were separately dissolved in 1 ml distilled water. One milliliter per kilogram BW dose volume was administered to rats. This means that, for example, a pub weighing 10 g had 10 μl of CTD and a pub weighing 30 g had 30 μl of CTD by oral dosing during the weanling period.

Animals and subchronic 90-day oral toxicity study

The animals were randomly divided into four groups with six animals in each group. The first group was taken as:

1. *control group*: rats received 0 mg/kg BW CTD in distilled water daily for a period of 3 months by gavage, and the other groups were treated as in the following way:
2. *2 mg CTD group*: rats received CTD dissolved in distilled water daily for a period of 3 months at a dose of 2 mg/kg BW by gavage.
3. *8 mg CTD group*: rats received CTD dissolved in distilled water daily for a period of 3 months at a dose of 8 mg/kg BW by gavage.
4. *32 mg CTD group*: rats received CTD dissolved in distilled water daily for a period of 3 months at a dose of 32 mg/kg BW by gavage.

In the present study, a maximum dose of 32 mg/kg for CTD was selected based on the reported reproductive NOAEL for rats (Federal Register 2003). The dose was adjusted daily according to the body weight of the individual animals. Animals were 7 days of age at the start of treatment.

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. Right testicles were fixed in Bouin's fluid. Left testicles were frozen in liquid nitrogen and stored at −70°C until use for TBARS, GSH, fatty acids, cholesterol and α-tocopherol analyses. 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 labelling (TUNEL) assay with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA). Briefly, the fixed testicular tissue was embedded in paraffin, and sectioned at 4 μm thickness. 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. Negative controls were performed using distilled water in the place of the TdT enzyme. Sections were counterstained with Mayer's hematoxylin, dehydrated in graded alcohol, and cleared.

Sperm analyses

All sperm analyses were performed by using the modified methods described by Türk et al. (2008). The epididymal sperm concentration in the right cauda epidymal tissue was determined with a hemocytometer. Freshly isolated left cauda epididymal tissue was used for the analysis of sperm motility. The percentage sperm motility was evaluated using a light microscope with heated stage. 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 prepared. The slides were then viewed under a light microscope at 400× magnification. A total of 300 spermatozoa were examined on each slide (1,800 cells in each group), and the head, tail and total abnormality rates of spermatozoa were expressed as percentage. An observer who analysed sperm parameters was blinded to treatment groups.

Determination of TBARS level

The concentration of thiobarbituric acid-reactive substances (TBARS) in the testis samples was estimated by the method of Niehaus and Samuelsson (1968). In brief, 1 ml of tissue homogenate (supernatant; Tris–HCl buffer, pH 7.5) was mixed with 2 ml of (1:1:1 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. 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 TBARS was analysed using the 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 (de las Heras et al. 2003). The TBARS values were expressed as nanomoles per gram tissue.

Determination of GSH level in tissue samples

Reduced glutathione (GSH) was determined by the method of Ellman (1959). Briefly 1 ml testis tissue homogenate was treated with 1 ml of 5 % metaphosphoric acid (Sigma, St. Louis, MO), the mixtures were centrifuged in 5,000 rpm and were taken the supernatant. After deproteinization, the supernatant was allowed to react with 1 ml of Ellman's reagent (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 (Akerboom and Sies 1981).

Lipid extraction

Lipid extraction of tissue samples were extracted with hexane–isopropanol (3:2 *v*/*v*) by the method of Hara and Radin (1978). A 1-g testis tissue sample 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 (Christie 1992). 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, 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 was 1 ml/min. The methyl esters of fatty acids were identified by comparison with authentic external standard mixtures analysed under the same conditions. The concentrations of fatty acid were calculated with *Class GC 10* software (version 2.01) based on the external standard methods. The results were expressed as microgram per gram tissue.

Saponification and extraction

Alpha-tocopherol and cholesterol were extracted from the lipid extracts by the method of Sánchez-Machado et al. (2004) with minor modifications. Five milliliters *n*-hexane/isopropyl alcohol mixture was treated with 5 ml of KOH solution (0.5 M in methanol) were added and 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 5,000 rpm. The supernatant phase were 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 analysis

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 (Lopez-Cervantes et al. 2006).

Serum testosterone

The serum testosterone level was measured by ELISA method using 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. The sensitivity of the kit was 0.083–16 ng/ml, 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 via nylon mesh to separate the supernatant from tissue particles and stored at −20°C until use. The supernatant fluid containing all epididymal spermatozoa was then thawed and 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 analysed 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 (ANOVA) and post hoc Tukey-HSD test were used to determine differences between groups. 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 CTD on body weight

Table 1 demonstrates the effects of CTD at daily doses of 2, 8 and 32 mg/kg BW on final body weights of male rats. The final body weights were significantly less in only CTD-32 group when compared to control group ($p<0.001$).

Effect of CTD on reproductive organ weights

Organ weights of testis, epididymis, right cauda epididymis, seminal vesicles and prostate of control and CTD-treated groups are shown in Fig. 1 as bar graph. The relative organ weights were estimated by dividing the absolute reproductive organ weights to body weight. Administration of CTD at only 32 mg/kgBW/day resulted in significant decreases in the absolute weights of right cauda epididymis ($p<0.05$) and seminal vesicles ($p<0.05$), but did not change the relative weights of all reproductive organs.

Effect of CTD on epididymal sperm characteristics

Epididymal sperm characteristics of control and CTD-2, CTD-8 and CTD-32 groups are presented in Table 2. CTD at 2 mg/kg BW/day had no significant change in sperm motility, epididymal sperm concentration and abnormal sperm rate compared to control. However, CTD at 8 and 32 mg/kgBW/day increased significantly the head ($p<0.05$), tail ($p<0.05$) and total ($p<0.01$) abnormality rates in sperm compared to the control group. In addition, CTD at 32 mg/kgBW/day appeared to reduce epididymal sperm concentration significantly ($p<0.05$).

Effect of CTD on testicular apoptosis

Figure 2 illustrates apoptosis, demonstrated by TUNEL staining, in the testis of rats of control and

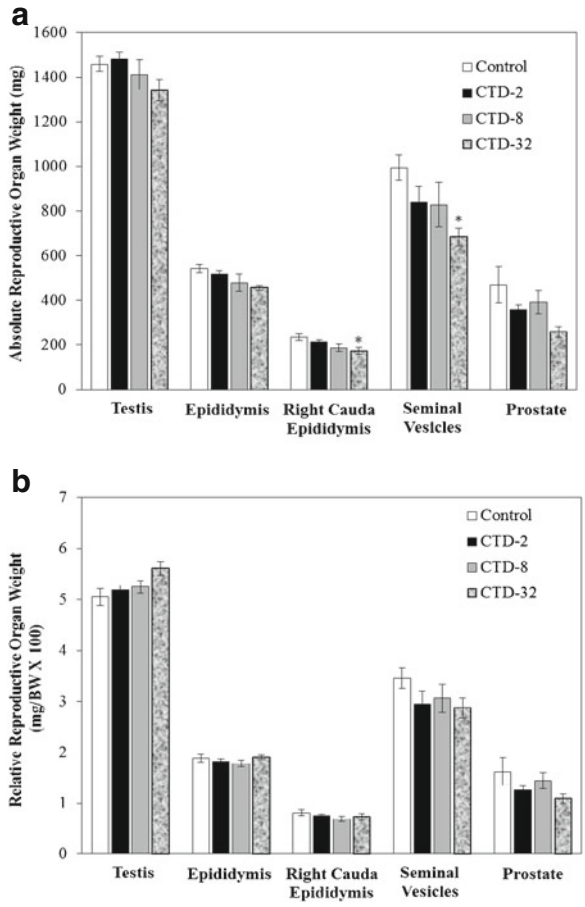

Fig. 1 Effect of CTD on absolute (**a**) and relative (**b**) weights of reproductive organs including testis, epididymis, right cauda epididymis, seminal vesicles and prostate. Data are presented as mean ± SEM. The absolute weights of right cauda epididymis and seminal vesicles of CTD-32 group are significantly different from only control group but not CTD-2 and CTD-8 groups. *$p<0.05$

CTD-treated groups. TUNEL-positive cells had 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 cells with TUNEL positivity. The

Table 1 Effects of CTD on final body weight

	Control	CTD-2	CTD-8	CTD-32
Final body weight (g)	280.0±2.9	283.0±2.5	268.3±6.7	239.0±4.4***

Data are presented mean ± SEM values

***$p<0.001$; significantly different from control, CTD-2 and CTD-8 groups

Table 2 Effects of CTD on sperm parameters

Groups	Sperm motility (%)	Epididymal sperm concentration (million/cauda epididymis)	Abnormal sperm rate (%)		
			Head	Tail	Total
Control	79.9±2.8	104.4±8.9	2.4±1.0	2.6±0.6	5.0±0.9
CTD-2	62.5±8.6	99.3±3.6	3.5±0.6	7.0±1.7	10.5±1.6
CTD-8	66.6±5.3	77.3±10.2	6.0±0.6*	8.8±2.1*	14.8±1.8**
CTD-32	69.2±7.9	70.3±8.5*	7.5±0.9*	9.3±1.1*	16.8±1.2**

Data are presented mean ± SEM values

The epididymal sperm concentration of CTD-32 group is significantly different from only control group but not CTD-2 and CTD-8 groups

The head abnormality rate of CTD-32 group is significantly ($p<0.05$) different from control and CTD-2 groups but not CTD-8 group, and also this rate in CTD-8 group is significantly different from only control group but not CTD-2 and CTD-32 groups

The tail abnormality rates of CTD-8 and CTD-32 groups are significantly different from only control group but not CTD-2 group

The total abnormality rates of CTD-8 and CTD-32 groups are significantly different from only control group but not CTD-2 group

*$p<0.05$; **$p<0.01$

apoptotic index in the testis of control rats was found to be 0.14±0.09 %. CTD treatment resulted in increases in the number of TUNEL-positive cells in a dose-dependent manner. The apoptotic index was 0.57±

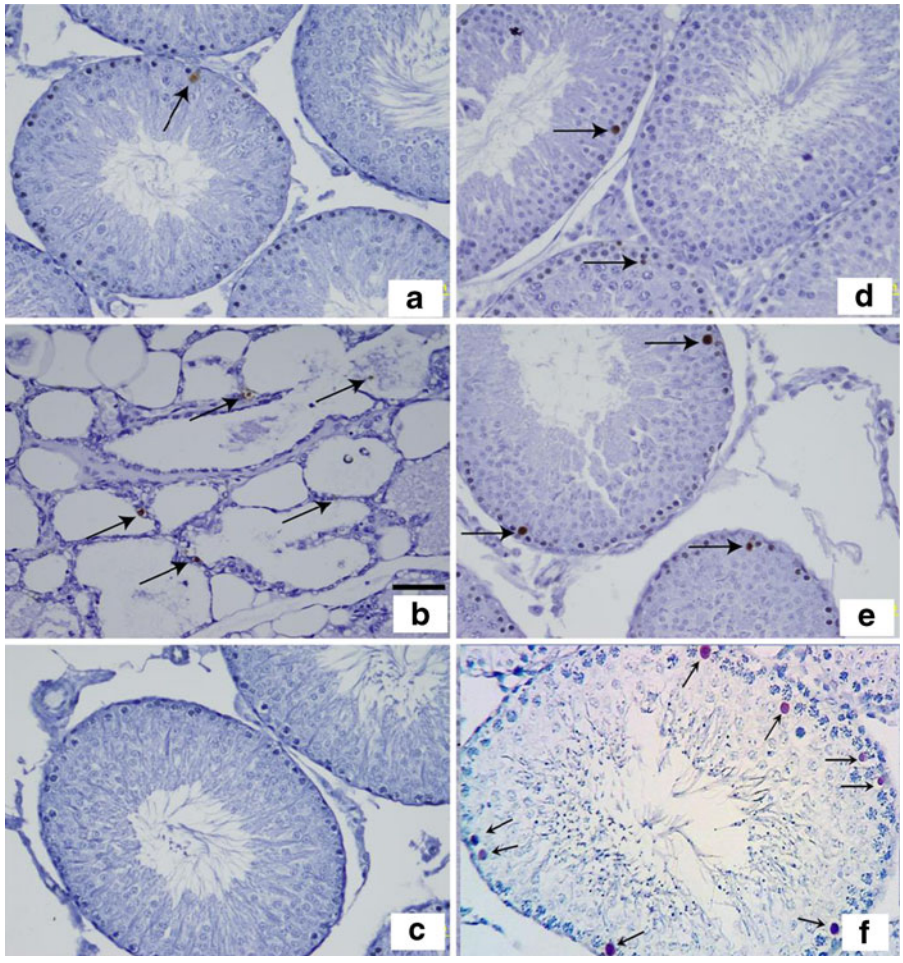

Fig. 2 Representative photomicrographs of TUNEL staining in the testes of control (**a**), CTD-2 (**d**), CTD-8 (**e**) and CTD-32 groups (**f**). **b**: Positive control: TUNEL-stained cells in breast tissue where continuous apoptosis takes place. **c**: Negative staining control is also illustrated to ensure the staining method is working well. Note that there were no detectable signals in the negative control. *Arrows* indicate candidate apoptotic cells. *Calibration bar* 50 μm

0.42 %, 1.83±0.83 % and 3.16±1.04 % in CTD-2, CTD-8 and CTD-32 groups, respectively. The increase in the apoptotic index was statistically significant in CTD-32 group compared to control group ($p<0.05$).

Effect of CTD on sperm DNA fragmentation

Apoptotic cells usually possess fragmental DNA which can be visualized by DNA-agarose gel electrophoresis. Therefore DNA fragmentation was used as the criterion for apoptosis in the current study. DNA isolated from the sperm of rats exposed to CTD at doses of 2 and 8 mg/kgBW 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. Whereas, in rats treated with CTD at 32 mg/kgBW dose, a clear DNA fragmentation was observed (Fig. 3).

Effect of CTD on biochemical parameters

Levels of serum testosterone, testicular tissue lipid peroxidation (TBARS) and antioxidant (GSH), fatty acid compositions (palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, dihomo-γ-linolenic acid, arachidonic acid and docosapentaenoic acid), cholesterol and α-tocopherol are presented in Table 3. Administration of CTD at 2 and 8 mg/kgBW/day for 3 months did not change the serum testosterone level significantly. Yet, CTD administration at 32 mg/kgBW/day resulted in statistically significant reduction in the serum testosterone level when compared to control group ($p<0.05$).

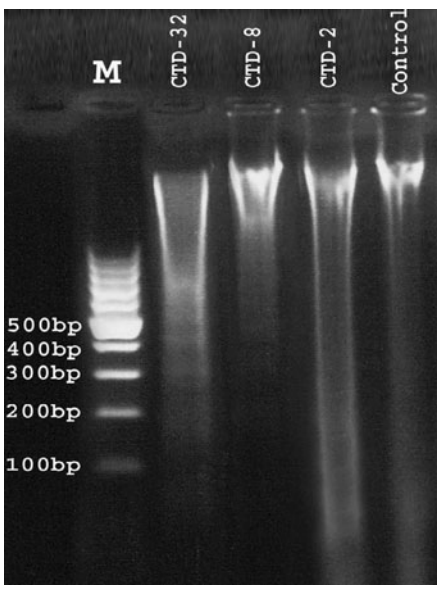

Fig. 3 Effect of CTD on sperm DNA fragmentation in male rats. CTD exposures at particularly 32 mg/kg body weight (BW) dose induce the cleavage of DNA into oligonucleosome-length fragments, a characteristic of apoptosis. *Marker (M)* Mol weight standards; *CTD 2* 2 mg/kg body weight (BW); *CTD 8* 8 mg/kg body weight (BW); *CTD 32* 32 mg/kg body weight (BW)

Administration of CTD up to 32 mg/kgBW/day to rats caused the numerical but not statistically significant increase in the lipid peroxidation measured as TBARS levels when compared to control rats. Testicular tissue GSH level was significantly lower in the rats exposed CTD up to 32 mg/kgBW/day than that in the controls ($p<0.001$). CTD exposure did not change the level of α-tocopherol when compared to that of control.

CTD administration at doses of 2, 8 and 32 mg/kgBW/day for 3 months resulted in increases in testicular tissue fatty acids including palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, dihomo-γ-linolenic acid, arachidonic acid and docosapentaenoic acid in a dose-dependent manner. But the increases were significant only in palmitic acid at 8 mg/kgBW/day ($p<0.05$), in palmitoleic acid at 2, 8 and 32 mg/kgBW/day ($p<0.05$), in arachidonic acid at 8 and 32 mg/kgBW/day ($p<0.01$) and in docosapentaenoic acid at 8 and 32 mg/kgBW/day ($p<0.01$). Administration of CTD at 2, 8 and 32 mg/kgBW/day doses resulted in significant increases in the total lipid in testis ($p<0.05$). Oral administration of CTD at 32 mg/kgBW/day caused testicular tissue cholesterol level to increase significantly ($p<0.01$).

The ratios of 20:4/18:2 in the testis of rat treated with CTD at 0, 2, 8 and 32 mg/kgBW/day, were in ~4, ~3.5, ~3.3 and ~3.1, respectively. The ratios of 18:1/18:0 in control and all CTD groups were found to be ~1.9 and ~1.4, respectively. The decreases in these ratios were found in all CTD-treated groups when compared to the control group.

Discussion

In the present study, in order to understand the effects of CTD during critical stages of testicular development (particularly from 7 to 56 days of age), rats were exposed to CTD at comparable doses (2, 8 and 32 mg/kgBW/day) to the reported NOAEL for

Table 3 Effects of CTD on levels of serum testosterone and testicular tissue thiobarbituric acid-reactive substances (TBARs), glutathione (GSH), cholesterol, α-tocopherol and some fatty acids including palmitic, palmitoleic, stearic, oleic, linoleic, arachidonic and docosapentaenoic acids

	Control	CTD-2	CTD-8	CTD-32
Testosterone (ng/dl)	103.5±17.9	105.9±43.5	102.2±26.8	77.5±19.0*
TBARS (nmol/g prot.)	20.2±2.1	27.2±1.2	26.8±1.4	29.1±2.3
GSH (μg/g prot.)	548.0±27.8	187.0±22.5***	155.0±16.4***	141.0±12.8***
Palmitic acid (16:0) (μg/g tissue)	3213.9±230.6	5155.1±685.8	5558.6±472.7*	5133.2±570.1
Palmitoleic acid (16:1) (μg/g tissue)	103.7±44.1	308.6±41.2*	333.0±25.1*	340.0±46.5*
Stearic acid (18:0) (μg/g tissue)	598.4±128.7	1286.3±428.7	1378.9±204.7	1506.8±220.3
Oleic Acid (18:1) (μg/g tissue)	1151.6±81.5	1752.8±248.5	1910.0±134.4	2082.6±335.4
Linoleic acid (18:2 *n*6) (μg/g tissue)	409.7±61.1	719.9±171.7	838.7±83.1	893.2±212.6
Dihomo-γ-linolenic acid (20:3*n*6) (μg/g tissue)	88.6±19.8	149.2±19.8	150.8±16.4	161.0±16.1
Arachidonic acid (20:4 *n*6) (μg/g tissue)	1663.7±84.5	2524.1±254.2	2803.4±254.2**	2825.8±254.2**
Docosapentaenoic acid (22:5*n*6) (μg/g tissue)	1741.2±190.9	2781.3±304.6	3063.8±280.2**	3156.0±258.0**
Total lipid (μg/g tissue)	8771.3±362.1	15786.7±206.0*	17119.0±1463.3*	15435.0±2201.9*
Cholesterol (μg/g tissue)	5009.2±307.3	5263.4±201.1	5630.4±180.8	6480.6±261.7**
α-tocopherol (μg/g tissue)	124.4±11.2	120.5±10.7	121.8±15.1	117.2±13.9

Data are presented as mean ± SEM values. (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)

The testosterone concentration of CTD-32 group is significantly different from control, CTD-2 and CTD-8 groups

The GSH levels of CTD-2, CTD-8 and CTD-32 groups are significantly different from control group

The palmitic acid concentration of CTD-8 group is significantly different from only control group, but not CTD-2 and CTD-8 groups

The palmitoleic acid concentrations of CTD-2, CTD-8 and CTD-32 groups are significantly different from control group

The arachidonic acid concentrations of CTD-8 and CTD-32 groups are significantly different from only control group but not CTD-2 group

The docosapentanoic acid concentrations of CTD-8 and CTD-32 groups are significantly different from only control group but not CTD-2 group

The total lipid levels of CTD-2, CTD-8 and CTD-32 groups are significantly different from control group

The cholesterol concentration of CTD-32 group is significantly different from control, CTD-2 and CTD-8 groups

*$p<0.05$; **$p<0.01$; ***$p<0.001$

90 days starting from 7 to 97 days of age. The results of the current study showed that ingestion of CTD by gavage for 90 consecutive days induced some adverse effects on male reproductive system, such as the changes in reproductive organ weights, decreased epididymal sperm concentration, increased abnormal sperm rate, decreased GSH level, disturbance in fatty acid composition and increase in apoptotic index in testicular tissue and increased sperm DNA fragmentation. This is the first report about the adverse effects of the recently marketed neonicotinoid insecticide, CTD, on reproductive system of developing male rats.

With respect to general health levels, CTD as a neonicotinoid is considered far less toxic to mammals when compared to invertebrates (Bal et al. 2010; Tomizawa and Casida 2005). However, it has been reported that NOAEL dose imidacloprid, is a neonicotinoid insecticide, administration causes immuno- (low numbers of lymphocytes in spleen, lymphocytic depletion in thymus, decreased phagocytic activity, decreased chemokinesis and chemotaxis) and hepatotoxic effects in adult male rats (Mohany et al. 2011). Although it has no teratogenic and developmental effects on fetuses at NOAEL doses, however, fetal death, decrease in fetal weight or ossifying delay in sternebrae have been reported (Sumitomo Chemical Takeda Agro Company, Ltd 2003). Tanaka (2012) have reported that CTD exposure causes some neurobehavioral effects without litter size, litter weight or sex ratio in mice during developmental period. Based

on the reduced sperm number, increased abnormal sperm rate and increased sperm DNA fragmentation obtained in the present study, less offspring but not developmental delay may be expected from CTD exposed animals in their sexual life due to the negative correlation between some morphological sperm abnormalities and fertilization rate (Kawai et al. 2006).

It is known that monitoring body weight provides information on the general health level of animals, which can be important to interpretation of reproductive effects. Androgens stimulate the growth by inducing the protein synthesis (Fernandes et al. 2007). In addition, it is well known that the epididymis and accessory sex organs need a permanent androgenic stimulation for their normal growth and functions (Klinefelter and Hess 1998). Zhang et al. (2011) have reported that acetamiprid, a chlorinated nicotimine pesticide, causes significant decreases in body and reproductive organ weights, and also causes disorders of mitochondria and endoplasmic reticulum of Leydig cells, which mainly produces testosterone, in male mice. Decrease in reproductive organ weights were also reported for some other insecticides (Barkley and Goldman 1977; Mallick et al. 2007). The significant decreases in body weight and the absolute weights of right cauda epididymis and seminal vesicles could possibly be accounted for by the decreased serum levels of testosterone at 32 mg/kgBW/day in the current study.

The use of oxygen during normal metabolism in living cells can cause normal production of ROS. In a healthy body, ROS and antioxidants remain in balance. When the balance is disrupted towards an overabundance of ROS and a reduction of antioxidants, oxidative stress occurs. Spermatozoa and Leydig cells in mammalians are rich in polyunsaturated fatty acids (PUFAs) and are more susceptible to oxidative damage resulting in impairment of sperm. ROS can attack the unsaturated bonds of the lipids of sperm membrane, and destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability and increased mid-piece morphological defects, and even it completely inhibits spermatogenesis in extreme cases (Agarwal et al. 2008). Besides, some nAChR subunits 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 the calcium influx mechanisms, and mice deficient in nAChR subunit $\alpha 7$ produce impaired sperm motility. Li et al. (2011) have also reported that although neonicotinoids imidacloprid but not CTD strongly inhibits the $\alpha 4$ and $\beta 2$ subunits of nAChR activated by acetylcholine in human embryonic kidney cells. Therefore, any drug affected nicotinic system may cause disturbed function of the testicular tissue and sperm. The findings that epididymal sperm concentration decreased and the rates of abnormal sperm increased in response to CTD administration in the present study are consistent with the findings reported by some other researchers investigating effects of some neonicotinoids on reproductive system (Najafi et al. 2010; Zhang et al. 2011). The significant decrease in sperm concentration, significant increase in abnormal sperm rate and insignificant decrease in sperm motility observed in the present study could be explained by either the oxidative damaging of spermatogenic cells as demonstrated by decreased GSH level or inhibition of testosterone biosynthesis as evident by decreased testosterone level, or apoptosis in spermatogenic cells as demonstrated by increased TUNEL staining and increased sperm DNA fragmentation, or affected nAChR subunits. In this study, decreases in tail abnormality rate of sperm were statistically significant, whereas, decreases in sperm motility were statistically insignificant. In fact, numerical decreases (21.1 % in CTD-2, 16.6 in CTD-8 and 13.3 in CTD-32 groups versus control) were observed in all CTD-treated groups. However, as the observed decreases in each CTD-treated group were individual, differences in sperm motility did not reach the statistical significance. This status was also confirmed with that SEM values of all CTD groups were found to be higher than in the control values.

In the current study, we showed that administration of CTD resulted in reductions in the level of serum testosterone in a dose-dependent manner. Testosterone, the principal male hormone, is produced almost exclusively by Leydig cells in the testis. In rats, Leydig cell development begins by day 14 postpartum and differentiation of Leydig cells are finalized by day 56 (Akingbemi et al. 1999). While in the prepubertal period, testosterone is responsible for the development of male secondary sex characteristics and hormonal imprinting of the liver, prostate and hypothalamus, in

the adult, testosterone supports spermatogenesis, sperm maturation and sexual function (Ewing and Keeney 1993). For that reason, impairment of the testosterone biosynthesis may consequently results in dysfunction of the reproductive system. The underlying mechanism for this hormonal disturbances may attributed to either direct damaging effect of pesticide on Leydig cells (Zhang et al. 2011) or high testicular cholesterol concentration, which damages Leydig cells (Yamamoto et al. 1999), as evident by increased cholesterol level in the present study.

The reduction of GSH in the rats exposed to CTD might underlie the changes mentioned in the result section, even though TBARS level did not increased significantly. Some neonicotinoid pesticides induce oxidative stress in the serum (Mohany et al. 2011), liver (El-Gendy et al. 2010) and testis (Zhang et al. 2011). The subcellular membranes, which are rich in unsaturated fatty acids and contain low levels of antioxidants, are highly susceptible to lipid peroxidations. Enhanced lipid peroxidation and GSH depletion cause the subcellular membranes to change permeability. Male reproductive organs are especially susceptible to the deleterious effects of ROS and lipid peroxidation, which results in impaired fertility (Agarwal et al. 2008; Türk et al. 2008, 2010). The numerical but not statistical increase in testicular TBARS level is probably due to the CTD-induced excessive production of ROS and consequently elevated lipid peroxidation. The significant decrease in testicular GSH level observed in this study may be attributed to its excessive utilization in order to scavenge the overproduction of ROS.

TUNEL staining and DNA fragmentation analysis were performed to detect programmed cell death. The increased apoptotic index in the germinal epithelium of testis and the increased sperm DNA fragmentation in rats exposed to CTD are consistent with the decreased GSH level, which is well correlated with the decreased sperm count and increased sperm abnormalities. Apoptosis is an oxidative stress-mediated process involving lipid peroxides and lipid peroxidation of PUFAs of the membrane structures (Altuğ et al. 2008; Yener et al. 2009). Some authors (Bian et al. 2004; Saradha et al. 2009; Vaithinathan et al. 2010) have reported that exposure of different pesticide results in sperm DNA damage and testicular apoptosis via different pathways. ROS generation (El-Gohary et al. 1999) or direct DNA, mitochondria and chromatin damages (Saradha et al. 2009; Vaithinathan et al. 2010) might play a critical role in the initiation of CTD-induced apoptosis in spermatogenic cells.

The effects of CTD mentioned above might be due to the fact that the experimental animal used here was in their developing stages, not matured ones, since the testis of developing rats differ from that of the mature one. Prominent changes occur in terms of morphological, physiological and biochemical point of view during sexual maturation. For example, dynamic changes take place in oxidant/antioxidant profile of the testis during sexual maturation and ageing (Peltola et al. 1992). Samanta et al. (1999) demonstrated that susceptibility to peroxidation of PUFAs increased gradually from birth till 21 days of age and remained unchanged till 600 days of age. They related this differential susceptibility to the qualitative and quantitative changes occurring in phospholipid and fatty acid compositions of the testis during maturation (Johnson 1970), since the amount of phospholipid and fatty acid composition are among the essential factors that control lipid peroxidation (Hammer and Wills 1978). On the one hand, it has been demonstrated that the level of GSH in the testicular tissue increases gradually till 30 days of age and did not change much thereafter till 45 days of age. Its level is sharply rises when they are 3 months old, after which it remained unchanged till 365 days of age. On the other hand, in testicular tissue, endogenous lipid peroxidation was very high at the time of birth. Its level declined significantly on the 3rd day of age and remained unchanged till 365 days of age. But, its level markedly increases when they are nearly 2 years old (Peltola et al. 1992; Samanta et al. 1999). Consistent with these, one of the organochlorine pesticide, hexachlorocyclohexane, induces oxidative stress in the testis, whose magnitude is higher in immature rats than the mature ones (Samanta and Chainy 1997). Similarly, Akingbemi et al. (2000) have presented evidence indicating that Leydig cells have been found more sensitive to an insecticide, by a biologically active metabolite of methoxychlor, during pubertal differentiation versus adulthood.

The fatty acid profiles of testis tissue of control rats in this study is comparable to the one reported previously (Bal et al. 2011). In developing rats exposed to CTD, all fatty acids analysed were elevated when compared to control, but only docosapentaenoic acid, arachidonic acid, palmitic acid and palmitoleic acid

reached to significant levels. Rat testes contain relatively substantial amounts of PUFAs mainly arachidonic acid (20:4 *n*6) and docosapentaenoic acid (22:5 *n*6), which are involved in spermatogenesis and androgenic activity of Leydig cells (Ayala et al. 1977; Davis and Coniglio 1966). Our finding related to increased fatty acid is consistent with previous report that herbicide, atrazine, stimulates fatty acid synthesis (El-Sheekh et al. 1994). The dominance of PUFAs in the membrane of organelles present in testis cause susceptibility to lipid peroxidative degradation (Gutteridge et al. 1998). It has been suggested that excessive PUFAs may be deleterious for testosterone production (Bourre et al. 1997; Meikle et al. 1989, 1996). This might explain the decrease in testosterone level in the present study. The elevation in fatty acids, especially PUFAs such as arachidonic acid could possibly account for the increased apoptotic index, since they can be cytotoxic and induce apoptosis (Pompeia et al. 2002; Vento et al. 2000). Although the reason of increase observed in fatty acids after CTD treatment is exactly unknown, their non-utilization leading to disturbed spermatogenesis as evidenced by decreased sperm concentration in this study may be responsible for the increased levels of PUFAs

The decreases in 20:4/18:2 and 18:1*n*−9/18:0 ratios in the testis of rat treated with CTD in our study indirectly show decreased activities of Δ6 desaturase and Δ9 desaturase, respectively. Consistent with our result, Hurtado de Catalfo and de Gomez Dumm (2005) demonstrated the decrease of Δ6 desaturase activity by testosterone in cultured Sertoli cells. CTD appear to modify the PUFAs biosynthesis, modulating the activities of Δ6 and Δ9 desaturases in the testicular tissue.

Administration of CTD to developing rat caused the total cholesterol level to increase in the testicular tissue. Cholesterol is the main precursor for steroidogenesis and it is produced mostly in the liver from LDL and HDL (Catala 2007; Walsh et al. 2000; Wang et al. 2002). The increase of cholesterol level in the testicular tissue may result from its non-utilization for production of testosterone. Similar observations were reported by Ngoula et al. (2007) who tested pirimiphos-methyl, an organophosphorous pesticide in rats. In addition, Yamamoto et al. (1999) have reported that high testicular cholesterol concentration have a detrimental effect on Leydig and Sertoli cell secretory function, spermatogenesis, epididymal sperm maturation process (decreased sperm motility and concentration), and the overall sperm fertilizing capacity. One of the reasons of the significant decrease in sperm concentration and significant increase in abnormal sperm rate after exposure of CTD in the present study may be due to the CTD-induced increased testicular cholesterol concentration that affects negatively spermatogenesis.

In conclusion, the reduction in the level of GSH, disturbed sperm parameters, increased apoptotic index and sperm DNA fragmentation, and change in fatty acid composition could reflect an adverse effect of CTD on the reproductive system of developing male rats. Therefore, these results of the current study suggest that CTD exposure to during critical stages of sexual maturation produced some damage, possibly through induction of oxidative stress, and as a consequence, this could cause testicular dysfunction in adulthood. Exposure of rats to NOAEL dose levels of CTD had moderate detrimental effects on reproductive organ system and more severe effects are likely to be observed at higher dose levels. In addition, the reproductive system may be more sensitive to exposure of CTD even earlier in development (prenatal and early postnatal), and therefore it could be expected that more severe effects could be observed at the NOAEL dose levels, if dosing had occurred in utero or early postnatal.

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