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Influence of fipronil intoxication on thyroid gland ultra-structure and hepatic microsomal enzymes expression in male albino rats

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Abstract

The aim of the current study is to investigate the impact of fipronil (FPN) insecticide oral exposure on thyroid gland and its related hormones in male albino rats. FPN was administered at dose of (4.85 mg/kg b.wt) twice weekly for 90 days. FPN exposed rats showed non-significant decrease in serum TSH level, while serum T_3 and T_4 levels showed significant decrease, significant increase in mRNA expression of hepatic uridine 5′- diphospho-glucuronosyl transferases (Ugt1a1) and sulfotransferases *(Sult1b1)* enzymes, Thyroid gland showed follicular disorganizations with severe vacuolation of the lining epithelium and extremely reduced sizes and colloid content. Ultra-structure of follicles showed damaged cells with detached microvilli and pyknotic nucleus. Mitochondria were degenerated with loss of cristae. It is concluded that alterations in thyroid hormones by FPN not only due to distress on physiology of the gland but more specifically due to its injurious effect on thyroidal tissue.

Introduction

Pesticides are group of hurtful chemicals which liberated accidentally in the environment. The phenyl-pyrazole insecticide fipronil (FPN), is one of a newly introduced insecticides. FPN has been used in public health to control the main vectors of anthropozoonosis[17]. FPN is used either as a veterinary medicine[13] or as a pesticide on large scale in agriculture, in topical pet care products, households, and health care in many countries of the world[24]. Additionally, the growing use of FPN-containing insecticides versus the limitations on use of organochlorine and organophosphorus pesticides[11] resulted in higher environmental pollution[20]. Despite numerous benefits from pesticide use, a great evidences point to their role in endocrine disruption[25]. Besides being a potent inhibitor of the γ-aminobutyric acid (GABA) gated chloride channel[28], FPN also, acts as an inducer of microsomal enzymes[1], and as a thyroid disruptor, decreasing thyroid hormones in rats[19]. Observations of FPN cytotoxicity at doses lower than those observed for many pesticides prompted further investigations[3]. Thus, it is critical to explore the mechanisms of FPN induced thyroid disruption. So the aim of the current study is to spot the light on the impact of prolonged exposure to low dose of FPN on male rat thyroid gland and its related hormones, hepatic microsomal enzymes gene expression, histological and ultra-structural investigation.

Materials and Methods

All the experimental protocols were conducted as stipulated in the Guide for Care and Use of

Laboratory Animals Guidelines of the National Institutes of Health (NIH), and approved by the local authorities of Zagazig University, Egypt.

Chemicals: Firogen containing FPN 80% WG was obtained from Mytrade, inc. Egypt. CAS Registry Number: 120068-37-3. RNA extraction kit (Thermo Scientific, Fermentas, #K0731). Reverse transcription kits (Thermo Scientific, Fermentas, #EP0451). Kits used for estimation of serum TSH, T_3, and T_4 was obtained from Monobind Inc, Lake Forest CA 92630, USA. All other chemicals were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

Animals and experimental design: Eighteen mature *Sprague–Dawley* rats weighing (150-200g) were obtained from the Laboratory Animal Housing Unit, Faculty of Vet. Medicine, Zagazig Univeristy. Rats were housed separately in stainless steel cages in 12 h light/dark cycle. The animals were fed a standard diet beside tape water *ad-libtum*. All animals were acclimated to our facilities for two weeks. Rats were randomly divided into three equal groups, 1st group serve as control, 2nd group received corn oil and 3rd one received FPN dissolved in corn oil via stomach tube at dose level of 4.85 mg/kg b.wt (1/20 LD_{50}) twice weekly for 90 days based on oral LD_{50} of FPN for male albino rats is 97 mg/kg body weight[23].

Samplings: Blood samples were collected from retro-orbital venous plexus. The sera were separated and stored at -20° for measurement of TSH, total T_3 and T_4. Liver specimens were collected and snap frozen in liquid nitrogen for mRNA expression of *Ugt1a1- Sult1b1* enzymes. Samples of thyroid glands were dissected, immediately fixed in 3% gluatraldehyde buffer for electron microscopic examination and the other part was preserved in 10% buffered neutral formalin for histopathological examination.

Biochemical analysis: Determination of serum TSH using TSH AccuBind™ ELISA test, product code 325-300, Serum T_3 was determined using T_3 AccuLiteTM Microplate Chemiluminescence Immunoassay (CLIA) kit, product code 175-300 Serum T_4 determined using T_4 AccuBind™ ELISA test kit, product code 225-300.

Real time PCR: Pure RNA was extracted using total RNA Purification Kit following the manufacturer protocol. After reverse transcription, real-time PCR with SYBR Green was used to measure mRNAs expression of target genes with β-actin as an internal reference. The isolated cDNA were amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer protocol (Thermo scientific, USA,# K0221) and gene specific primers. The primers used in the amplification are shown in table (1). The web based tool, Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/ primer/primer3_www.cgi) was used to design these primers based on published rat. The final reaction mixture was placed in a Step One Plus real time thermal cycler (Applied Biosystems, Life technology, USA). The amplification conditions and cycle counts were carried out as follows: 95°C for 15 minutes for the initial activation, then 40 cycles of denaturation at 94°C for 15 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 30 seconds.

Histo-pathological examination: Samples of thyroidal tissue were fixed in 10% neutral-buffered formalin for 12 h, transferred to 70% ethanol, embedded in paraffin, sectioned (5μm) and stained with hematoxylin and eosin (H&E) then examined under light microscopy. Specimens of thyroid gland were fixed in 3% gluatraldehyde, followed by post-fixation in 1.3% osmium tetroxide then dehydrated with ascending grades of acetone and embedded in epoxy resin mixture. Ultrathin sections (70–90nm) were prepared and mounted on hexagonal copper grids, stained with uranyl acetate and lead citrate[8]. Stained grids were then examined by a JEOL JEM-2100 in El- Mansoura University, Egypt.

Statistical analysis: Obtained data were statistically analyzed using SPSS/PC+2001. The statistical method was one way ANOVA test, followed by Duncan's multiple range test[4]. Data are presented as means plus or minus the standard

Table (1): Primer sequences of target genes used for real time PCR in this study.

Target	Sequence
Ugt1a1	*F 3'* -CTTCAGAAAAAAGCCCTATCCCA-5' R *3'* -CCAAAGAGAAAACCACGATGC-5'
Sult1b1	*F 3'* -GAACAAAATGTTCCTGGAGCAAG-5' R *3'* -TGATGGAGTTTTCTTCAAGAGTTCAA-5'
β-actin	*F 3'* -CCTCTATGCCAACACAGTGC-5' R *3'* -GTACTCCTGCTTGCTGATCC-5'

Table (2): Changes in serum TSH (μIU/ml), total T_3 (ng/dl), total T_4 (μg/dl) and hepatic Ugt1a1, *Sult1b1* mRNA gene expression of male albino rats orally administered 1/20 LD_{50} FPN for 90 days (mean ± SE)

Parameter / *Group*	*Ugt1a1*	*Sult1b1*	*TSH (μIU/ml)*	*T_3(ng/dl)*	*T_4 (μg/dl)*
Control	1 ± 0.03^{c}	1 ± 0.04^{c}	0.051 ± 0.011^{a}	46.63 ± 1.64^{a}	4.05 ± 0.07^{a}
Corn oil	1.6 ± 0.13^{b}	1.53 ± 0.12^{b}	0.046 ± 0.010^{a}	41.20 ± 1.56^{ab}	3.60 ± 0.55^{ab}
FPN	4.8 ± 0.15^{a}	3.49 ± 0.08^{a}	0.033 ± 0.018^{a}	40.80 ± 2.97^{b}	2.833 ± 0.12^{b}

Means in the same column with different superscripts were significantly different at $P < 0.05$.

error. The minimum level of significance was set at $P \leq 0.05$.

Results and Discussion

Introduction of new, more toxic and rapidly disseminating pesticides into the environment has necessitated accurate identification of their potential health hazards.

Regarding to the effect of FPN on serum TSH, total T_3 and T_4; level of serum TSH showed non-significant decrease in FPN exposed rats, while levels of total T_3 and T_4 showed a significant decrease as compared with the control one and non significant decrease when compared with corn oil administered group as shown in table (2). The non significant decrease in serum TSH is controversy with those obtained by[12,19,20,24]. Despite decreased TSH level, there was a decrease in T_4, T_3. This may be owed to impairment of hypothalamus pituitary thyroid (HPT) axis as a negative feedback against decreased plasma T_4 level as mentioned by[15]. Moreover, FPN has damaging effect on nervous system[22] and pituitary gland in rodents[6]. Many of thyroid endocrine disruptors producing marked to dramatic reductions in serum T_4 in rats, with no change in serum TSH and little stimulation of the thyroid gland[9]. Concerning to the significant decrease in serum T_3 and T_4 levels in FPN exposed, these results are in accordance with those previously obtained by[12,19,20,24]. This may be attributed to the induction of hepatic enzymes responsible for T_3 and T_4 catabolism by FPN resulting in their increased clearance and reduced serum concentrations[12]. Moreover, hepatic microsomal enzymes play an important role in thyroid hormone economy because glucuronidation is the rate-limiting step in the biliary excretion of T_4 and sulfonation primarily by phenol sulfotransferase for the excretion of T_3[5,21] which is confirmed by our obtained results. Hormone levels are not only regulated by synthesis and secretion but also by catabolism and elimination. Thus, the hepatic-endocrine axis is an important component in the homeostatic control of a number of hormone18). Furthermore, Thyroid-disrupting effect of FPN is not limited to the increase in the clearance of T3 and T_4[24], but also may be due to significant changes in thyroid tissue structure[6].

Regarding to the effect of FPN on hepatic Ugt1a1, Sult1b1 gene expression: the hepatic Ugt1a1 and

Sult1b1 activity was up-regulated significantly in FPN exposed male albino rats in comparison with those of control and corn oil groups as shown in table (2). These results are in agreement with that obtained by[19] who suggest that FPN increase T_4 conjugation pathway via increased activity of phase II hepatic enzymes thus; increasing clearance of T_4 which mentioned also by[12]. Chemicals that induce UGTs are of concern because of their disruptive effect on thyroid hormone homeostasis[2]. Expression of Sult1b1 was significantly increased as iodothyronines may be sulfonated to facilitate their excretion where Sult1b1 has shown to be the major thyroid hormone sulfotransferases (SULT)[10]. Regarding to FPN induced histo-pathological and ultra-structural alterations of thyroid follicles: Light microscopic examination of thyroid glands of control rats showed intact thyroid follicles were lined by cuboidal epithelium with rounded nuclei and colloid fig.1 (A). While, those of corn oil administered group showed some irregularity in the follicles with reduction in its size fig.1 (B). However, thyroid glands of FPN exposed rats showed prominent follicular disorganization with severe vacuolation of the lining epithelium and were extremely reduced in size and colloid fig.1 (C), focal hypertrophy of lining epithelium. Sometimes, the follicles were ruptured forming large and irregular ones fig.1 (D). Severe congestion, rarely hemorrhage and lymphocytes infiltrations were also seen. FPN induced alteration in thyroid gland ultra-structure as thyroid gland of control rats revealed normal follicular cell with cuboidal epithelium composed of rough endoplasmic reticulum, mitochondria and euchromatic nucleus with regular and well formed nuclear membrane and prominent nucleolus. The apical surface showed microvilli protruding to the colloidal lumen fig.2 (A) while in FPN exposed rats follicles showed damaged cells and marked decrease in height. Exfoliated debris of follicular cells in lumen was seen fig.2 (B). Two adjacent follicular cells, one of them showed damaged cells with pyknotic nucleus and other showed indistinct organelles and vacuoles fig.2 (C). Follicular cells with marked decrease in height with detached microvilli, the mitochondria were degenerated with loss of cristae. The nuclei appear heterochromatic, flattened and irregular with peripheral condensation of chromatin fig.2 (D). Finally, these alterations may be attributed to FPN induced oxidative stress as supported by[14], or occurs secondary to the degenerative and apoptotic changes seen in many cells.

Different toxicants induce cell death through apoptosis or necrotic events[27]. Apoptotic events include; dissipation of the mitochondrial trans-membrane potential, nuclear and chromatin condensation as mentioned by[16].

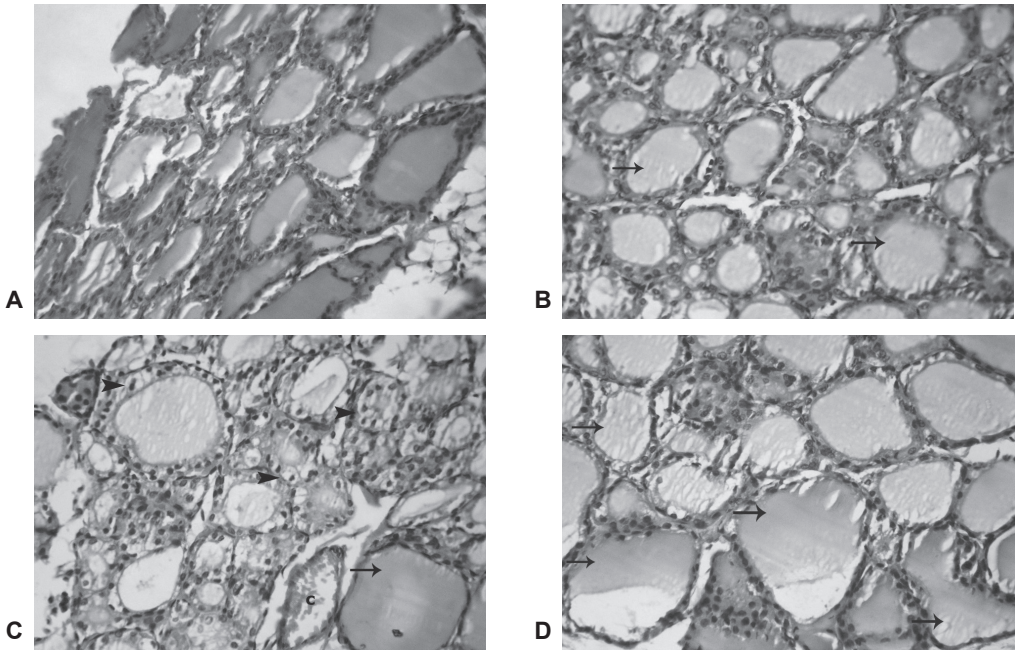

Figure (1): Photomicrograph of thyroid gland section of male albino rats FPN exposed rats showing: (A) control; normal intact follicles and lining epithelium. **(B)**; corn oil exposed rats showing slight irregularity in the follicles (arrows). **(C)**; FPN exposed rats showing follicular disorganization, severe vacuolation of the lining epithelium (arrowheads), congestion **(c)** and extremely reduced sizes and colloid. **(D)** large and irregular follicles in FPN exposed rats. (HEX400).

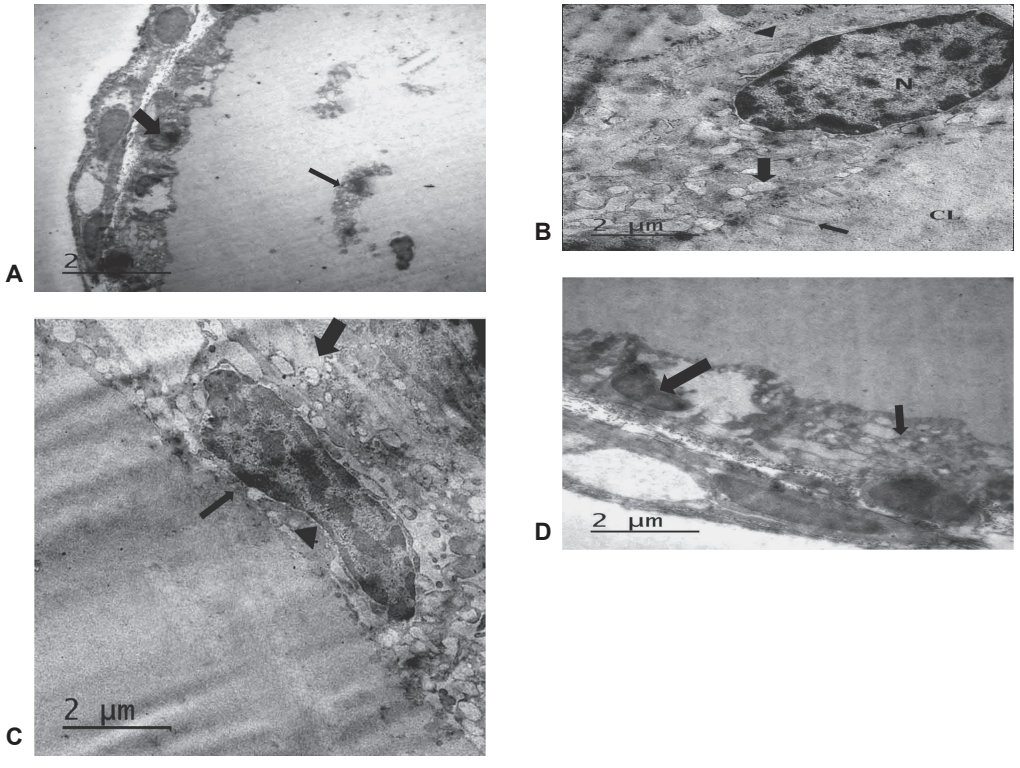

Figure (2): An electron photomicrograph section of rat thyroid gland showing: (A); follicular cell with microvilli of apical border (MV) (narrow arrow) projecting to the colloidal lumen (CL), rough endoplasmic reticulum (ER) (arrow head) and mitochondria (M) (thick arrow). The nucleus (N) **(B)**; FPN exposed rats showing: marked decrease in height (thick arrow), cellular debris (narrow arrow). **(C)**: High power of the previous fig to two adjacent follicular cells, one with pyknotic nucleus (thick arrow) and other with indistinct organelles (thin arrow) **(D)** Follicular cells of FPN exposed rats showed marked decrease in height with detached microvilli (MV) (narrow arrow), rounded mitochondria (thick arrow). The nuclei are heterochromatic, flattened and irregular (arrow head) (2μm).

These apoptotic features were detected in follicular cells after FPN exposure; these are in agreement with that observed by[3,26] who found that FPN triggers apoptosis in HepG2 cells; human hepatocytes and SHSY5Y cells; respectively. In addition, nuclear condensation takes place at the end of the apoptotic process[7]. All of these data indicate that FPN induced lesions in thyroid follicles occurred mainly via an apoptotic mechanism. In conclusion, FPN exposure induced apoptotic changes in thyroid follicle ultra-structure and altered thyroid hormones. Consequently, strict limitations on its use are required and replaced with the most safety ones.

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