



Study of Zinc Effects on Fipronil Induced Thyroid Toxicity in Adult Male Albino Rats

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Abstract

Background: Fipronil has also become a widespread environmental contaminant detected in both soil and water. Zinc retards oxidative processes on a long-term basis by inducing the expression of metal-binding cysteine-rich proteins called metallothioneins, and in mammals. In mammals, the thyroid is an endocrine gland of critical importance for the regulation of the metabolism in general.

Objective: to study protective effect of zinc on thyroid toxicity induced by fipronil in adult male albino rats through biochemical, histopathological and genotoxic studies.

Materials and methods: In the present study, forty adult healthy male albino rats weighing 180 - 200 gm, were used. They were divided into 4 groups as following: Group I (control group): It was subdivided into 2 groups each of 8 rats; Group IA (negative control group) 8 rats:

Rats received no medication, only regular diet and water to measure the basic parameters for 6 weeks, Group IB (positive control group) 8 rats: Each rat treated with 1 ml distilled water (the vehicle of fipronil and zinc) by oral gavage once daily for 6 weeks. Group II (zinc group) 8 rats:

Each rat was gavaged orally with 2mg/kg bw zinc as powder dissolved in 1 ml distilled water once daily for 6 weeks. Group III (fipronil group) 8 rats: Each rat was gavaged orally with 9.7 mg/kg bw fipronil dissolved in 1 ml distilled water once daily for 6 weeks which equals 1/10 of LD50. Group IV (zinc+fipronil) 8 rats:

Each rat was gavaged orally with 2mg/kg bw zinc as powder dissolved in 1 ml distilled water. An hour later; Fipronil was gavaged orally with 9.7mg/kg bw fipronil dissolved in 1 ml distilled water once daily for 6 weeks. At the end of 6 weeks, all rats were anesthetized then subjected to blood samples collection for estimation of serum biomarkers of thyroid functions (TSH, T3 & T4), oxidative stress (MDA) and Antioxidant biomarkers SOD & GPX).

Results: The present findings showed that zinc administration significantly decreased thyroid function tests (T3, T4) & increased TSH in Zinc with fipronil treated group when compared with FPN treated group. that zinc administration with FPN resulted in a very highly significant decrease in the mean values of serum (MDA, GPX & SOD) of Zinc with fipronil group when compared with FPN treated group. Comet assay performed on thyroid specimens of zinc with fipronil treated group revealed a significant decrease in % of tailed nuclei, tail length, tail DNA % and unit tail moment and a significant increase in % of untailed nuclei when compared with those of the negative control group.

Conclusion: Zinc plays an important role in protection against fipronil thyroid toxicity evidenced by improving the biochemical, histopathological and comet assay results in groups treated with zinc and fipronil

Keywords: Zinc, Fipronil, Thyroid

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1. Introduction

Phenylpyrazoles are a class of chemically-related broad-spectrum insecticides. The chemical structures of these insecticides are characterized by a central pyrazole ring with a phenyl group attached to one of the nitrogen atoms of the pyrazole. There are many examples including fipronil, acetoprole, ethiprole, flufiprole, pyraclofos, pyrafluprole, pyriprole, pyrolan and vaniliprole(1).

Fipronil is a relatively new insecticide that belongs to the phenylpyrazole family, classified by WHO as class II moderately hazardous pesticide (2). It came to broad public attention in 2017, when fipronil-contaminated eggs were found in several European countries (3).

Fipronil has also become a widespread environmental contaminant detected in both soil and water (4), indoor and outdoor dust (5) as well as in various food matrices as highlighted by fipronil egg contamination scandal (6).

Acute poisoning in response to fipronil exposure in humans causes headache, dizziness, sweating, nausea, vomiting, agitation, and seizures (7).

Although fipronil showed lower toxicity in mammals than in insects, fipronil was documented to have adverse effects to organs such as liver, thyroid, and reproductive function in non target species ranging from frogs to mammals, also Chronic fipronil exposure in rats led to injury of the thyroid, liver, and kidney (8).

Fipronil impedes the metabolic enzymatic systems that are often known to contain sulfhydryl groups causing uncoupling of oxidative phosphorylation in the mitochondrial complex. This process results in depletion of sulfhydryls, increased lipid peroxidation, DNA damage, altered calcium

homeostasis leading to ischemia, hypoxia of vital organs and death (9).

In mammals, the thyroid is an endocrine gland of critical importance for the regulation of the metabolism in general. It is located at the anterior cervical region of the neck and synthesizes the hormones triiodothyronine (T3), thyroxine (T4). This organ is an excellent bio-indicator in toxicology studies, as it is very sensitive to toxic exposure, even in minimum dosages (10).

Zinc is a nutritionally fundamental trace element, essential to the structure and function of numerous macromolecules, including enzymes regulating cellular processes and cellular signaling pathways. Zinc also modulates immune response and exhibits antioxidant and anti-inflammatory activity. Zinc retards oxidative processes on a long-term basis by inducing the expression of metal-binding cysteine-rich proteins called metallothioneins. Furthermore, zinc increases the activation of antioxidant proteins and enzymes, such as glutathione and catalase (11).

2. Subjects and methods

➤ Material:-

I-Chemicals: -

A) -Fipronil:

Fipronil (Contrado powder 5%) a preparation obtained from **StarChem Industrial Chemicals Company** and manufactured by Zhejiang Yongnong Chem.Co., China. It was in the form of white powder, dissolved in water, stable only for 24 hours after dissolution so, it was freshly prepared before every use.



B) -Zinc:

Zinc was obtained from Elgomhoria Pharmaceuticles Co (Elsawaf st., Zagazig, Egypt) in the form of White odorless powder of zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$) soluble in distilled water.

C)- Distilled water:

Obtained from El- Nasr Co, Egypt and used as a solvent for both fipronil and zinc.

D)- Reagents and commercial kits:

-Kits for estimation of Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline phosphatase (ALP), Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Malondialdehyde (MDA), ELIZA kits for estimation of triiodothyronine (T3) and thyroxine (T4), were purchased from Bio diagnostic Co. ELIZA Kits for estimation of thyroid stimulating hormone (TSH) were purchased from Calbiotech chemical company in Cairo

-Kits for estimation of lactate dehydrogenase (LDH) were purchased from Egyptian Company for Biotechnology.

II-Animals: -

Forty male adult albino rats, aged about 6 weeks and weighing 180 - 200 gm, were obtained from the animal house of Faculty of Medicine, Zagazig University.

The adult male albino rat was the animal of choice for this experiment because of metabolic similarities with human (12). It is extremely valuable in duplicating the response of human to drugs. Its numerical availability provides ground for its use in order to obtain relevant statistical evaluation (13).

N.B: One month injection in rats is equivalent to 24 months in human being (14).

The Institutional Animal Care and Use Committee (IACUC), Zagazig University has approved the design of the experiment. According to mean difference, sample size was calculated to be 40 rats using open Epi program in Community Medicine department, Faculty of Medicine, Zagazig University.

Before starting the experiment, all animals subjected to 2 weeks of passive preliminaries for house acclimatization, to ascertain their physical wellbeing and to exclude any diseased animal.

All animals received human care in compliance with the animal guidelines and ethical regulations in accordance with "The Guide for The Care and Use of Laboratory Animals (15).

The animals were kept in plastic mesh cages with solid bottom. These cages contained wood shavings in the bottom as bedding which were changed frequently to keep the animals clean. Overcrowdness and isolation were avoided. Animals were allowed free access to solid food and water in their home cages. There was proper ventilation in the animal house and in the cage. The room was maintained with 12h-light/dark cycle.

All experimental procedures were ethically approved by The Ethical committee for scientific research of Faculty of Medicine, Zagazig University and performed according to the institutional guidelines for the care and use of laboratory animals, which are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.



➤ **Methods:-**

Experimental design: -

The rats were divided into 4 groups as following:

***Group I(control group):** contains 16 rats which was subdivided into 2 groups each of 8 rats

• **Group IA (negative control group):**

Rats will receive no medication, only regular diet and tap water to measure the basic parameters for 6 weeks.

Group IB (positive control group):

Each rat was treated with 1 ml distilled water (the vehicle of fipronil and zinc) by oral gavage once daily for 6 weeks.

***Group II (zinc group) (8 rats):**

Each rat was gavaged orally with 2mg/kg zinc as powder dissolved in distilled water once daily for 6 weeks according to **Goel et al. (16)**.

***Group III (fipronil group) (8 rats):**

Each rat was gavaged orally with 9.7 mg/kg bw fipronil dissolved in distilled water once daily for 6 weeks which equals 1/10 of LD₅₀ according to **Tomlin (17)** who reported that oral LD₅₀ of fipronil is 97 mg/kg in rats.

***Group IV (zinc+fipronil) (8 rats):**

Each rat was gavaged orally with 2mg/kg zinc as powder dissolved in distilled water. An hour later; Fipronil was gavaged orally with 9.7mg/kg bw dissolved in distilled water once daily for 6 weeks according to **Swelam et al., (18)**.

At the end of 6 weeks, all rats were subjected to the following:

Each rat was anesthetized by ether inhalation and blood samples was collected from the venous retro-orbital plexus for estimating: -

❖ **thyroid function tests:** thyroid stimulating hormone (TSH), triiodothyronine (T3) & thyroxine (T4).

❖ **oxidative stress biomarkers assay:** Malondialdehyde (MDA), superoxide dismutase (SOD) and Glutathion peroxidase (GPX).

Then rats were sacrificed by cervical dislocation, thyroid were immediately dissected out and grossly inspected to assess any gross abnormalities. The thyroids and were divided into two parts, the first part was fixed in 10% formalin for histopathological examination under light microscope. The second part was put in normal saline then kept frozen at -20°C for Comet assay to evaluate genotoxic effects of fipronil.

• **Biochemical Studies:**

Method used for blood samples collection:

Venous blood samples were collected from animals by means of micro-capillary glass tubes from the retro-orbital plexuses in accordance with the procedure described by **Johnson, (12)**.

The animal was held in the left hand and grasped from the back, while enclosing the neck and exerting slight pressure by the thumb and index fingers. This would cause engorgement to the veins of the retro-orbital plexuses with slight protrusion of the eyeball. A glass tube with capillary orifice of 0.6 mm was inserted into the orbit of the eye at an anterior angle. Then it was rotated to drill through the conjunctiva in the direction of



the site of optic nerve. The plexus would be reached at depth of 4.5 mm, and because of venous congestion, the blood spontaneously shoots into the capillary tube.

Evaluation of DNA damage:

(1) For viewing the DNA damage, observations were made of EtBr-stained DNA using a 40x objective on a fluorescent microscope.

(2) A Komet 5 image analysis software developed by Kinetic Imaging Ltd. (Liverpool, UK) linked to a charge coupled device (CCD) camera was used to determine the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration [tail length] and the percentage of migrated DNA in the tail [tail DNA %]. Finally, the program calculates tail moment [correlation between tail length and tail DNA %]. Generally, images of 100 (50 X 2) randomly selected cells are analyzed per sample. The mean value (for 100 cells) was calculated.

Statistical analysis

The obtained results were tabulated as mean \pm SE. Statistical analysis was performed using one-way analysis of variance (ANOVA), One Way Analysis of variance (ANOVA) test for comparison of means of multiple independent groups of normally distributed data, Least Significance Difference (LSD) test is used for comparison between different groups, Paired t-test is used for comparison of means of one group at different time interval, Chi-square test (χ^2) is used to find the association between row and column variables, For all above-mentioned statistical tests, the threshold of significance was fixed at 5% level (P-value). P value of > 0.05 indicates non-significant results. P value of < 0.05 indicates significant results. P value of < 0.01 indicates high significant results. P

value of < 0.001 indicates very high significant results

3. Results

The present study was carried out on 40 adult male albino rats. The rats were divided into 4 groups: control group which comprised both negative control group (received only regular diet and water) and vehicle control group (received 1 ml of distilled water), zinc treated group (with a dose 2mg/kg once daily by oral gavage), fipronil group (treated with a dose 9.7 mg/kg once daily by oral gavage) and zinc with fipronil group treated with zinc and after one hour treated with fipronil with the same previously mentioned doses. The study extended for 6 weeks; all rats were sacrificed.

I- Biochemical results:

(A) Biochemical parameters of control groups:

As comparing the laboratory results of biochemical tests regarding serum biomarkers of thyroid functions and oxidative stress biomarkers of the negative control (group Ia) and the vehicle control (group Ib) were within normal values. There was no statistically significant difference between them as well as within the same group all over the period of the study ($p > 0.05$). So we used negative control group as a standard reference for comparison with other groups (Tables 1, 2, 3).

Thyroid function tests:-

1) Thyroid stimulating hormone (TSH):

There was a very highly significant difference ($p < 0.001$) in the mean values of serum TSH among negative control, Fipronil, Zinc and zinc with fipronil groups by ANOVA test as shown in (Table 4 & Figure 1).

There was a very highly significant increase ($p < 0.001$) in the mean values of serum TSH of fipronil treated group when compared with negative control group. Also there was a very highly significant decrease ($p < 0.001$) in the mean values of serum TSH of zinc with fipronil group when compared with fipronil treated group by LSD test as shown in **(Table 5 & Figure 1)**.

Moreover, there was a non significant difference ($p > 0.05$) in the mean values of serum TSH of zinc with fipronil group when compared with negative control group by LSD test as shown in **(Table 5 & Figure 1)**.

2) Triiodothyronine (T3):

There was a very highly significant difference ($p < 0.001$) in the mean values of serum T3 among negative control, Fipronil, Zinc and zinc with fipronil) groups by ANOVA test as shown in **(Table 4 & Figure 1)**.

There was a very highly significant decrease ($p < 0.001$) in the mean values of serum T3 of fipronil treated group when compared with negative control group. Also, there was a very highly significant increase ($p < 0.001$) in the mean values of serum T3 of zinc with fipronil group when compared with fipronil treated group by LSD test as shown in **(Table 5 & Figure 1)**.

Moreover, there was a non-significant difference ($p > 0.05$) in the mean values of serum T3 of zinc with fipronil group when compared with negative control group by LSD test as shown in **(Table 5 & Figure 1)**.

3) Thyroxine (T4):

There was a very highly significant difference ($p < 0.001$) in the mean values of serum T4 among negative control, Fipronil, Zinc and zinc with fipronil groups by ANOVA test as shown in **(Table 4 & Figure 1)**.

There was a very highly significant decrease ($p < 0.001$) in the mean values of serum T4 of fipronil treated group when compared with negative control group. Also there was a very highly significant increase ($p < 0.001$) in the mean values of serum T4 of zinc with fipronil group when compared with fipronil treated group by LSD test as shown in **(Table 5 & Figure 1)**.

Moreover, there was a non-significant difference ($p > 0.05$) in the mean values of serum T4 of zinc with fipronil group when compared with negative control group by LSD test as shown in **(Table 5 & Figure 1)**.

Oxidative stress biomarkers:-

Malondialdehyde (MDA):

There was a very highly significant difference ($p < 0.001$) in the mean values of serum MDA among negative control, Fipronil, Zinc and zinc with fipronil groups by ANOVA test as shown in **(Table 6 & Figure 2)**.

There was a very highly significant increase ($p < 0.001$) in the mean values of serum MDA of fipronil treated group when compared with negative control group. Also, there was a very highly significant decrease ($p < 0.001$) in the mean values of serum MDA of zinc with fipronil group when compared with fipronil treated group by LSD test as shown in **(Table 7 & Figure 2)**.

Moreover, there was a non-significant difference ($p > 0.05$) in the mean values of serum MDA of zinc with fipronil group when compared with negative control group by LSD test as shown in **(Table 7 & Figure 2)**.



-Serum Glutathione peroxidase (GPX):

There was a very highly significant difference ($p < 0.001$) in the mean values of serum GPX among negative control, Fipronil, Zinc and zinc+fipronil groups by ANOVA test as shown in **(Table 6)**.

There was a very highly significant decrease ($p < 0.001$) in the mean values of GPX of fipronil treated group when compared with negative control group. Also there was a very highly significant increase ($p < 0.001$) in the mean values of GPX of zinc with fipronil group when compared with fipronil treated group by LSD test as shown in **(Table 7)**.

Moreover, there was a non-significant difference ($p > 0.05$) in the mean values of serum GPX of zinc with fipronil group when compared with negative control group by LSD test as shown in **(Table 7)**.

Superoxide dismutase (SOD):

There was a very highly significant difference ($p < 0.001$) in the mean values of serum SOD among negative control, Fipronil, Zinc and zinc with fipronil groups by ANOVA test as shown in **(Table 6)**.

There was a very highly significant decrease ($p < 0.001$) in the mean values of serum SOD of fipronil treated group when compared with negative control group. Also, there was a very highly significant increase ($p < 0.001$) in the mean values of SOD of zinc with fipronil group when compared with fipronil treated group by LSD test as shown in **(Table 7)**.

Moreover, there was a non-significant difference ($p > 0.05$) in the mean values of serum SOD of zinc with fipronil group when compared with negative control group by LSD test as shown in **(Table 7)**.

II- Histopathological results:

Thyroid

Control group (I) and zinc treated group (II):

Histopathological examination of the thyroid sections of adult male albino rats of negative control group (IA), vehicle control group (IB) and zinc treated group (II) showed the same histological features without any observable histopathological finding.

Macroscopic features:

Normal appearance of the thyroid gland was noticed with no cystic changes or abnormal masses, as well as cut sections were normal.

Microscopic features:

Examination of H&E-stained thyroid sections of these groups showed the presence of normal tissue, where the thyroid follicles could be observed surrounded by a cubic and mono-nucleated follicular cells epithelium. Inside the follicles, the colloids also showed an intact aspect. In the extra follicular region, the connective tissue filled the spaces and brought many blood vessels to the glandular tissue, responsible for collecting gland's secretion.

- **Fipronil treated group (III):**

Examination of H&E stained thyroid sections of this group showed more significant alterations which can be represented by loss of the original follicular rounded form of the thyroid, since some of them acquired a totally irregular appearance, fusion of more than one follicle causing a total structural disarrangement in the gland, increased interfollicular spaces with consequent occupation by edema. Increased the vascularity of the gland, as well as the caliber of blood vessels. Follicular cell vacuolation,

which led to displacement of the nucleus from the central regions to the cell periphery.

zinc with fipronil treated group (IV):

Thyroid sections from zinc with fipronil treated group revealed marked improvement of thyroid structure. It nearly retained its normal architecture. The colloid material filled most follicle lumen and the follicular cells exhibited normal shape and arrangement except some vacuolation at follicular lining epithelium (**Fig 27**).

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III- Comet assay results:

Regarding the oxidative DNA damage caused by fipronil, the present study tested the *in vivo* genotoxic potential of fipronil in rats using the single cell gel electrophoresis (comet assay). The result revealed that oral administration of fipronil causes a time dependent increase in DNA damage in the thyroid of adult male albino rats indicated by the damaged nuclei. The parameters used to measure DNA damage in the cells were the following: % of tailed nuclei, % of untailed nuclei, tail length (length of DNA migration), tail DNA % (percentage of migrated DNA in the tail) and unit tail moment (correlation between tail length and tail DNA %).

Comet assay of the thyroid:

The results of the present study showed no significant difference ($P>0.05$) among negative control group IA and the vehicle control group IB as well as within the same group as regard mean values of % of tailed nuclei, % of untailed nuclei, tail length, tail DNA % and unit tail moment in the thyroid gland after 6 weeks of exposure. So, we used negative control group as a standard reference for comparison with other groups.



Table 1: Descriptive statistics base line clinical data

Table (1): Statistical comparison between negative and positive control groups as regard mean values of serum alanine amino transferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) at the end of six weeks by using student t- test.

Group N=8 parameter	-ve Control	+ve Control	t	p-value
	Mean±SD			
ALT (U/L)	28.88±4.7	31.25±4.65	1.015	0.327 NS
AST (U/L)	26.88±3.91	26.00±3.74	0.457	0.654 NS
ALP (U/L)	53.75±9.36	53.25±7.21	0.120	0.907 NS
LDH (U/L)	261.88±25.06	263.75±22.79	0.157	0.878 NS

N.B All values are expressed as mean±SD. (SD: standard deviation)

N : Number of rats in each group was 8 rats.

NS: P >0.05 =non significant. t:t- test

U/L= unit per litre

Table (2): Statistical comparison between negative and positive control groups as regard mean values of serum thyroid stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4) using at the end of six weeks by student t- test.

parameter	-ve Control	+ve Control	t	p-value
	Mean±SD			
TSH (ng/ml)	0.91±0.09	0.92±0.06	0.228	0.823 NS
T3 (ng/ml)	1.52±0.39	1.47±0.37	0.267	0.793 NS
T4 (µg/dl)	8.55±1.12	8.21±1.07	0.625	0.542 NS

N.B All values are expressed as mean±SD. (SD: standard deviation)

N: Number of rats in each group was 8 rats.

NS: P >0.05 =non significant.

t: t- test

µg/dl = microgram per deciliter.

ng/mg = nanogram per milligram

Table (3): Statistical comparison between negative and positive control groups as regard mean values of serum malondialdehyde (MDA), Glutathione peroxidase (GPX) and superoxide dismutase (SOD) at the end of six weeks by using student t- test.

parameter	-ve Control	+ve Control	t	p-value
	Mean±SD			
MDA (nmol/ml)	0.66±0.18	0.61±0.15	0.617	0.547 NS
GPX (µu/ml)	170.38±11.33	170.63±11.89	0.051	0.960 NS
SOD (U/ml)	292.13±19.13	292.63±20.26	0.043	0.966 NS

N.B All values are expressed as mean±SD. (SD: standard deviation)

N: Number of rats in each group was 8 rats.

NS: P >0.05 =non significant.

t: t- test

nmol/ml = nanomole per millilitre.

U/ml= unit per millilitre.



Table (4): Statistical comparison among negative control, zinc, fipronil and zinc with fipronil groups as regard mean values of serum thyroid stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4) at the end of six weeks by using ANOVA test.

Group N=8 Parameter	-ve Control	Zinc	Fipronil	Zinc+fipronil	F	P-value
	Mean ± SD					
TSH (ng/ml)	0.91±0.09	0.94±0.16	7.60±1.56	1.70±0.82	106.28	<0.001**
T3 (ng/ml)	1.52±0.39	1.48±0.42	0.52±0.15	1.33±0.34	15.051	<0.001**
T4 (µg/dl)	8.55±1.12	8.40±1.84	3.01±0.82	7.52±1.14	33.177	<0.001**

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N.B: All values are expressed as mean±SD. (SD: standard deviation)

N.B: All values are expressed as mean±SD. (SD: standard deviation)

N: Number of rats in each group was 8 rats.

** : statistically highly significant (P <0.001)

µg/dl = microgram per deciliter

ng/mg = nanogram per milligram.

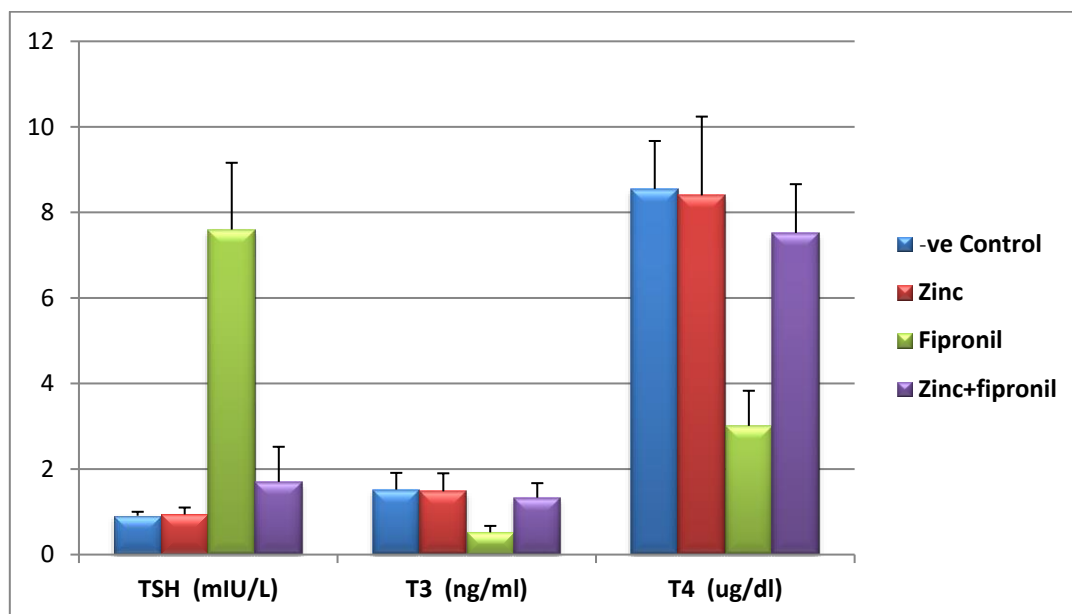


Fig (1): Bar chart showing comparison among negative control, zinc, fipronil and zinc with fipronil groups as regard mean values of serum thyroid stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4)

Table (5): Least significance difference (LSD) for comparison between mean values of serum thyroid stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4) of negative control, zinc, fipronil, and zinc with fipronil groups at six weeks of the study.

Parameters	Group N=8	Zinc (0.94±0.16)	Fipronil (7.60±1.56)	Zinc+fipronil (1.70±0.82)
TSH (ng/ml)	-ve control (0.91±0.09)	0.944 NS	<0.001**	0.085 NS
	Zinc (0.94±0.16)		<0.001**	0.098 NS
	Fipronil (7.60±1.56)			<0.001**
T3 (ng/ml)	Group	Zinc (1.48±0.42)	Fipronil (0.52±0.15)	Zinc+fipronil (1.33±0.34)
	-ve control (1.52±0.39)	0.801 NS	<0.001**	0.267 NS
	Zinc (1.48±0.42)		<0.001**	0.377NS
	Fipronil (0.52±0.15)			<0.001**
T4 (µg/dl)	Group	Zinc (8.40±1.84)	Fipronil (3.01±0.82)	Zinc+fipronil (7.52±1.14)
	-ve control (8.55±1.12)	0.812 NS	<0.001**	0.119 NS
	Zinc (8.40±1.84)		<0.001**	0.182 NS
	Fipronil (3.01±0.82)			<0.001**

NS: statistically non significant (p>0.05) ng/mg = nanogram per milligram.
 *: statistically significant (p<0.05). µg/dl = microgram per deciliter.
 **: statistically highly significant (P<0.001).
 N: Number of rats in each group was 8 rats.

Table (6): Statistical comparison among negative control, zinc, fipronil and zinc with fipronil groups as regard mean values of serum malondialdehyde (MDA), Glutathione peroxidase (GPX) and superoxide dismutase (SOD) at the end of six weeks by using ANOVA test

Group N=8	-ve Control	Zinc	Fipronil	Zinc+fipronil	F	P-value
Parameter	Mean ± SD					
MDA (nmol/ml)	0.66±0.18	0.72±0.21	6.38±1.13	0.80±0.15	187.217	<0.001**
GPX (µu/ml)	170.38±11.33	172.00±11.55	100.50±15.98	159.50±9.55	60.293	<0.001**
SOD (U/ml)	292.13±19.13	291.25±21.67	106.13±10.95	275.63±24.85	166.360	<0.001**

N.B: All values are expressed as mean±SD. (SD: standard deviation)
 N: Number of rats in each group was 8 rats.
 **: statistically highly significant (P <0.001)
 nmol/ml = nanomole per millilitre.
 µu/ml = milliunit per millilitre.
 U/ml= unit per millilitre.



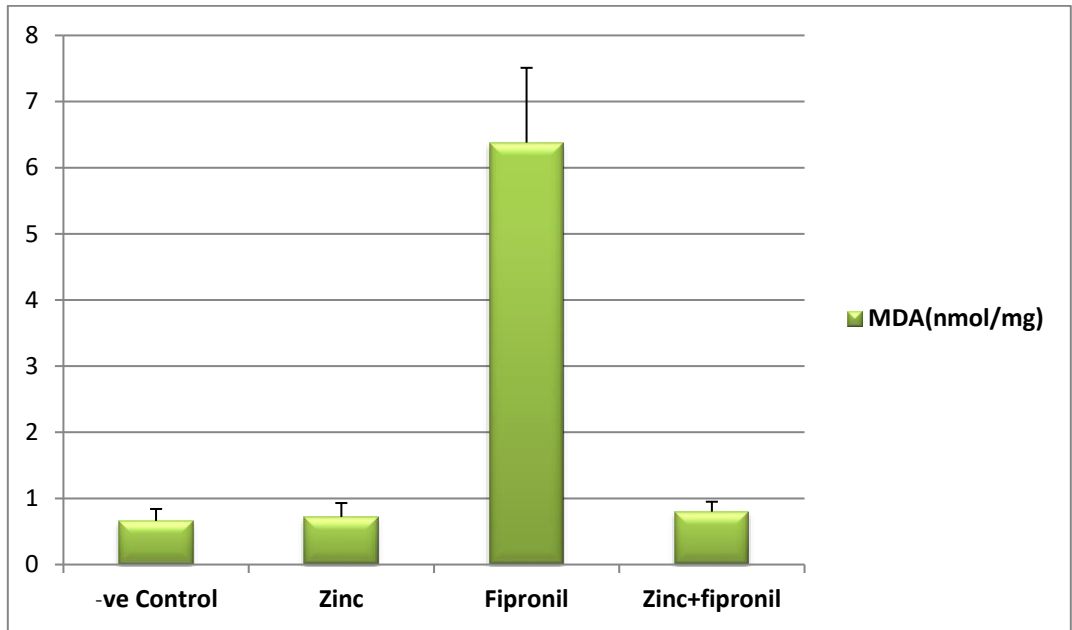


Figure (2): Bar Chart showing comparison among negative control, zinc, fipronil and zinc with fipronil groups as regard mean values of serum malondialdehyde (MDA).

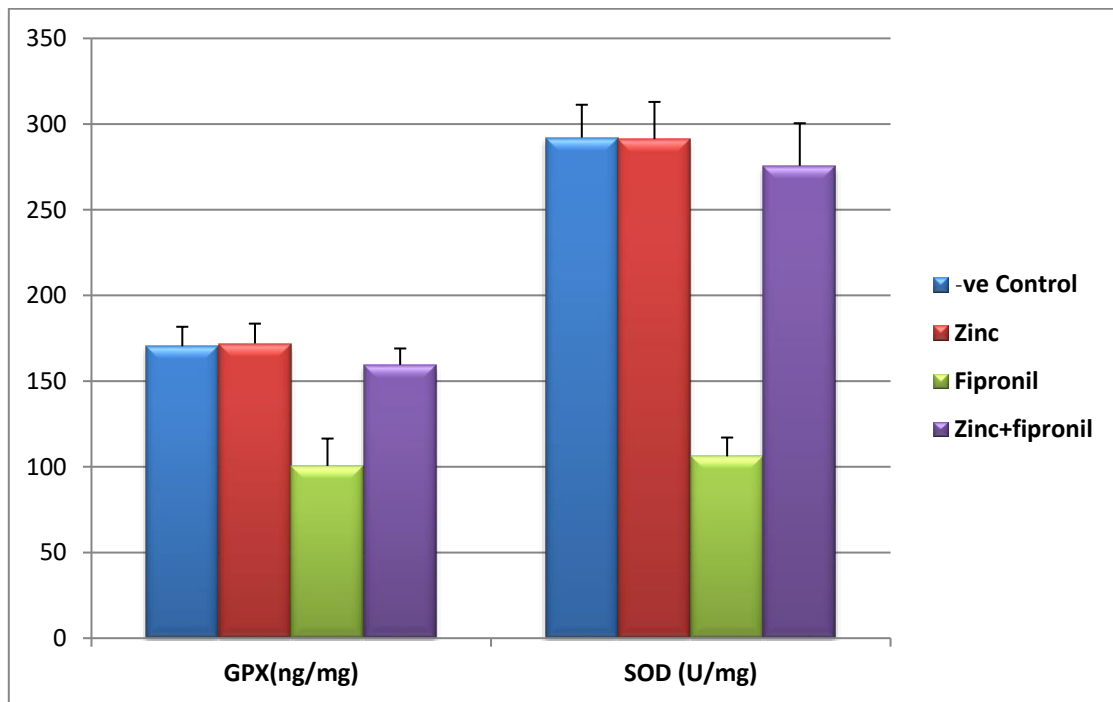


Figure (3): Bar Chart showing comparison among negative control, zinc, fipronil and zinc with fipronil groups as regard mean values of serum glutathione peroxidase (GPX) and superoxide dismutase (SOD).

Table (7): Least significance difference (LSD) for comparison between mean values of serum malondialdehyde (MDA), Glutathione peroxidase (GPX) and superoxide dismutase (SOD) of negative control, zinc, fipronil, and zinc with fipronil groups at six weeks of the study.

Parameters	Group N=8	Zinc (0.72±0.21)	Fipronil (6.38±1.13)	Zinc+fipronil (0.80±0.15)
MDA (nmol/ml)	-ve control (0.66±0.18)	0.836 NS	<0.001**	0.629 NS
	Zinc (0.72±0.21)		<0.001**	0.795 NS
	Fipronil (6.38±1.13)			<0.001**
GPX (mu/ml)	Group	Zinc 172.00±11.55)	Fipronil 100.50±15.98)	Zinc+fipronil 159.50±9.55)
	-ve control (170.38±11.33)	0.946 NS	<0.001**	0.089 NS
	Zinc (172.00±11.55)		<0.001**	0.052 NS
	Fipronil (100.50±15.98)			<0.001**
SOD (U/ml)	Group	Zinc (291.25±21.67)	Fipronil (106.13±10.95)	Zinc+fipronil (275.63±24.85)
	-ve control (292.13±19.13)	0.817 NS	<0.001**	0.107 NS
	Zinc (291.25±21.67)		<0.001**	0.126 NS
	Fipronil (106.13±10.95)			<0.001**

NS: statistically non significant (p>0.05). **mu/ml** = milliunit per millilitre.
 : statistically highly significant (P<0.001). **nmol/ml = nanomole per millilitre.
 N: Number of rats in each group was 8 rats **U/ml**= unit per millilitre



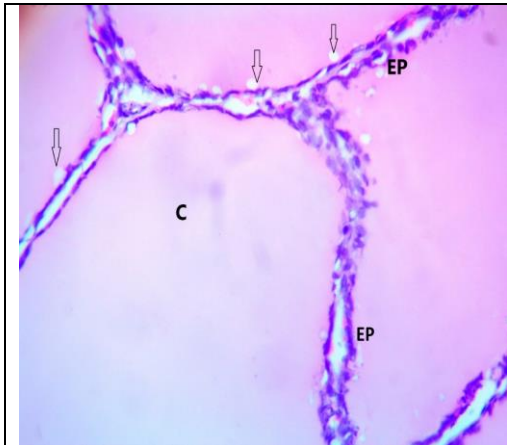


Fig. 4 A photomicrograph of a section in thyroid gland obtained from an adult male albino rat of control group (I) showing normal thyroid architecture with follicles lined with cuboidal follicular epithelium (EP) with rounded nuclei. The follicular lamina is filled with homogenous acidophilic colloid (C) exhibiting peripheral small vacuoles (arrow). (H&E 400X).

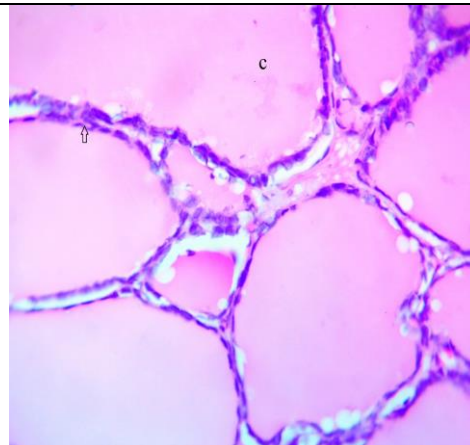


Fig. 5 A photomicrograph of a section in thyroid gland obtained from an adult male albino rat of zinc group (group II) showing the normal histopathological structure of the thyroid. Thyroid follicles lined with cuboidal epithelium (arrow) and homogenous colloid (C) (H&E x400).

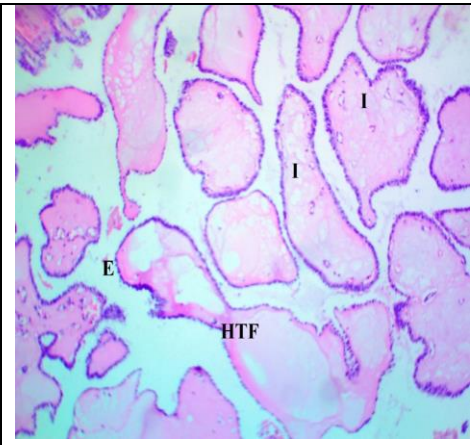


Fig. 6 A photomicrograph of a section in thyroid gland obtained from an adult male albino rat of fipronil treated group (group III) showing hypertrophy of some follicles (HTF) causing loss of the original rounded form, irregular follicles (I) and increased interfollicular spaces due to edema (E) (H&E x400).



Fig. 7 A photomicrograph of a section in thyroid gland obtained from an adult male albino rat of fipronil treated group (group III) showing fusion (F) of more than one follicle, causing a total structural disarrangement in the gland (H&E x400).

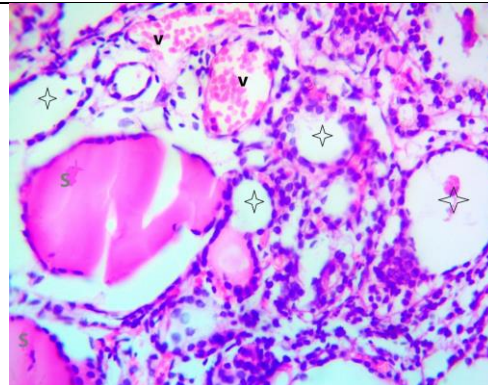


Fig. 8 A photomicrograph of a section in thyroid gland obtained from an adult male albino rat of fipronil treated group (group III) showing increased vascularity (V) of the gland, as well as increased caliber of blood vessels. The colloid within the follicles was also stained with a greater intensity (S), and some follicles were empty (cross) (H&E x400).

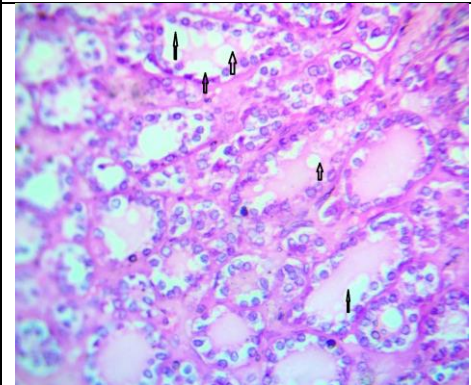


Fig. 9 A photomicrograph of a section in thyroid gland obtained from an adult male albino rat of fipronil treated group (group III) showing increased follicular cell vacuolation (arrow) (H&E x400).

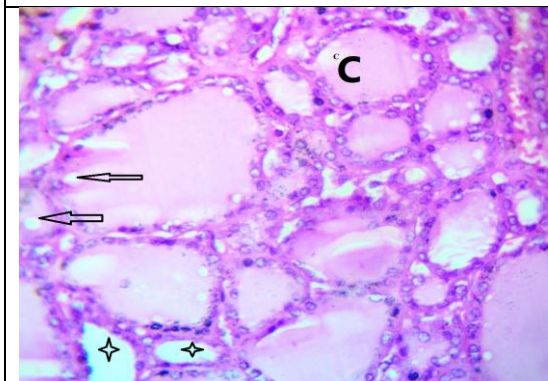


Fig. 10 A photomicrograph of a section in thyroid gland obtained from an adult male albino rat of zinc with fipronil group (IV) showing nearly normal thyroid follicles with colloid (C), some vacuolation (arrow) in the cytoplasm of follicular cells and some empty follicles (cross) (H&E 400X).

Table (8): Statistical comparison between negative and positive control groups as regard mean values of comet parameters in the thyroid at the end of six weeks by using student t- test.

Group N=8 parameter	-ve Control	+ve Control	t	p-value
	Mean±SD			
Tailed nuclei %	4.53±0.82	3.91±0.69	1.616	0.128 NS
Un tailed nuclei %	95.48±0.82	96.09±0.69	1.616	0.128 NS
Tail length (μm)	1.17±0.10	1.16±0.11	0.190	0.852 NS
Tail DNA %	1.27±0.13	1.28±0.13	0.096	0.925 NS
Unit tail moment	1.484±0.21	1.480±0.23	0.036	0.972 NS

N.B All values are expressed as mean±SD. (SD: standard deviation)

N: Number of rats in each group was 8 rats.

NS: P >0.05 =non significant. %: percent.

μm: Micrometer

Table (9): Statistical comparison among negative control, Zinc, fipronil and zinc with fipronil groups as regard mean values of comet parameters in the thyroid using at the end of six weeks by using ANOVA test.

Group N=8 Parameter	-ve Control	Zinc	Fipronil	Zinc+fipronil	F	P-value
	Mean ± SD					
Tailed nuclei%	4.53±0.82	4.28±1.04	14.13±1.25	5.04±0.55	201.469	<0.001**
Un tailed nuclei%	95.48±0.82	95.73±1.04	85.88±1.25	94.96±0.55	201.469	<0.001**
Tail length (μm)	1.17±0.10	1.22±0.11	3.04±0.29	1.27±0.13	210.307	<0.001**
Tail DNA %	1.27±0.13	1.23±0.08	2.91±0.40	1.34±0.26	84.569	<0.001**
Unit tail moment	1.48±0.21	1.49±0.14	8.87±1.53	1.68±0.32	169.517	<0.001**

N.B All values are expressed as mean±SD. (SD: standard deviation)

N: Number of rats in each group was 8 rats.

** : statistically highly significant (P <0.001)

%: percent

μm: Micrometer



Table (10): Least significance difference (LSD) for comparison between mean values of comet parameters in the thyroid of negative control, Zinc Fipronil, and zinc with fipronil groups at six weeks of the study.

Parameters	Group N=8	Zinc (4.28±1.04)	Fipronil (14.13±1.25)	Zinc+fipronil (5.04±0.55)
Tailed nuclei%	-ve control (4.53±0.82)	0.660 NS	<0.001**	0.290 NS
	Zinc (4.28±1.04)		<0.001**	0.120 NS
	Fipronil (14.13±1.25)			<0.001**
Un tailed nuclei %	Group	Zinc (95.73±1.04)	Fipronil (85.88±1.25)	Zinc+fipronil (94.96±0.55)
	-ve control (95.48±0.82)	0.660 NS	<0.001**	0.290 NS
	Zinc (95.73±1.04)		<0.001**	0.120 NS
Tail length (μm)	Group	Zinc (1.22±0.11)	Fipronil (3.04±0.29)	Zinc+fipronil (1.27±0.13)
	-ve control (1.17±0.10)	0.685 NS	<0.001**	0.289 NS
	Zinc (1.22±0.11)		<0.001**	0.591 NS
Tail DNA %	Group	Zinc (1.23±0.08)	Fipronil (2.91±0.40)	Zinc+fipronil (1.34±0.26)
	-ve control (1.27±0.13)	0.712 NS	<0.001**	0.609 NS
	Zinc (1.23±0.08)		<0.001**	0.400 NS
Unit tail moment	Group	Zinc (1.49±0.14)	Fipronil (8.87±1.53)	Zinc+fipronil (1.68±0.32)
	-ve control (1.48±0.21)	0.988NS	<0.001**	0.623 NS
	Zinc (1.49±0.14)		<0.001**	0.634 NS
	Fipronil (8.87±1.53)			<0.001**

N.B All values are expressed as mean±SD. (SD: standard deviation)

N: Number of rats in each group was 8 rats. %: percent **: statistically highly significant (P <0.001) (μm): Micrometer



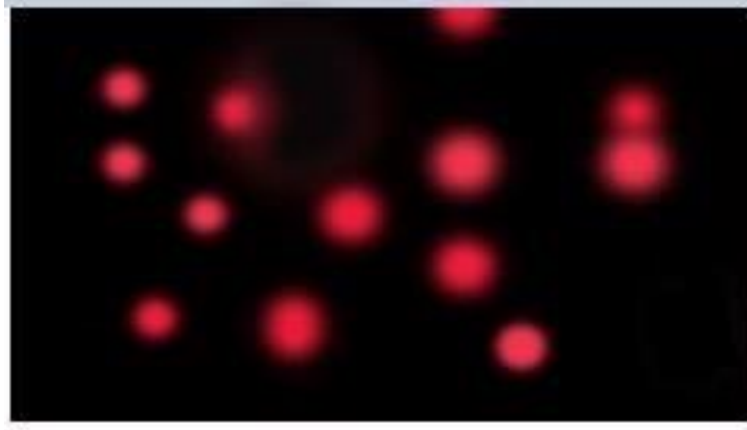


Figure (11): A photomicrograph by fluorescent microscope showing thyroid cells nuclei from adult male albino rats of control group (group I), DNA in most cells is tightly compressed and maintains the circular disposition of normal nucleus.

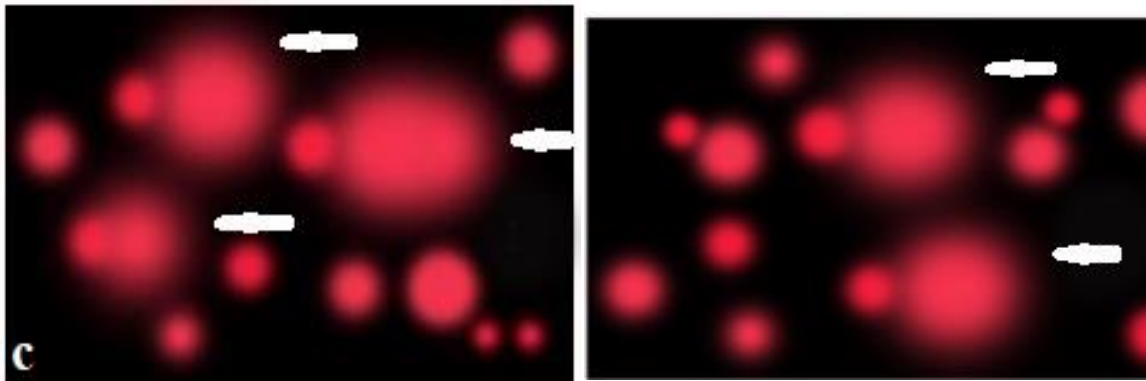


Figure (12): A photomicrograph by fluorescent microscope showing thyroid cells nuclei from adult male albino rats of fipronil group (group III), the comet has residual head and long dense tail pattern since most DNA migrated to tail (white arrow).

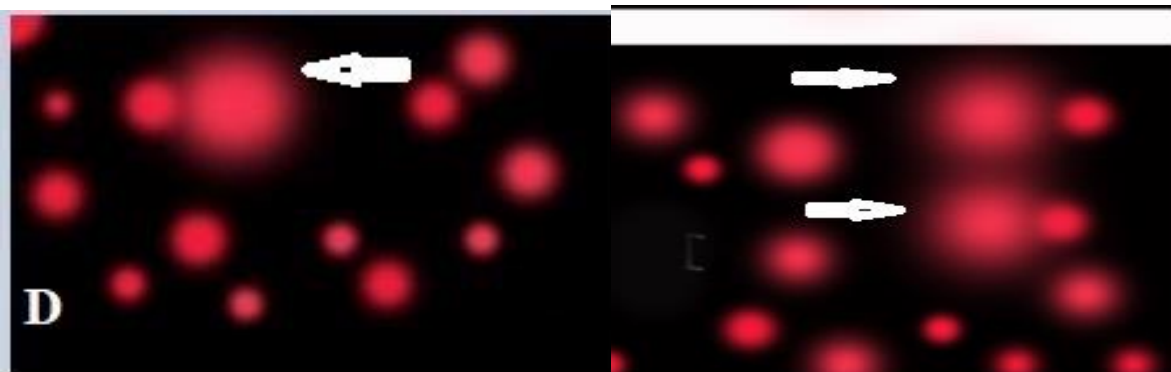


Figure (13): A photomicrograph by fluorescent microscope showing thyroid cells nuclei from adult male albino rats of zinc with fipronil group (group IV), the comet has residual head but shorter and less dense tail pattern as compared to fipronil treated group (white arrow).

4. Discussion

Fipronil or its metabolites, exert neurotoxic effects through suppression of the inhibitory effect of gamma aminobutyric acid (GABA) by targeting GABA_A-regulated chloride channels. This inhibits chloride influx into nerve cells leading to hyperexcitation, paralysis, and death of insects (19).

Zinc is an essential mineral. It is a component of numerous metalloenzymes. It is important for cell growth, replication, osteogenesis and immunity (20).

Zinc has close interrelationships with the endocrine system, and is essential for normal growth, reproductive function, thyroid function and glucose metabolism (21).

Effect of Fipronil on thyroid function tests (T3, T4 & TSH):

In this study, there was a very highly significant decrease ($P < 0.001$) in the mean values of T3 and T4 and a highly significant increase in the mean values of TSH of FPN treated group when compared with negative control group.

These results are in agreement with Leghait et al., (22) who demonstrated that fipronil treatment decreased total T4 plasma concentrations, total T3 and increases TSH plasma concentrations in thyroid-intact female rats, receiving fipronil in a dose of 3mg/kg/day per os for 14 days and explained that fipronil-induced thyroid disruption occurs by enhancing the rate of T4 elimination via increased hepatic enzyme activity.

Also, Roques et al., (23) demonstrated that both fipronil and fipronil sulfone treatment at a dose (3.4 $\mu\text{mol/kg/day}$ per os, 14 days decreased T4 levels by enhancing its total and free clearance through hepatic enzyme induction in rats. Fipronil sulfone is the main metabolite of fipronil and its role as a thyroid disruptor is more critical

because it persists much longer in organism than fipronil itself.

The results of the present study are in agreement with the results of Abu Zeid, (24) regarding the effect of FPN on serum levels of total T3 and T4 but in contrast to this study regarding the FPN effect on serum TSH. Abu Zeid.(24) study showed significant decrease in the serum levels of total T3 and T4 in FPN exposed rats when compared with the control ones, while the level of serum TSH showed non-significant decrease in FPN exposed rats, as compared with the control ones.

Tukhtaev et al. (25) reported that prolonged exposure to low doses 3,6 mg / kg / day, this corresponded to 1/100 of LD₅₀ of the drug of FPN) for 30 days had different effects on thyroid function of pregnant and non-pregnant female rats. In non-pregnant female rats FPN exposure resulted in a moderate decrease of both T3 and T4 hormones, but it significantly increased the concentration of TSH but in pregnant and lactating female rats, there was a marked reduction in TH concentration with a significant increase in TSH levels.

In contrary, Herin et al. (27) reported that fipronil-exposed workers have been negatively associated with serum TSH concentrations, raising the possibility that fipronil has a central inhibitory effect on TSH secretion in humans

Effect of zinc and fipronil on thyroid function tests (T3, T4 & TSH):

The present findings showed that zinc administration significantly increased thyroid function tests (T3, T4) & decreased TSH in Zinc with fipronil treated group when compared with FPN treated group.

Zinc is required for the synthesis of the thyrotropin-releasing hormone (TRH), which plays an important role in binding of T3 to its

nuclear receptor, participates in the synthesis of the thyroid-stimulating hormone (TSH) in the anterior pituitary and acts as an inhibitor or cofactor of type 1 and type 2 deiodinases (28).

In agreement with the current results, **Salah et al. (29)** detected that zinc administration ameliorated nickel chloride (NiCl₂-) induced thyrotoxicity as it significantly improved plasma T₄, T₃, and TSH levels in pregnant Wistar rats.

In accordance with the present results, **El-Banna et al., (30)** study showed that simultaneous zinc sulphate and Aroclor 1254 administration for 28 days restored the levels of T₃, T₄, and TSH in Aroclor 1254 exposed rats.

Moreover, **Abbas & Zaki, (31)** results revealed that Zinc supplementation counteract the thyroid toxicity of Aluminum by elevated serum levels of T₃, T₄ near to the normal level, but in contrary to our study zinc also could elevate the decreased TSH.

In contrast to the current results, **Giray et al. (32)** and **Kuriyama et al.(33)** reported no marked difference in the plasma Zn levels between patients with thyroid diseases and healthy controls.

Effect of fipronil on histopathological changes of thyroid:

The thyroid is an endocrine gland of critical importance for the regulation of the metabolism in general. It is an excellent bioindicator in toxicological studies since it is very sensitive to toxic exposure, even in minimum dosages (34).

The thyroid histopathological findings in the current work confirmed the results obtained from the effect FPN on alteration of thyroid function tests (T₃, T₄ & TSH).

As compared to control groups FPN exposure induced several histopathological alterations in the thyroid as structural disarrangement in the

gland due to hypertrophy of some follicles, irregular appearance of others, fusion of more than one follicle, follicular cell vacuolation, increased interfollicular spaces due to the occurrence of edema, increased caliber of blood vessels and vascularity of the gland. The colloid within the follicles was also stained with a greater intensity and some follicles were empty.

These results are in agreement with **Cunha et al. (35)** who observed significant histopathological changes in the thyroid of all treated animals with FPN such as loss of the rounded form of thyroid follicles, fusion of more than one follicle, interfollicular edema, follicular cell vacuolation causing displacement of the nucleus from the central regions to cell periphery and loss of colloid integrity within the follicles, since they were stained with a greater or lesser intensity.

Also, our results are supported with **Ferreira et al., (26)** who demonstrated similar effects of different fipronil concentrations on the thyroid of non-target organisms, including disorganization and vacuolated areas, fusion of two or more follicles, hypertrophic follicles, enlargement of the spaces permeating the follicles and the presence of swollen regions.

In addition, **Leghait et al., (22)** demonstrated that fipronil would chemically interact with the thyroid hormone production, causing morphophysiological alterations and overstimulating the gland.

Moreover, **Hurley, (37)** studied the toxic potential of fipronil, a physiological imbalance between the thyroid and the hypophysis would occur, causing a decrease in hormones T₃ and T₄ circulation and an increase in the concentration of TSH (thyroid stimulating hormone). The consequences of such imbalance would be due to inhibition of inorganic iodate transportation to the interior of follicular cells; lesions to the follicular cells; inhibition of thyroid hormones release; inhibition of colloid (prehormone) conversion into T₃ and T₄; increase in the metabolic rate.

Effect of zinc and fipronil on histopathological changes of thyroid:

The results revealed marked improvement of thyroid structure. It nearly retained its normal architecture. The colloid material filled most follicle lumen and the follicular cells exhibited normal shape and arrangement except some vacuolation at follicular lining epithelium.

These findings are in line with those obtained by **Salah et al., (29)** who found that thyroid pathological lesions induced by nickel chloride (NiCl₂) in pregnant rats have been remarkably reduced by the administration zinc and attributed that to the antioxidant roles of zinc.

In agreement with our results **Fedala et al., (38)** have reported that the administration of ZnCl₂ has almost restored the thyroid histoarchitecture of pregnant Wistar rats exposed to potassium dichromate.

The results of the present study are in accordance with **El-Banna et al. (30)** who noticed marked improvement in the histological features of thyroid follicular cells in rats received zinc with Aroclor 1254 as the follicles were lined with low cuboidal epithelial cells or even flat squamous cells, most of the follicles appeared with homogenous colloid, with minimal cytoplasmic was depicted.

Comet assay results:

The interaction of free radicals, aldehydes derived from lipid peroxidation and protein carbonyls with DNA may lead to the hydrolysis of chemical bonds, resulting in DNA fragmentation **(39)**. Hence, metals tend to bind primarily with DNA and nuclear proteins, thus leading to the oxidative deterioration of biomolecules **(40)**.

Effect of fipronil on comet assay results:

One of the key disciplines governing risk assessment of substances for human health is genotoxicology due to the fact that classic genotoxic substances lead to carcinogenesis **(41)**. Genotoxicity testing, the evaluation of the carcinogenicity and mutagenicity of substances are the most important part of the safety testing of chemical compounds **(42)**.

In recent years, single cell gel electrophoresis "the comet assay" has been shown to be a very sensitive technique and a useful tool that is being widely used to detect genetic damage at individual cell level and in human biomonitoring **(43)**.

In the present study, comet assay performed on thyroid specimens of FPN treated group revealed a very highly significant increase in % of tailed nuclei, tail length, tail DNA % and unit tail moment and a very highly significant decrease in % of untailed nuclei when compared with those of the negative control group.

The present study showed that FPN is a genotoxic substance manifested by thyroid DNA damage which was evaluated by comet assay. Densitometric and geometric parameters of the comets as determined using image analysis software revealed a very highly significant increase in % of tailed nuclei, tail length, tail DNA % and unit tail moment and decrease in % of untailed nuclei after FPN administration.

An excess of ROS could significantly increase DNA fragmentation **(44)**. Similarly, **Khan et al., (45)** have found that higher doses of fipronil (5 and 10 mg/kg b.w.) orally for 4 weeks markedly reduced the DNA integrity of spermatozoa along with excessive ROS generation suggesting that FPN causes male reproductive toxicity through oxidative stress-induced DNA damage to spermatozoa.

In agreement with these results, **Al-Harbi , (46)** reported that FPN exposure to male rats at concentration 10 mg/L in drinking water for 30days resulted in damaged DNA strand breaks and damaged nuclei with appearance of more than one apoptotic cells with large tail and small head.

Similar toxic effects were observed by **Aldayel et al., (47)**, who detected a significant elevation in comet assay DNA damage %, DNA% in tail, and tail length in the lymphocytes of male rats received 9.7 mg/kg FPN for 30 days.

In addition, **Çelik et al., (48)** reported that by using the alkaline comet assay, all the doses of the FPN (0.7, 0.3, 0.1 mg/mL) induced DNA damage in human peripheral blood lymphocytes in vitro.

Moreover, the results of the present study are in a line with those of **Badgujar et al., (49)** who gavaged adult male and female mice with various doses of fipronil (2.5, 12.5, and 25 mg/kg body weight) to evaluate micronucleus test, comet assay and chromosome aberration (CA) and treated another group of animals with vitamin E orally (400 mg/kg bw) for 5 days prior to administration of fipronil (12.5 mg/kg). They reported that fipronil insecticide has clastogenic and mutagenic effects in male as well as female mice and attributed that to ROS-mediated oxidative stress.

In the same context, **Zhou et al., (50)** demonstrated FPN induced apoptosis and cell cycle arrest in porcine oocyte maturation because of increased ROS levels and DNA damage suggesting that the FPN may have potential detrimental effects on the female mammalian reproductive system.

In contrast, the study performed by **de Oliveira et al., (51)** revealed that doses of 15 mg/kg and 25 mg/kg of fipronil did not have genotoxic effects. Only the highest dose tested (50 mg/kg) induced DNA damage 24 h after exposure, indicating the mutagenic potential of fipronil.

2) Effect of zinc and fipronil on comet assay results:

In the present study, comet assay performed on thyroid specimens of zinc+fipronil treated group revealed a significant decrease in % of tailed nuclei, tail length, tail DNA % and unit tail moment and a significant increase in % of untailed nuclei when compared with those of the negative control group.

The results were in accordance with **Garcia et al., (52)** who stated that zinc therapy was able to prevent the increased DNA damage observed in spermatozoa from rats of the cigarette-smoking group as seen in the comet assay results and explained that by zinc antioxidant properties which can protect cells against ROS-provoked oxidative and electrophilic stress, damaging DNA or other important structures such as proteins or cell membranes.

These results are concomitant with the results of **Fedala et al., (53)** who found that zinc treatment has genoprotective effect against $K_2Cr_2O_7$ -induced thyroid DNA damage.

In addition, the study of **Song, et al., (54)** confirmed that both severe and marginal zinc deficiencies in vivo increased oxidative stress, impair DNA integrity and increase DNA damage in rat peripheral blood cells and that damage could be reversed upon zinc repletion.

Furthermore, zinc exhibited a marked impact in maintaining DNA integrity by preventing its oxidative damage and promoting its repair **(55)**. Thereby, the prophylactic treatment with Zn promoted DNA repair in HeLa cells by revoking the inhibition of DNA-protein interactions exerted by cadmium **(56)**.

5. Conclusion

Fipronil is hepatotoxic and thyrotoxic substance as indicated by a significant increase in thyroid function tests along with marked histopathological changes. Zinc plays an important role in protection against fipronil

thyroid toxicity evidenced by improving the biochemical, histopathological and comet assay results in groups treated with zinc and fipronil

Conflict of Interest: None

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