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Sub-acute oral toxicity of Imidacloprid and Fipronil pesticide mixture in male albino rats; biochemical and reproductive toxicity evaluation

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Abstract

Due to recent registration of the Imidacloprid and FIpronil pesticides formula as a termiticide/insecticide,28 days daily oral toxicity study of Imidacloprid and Fipronil pesticide mixture was conducted in three groups of male rats with doses of 1/200LD$_{50}$ (0.547mg/bw/day), 1/150LD$_{50}$ (0.409mg/bw/day), 1/100LD$_{50}$ (0.820 mg/bw/day) plus control group. No mortality occurred during treatments period while food intake was reduced significantly when compared to the control group. White blood cells (WBC),red blood cells (RBC), haemoglobin (Hb) and hematocritlevels were significantly decreased in a dose dependent manner while there were significant dose increases in blood platelet values. The administration of mixture induced impairment of biochemical parameters like serum alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) ,alkaline phosphatase (ALP), Ɣ-Glutamyltransferase (GGT), urea, uric acid, Albumin, Globulin and total protein levels in all treated groups. Fipronil-Imidacloprid mixture potentiate effects on antioxidant system and oxidative stress biomarkers as observed by the significant decrease in testis DNA, RNA concentrations and reduced GSH accompanied by the significant increase in testicular Malondialdehyde (MDA) for the treated mixture groups thus side effects of pesticide mixture may need more investigations before applying.

1. Introduction

Imidacloprid[1-(6-chloro-3-pyridylmethyl) - Nnitroimidazolidin-2-ylidene-amine] is an extensively used insecticide for crop protection in the world from the last decade due to its' low soil persistence and insecticidal activity at low application rate[1-2]. It is fastest growing in sales as insecticide globally because of its low selectivity for insects and apparent safety for humans [3-5]. Its' selective toxicity result from its' high affinity to insects nicotinic acetylcholine receptors compared to mammals [1, 4, 6]. Reactive Oxygen Species (ROS) may be involved in the toxicity of various pesticides [7]

Imidacloprid acts on several types of post-synaptic nicotinic acetylcholine receptors in the nervous system of insects [8-9], these receptors are located only within the central nervous system. Following binding to the nicotinic receptor, nerve impulses are spontaneously discharged at first, followed by failure of the neuron to propagate any signal[10-11] Sustained activation of the receptor results from the inability of Acetylcholinesterases to break down the pesticide[9], this binding process is irreversible [12].

Fipronil,5-amino-1-(2,6-dichloro-4-(trifluoromethyl)phenyl)-4-((1,R,S)trifluoromethyl)sulfinyl)-1-H-pyrazole-3 -carbonitrile, is a phenyl pyrazole, broad-spectrum insecticide.It is particularly effective by way of ingestion and causes neural excitation and convulsions in insects, resulting in death [14,5]. It is used to control ants, beetles, cockroaches, fleas, ticks, termites, mole crickets, thrips, rootworms, weevils, and other insects. [13].Pathways of pyrethroids (sodium channel blockers), organophosphates, and carbamate (cholinesterase inhibitors) which are classical insecticides to which some insects have developed resistance [14]. FPN found that it interferes with the γ-aminobutyric acid (GABA)-gated channels; FPN disrupts normal nerve influx transmission (e.g., passage of chloride ions) by targeting the GABA-gated chloride channel and at sufficient doses, causes excessive neural excitation[15], severe paralysis, and insect death [14,16].

Free radicals and reactive oxygen species (ROS) generated by pesticides toxins are highly reactive and they can predispose the tissues to lipid peroxidation and tissue damage. To maintain thephysiological redox balance,

enzymatic and non-enzymatic antioxidants are essentials in defending tissues against the deleterious effects of (ROS)[17].Generally, animal tissues possess the activities of the enzymatic and non –enzymatic antioxidant like reduced glutathione (GSH) in addition; these antioxidants are also valuable indicators of oxidative damage and toxicities from environmental toxicants [18]. Currently,Fipronil and Imidacloprid has been intensively used separately in many forms while at the same time a mixture of the two active ingredients has been registered since 2013 to be used as a termiticide /insecticide which increases the exposure probability of the twocomponents in their mixture form in our environment however, there are little or no information exists on the acute & sub-acute toxicity of the mixture on non-target organisms such as mammals considering the intensive application of this mixture components for domestic and agriculture purposes and evaluation of its toxicological effects is of a great importance for public health therefore, the study has been carried out to evaluate the haematological, biochemical ,testicular effects on male albino rats .

2. Material and Methods

2.1. Chemicals and reagents

Imidacloprid technical 97% pure, 1[(6-chloro-3-pyridinyl) methyl]-N-nitro-2-imidazolidinimine, [CAS No. 138261-41-3] was obtained from BAYER company, Germany, Fipronil technical 97% pure,5-amino-1-(2,6-dichloro-4-(trifluoromethyl)phenyl)-4-(1,R,S)trifluoromethylsulfinyl)-1-H-pyrazole-3-carbonitrile , [CAS No. 120068-37-3] was obtained from ISAGRO company, USA. The assay kits used for biochemical measurements of Aspartate aminotransferases (AST,EC 2.6.1.1.), Alanine aminotransferases (ALT, EC 2.6.1.2),Alkaline phosphatase (ALP, EC 3.1.3.1), Albumin, Globulin ,uric acid and other chemicals(reagent grades)were purchased from Biodiagnostic Company, Dokki, Giza, Egypt.

2.2 Animals and managements

Twenty eight adult male rats (*Rattusnorvigicus*Wistar strain) weighing 150-180 g were obtained from the Faculty of agriculture, Alexandria, Egypt ,breeding colony were maintained under condition of controlled temperature (22 ± 3 _C) and humidity (30–70%) with 12 h light and dark cycle. The animals were given standard diet and water. Rats were acclimatized for 2 weeks prior the start of experiment. All experimental rats are complying with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

2.3. Experimental designs

Oral LD_{50} values for Imidaclopridin rats were estimated to be 450 mg/kg for both sexes [17], while oral LD_{50} for Fipronil in rats were estimated to be 97 mg/kg in rats and an LD_{50} of 95 mg/ kg in rats [18].Adult male rats were divided into four groups. One group was served as control and was given corn oil as vehicle through gavage. The other three groups were given doses of Imidacloprid-Fipronil technical mixture suspended in corn oil, tested doses were 1/100LD_{50} Fipronil+1/100LD_{50} Imidacloprid (0.820mg/bw/day), 1/150LD_{50} Fipronil+1/150 LD_{50} Imidacloprid (0.547mg/bw/day), 1/200LD_{50} Fipronil+1/200LD_{50} Imidacloprid (0.409mg/bw/day) to male rats for 28days. At the end of the exposure period, on day 29, rats were sacrificed by general anaesthesia of Ethyl ether.

2.4. Blood and tissue sample

Blood was collected from heart vena cava within 1 minute tubes was used to compile 1 ml were collected on sodium heparin for haematological studies .The rest of the blood was collected in glass tubes for coagulation and serum formation, blood was allowed to set for 30 min at at 4 °C to clot,then centrifuged at 1000 xg for 5 min. Packed cells were discarded and the supernatant samples were decanted and stored in to capped sterile polyethylene tubes at -20 °C until used (within 24 hours).the abdominal cavity of each rat was opened where testis were excised .

Testis were collected, tissue homogenate prepared in tubes with 5 volumes of phosphate buffer 0.1 M pH 7.4 then centrifuged at 3000 r.p.m for 15 mints and then supernatant were kept frozen at -80°C until analysed,One millilitre of Blood collected in to heparinised sample bottle were analysed for haematological parameters evaluation by coulter (hemat 8 analyser ; SEAC) white blood cells(WBC), red blood cells (RBC), haemoglobin (Hb),platelet and hematocrit(HCT) through automated cell counter (MeletSchloesing MS 9-3, France).

2.5.Biochemical analysis

Alanine aminotransferase (ALT), Aspartate aminotransaminase (AST),alkaline phosphatase (ALP) ,Ɣ-Glutamyltransferase (GGT), serum urea, Uric acid, albumin, Globulinandtotal protein in serum were measured through fully automated biochemical analyser (CHEMWELL 1520, USA) using standard kits Marketed by (BioMerieuxFrance) [19-23].

2.6 Determination of Nucleic acid concentration and Malondialdehyde in testicular tissues
The RNA concentration in the samples was determined by precipitation of the nucleic acids in 0.5 M $HClO_4$, after which the RNA was hydrolysedinice by incubation in 0.3 M KOH at 60°C for 1 h. After pre removal of DNA by 0.5 M $HClO_4$ precipitation, the RNA concentration was determined by boiling the samples for 30 min in 6 M HC1, 0.01% $FeCl_3$ and 0.3% orcinol. Absorption was measured at 660 nm. RNA concentrations were calculated using a calibration curve with highly purified total adult heart RNA that was isolated by ultracentrifugation through a caesium chloride cushion as a standard [24].
The DNA concentration in the samples was determined by hydrolysing the RNA in 0.1 M NaOH after which the DNA was precipitated with half volume 10% $HClO_4$. The DNA was re suspended in 10% 32PHClO4 and incubated at 70°C to hydrolysed the DNA. After clearing the solution by centrifugation, dip- henylamine and acetaldehyde were added to a final concentration of 2% and 0.01%, respectively. After an overnight incubation at 30°C the absorbance was determined at 560 and 700 nm. The concentration in the samples was calculated relative to the included calibration curve that was prepared using highly purified herring testis DNA [25].
The extent of lipid peroxidation was assayed by the measurement of thiobarbituric acid reactive substances [TBARS] the method is based on the determination of malondialdehyde MDA as an end product of lipid per oxidation, which can react with thiobarbituric acid in acidic medium to yield a pink coloured trimethine complex exhibiting absorption maximum at 532 nm. The concentration of RBARS in the samples is calculated as nmole/g fresh tissue [26].

2.7 *Determination of glutathione concentration*
The level of GSH in the testicular homogenate was determined spectrophotometrically the colored product (2-nitro-5-thiobenzoic acid) produced from the reaction of Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB with GSH has a molar absorption at 412 nm[27].

2.8 Statistical analysis
All data were expressed as mean ± standard error (SE).Data were statistical analysed by one-way analysis of variance ANOVA analysis followed by Tukey's post-hoc test. Results were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Signs of toxicity
No mortality occurred during the experimental period. Generally, signs of toxicity included a change in activity and abnormal gait were observed in rats exposed to higher concentration (0.820mg/bw/day) from the second week.

3.2. Serum biochemical parameters
The activity of serum enzymes; AST, ALT, ALP, urea and GGT significantly increased after 28 days of exposure to FPN-IMI mixture when compared to the control group (Table 1& figure 1).

Table 1: Effect of Fipronil and Imidacloprid mixture on serum ALT,AST, ALP, GGT in male albino rats

Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)
I	*33.52*^{bcd} ±4.861	*82.8*^{bcd}±4.604	113.8^{bcd}±13.21	11.48^{bcd}±0.409
II	39.6^{a}±2.702	*99.6*^{ac}±5..504	163.8^{acd}±21.029	13^{a}±0.735
III	*40.4*^{a} ± 2.408	130.6^{ab}±24.835	193.4^{abd}±17.672	13.08^{ad} ±0.268
Iv	43.6^{a} ± 5.004	*128.4* ^{a}±27.519	230^{abc} ±10.863	12.48^{ac} ±0.402

(*) Each value is a mean of six animals ±SE having the same group of letters are not significantly different from each other P< 0.05 I : control group ;II,III and IV ;groups that received 1/100 ,1/150 and 1/200 LD_{50} of FPN-IMI mixture .each parameter has been statistically done separately

Also there were significantdecrease in the level of serum uric acid in the mixture treated groups when compared to the control one as shown in Figure 2. The study recorded higher total protein, Albumin and Globulin concentrations for the treated FBN- IMI pesticide mixture when compared to control group concentration after the oral administrations for 28 days as shown in Table 2.

3.3 *Haematological parameters*

Table 3 shows that there were significant changes in the haematological parameters of the mixture treated groups when compared to untreated group, The study showed that there were a significant decrease in the level of white blood cells (WBC), red blood cells count (RBC), haemoglobin (Hb) and hematocrit % respectively in a dose dependent manner for the Fipronil-Imidacloprid mixture treated groups, While there were a significant increase in the blood platelets count in treated groups when compared to the control group .

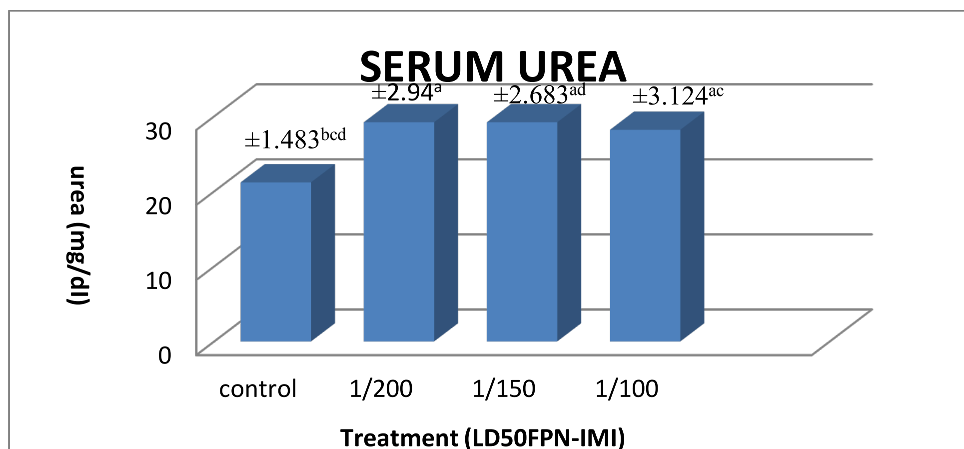

Figure1: Serum UREA (mg/dl) of rats treated with Fipronil- Imidaclopridmixture (1/200 ; 1/150 and 1/100LD_{50}) Significance at $P< 0.05$

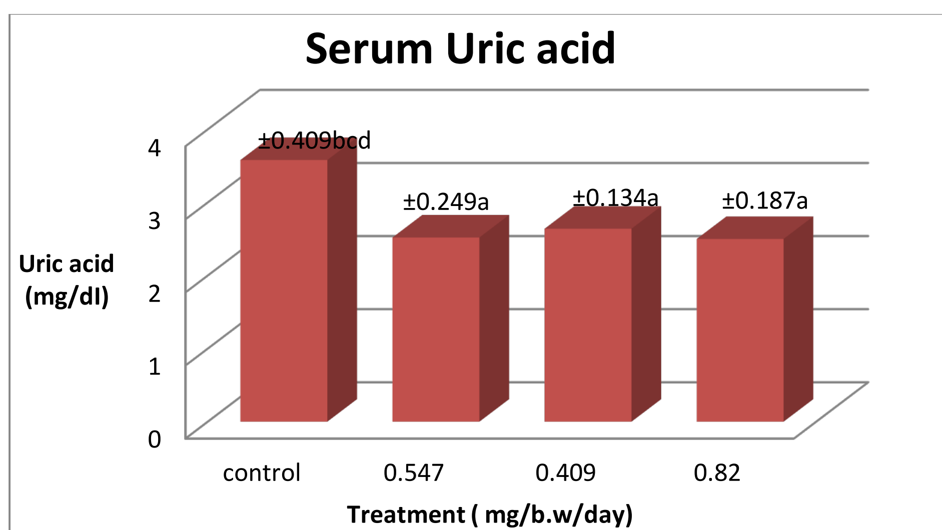

Figure 2: Serum Uric acid (mg/dl) of rats treated with Fipronil-Imidaclopridmixture(1/200; 1/150 and 1/100 LD_{50}) Significance at $P< 0.05$

Table 2: The activity of some enzymes in the sera of male rats exposed to the sub-acute concentrations of Fipronil –Imidaclopridmixture for 28 days.

Treatment	Total protein (g/dI)	Albumin (g/dI)	Globulin (g/dI)
I	*8.22*^{bcd}±0.622	4.64^{bcd}±0.134	3.580^{bcd}±0.753
II	9.90^{ac}±0.956	5.28^{a}±0.432	4.62^{a}±1.028
III	12.36^{abd}±1.607	*5.06* ^{a}±0.251	5.50^{a}±0.659
IV	10.50^{ac}±1.155	*5.20* ^{a}±0.3	5.30^{a}±o.977

(*) Each value is a mean of six animals ±SE having the same group of letters are not significantly different from each other $P< 0.05$I: control group ;II,III and IV ;groups that received 1/200 ,1/150 and 1/100 LD_{50} of FPN-IMI mixture. Each parameter has been statistically done separately.

3.4 *Peroxidation and Reproductive toxicity parameters*

The effect of different doses of FPN-IMI mixture on lipid peroxidation(LPO) in the testis of examinedrats. LPO was measured in terms of malondialdehyde (MDA) produced in the testis of rats. , MDA formed was significantly ($p < 0.05$) higher in FPN-IMI treated rats in a dose dependent manner when compared with control as shown in Figure 3.

Table 3: The effect of sub-acute exposure to FipronilandImidaclopridmixture on some haematological Parameters; white blood cells (WBC), platelet, red blood cells (RBC), haemoglobin and Hematocrit

Treatment	WBC (m/mm^3)	Platelet (m/mm^3)	RBC (m/mm^3)	HB (g/dI)	Hematocrit (%)
I	15.020^{bcd}±2.872	427.4^{bcd}±72.806	7.768^{bcd}±0.547	14.320^{bcd}±1.026	39.28^{bcd}±2.819
II	9.740^{acd}±1.471	534.8^{a}±33.003	4.996^{acd}±0.924	8.820^{ad}±2.609	24.22^{ac}±5.370
III	6.320^{abd}±1.119	595.8^{a}±22.554	3.370^{ab}±1.073	7.280^{a}±0.507	17.48^{ab}±3.415
IV	3.640^{abc}±0.483	693.4^{a}±30.558	2.876^{ab}±0.568	5.940^{ab}±2.11	18.72^{a}±7.545

(*) Each value is a mean of six animals ±SE having the same group of letters are not significantly different from each other's P< 0.05 I : control group ;II,III and IV ;groups that received 1/200 ,1/150 and 1/100 LD_{50} of FPN-IMI mixture, Each parameter has been statistically done separately.

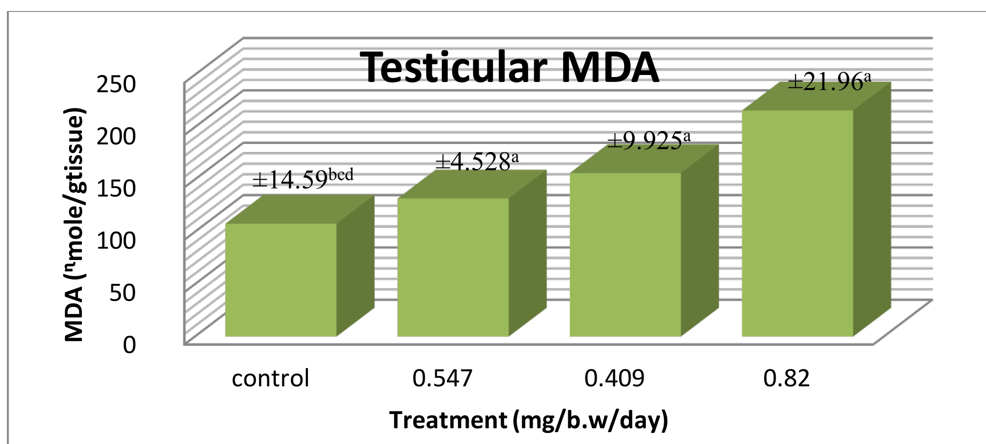

Figure 3: testicular MDA concentration (ⁿmole/g tissue)of rats treated with Fipronil -Imidacloprid mixture (1/200; 1/150 and 1/100 LD_{50}) Significance at P< 0.05

Sub-acute exposure effect of FPN-IMI mixture in the concentration of DNA and RNA of male rate testis are shown in Table 4, there were a significant decrease ($p < 0.05$) in treated groups when compared to the control untreated rats in a dose dependent manner in both DNA and RNA concentrationsaccompanied by the Testicular GSH significantly decreased ($p<0.05$) in treated groups when compared to the control group as shown in table 4.

Table 4: Testis DNA, RNA and GSH concentration s (µg/gm tissue) of rat treated with Fipronil – Imidacloprid mixture

Treatment	DNA µg/gm tissue	RNA µg/gm tissue	GSH Reduced µg/gm tissue
I	*60.8*^{bcd}±3.899	47.0^{bcd}±4.123	2.286^{bcd}±0.104
II	50.6^{acd}±3.847	37.8^{acd}±3.271	1.560^{ad}±0.674
III	33.0^{abd}±3.162	*23.6* ^{abd}±4.505	1.496^{ad}±0.230
IV	21.0^{abc}±3.808	*11.8*^{abc}±2.774	1.328^{abc}±0.965

Each value is a mean of six animals ±SE having the same group of letters are not significantly different from each other's P< 0.05 I : control group ;II,III and IV ;groups that received 1/200 ,1/150 and 1/100 LD_{50} of FPN-IMI mixture, Each parameter has been statistically done separately.

Our study is the first report on the effect of Fipronil-Imidacloprid pesticides mixture on oxidative stress in the animal model (rats).The results of the present study indicated that sub-acute exposure to 0.82, 0.547 and 0.409 mg/bw/day of FPN-IMI (groups II, III and IV)probably causes liver and kidney damage in treated rats compared to control by increases in serum marker enzymes AST, ALT, ALP, Urea and GGT along with decrease in uric acid level. Transaminases (AST and ALT) play an important role in amino acids catabolism and biosynthesis, They are responsible for detoxification processes, metabolism and, biosynthesis of energetic macromolecules for different essential functions [28] and used as specific indicators for liver damage [29].The increase in these enzymes may be due to liver dysfunction and disturbance in the biosynthesis of these enzymes with alteration in the permeability of the liver membrane [30].

Serum urea concentrations were significantly increased in FPN-IMI treated rats (groups, III and IV). The increased in urea level is a marker indicator of impaired kidney function while a decrease in the serum uric acid of rat treated with FPN-IMI mixture compared to the control is probably a symptom of liver or kidney disease.

It's also a symptom of Fanconi syndrome, a disorder of the kidney tubules that prevent the absorption of substances such as glucose and uric acid. [31-32]

In addition, the increase of total protein in FPN-IMI treated rats may be due to the liver and kidney dysfunctions, partially because of the high elevation of the serum enzymes [33-34] ,the changes produced to protein suggested some level of hepatocellular injuries[36-38].Hyperalbuminemia is an increased concentration of albumin in the blood [39].

The Globulins are a family of globular proteins that have higher molecular weights than Albumins and are insoluble in pure water but dissolve in dilute salt solutions. Some Globulins are produced in the liver, while others are made by the immune system ,High serum Globulin levels may be indicative of some chronic inflammatory or infectious disease or condition, Leukemia or other bone marrow disease, an autoimmune disease such as Lupus or Rheumatoid arthritis, liver or/and kidney disease, or carcinoid tumours. Which is the case observed in this experiment. The administration of FPN-IMI for 28 days resulted in haematological changes where significant decreases ($P< 0.05$) in red blood cells count (RBC), white blood cells (WBC) Haemoglobin (HB) and Hematocrit percentage in rat treated with FPN-IMI mixture compared to the control group, however, there was a significant increase in the level of platelets in treated groups.

The decrease in the level of white blood cells (WBC) is frequently a sign of an inflammatory response, most commonly as a result of infection, but may also occur following certain parasitic infections or bone tumours. [40]. Platelets play an important role in coagulation and any defects in the structure, numbers and, stability would lead to bleeding or thrombotic disorders ; ultimately resulting in death,the Excessive numbers of platelets, may lead to venous thrombosis and arterial thrombosis. The symptoms depend on the site of thrombosis [41]. A decline in the Haemoglobin content shown may be due to either decrease in the rate of haemoglobin synthesis or increase in the rate at which the haemoglobin is destroyed. Thissynthesis requires iron which is generally obtained from stored ferritin and from dietary sources, the reduction in general food intake by experimental rats and no supplementary supply of extra iron might be the reason for the iron deficiency which is essential for the haemoglobinsynthesis [41]. Diminished Hb content can also be correlated to the reduction in the size of red blood cells or the impede biosynthesis ofHeme in bone marrow [42]. The Hematocrit values are directly correlating with the RBC's count [41]. Thereduction in the size or number of erythrocytes may be have resulted due to the decrease in Hematocrit count in the animal treated with FBN-IMI mixture.

In the present investigation, oxidative stress biomarkers were evaluated in the testis tissue of male rats dosed FPN-IMI mixture for 28 consecutive days ,Testis was thought to be the target organ for this investigation due to the large amount of polyunsaturated fatty acids in it, thus highly vulnerable to oxidative damage ,Pesticides mediated toxicity involves excessive production of ROS leading to alteration in the cellular antioxidant defence system leading to susceptibility to oxidative stress [44]. ROS if unchecked will modify lipids, nucleic acids and proteins leading to cell disruption and increased production of lipid peroxidation LPO [45]. LPO has been extensively used as a marker of oxidative stress, In the present study,FPN-IMI treatment caused a significant increase in the level of MDA in testis tissues. The severe increase in the concentration of MDA is an indicator of FPN-IMI-induced LPO leading to tissue injury. These results support earlier in vitro findings with Fipronil to induce oxidative stress [12]. Glutathione (GSH) is the most abundant non-protein thiol (-SH) in organisms and it plays a key role in intracellular protection against toxic compounds [46]. GSH with its -SH group functions as a catalyst for disulfide exchange reaction, and contributes in H_2O_2 detoxification. GSH plays a major role in antagonizing the oxidative action of the herbicides or insecticides [47]. This mechanism explains the significant decrease in the level of non-enzymatic antioxidant molecules, i.e., GSH in testis tissues after the mixture of FPN-IMI treatment and the study showed a significant decrease in the testis DNA &RNA concentration in the treated groups when compared to the control one.

Conclusion

Mixtures in the environment are usually composed of multiple components from a range of sources with dissimilar chemical structures and modes of action. Unfortunately, this is exactly the type of mixture that has been least frequently studied. Hence, more empirical evidence on the joint action of environmentally realistic mixtures composed of agents from different chemical and functional classes are needed in order to further substantiate the conjecture that concentration or dose addition might be applicable as a general "rule of thumb" for describing the risk assessment of chemical mixtures and to explore its limitations. In view of the data of the present study it can be suggested that using Fipronil-Imidacloprid as a mixture may pose high toxicity at low exposure levels that corroborated with haematological, biological and reproductive impairment which increase the need and the importance of testing the side effects of pesticides mixtures to preserve our environment and to avoid the potential health hazardous that may be caused.

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