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Evaluation of The Mutagenicity and Oxidative Stress of Fipronil After Subchronic Exposure in Male Albino Rats

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ABSTRACT

Fipronil (FPN) is a wide-ranging effective N-phenylpyrazole insecticide that is commonly used in agriculture and public health for a long time but until now no adequate data available concerning its side effect. The present study was to evaluate the oxidative stress, cytotoxicity, and mutagenic effect of FPN subchronic exposure. Twenty male Albino rats (120g±10%) were randomly assigned into four groups each has five animals, a control group, two FPN treated groups with doses of 3.9, and 2.3 mg kg^{-1} b.w. per day for 90 days and the fourth group is a positive control group. Superoxide Dismutase (SOD), Catalase (CAT), and Malondialdehyde level (MDA) were used as oxidative stress biomarkers. The cytotoxicity was estimated by micronucleus assay, while the mutagenic effect was studied on the P53 tumor suppressor gene using the single-strand conformation polymorphism (SSCP) technique. The results showed that SOD significantly decreased in the high and low doses treatments and insignificant changes in CAT compared to control. MDA was significantly elevated in the high dose group and remained insignificant in the low dose. The high dose of FPN was showed an increased number of micronuclei in the Normochromatic Erythrocytes (NCE) in the bone marrow. PCR-SSCP analysis by acrylamide gel didn't show mutations in the P53 gene. This study demonstrated that FPN induces oxidative stress and micronuclei but did not show alternation of the P53 gene. The study indicated the relative safeness of fipronil to be used in the public health field with taking all use precautions.

INTRODUCTION

Fipronil (FPN) is a pesticide that belongs to the phenylpyrazole chemical group. It is an insecticide with widespread use in the control of many agricultural and domestic pests (Badgujar *et al.*, 2017). The usage of FPN has increased considerably as it is replacing conventional pesticides, such as organophosphates, carbamates, and pyrethroids insecticides, which become less effective against resistant pest strains (Narahashi *et al.*, 2010).

FPN is a noncompetitive inhibitor to the Gamma-aminobutyric acid (GABA) gated chloride channel receptor reducing neuronal excitability throughout the central nervous system, which leads to hyperexcitation of the central nervous system, convulsions, and death (Zhao *et al.*, 2004).

One of the most prominent adverse effects of pesticide detoxification is oxidative stress, the increase of free radicals such as reactive oxygen species (ROS) over the antioxidant d defense system capacity (Mansour & Mossa, 2009).

The Reactive Oxygen Spices (ROS) interact with various critical cellular macromolecules, including DNA, causing damage (Cicchetti & Argentin, 2003). Pesticides alter antioxidant levels and induce oxidative stress in animals (Hendawy *et al.* 2012; Mohamed e*t al.,* 2004), which are one of the common methods for indicating pesticide toxicity (Arnal *et al.,* 2011).

Despite the moderate toxicity of FPN to mammals, researches have shown that it causes oxidative stress by inducing significant variations in catalase, superoxide dismutase activities, and levels of malondialdehyde in rat liver after 45 and 90 days from treatment (Kartheek and David 2016). (Al-Harbi 2016) reported that FPN induces a decrease in oxidative stress biomarkers. On the other hand, the mutagenicity of the FPN is not cleared. Little research found the mutagenic effect of FPN at the cytogenetic level but not at the molecular genetic level.

The micronucleus assay MN has been established to assess the mutagenicity of compounds at the cytogenetic level. The MN assay is commonly used for reliable measurement of genetic damage, e.g. chromosome loss and chromosome breakage (Krishna and Hayashi 2000; Tucker and Preston 1996). Moreover, it is recommended, with other examinations, for evaluating and releasing new chemical products (Choy 2001). Also, at the molecular genetic level e.g. P53 gene mutation may give additional information about the mutagenic effect at the gene level.

The specific type and location of P53 gene mutation result from exposure to certain types of carcinogenic agents (Wang *et al.,* 1996). Genetic mutations leading to inactivation of tumor suppressor genes and oncogene activation is the first cause for the onset of cancer (Calaf, Echiburu-Chau, and Roy 2009).

P53 gene is an important tumor suppressor gene in normal cells (Nigro *et al.*, 1989), its mutation reflects an early cancer occurrence is the most common genetic alteration in human cancers (Hollstein *et al.,* 1991; Perri *et al.,* 2016). Therefore, P53 mutation is a useful biomarker for etiology, molecular mechanisms, and, hopefully, the prevention of environmental cancers (Hollstein *et al.,* 1997; Harris, 1996; Sidransky & Hollstein, 1996).

On the other hand, with the steady increase in the use of FPN, especially in public health, there was an urgent necessity to identify the potential adverse effects with long-term exposure. Therefore, our study was to investigate the mutagenic effects of FPN at cytogenetic and molecular genetic levels as well as the capability to oxidative stress induction in male albino rats, after a sub-chronic oral toxicity study.

MATERIALS AND METHODS

The Tested Insecticide:

The insecticide is a commercial formulation containing 20% Fipronil (FPN) active ingredient purchased from a local distributor. The chemical name is (5-amino-1-(2,6-dichloro-4-(trifluoro-methylphenyl)-4-(trifluoro-methylsulfinyl) pyrazole-3-carbonitrile).

Animals:

Twenty male adult albino rats (Wistar strain) weighing 120g±10% were obtained from the Mammalian and Aquatic Toxicology department, Central Agricultural Pesticides Lab, Giza. The animals were housed for two weeks before starting the experimental work as five rats per cage and an excess amount of fresh water and fresh well-balanced diet was available. The house condition is adjusted at 25±2^{o}C, a relative humidity of 50-60%, and a normal light/dark cycle.

Experimental Design:

Two groups were administered a dose of 3.9 and 2.3 mg/kg b.w respectively of FPN. A third group was kept as negative control and given just (1 ml (d/w)/100g b.w.). The fourth group is a positive control and was injected intraperitoneally with a dose of 250 mg/kg b.w. of Ethyl Methane Sulfonate (EMS) group 24 hrs before collecting the samples. The administrations of tested insecticides were orally by gavage for 90 consecutive days according to OECD 408 guidelines. The high and low doses were equivalent to 1/30, and 1/50 of the estimated LD_{50} of this tested insecticide (data not shown). The signs of toxicity and the body weights of rats were measured regularly.

Samples Collection:

At the end of treatment, the rats were euthanized by sodium pentobarbital to collect the blood from the eye vein and then sacrificed by cervical dislocation. The experiment was approved by the Ethics Committee, Institutional Animal Care and Use Committee (IACUC) of Cairo University (CU. II. F.33.19). Blood samples were collected in sodium heparin tubes for plasma samples, and then the samples were centrifuged at 3000 rpm for 10 min and stored at −20°C to be used for biochemical parameters. The liver was immediately removed and washed using chilled saline solution then were preserved at −20°C to do DNA extraction. Also, both femurs were excised out and, the bone-marrow cells were collected from both femurs for mutagenicity assays according to the technique described later.

Oxidative Stress Parameters:

Oxidative stress parameters were determined in plasma samples according to the details given in the kit's instructions of Biodiagnostic Company. All assays were performed by using a Jasco UV-VIS spectrophotometer V-630 PC (Japan).

Mutagenicity Tests:

1-Micronucleus Test:

The micronucleus assay MN has been established to assess the mutagenicity of compounds at the cytogenetic level. The MN assay is commonly used for reliable measurement of genetic damage, e.g. chromosome loss and chromosome breakage (Krishna and Hayashi 2000). The procedure was performed according to the method described earlier by (Schmid 1975) and (Adler 1984).

2-DNA Extraction:

Genomic DNA was extracted from liver tissue using Quick-gDNA™MiniPrep Kit (Zymo Research, USA). DNA quality and quantity were estimated at 260 nm and 280 nm using a Jasco UV-VIS spectrophotometer.

3-PCR amplification of P53 gene:

In the present study, the primers were used according to (Deng *et al*. 2004), the rat P53 gene differs from the human gene, having processed pseudogenes in its genome but pseudogenes lack intronic sequences so, the PCR primers designed based on the intron sequence in true P53gene. (Table 1)

PCR reactions were performed in a 50µl volume as detailed by (Noaishi, Haggag, and Afify 2011), These modifications were in PCR conditions, as the denaturation was at 94°C for 30 sec, the annealing temperature (below 2 degrees than melting temperature)

was for 30 sec, and 72°C extensions for 30 sec. followed by 39 cycles. The final extension was conducted at 72 °C for 5 min. the molecular weight of the P53 exons was checked by a 1% agarose gel.

Table 1. Primers sequences for exons 6, 7, and 8 of P53 gene

Primers	Sequences	Length (bp)	Annealing Temp.
Exon 6/7 Sense	5` GCCTCTGACTTATTCTTGCTC 3`	273	60°C
Antisense	5` CCCAACCTGGCACACAGCTTC 3`		
Exon 8 Sense	5` CTGTGCCTCCTCTTGTCCCG 3`	188	61°C
Antisense	5` CCACCTTCTTTGTCCTGCCTG 3`		

Single-Strand Conformation Polymorphism (SSCP):

The single-strand conformation polymorphism (SSCP) analysis of PCR products used in our study was as firstly described by (Hongyo *et al*. 1993). According to this method, the PCR-amplified products could be separated into single strands by denaturation then it was electrophoresed on polyacrylamide gels.

Statistical Analysis:

Statistical analyses were performed using one-way variance ANOVA and followed by Duncan's test to determine differences between groups for all parameters. The results are presented as mean ± Standard deviation (SD). Values were considered statistically significant if $P<0.05$. The SPSS statistics software, Ver.15 was used for the statistical analysis.

RESULTS

Signs of Toxicity:

It was observed that no mortality was recorded in treated rats. Additionally, no symptoms of toxicity were noticed in rats exposed to FPN after subchronic exposure.

Effect on the Body Weights:

After 30 days of treatment, the body weight increased by the rate of 68, 62, and 58 gm in the control group, high dose of FPN, and the low dose of FPN, respectively. After 60 days, the control group's body weight increased by the rate of 67 gm, while this rate decreased to 3 and 17 gm in high and low doses of FPN, respectively. After 90 days, the rate of body weight was 43 gm in the control group, and it was -1 and 3 gm in the two groups, respectively (Fig 1).

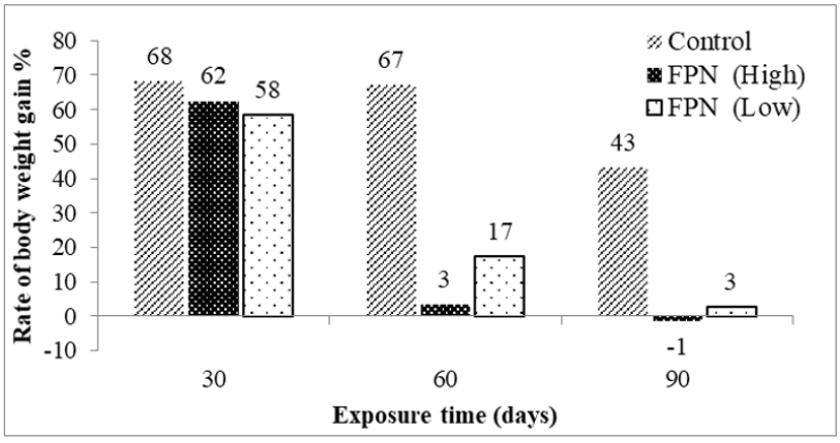

Fig. 1. The correlation between the rate % of body weight gain and the exposure time (days) of the control group and treatment groups (FPN high and low doses).

Effects on the Oxidative Stress Parameters:

Significant differences in enzymatic activity of antioxidant enzymes were observed in the animal group of both the high and low doses of FPN compared to control groups (Table 2). The SOD activity was significantly reduced in both high and low doses to -73 and -54%, respectively compared to the control group. Catalase Activity decreased insignificantly in the high dose of FPN to -25% but elevated insignificantly to 32% in the low dose group compared to control. Lipid peroxidation (LPO) was a significant increase of MDA in the high dose of FPN treatment to 101% while decreased insignificantly to -22% in the low dose group compared to control. (Fig 2).

Table 2. Changes in antioxidant enzymes of Wistar rats exposed to select doses of FPN**.**

Treatments	SOD (U/g tissue)	Catalase (U/ L)	MDA (nmol/ ml)
Control	$317.79^{a}\pm3.29$	$305.85^{ab}\pm 55.48$	$6.64^{b}\pm0.846$
FPN (high dose)	$85.91^{c}\pm12.80$	$227.94^{b}\pm 35.44$	$13.34^{a}\pm2.37$
FPN (Low dose)	$145.66^{b}\pm5.82$	$403.18^{a}\pm45.70$	$5.197^{b}\pm0.162$

Values represented are groups means ± SE; Means within the same column followed by the same letter are not significantly different (Duncan test at $P < 0.05$) comparing to the control group.

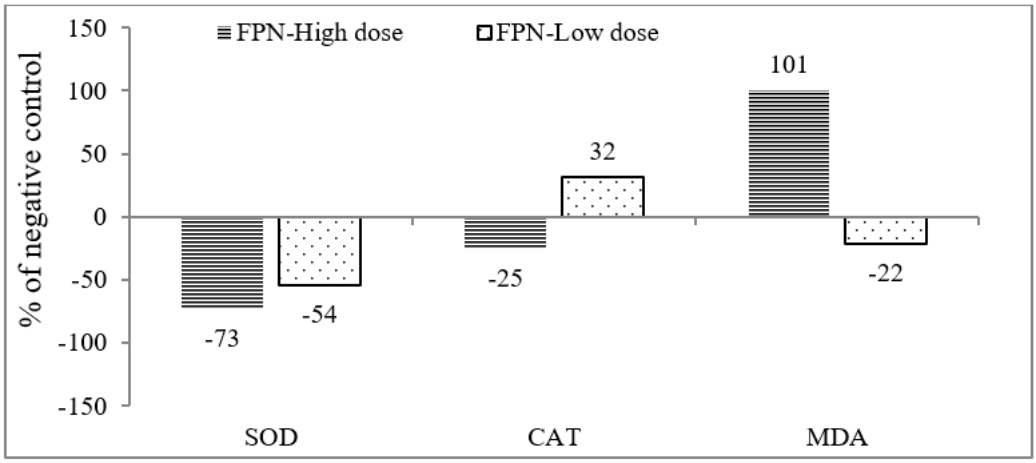

Fig. 2. Percent of changes in the SOD, CAT, and MDA in rats exposed to different doses of FPN compared to the negative control group.

Mutagenicity Effects:

1-Micronucleus Assay:

Both groups of FPN caused increases in the number of micronuclei (MN). High dose treatment increased the MN significantly, while at the low dose the change was not significant compared with control (Table 3). The positive control group treated with Ethyl Methane Sulfonate (EMS) for 24hrs shows a significant increase in the frequency of MN compared to the control. The average number of MN cells of the negative control group was only 8.2 while the positive control group increased to 36.0. In the FPN-treated groups (both high and low dose treatments), higher frequencies of MN were observed compared to control. The average frequency of MN was significant in high dose (27.8), and insignificant in low dose (14.2), as shown in Figure 3.

Table 3. The numbers of micronuclei frequency in rat's bone marrow cells after FPN treatments. Values were calculated and expressed as mean ± SE (standard error) of 5 replicates. Means followed by the same letter are not significantly different (Duncan test at $P < 0.05$).

Treatments	Total No. of MN cells	Mean ± SE
Control group	41	8.2^{b}± 1.32
FNP-High dose	139	27.8^{a}±2.26
FNP-Low dose	71	14.2c± 1.93
Positive control	180	36.0^{a}±2.5

PCR-SSCP Analysis of Rat P53 gene:

PCR-SSCP analysis of P53 exon 6, 7 and 8 on a 10% PAGE showed no alteration in-band mobility of both treatment groups compared to the negative control in Figure 4.

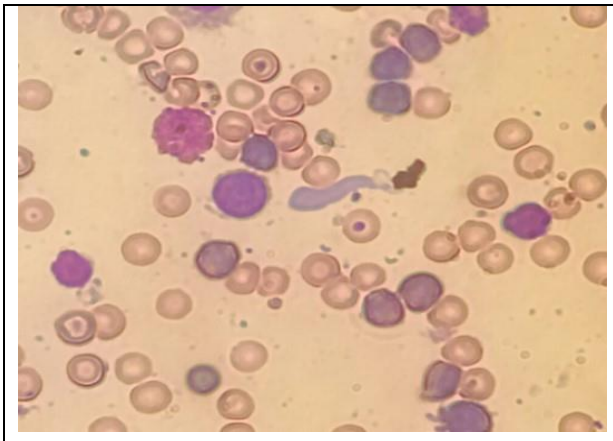

Fig. 3. Micronuclei formation in rat's bone marrow cells after 90 days of high dose treatment.

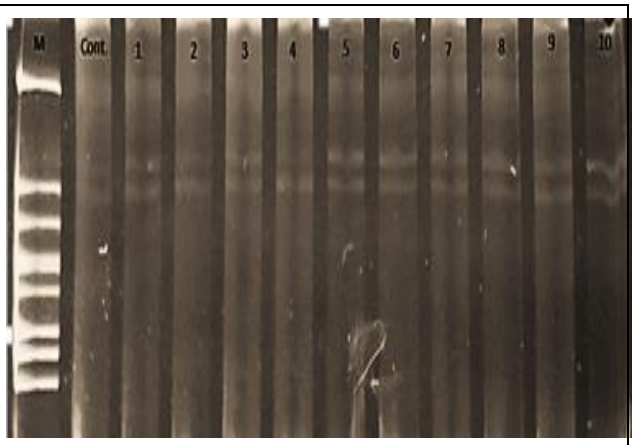

Fig. 4. Mobility of exon 6 and 7 of P53 gene in five male albino rats treated with Beta-cyfluthrin high dose for 90 days.

DISCUSSION

In our study, Fipronil (FPN) caused a reduction in body weight in the two subchronic doses compared to the control group. The decrease in body weight may result from the oxidative stress and neurotoxicity of FPN (Mansour and Mossa 2009).

The adverse effects of many xenobiotics and pesticides are caused by the excessive generation of free radicals in the biological systems (Henkler *et al*., 2010; Kartheek and David, 2016). Enzymatic and non-enzymatic antioxidants are working together to eliminate these radicals and prevent the negative impacts on the cell (Abdollahi et al. 2004). Superoxide dismutase (SOD) and Catalase (CAT) are enzymatic antioxidants considered to be scavengers of the reactive oxygen species (ROS) that produces after the chemical stress i.e. pesticides (Hu & Tirelli, 2012; Afonso *et al.,* 2007). Consequently, activities of these enzymes are used as biomarkers for the ROS capacity in the body (Monteiro *et al.,* 2006).

The levels of SOD enzyme activity were decreased following the two treated doses of FPN. The ingestion of FPN could be caused by an excess generation of superoxide radicals ($O2^{\bullet-}$) more than the required amounts (Sheng *et al.,* 2014). (Kartheek and David 2018) reported a significant decrease in SOD enzyme activity in rats after 90 days of daily

oral ingestion of different doses of FPN. Furthermore, the high dose of FPN decreased the CAT activity which could also be attributed to the long-term FPN detoxification cycle of 90 days (Kartheek and David 2018). These results agreed with (Al-Harbi 2016) who found FPN induced a significant reduction of SOD and CAT in rats treated with 10 mg/L of FPN in drinking water**.**

Surprisingly, the low dose of FPN showed a moderate increase in CAT activity compared with the control group. These results are following the findings of (Lukaszewicz-Hussain 2001) with Clofenvinphos. Also, (Kartheek and David 2018) found that the CAT activity mild elevation after the treatment of (6.46 mg/kg body weight/day) of FPN after 90 days. This increase could be attributed to the survival mechanism (Kartheek and David 2016). Another study suggested that elevated CAT activity could be attributed to a defense mechanism induced by high levels of ROS to protect the cell from oxidative insults (Xiao *et al.,* 2015).

Malondialdehyde (MDA) is one of the low-molecular-weight end products of lipid peroxidation products (LPO). It is widely used as a biomarker of pesticides afforded oxidative stresses (Kelly *et al.,* 1998). The MDA was significantly elevated especially in the high dose group compared to the control group. Several studies reported that the metabolites of FPN increased the formation of several reactive oxygen species (ROS) in cells, which in turn increases the lipid peroxidation rate (Bolton *et al.,* 2000; Tukhtaev *et al.,* 2013). The decline in antioxidant enzyme activity and elevation of MDA levels in the animals treated with subchronic doses of FPN indicate obvious oxidative stress as previously reported by (Kartheek & David, 2016; Mossa *et al.,* 2015; Tukhtaev *et al.,* 2013).

The significant increase in micronucleus is the limiting factor of mutation incidence. Consequently, the micronucleus test quantifies cytogenetic damage that is already fixed in the cell genome that cannot be repaired (Antunes *et al.,* 2000). Excessive production of free radicals increases the chance of DNA damage, especially during DNA duplication and cell division. Therefore, we further study the micronuclei and P53 mutations. The micronuclei are normally inducing during cell divisions, by clastogenic or an-eugenic action, producing chromosome fragments and/or entire chromosome loss during the cell division. In the telophase, these fragments or entire chromosomes are enclosed in a smaller nucleus separated from the main nucleus, thus being named "micronucleus" (al-Sabti & Metcalfe, 1995; Kirsch-Volders *et al.,* 2002)**.**

The MN was elevated significantly in the high dose group and only a moderate increase in the low dose group. The genetic damage and MN formation may be mediated by free radicals generation as reported by (Vijayalaxmi *et al.,* 1999). The previous study also reported a high percentage of MN in the peripheral blood of Swiss albino mice one day after the treatment of FPN (de Oliveira et al. 2012). Furthermore, (Girgis and Yassa 2013) recorded a micronucleus in bone marrow cells of albino rats that were treated with FPN 1/4 LD_{50}, and 1/2 LD_{50} for 24, 48, and 96 hr. This suggests that FPN disrupts the DNA duplication during the first cell cycle. The moderate increase of the MN in the low dose group could be attributed to an adaptive mechanism increasing in the replacement rate of dead or damaged cells to maintain normal physiological conditions (Mersch, Beauvais, and Nagel 1996)

The pathogenesis of a wide variety of human neoplasms involved mutations in the P53 tumor suppressor gene (Hollstein *et al.,* 1991). The P53 gene plays a crucial role in maintaining genomic stability by inducing a transient G1 arrest to allow damaged DNA to be repaired or by activating apoptosis of cells with severe damage (Nigro *et al.,* 1989; Olivier *et al.,* 2010). Therefore, the mutations in the P53 gene have a great impact on cancer development and maybe a clinically useful predictor of prognosis in mammals

(Nakamori *et al.,* 1995). The analysis by single-strand conformation polymorphism (SSCP) method allows the detection of different types of mutations such as base substitutions, small insertions, deletions, and rearrangements (Peltonen, Welsh, and Vahakangas 2007).

However, in our study, there were no altered banding patterns detected in exon 6, 7, and 8 of the P53 gene in both groups compared to the negative control sample indicating no mutation. One explanation for not detecting P53 gene mutation could be that the oxidative stress signals for the induction of apoptosis through activation of an array of cell signaling molecules, such as caspases and kinases (Junn & Mouradian, 2001; Kannan and Jain 2000; Simon *et al.,* 2000). Some studies suggested that the rat liver is resistant to P53 mutations caused by chemical agents (Bressac *et al.,* 1991; Hsu *et al.,* 1991; Raycroft, Wu, and Lozano 1990).

Although no mutation was detected in the studied exons, this does not exclude that other exons or other genes might have been affected (Bumroongkit *et al.,* 2008). Besides, the sensitivity of the SSCP method for base substitution mutation detection very efficient, although many parameters might affect its results. For example, its sensitivity varies dramatically with the DNA fragment size. Small fragments as approximately 150 bp are the optimal fragment size. Furthermore, the method is more sensitive to mutations in purine-rich sequences (Glavac and Dean 1993; Sheffield *et al.,* 1993).

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Conclusion

Although the micronucleus test results show a significant advert effect on genome stability, the PCR-SSCP analysis of P53 did not show detectable mutations, indicated that our pesticide is relatively safe compared to other alternatives used for the same purposes. More studies are needed using another PCR technique to give a clear picture of FPN genotoxicity. But until this happens the adverse effects i.e. oxidative stress and MN that appeared in the case of FPN high dose confirm the obligation to take care when applying this pesticide especially against indoor pests. A complementary PCR-SSCP on the wight blood cells DNA samples using the same primers to confirm the results obtained from tissue liver samples.

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ARABIC SUMMARY

عنوان البحث: تقييم التاثير الطفري والاجهاد التاكسدي للفيبرونيل بعد التعرض تحت المزمن في ذكور الفئران البيضاء

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يستخدم مبيد الفيبرونيل التابع لمجموعة N-phenylpyrazole علي نطاق واسع لمكافحة الافات الزراعية وآفات الصحة العامة، ولكن حتى الآن لا تتوفر بيانات كافية بشأن آثاره الجانبية. هدفت الدراسة الحالية إلي تقدير التأثير الطفري والإجهاد التأكسدي بعد التعرض تحت المزمن subchronic للفيبرونيل.

تم استخدام عدد عشرين فأر تجارب ذكر 120جرام ± 10% والتي قسمت الي 4 مجموعات بحيث تحتوي كل مجموعة علي 5 فئران، المجموعة الاولي استُخدمت للمقارنة، والمجموعتان الثانية والثالثة استُخدمت للمعاملة بالفيبرونيل بجرعات تحت مميتة (3.9 ملجم/كجم من وزن الجسم كجرعة عالية و 2.3 ملجم/كجم من وزن الجسم كجرعة منخفضة) ، المجموعة الرابعة استُخدمت كـ positive control.

تم تقدير Superoxide Dismutase (SOD) و Catalase (CAT) وMalondialdehyde (MDA) كمؤشرات حيوية للاجهاد التأكسدي، بينما تم استخدام اختبار ظهور النوية micronucleus كمؤشر للسمية علي مستوي الخلية والتأثيرات الطفرية علي جين p53 كأحد الجنيات الكابته للورم كمؤشر علي حدوث طفرات وذلك باستخدام تكنيك single-strand conformation polymorphism (SSCP) .

أظهرت النتائج حدوث انخفاض معنوي في SOD في الجرعات العالية والمنخفضة وتغيرا غير معنويا في في الجرعات العالية والمنخفضة في CAT بالمقارنة بمجموعة الكنترول. كما أظهرت النتائج حدوث زيادة معنوية في MDA في الجرعات العالية وغير معنوية في الجرعات المنخفضة بالمقارنة بمجموعة الكنترول.

اظهرت الجرعة المرتفعة من الفيبروبيل سمية خلوية عن طريق الزيادة المعنوية في عدد النويات في كريات الدم الحمراء (NCE) في النخاع العظمي ، بينما كانت الزيادة غير معنويه في الجرعات المنخفضة.

لم يُظهر اختبار SSCP علي جيل الأكريلاميد طفرات في جين P53 .أشارت الدراسة إلى السلامة النسبية لاستخدام الفيبرونيل في مجال الصحة العامة مع اتخاذ جميع إحتياطات الاستخدام.