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Research Article

The Effect of Different Doses of Fluopyram on the Kidney Tissues of Mice

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Abstract

Fluopyram is a widely used new generation broad spectrum fungicide and its toxic effects are mostly unknown. In this study, it was aimed to observe the effect of fluopyram administration at different doses on mouse kidney tissues. 0.5, 1 and 2 mg/kg Fluopyram were administered to mice, their kidneys were taken and evaluated histopathologically and immunohistochemically. Hematoxylin-eosin staining and also immunostaining with caspase-3, TNF-α, NF-Kb antibodies was performed. When the control group and fluopyram administered groups were compared, significant differences were found ($p<0.05$). While the kidney tissue of the control group was in normal histological structure, histopathological findings were observed in the experimental groups. When fluopyram administration groups were compared among themselves, it was observed that toxicity increased in a dose-dependent manner ($p<0.05$). No positive staining was observed with caspase-3, TNF-α, NF-kB in the tissues of the control group, however, a dose-dependent increase in positive staining was observed for all three stainings in the all fluopyram-treated groups ($p<0.05$). In conclusion, fluopyram appears to cause dose-dependent increased toxicity in mouse kidney tissues.

Keywords: Caspase-3; Fluopyram; Histopathology; Kidney Toxicity; NF-kB; TNF-α

Farklı Dozlardaki Fluopyram'ın Farelerin Böbrek Dokuları Üzerine Etkisi

Öz

Fluopyram, yaygın olarak kullanılan yeni nesil geniş spektrumlu bir fungisittir ve toksik etkileri üzerine çalışma yok denilecek kadar azdır. Bu çalışmada farklı dozlardaki fluopyram uygulamasının fare böbrek dokuları üzerindeki etkisinin gözlemlenmesi amaçlanmıştır. Farelere 0.5, 1 ve 2 mg/kg fluopyram dozları uygulanmış ve böbrekleri alınarak histopatolojik ve immünohistokimyasal olarak değerlendirilmiştir. Hematoksilen eozin boyamanın yanı sıra kaspaz-3, TNF-α, NF-Kb antikorları ile immün boyama yapılmıştır. Kontrol grubuyla fluopyram uygulanmış gruplar karşılaştırıldığında anlamlı farklar bulunmuştur ($p<0.05$). Kontrol grubu böbrek dokusu normal histolojik yapıda iken fluopyram verilen deney gruplarına ait böbrek dokusunda histopatolojik bulgular gözlenmiştir. Fluopyram verilen gruplar histopatolojik açıdan kendi aralarında kıyaslandığında doz bağımlı olarak toksisitenin arttığı görülmektedir ($p<0.05$). Kontrol grubu dokularında kaspaz-3, TNF- α, NF-kB ile herhangi bir pozitif boyanma gözlenmemiş ancak fluopyram uygulanan gruplar da her üç boyama için de yine doz bağımlı olarak artan anlamlı pozitif boyanma gözlenmiştir ($p<0,05$). Sonuç olarak fluopyramın fare böbrek dokularında doz bağımlı olarak artan toksisiteye sebep olduğu görülmektedir.

Anahtar Kelimeler: Böbrek toksisitesi; Fluopyram; Kaspaz-3; NF-kB; TNF-α

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1. Introduction

Agricultural production relies on the use of pesticides to achieve maximum crop efficiency, and the use of these chemicals in modern agricultural practices is now considered an integral part of the agricultural industry. Although pesticides are known as hazardous chemicals due to their chronic and acute toxicity, their consumption is almost inevitable (Wei et al., 2016). Different variables affect the risk of pesticide contamination, such as active compounds of pesticides or final products of chemical or microbial transformation of the pesticides in the environment (C. Li et al., 2020). Exposure to these compounds has been associated with various adverse effects, including cardiotoxicity. Fluopyram (FL) is known as a broad spectrum fungicide and is a succinate dehydrogenase inhibitor, originally discovered and patented by Bayer, used for the management of fungal diseases in more than 70 crops including vines, table grapes, fruits etc. (C. Li et al., 2020; Tzatzarakis et al., 2020). FL was a relatively new pesticide which registered in 2014 (Faske & Hurd, 2015; J. Li et al., 2020; Rouquié et al., 2014). Sustained and excessive application of fluopyram may pose a risk to human health through instability of the associated agro-ecosystem (Anastassiadou et al., 2019; Mekonnen, Panne, & Koch, 2019; Storelli, Keiser, Eder, Jenni, & Kiewnick, 2020; Wei et al., 2016). Unlike plants, FL is extensively metabolized in animals and is known to have no acute toxic effects by inhalation, no skin or eye irritant or skin sensitizing effects. Although FL is classified as unlikely to pose a risk to human health (Wei et al., 2016), toxic effects are still a matter and mostly unknown. Kidney and liver are known to be the target organs most affected by pesticide toxicity. FL can cause liver toxicity in rodents and an increase in the incidence of hepatocellular adenoma and carcinoma has been observed in female rats exposed to FL (Colnot & Dekant, 2017; Klich et al., 2020; Tinwell et al., 2014). Likewise acute FL exposured cause thyroid tumors in mice and liver tumors in rats and transient, non-specific functional effects on the nervous system (Dong & Hu, 2016). However, there are no studies on the effect of fluopyram on the kidneys. In this study, the possible toxic effects of different doses of FL on mouse kidney tissues were evaluated by histochemical and immunohistochemical analyzes.

2. Material and Method

2.1. Experimental design

In the study; a total of 32 Swiss albino mice, 16 male and 16 female, 6-12 m old, 29 – 35 gr, were used after ethical approval (17.06.2021-05/07) by SDU Local Ethics Committee of Animal Experiments. 32 mice were randomly divided into four group (Table 1) and were given unlimited access to water and food (ad libitum) throughout the experiment and were allowed a preparation period for 2 weeks. 2 h light/12 h dark period and 25 °C heat were maintained. Doses (Table 1) were dissolved in 0.1 ml DMSO and administered subcutaneously every 2 days for 21 days (a total of 11 injections).

Table 1. Classification of Groups

Group	Dose	n
1	None / Control	8
2	0.5 mg Fluopyram	8
3	1 mg Fluopyram	8
4	2 mg Fluopyram	8

n: Number of rat used

Following experimental procedure, anesthesia was applied by xylazine (10 mg/kg) and ketamine (90 mg/kg) intraperitoneally, mice were sacrificed, kidney tissue were obtained and were placed in 10% neutral formalin for histochemical and immunohistochemical analyses.

2.2. Histochemical analyses

Tissue samples were washed in water over night and were dehydrated in ethanol (50-60-70-80-90-100%), then were made transparent in xylol. Following, samples were embedded into paraffin, were cut with a thickness of 4 μm by microtome (Leica SM2000R, Germany) and were stained by Hematoxylin–Eosin (H–E) than covered with entellan. Histopathological findings were graded and evaluated with photomicroscope by using the semi-quantitative method according to as following:

(-), negative score: No structural changes
(+), 1 positive score: Light structural changes
(++), 2 positive score: Middle structural changes
(+++), 3 positive score: Serious structural changes

2.3. Immunohistochemical analyses

Samples of 3– 4μm thicknesses were obtained and were stained with Caspase-3 primary ab (rabbit anti-caspase antibody, Abcam, Cambridge, USA), TNF-α primery ab (rabbit anti-TNF-α antibody, Abcam, Cambridge, USA), NF-kB primary ab (rabbit anti- NF-kB antibody, Abcam, Cambridge, USA) and were covered with entellan. After that, samples receptor densities were graded by the semi-quantitative evaluation method (Refaiy, Muhammad, & ElGanainy, 2011)

(-), negative score: No staining
(+), 1 positive score: Light staining
(++), 2 positive score: Middle staining
(+++), 3 positive score: Serious staining

2.4. Statistical analysis

Mann-Whitney U test were conducted as described in (Gibson-Corley, Olivier, & Meyerholz, 2013) via SPSS 18 software for all analysis. All findings were considered significant at $p<0.05$.

3. Result and Discussion

3.1. Histochemical results

Normal histological structures were observed in the control group (group 1), (Fig. 1 a-a1). When control group were compared the FL. groups (group 2, group 3, group 4), significantly changes such as, mononuclear cell infiltration, hemorrhagic areas, vascular congestions, vacuolar and granular degeneration, tubular dilatations in medulla were observed ($p<0.05$), (Fig. 1, b-b1, c-c1, d-d1), (Table 2) However, histological structural changes were higher in group 4, group 3 and group 2, respectively ($p<0.05$).

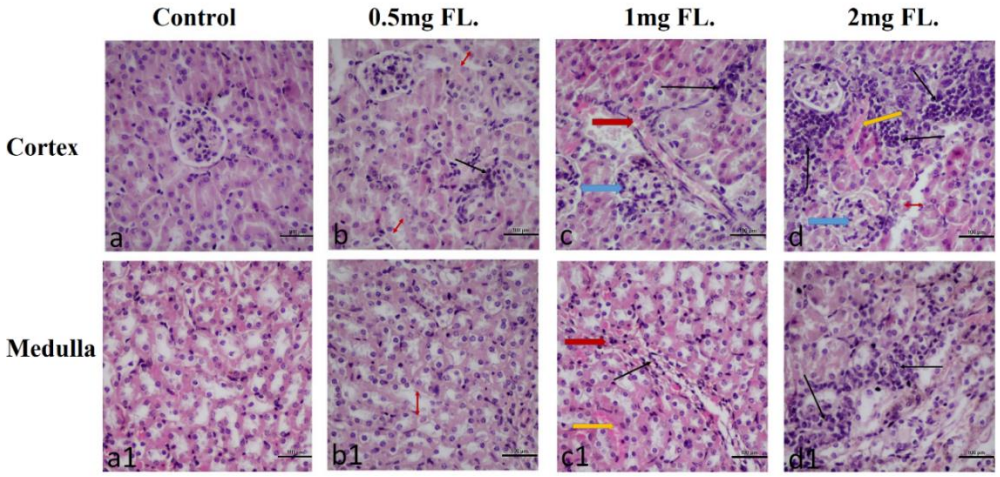

Figure 1: Kidney tissue sections from control and fluopyram groups. Control group showed normal kidney histology (a-a1). In the samples belonging to the groups given 0.5-1- 2 mg/kg FL, histopathological findings were observed in the kidney tissue (b-b1, c-c1, d-d1); mononuclear cell infiltration (black arrows), dilatations and degenerations in the proximal - distal tubules (red two-pointed arrows), vascular congestions (red thick arrows), glomerular degenerations (blue thick arrows), hemorrhagic areas (yellow arrows). These findings at least were in the group given 0.5 mg/kg FL and at most in the group given 2 mg/kg FL, H-E, a,b,c,b: cortex, a1,b1,c1,d1: medulla, x20.

Table 2. Average Score of Histopathological Findings between All Groups

Groups	Mononuclear Cell Infiltration	Hemorrhagic Areas	Vascular Congestions	Vacuolar - Granular Degeneration	Tubular Dilatations in Medulla
1	-/+	-	-	-/+	-/+
2	+/++	+	+	+/++	+/++
3	++/++	++	++	+++	++
4	+++	+++	+++	+++	+++
The significance status obtained as a result of the comparison of the groups					
1-2	p = 0.003	p = 0.001	p = 0.001	p = 0.002	p = 0.002
1-3	p = 0.000	p = 0.000	p = 0.000	p = 0.001	p = 0.001
1-4	p = 0.001	p = 0.000	p = 0.000	p = 0.001	p = 0.001
2-3	p = 0.003	p = 0.004	p = 0.001	p = 0.008	p = 0.117
2-4	p = 0.008	p = 0.001	p = 0.001	p = 0.008	p = 0.008
3-4	p = 0.747	p = 0.003	p = 0.015	p = 1.000	p = 0.015

$p<0.05$ were considered statistically significant. The relationships between groups and results of histopathological markers are assessed by Mann-Whitney U test. FL: Fluopyram.

(-), negative score: No structural changes

(+), 1 positive score: Light structural changes

(++), 2 positive score: Middle structural changes

(+++), 3 positive score: Serious structural changes

3.2. Immunohistochemical results

Results indicated that group 1 were poorly stained with Caspase-3, TNF-α and Nf-kB antibodies (Fig. 2, a-a5) while staining were intense in FL treated groups. When the FL treated groups were compared among themselves, staining levels were determined as group 4 > 3 > 2 for all antibodies ($p<0.05$, Fig. 2, d-d5, c-c5, b-b5, Table 3).

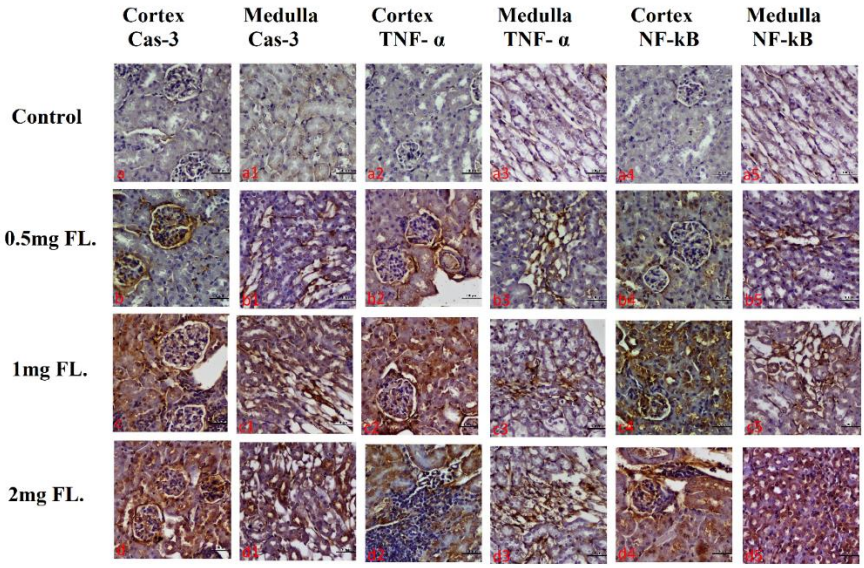

Figure 2: Kidney tissue sections with immunohistological staining from control and fluopyram groups. Sections from control showed very less positive staining (a-a5). Conversely, sections from the FL. groups showed intense positive staining with Caspase 3, TNF- α ve NF-kB staining in cortex and medulla (b—b5, c-c5, d-d5). The highest positive staining for all three antibodies was observed in group 4 (d-d5), group 3 (c-c5) and group 2 (b-b5) respectively, immün satining x20.

Table 3. Caspase 3, TNF- α ve NF-kB Marking Average Degrees between All Groups

Groups	Caspase 3 Staining Degree	TNF- α Staining Degree	NF-kB Staining Degree
1	-/+	-/+	-/+
2	+	+	+
3	+/++	+/++	+/++
4	+++	+++	+++
The significance status obtained as a result of the comparison of the groups			
1-2	p = 0.117	p = 0.025	p = 0.318
1-3	p = 0.006	p = 0.006	p = 0.006
1-4	p = 0.001	p = 0.001	p = 0.001
2-3	p = 0.002	p = 0.025	p = 0.015
2-4	p = 0.001	p = 0.001	p = 0.001
3-4	p = 0.008	p = 0.008	p = 0.003

$p<0.05$ were considered statistically significant. The relationships between groups and results of immunohistochemical markers are assessed by Mann-Whitney U test. FL: Fluopyram.

(-), negative score: No staining
(+), 1 positive score: Light staining
(++), 2 positive score: Middle staining
(+++), 3 positive score: Serious staining

4. Conclusion

In this study, toxic effects of different doses of FL on the kidney tissues were investigated. FL, is widely used broad spectrum fungicide targeting pathogenic plant fungi. The toxic effects of pesticides, which are increasingly used, are the subject of experimental studies. However, there are not many studies in this sense (Colnot & Dekant, 2017; Gibson-Corley et al., 2013; Tinwell et al., 2014).

Fluopyram is absorbed after oral administration and extensively metabolized, and almost completely excreted after 168 h. Although the studies on FL is limited, it's known that it is not acutely toxic via oral, dermal and inhalation routes, not skin or eye irritant, nor skin sensitizer as a result of the analyzes. Studies shows that FL is not cause effects on fertility and not teratogenic and signs of fetotoxicity are confined to the high dose level with clear maternal toxicity in both rats and rabbits (Authority, 2013). However, there are not enough studies to reach a definite conclusion. Since FL is a fairly new pesticide, there are very few studies investigating its effects. Çelik et al., in their study with rats, implemented 5 mg/kg, 10 mg/kg and 20 mg/kg of Luna Experience SC 400 (FL) at 48 h intervals for 30 days by gavage and reported genotoxicity for all doses suggesting that FL may be genotoxic and cytotoxic in rat bone marrow (Çelik, Güler, Aktaş, & Yalin, 2019). Although little is known about the effects of FL, there are some studies on the effects of certain pesticides on the pathways investigated in this study. Owumi and Dim, investigated chlorpyrifos and manganese effects on oxidative stress, inflammation and caspase-3 activation in rats and reported chlorpyrifos-induced increase in biomarkers of hepatorenal toxicity were significantly alleviated ($p < 0.05$). In addition, chlorpyrifos mediated increase in TNF-α, IL-1β and caspase-3 activity were significantly diminished in the liver and kidney of rats co-exposed to chlorpyrifos and manganese. Light microscopic examination evidenced that the severity of histopathological lesions induced by CPF were alleviated in rats coexposed to chlorpyrifos and manganes. In conclusion of the study, they suggested that the results highlight that co-exposure to chlorpyrifos and manganes in rats assuaged chlorpyrifos -induced oxidative stress, inflammation and caspase-3 activation in the liver and kidney of the rats (Owumi & Dim, 2019). Ozmen and Mor, examined the toxic effects of endosulfan and vitamin C on New Zealand white rabbit kidneys with histopathological and caspase-3 stainings. Results revealed that hemorrhages, tubule cell necrosis, glomerular infiltration, glomerulosclerosis and proteinaceous material in the tubules, Bowman spaces and Caspase-3 reaction in the kidneys of endosulfan treated group (Ozmen & Mor, 2015). Guvenc et al., examined the caspase-dependent apoptotic and necrotic changes in rat kidney exposed to different doses of permethrin. Results showed that degenerative changes were observed in the epithelial lining

of the S1, S2, and S3 segments of the renal proximal tubules, apoptotic cells were seen in the inner stripe of the outer medulla and immunohistochemical staining of caspase 3 and caspase 9 also was observed in the same areas in permethrin treated groups. Authors suggested that damage to regions of the proximal tubules is dose-related, and caspase-9-dependent, mitochondria-related apoptotic cell death could play an important role in permethrin-induced nephrotoxicity in rats (Guvenc, Kabak, Atmaca, Aksoy, & Guvenc, 2013). Wang et al, reported enhanced expression of caspase-3, caspase-7 and PARP activity in HepG2 cells, which was 1.7, 1.3 and 1.6-fold higher than the control, respectively, along with significant protein cleavage; and induced apoptosis in a concentration-dependent manner after administration of certain widely used pesticides. Further, the pesticide mixtures significantly increased ROS level (up to 1.3-fold), induced DNA fragmentation (up to 1.8-fold), inhibited DNA synthesis (up to 53%), and damaged the cells by destroying the cell membrane and producing a large amount of LDH. Authors suggested that widely used pesticides could exhibit cytotoxicity and apoptosis through the ROS-related caspase pathway, providing a basis for evaluation of health risks from pesticide mixtures via food consumption (Wang, Ma, Chen, Yang, & Qian, 2021).

In this study, It was found that, FL was shown to induce toxicity on kidney tissues in mice with histological and immunohistological analyses especially with high dose. Caspase-3, TNF-α and Nf-kB staining was analyzed for observing FL toxicity via to immunohistological methods. Increased Caspase-3 staining; it suggests that FL induces mitochondrial damage and following that increased ROS activity. ROS starts the release of inflammatory mediators such as NF-kβ and TNF-α and inflammation increases in kidney. In addition, since there are not many studies on FL, the mechanism of its toxic effect in the kidney is not clearly understood yet. Ahmad et al., studied another fungicide, edifenphos in rat kidneys. Results showed that edifenphos promotes deleterious effects like oxidative stress, DNA damage, reduced mitochondrial membrane potential, generation of ROS production, activation of caspase 3/9 activities and causing hepato-renal histopathological changes (Ahmad, Kumari, & Ahmad, 2019).

Rouquie et al., observed that FL thyroid toxicity is mediated by activation of hepatic Car/Pxr receptorsand (Rouquié et al., 2014), in the other study; Tinwell et al., observed that FL is a threshold carcinogen and the resultant hepatocellular carcinomas in rat are due to hepatocellular proliferation mediated by CAR/PXR activation (Tinwell et al., 2014). In present study, it was observed that FL induced kidney toxicity mediated by Caspase-3, TNF-α and Nf-kB staining, and higher doses of FL leads to more damage.

The present study was tested effect of different dose of FL on kidney toxicity. Results suggests that FL induced toxicity on kidney tissues especially with high doses. Action mechanisms of FL should be investigated with more comprehensive studies.

Ethics

This is study was conducted after ethical approval (17.06.2021-05/07) by SDU Local Ethics Committee of Animal Experiments.

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