KSÜ Tarım ve Doğa Derg 26 (6), 1436-1442, 2023
KSU J. Agric Nat 26 (6), 1436-1442, 2023
https://doi.org/10.18016/ksutarimdoga.vi.1224789

The Effects of Fipronil on Glutathione and Histology of Freshwater Snails

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ABSTRACT

Fipronil ($C_{12}H_4Cl_2F_6N_4OS$, CAS No: 120068-37-3) is frequently used in agricultural fields and veterinary medicine as an insecticide and acaricide. It is known to contaminate aquatic ecosystems by mixing with surface waters and to accumulate in abiotic matrices. In this study, the effects of fipronil are investigated using freshwater snails *Viviparus contectus* (Millet, 1813). After exposure of snails to 1, 10 and 100 mg L^{-1} fipronil for 7 days, all body tissues were taken. As a result of the study of glutathione, one of the tissue antioxidant parameters, a significant increase was observed in the control group, which was administered 1 mg L^{-1} fipronil, compared to the other dose groups ($P<0.05$). Exposure to different concentrations of fipronil resulted with degenerations and necrosis of the digestive gland tubules of snails, histologically. The damages in the digestive gland tissue were increased with increasing of the concentration. Since snails are an important species for freshwater ecosystems, it can be emphasized that pesticides such as fipronil pose a potential risk to these organisms.

Zoology

Research Article

Article History
Received : 26.12.2022
Accepted : 07.04.2023

Keywords
Freshwater snail
Glutathione
Histopathology
Fipronil

Tatlı Su Salyangozlarında Fipronilin Glutatyon ve Histolojisine Etkileri

ÖZET

Fipronil ($C_{12}H_4Cl_2F_6N_4OS$, CAS No: 120068-37-3) insektisit ve akarisit olarak tarımsal alanlarda ve veteriner hekimlikte sıklıkla kullanılmaktadır. Sucul ekosistemler, yüzey sularına karışması yolu ile kontamine ettiği ve abiyotik matrikslerde birikim gösterdiği bilinmektedir. Bu çalışmada, fipronilin etkileri tatlı su salyangozları *Viviparus contectus* (Millet, 1813) kullanılarak incelenmektedir. Salyangozların 7 gün süreyle 1, 10 ve 100 mg L^{-1} fipronile maruz kalmasını takiben tüm vücut dokuları alınmıştır. Doku antioksidan parametrelerinden glutatyon incelemesi sonucunda 1 mg L^{-1} fipronil uygulanan grupta kontrol de diğer doz gruplarına göre önemli bir artış gözlenmiştir ($P<0.05$). Farklı fipronil konsantrasyonlarına maruz kalmak, salyangozların histolojik olarak sindirim bezi tübüllerinin dejenerasyonuna ve nekroza neden olmuştur. Konsantrasyonun artmasıyla sindirim bezi dokusundaki hasarlar artmıştır. Salyangozların tatlı su ekosistemleri için önemli bir tür olması nedeniyle fipronil gibi pestisitlerin bu canlılara karşı potansiyel bir risk oluşturduğu vurgulanabilir.

Zooloji

Araştırma Makalesi

Makale Tarihçesi
Geliş Tarihi : 26.12.2022
Kabul Tarihi : 07.04.2023

Anahtar kelimeler
Tatlı su salyangozu
Glutatyon
Histopatoloji
Fipronil

To Cite : Arslan, P., Yıldırım, M.Z., & Günal, A.Ç., *(2023)* The Effects of Fipronil on Glutathione and Histology of Freshwater Snails. *KSÜ Tarım ve Doğa Derg 26 (6),* 1436-1442. https://doi.org/10.18016/ksutarimdoga.vi.1224789

Atıf Şekli: Arslan, P., Yıldırım, M.Z., & Günal, A.Ç., (2023) Tatlı Su Salyangozlarında Fipronilin Glutatyon ve Histolojisine Etkileri. *KSÜ Tarım ve Doğa Derg 26* (6), 1436-1442. https://doi.org/10.18016/ksutarimdoga.vi.1224789

INTRODUCTION

The application of pesticides in agriculture, industry, domestic and veterinary medicine is very important in pest control and prevention of pest-borne diseases (Perkins et al. 2021; Tang et al. 2021a; Shen et al. 2022). After application of these areas, the pesticides are transported into aquatic environments by different

ways including run-off water (Perkins et al. 2021). Due to the bioaccumulation and biomagnification tendency of pesticides and their metabolites, they are important contaminant of the aquatic environment (Tongo et al. 2022). There are many studies in the aquatic environment where pollutants and their metabolites including pesticides, are encountered in water, sediment and tissues of aquatic organisms (Arisekar et al. 2019; Adeyeye et al. 2021; Tyohemba et al. 2021; Arslan & Ozeren 2022; Şimşek & Bilgili 2022). For example, fipronil and its metabolites ranged from 0.5 to 1.6 ng L^{-1} water samples, 4.05 ng g^{-1} of fish muscle and 19.91 ng g^{-1} of fish liver samples of River Elbe (Germany) (Michel et al. 2016). Likewise, fipronil, an insecticide, were found 0.69 ng g^{-1} in sediment samples of River Sutlej (India) (Kaur et al. 2019).

Fipronil, which is detected as a residue in different compartments in the aquatic ecosystem, has a wide range of use as an ectoparasite agent (Perkins et al. 2021). It shows its effect on insects by blocking the γ-aminobutyric acid (GABA)-gated chloride channel (Michel et al. 2016). It is reported that fipronil has toxic effects of non-target organisms such as fish and aquatic invertebrates (Wirth et al. 2004; Nillos et al. 2009; Qu et al. 2014; Qian et al. 2017).

Pesticides including fipronil are the main producers of reactive oxygen species (ROS) in the cells that damage on lipids, proteins and DNA (Stara et al. 2021; Dash and Rahman 2022; Sule et al. 2022). A multicomponent antioxidant complex largely regulates the balance between the production and elimination of ROS, as well as their potential detrimental effects (Gostyukhina et al. 2022). Glutathione, an antioxidant defense mechanism, is a main component to protect the cell from oxidative stress. As a reducing agent, it traps free radicals and acts as a substrate for some enzymes (Ali et al. 2020). Besides oxidative stress effects, research reported that pesticides also have adverse effects on histological alterations in the aquatic animal tissues (Ghaffar et al. 2018; Farhan et al. 2021; Tang et al. 2021b; Arslan & Ozeren 2022; Merola et al. 2022).

In studies with aquatic invertebrates, it has been observed that different subjects such as their ecology, biological properties, distribution and responses to aquatic pollutants are emphasized (Graf & Cummings 2021; Stara et al. 2021; Arslan et al. 2022). The freshwater snail *Viviparus contectus*, one of the aquatic invertebrates, is a cosmopolitan species that lives in freshwater ecosystems including rivers, lakes and swamps. In addition to feeding with detritus, they also feed by filtering the water. Thus, they show the feature of cleaning the abiotic parts of the aquatic ecosystem (Kocabaş et al. 2022; Kutluyer & Kocabaş 2022). Therefore, it may be used as an alternative non-target organism in toxicological studies in freshwater systems.

So far, to our knowledge, no studies have assessed the toxic effects of fipronil on freshwater snails. In this regard, the present study aimed to get knowledge about the biochemical and histological effects of fipronil on *V. contectus*.

MATERIALS and METHODS

Tested Organisms and Chemical

In the current study, the freshwater snails *Viviparus contectus* (Millet, 1813) was used as a model organism. Snails were collected from Hoyran, Eğirdir Lake located in Isparta, Turkey. A total of 100 snails (mean length 1.36±0.05 cm and mean weight 0.5±0.01 g) were acclimated to laboratory conditions as well as depuration period for two weeks.

24-h before the experiments, the stock solution of fipronil ($C_{12}H_4Cl_2F_6N_4OS$, CAS No: 120068-37-3, purity 98%) was prepared in dimethyl sulfoxide (DMSO) kept in +4ºC.

Experimental Design and Samples Collection

After the acclimation and depuration period, snails were randomly transferred to 15 L experimental aquariums (15 organisms/aquarium). There were three fipronil applied groups (1, 10, and 100 mg L^{-1}) and two control groups (control: water and snails; solvent control: water, snails and DMSO) in duplicate. The snails from each group were collected on the 7th day of exposure. For the biochemical analysis, 10 organisms in each group were dissected as whole body and immediately frozen in liquid nitrogen. Then, the samples were kept in -80ºC until the analysis. For the histological analysis, 5 organisms in each group were taken as whole body in tissue cassette and fixed into Davidson solution.

Biochemical Analysis

Frozen whole-body tissues were weighed as 100 mg on ice and homogenized in metaphosphoric acid (0.5 M, pH:8) using Miccra D-1 homogenizer (Germany). The homogenates were centrifuged in a refrigerated centrifuge (Hettich Zentrifugen Micro 220 R) at +4ºC 3500 rpm for 10 min. The levels of glutathione were evaluated according to the procedure of Ellman (1959). The main principle of this method is to determine the reaction of a thiol-selective DTNB reagent with free sulfhydryl groups to form a colored product at 420 nm. Total protein of tissues was measured at 595 nm using bovine serum albumin (BSA) as a standard and the Bradford method (Bradford, 1976).

Histological Analysis

After fixing the whole-bodies for 24-h, the tissues were put into ethyl alcohol series and embedded in paraffin blocks. The blocks were cut into 5 µm thickness and stained with hematoxylin and eosin (Luna 1968). The slides were observed under a light microscope

according to Benli et al. (2008).

Statistical Analysis

The glutathione values in the graphs were expressed as mean (µgM mg^{-1} protein) ± SEM. The GraphPad Prism program (version 5, USA) was used for the statistical analysis. One-way ANOVA was used to evaluate the differences between the control and exposed ($P < 0.05$).

RESULTS and DISCUSSION

Biochemical Assay

Glutathione activity increased in the 1 and 10 mg L^{-1} fipronil exposed groups compared to the control groups, while it decreased in the 100 mg L^{-1} fipronil exposed group. Glutathione activity was 2.8 times higher in the low-dose fipronil group compared to the control group ($P<0.001$). In addition, glutathione activity in the 1 mg L^{-1} fipronil exposed showed a significant increase of 1.9 ($P<0.01$) and 4.3 ($P<0.001$) times compared to the glutathione activities in the 10 mg L^{-1} and 100 mg L^{-1} fipronil exposed groups, respectively. Similarly, 10 mg L^{-1} fipronil exposed group compared to 100 mg L^{-1} fipronil exposed group significantly increased 2.2 times ($P<0.05$). The glutathione values of control and fipronil exposed groups is shown in Figure 1.

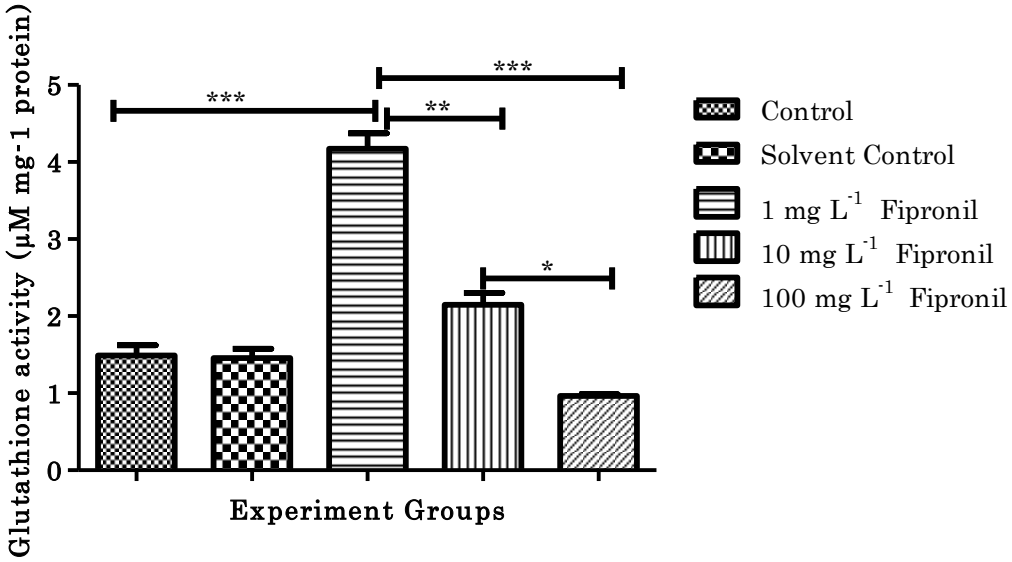

Figure 1. The glutathione activities of control and fipronil-exposed groups
(* indicates P<0.05, ** indicates P<0.01, *** indicates P<0.001)
Şekil 1. Kontrol ve fipronil uygulanan grupların glutatyon aktiviteleri
(P<0.05'i gösterir, ** P<0.01'i gösterir, P<0.001'i gösterir)*

By causing oxidative stress in organisms and speeding the generation of ROS, environmental pollutants including pesticides have impacts on antioxidant responses and harm cellular macromolecules (Stara et al. 2021). The results obtained in this study showed that the levels of glutathione, an antioxidant defense system, occur in freshwater snail tissues. The toxic effect of fipronil was more markedly increased in snails exposed to the lower concentration tested. This result obtained in the study differs from other ecotoxicological studies with freshwater snails in the literature. In a study conducted with another freshwater snail species, *Lymnaea luteola*, observed that the glutathione activities in digestive glands decreased in the azoxystrobin exposed groups for 24-h and 96-h (Ali et al. 2021). Similarly, another study reported that the glutathione levels decreased in the nanoparticle exposed groups of *L. luteola* (Al-Abdan et al. 2021). It has been reported that a decrease in glutathione level occurred in the freshwater snail *Pila virens* exposed to a nanoparticle substance for 24-h and 48-h (Srikanth et al. 2021). These results point to a critical role for antioxidants in the control of cell metabolism in the case of an imbalance brought on by xenobiotics injury.

Histopathological Analysis

The whole tissues of snails did not show any histological changes in the control and DMSO added control groups. Histopathological alterations were observed in the digestive gland tissues of *V. contectus* after exposure to different fipronil concentrations (Table 1). The control group digestive gland tissues were normal appearance with tubules (Fig 2a). The digestive gland tissues of snails were exhibited degenerations of the tubules and necrosis (Fig 2b, c,

and d) increased with increasing of the fipronil concentrations. There was no study regarding the histopathological effects of fipronil or xenobiotics to *V. contectus* in the open literature. The digestive gland, analogue of the liver in vertebrates, is beneficial tissue for determination of the health and toxicological exposures to aquatic invertebrates (Klobucar et al. 2001, Faggio et al. 2018). Exposure to toxic compounds has also previously been resulted with tubule degenerations in the digestive gland tissues of aquatic invertebrates and non-specific histological alterations to various xenobiotics (Cengiz et al. 2005, Karakaş & Otludil 2020, Tresnakova et al. 2020, Balamurugan et al, 2021). Similar to the results of the present study, exposure to 0.264 mgL^{-1} and 0.528 mgL^{-1} fipronil for 48-h and 7-d resulted with accumulated lipofuscin aggregates and caused mild degeneration of digestive tubules in freshwater mussels (Arslan & Gunal 2023). Exposure to fipronil also resulted with some tissue damages in other aquatic species and mamalian organisms. Qureshi et al. (2016) determined hemorrhagia, hyperplasia and nuclear hypertrophy of *Cyprinus carpio* after exposed to 400 µgL-1 for 4 days. Oral exposure to 6.46, 12.12 and 32.33 $mgkg^{-1}$ body weight/daily of fipronil for 90 days resulted with hypertrophy of hepatocytes in Wistar albino rats (Kartheek & David, 2019).

Table 1. Histopathological findings of digestive gland tissue of *V. contectus* after exposure to fipronil
Çizelge 1. Fipronile maruz kalan V. contectus'un sindirim bezi dokusunda histopatolojik bulgular

	Experimental Groups				
Histopathology	Control	Solvent control	1 mgL^{-1} fipronil	10 mgL^{-1} fipronil	100 mgL^{-1} fipronil
Tubul degeneration	-	-	+	++	++
Necrosis of digestive tubules	-	-	-	++	+++

* The histopathological alterations were scored as "(-) none (no histopathological alterations), which represents normal histological structure; (+) histopathology in > 20% of fields (mild); (++) histopathology in 20-60 % of fields (moderate) and (+++) histopathology in < 60% of fields (severe)"

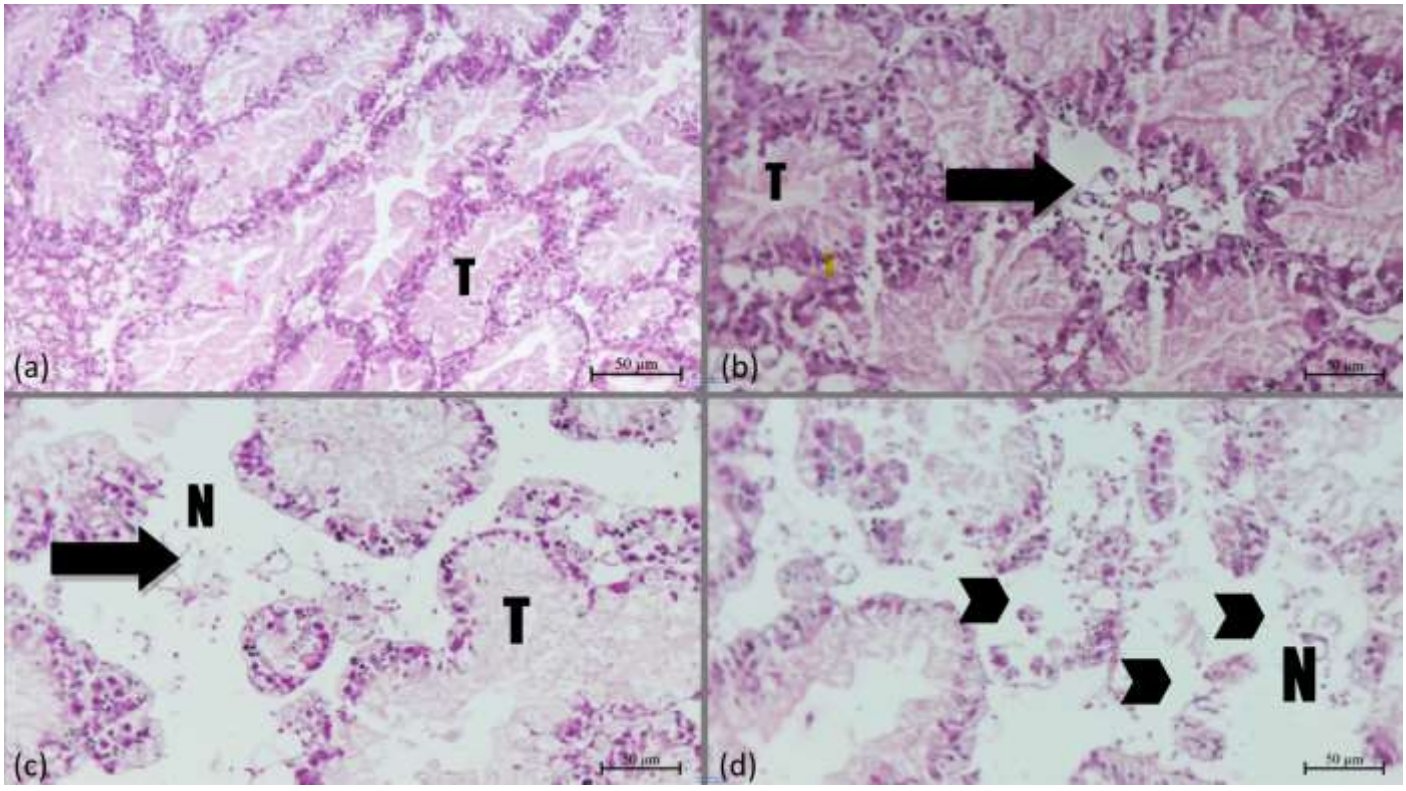

Figure 2. Digestive gland tissue of freshwater snail, *Viviparus contectus*, a) control, b) degenerations of the digestive tubules (black arrow) after exposure to 1 mg L^{-1} fipronil for 7 days, c) degeneration and necrosis of the digestive tubules (black arrow) after exposure to 10 mg L^{-1} fipronil for 7 days d) degeneration and necrosis of the digestive tubules after exposure to 10 mg L^{-1} fipronil for 7 days (black arrow heads) (H&E) T: tubule; N: necrosis

Şekil 2. Tatlı su salyangozu Viviparus contectus'un sindirim bezi dokusu, a) kontrol, b) 7 gün boyunca 1 mg L^{-1} fipronile maruz kaldıktan sonra sindirim tübüllerinin dejenerasyonu (siyah ok), c) 7 gün boyunca 10 mg L^{-1} fipronile maruz kaldıktan sonra sindirim tübüllerinin dejenerasyonu ve nekrozu (siyah ok) d) 7 gün boyunca 10 mg L^{-1} fipronile maruz kaldıktan sonra sindirim tübüllerinin dejenerasyonu ve nekrozu (siyah ok başları) (H&E) T: tübül; N: nekroz

CONCLUSION

The present study showed the first ecotoxicological effects of fipronil on the freshwater snail *V. contectus*. The glutathione activity results indicated that there was no dose-dependent concentration correlation in fipronil toxicity in snails. The histological results were revealed the effects of fipronil and *V. contectus* can be used as good candidates of determination of freshwater bodies as bioindicator organisms. The future experiments with other antioxidant stress parameters are required to measure the toxicological effects of fipronil on snails.

ACKNOWLEDGEMENT

The results of glutathione exposed to fipronil in this present study were presented as oral presentation at ESENIAS & DIAS Conference 2022.

Author's Contributions

The contribution of authors is equal.

Declaration of Interest

The authors declare that they do not have any competition and any conflicts of interest.

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