J. Black Sea/Mediterranean Environment
Vol. 28, No. 1: 63-77 (2022)

RESEARCH ARTICLE

Assessment of the genotoxic effect of thiamethoxam in *Cyprinus carpio* by the micronucleus and Comet assays

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Abstract

Pesticides are compounds formed by natural or various chemical processes that kill harmful organisms, remove pests from their environment, reduce population density or completely eliminate them. Monitoring for toxic effects and screening for different insecticides is vital and crucial for reducing adverse effects on aquatic organisms and public health. Therefore, in this study, we aimed to determine genotoxic effect of thiamethoxamine in a model fish species, *Cyprinus carpio*, using the micronucleus test and Comet assay. Common carp (average weight of 1.56±0.31g) were exposed to control and three different concentrations of thiamethoxamine (1.0, 1.5 and 2.0 mg L^{-1}) based on previously detected aquatic environmental concentrations for ten days. At the end of study, the Damage frequency (%), Arbitrary unit and Genetic damage index (%) were evaluated in gill and liver cells of carp by Comet assay. Micronucleus frequencies and erythrocyte abnormalities were also determined in erythrocytes cells of carp by micronucleus test. The highest micronucleus frequency and erythrocyte abnormalities were significantly observed in 2.0 mg L^{-1} group ($p<0.001$) and, the highest damage frequencies (%) as 74.000±1.732 and 68.000±1.732 were significantly observed in 2.0 mg L^{-1} group in gill and liver cells, respectively ($p<0.001$). Our results revealed significant increases in the frequencies of micronuclei and DNA strand breaks in *C. carpio*, following exposure to thiamethoxamine, thus demonstrated the genotoxic potential of this pesticide on fish.

Keywords: DNA damage, thiamethoxam, micronucleus test, Comet assay, pesticide

Received: 05.01.2022, **Accepted:** 05.04.2022

Introduction

Pesticides significantly contribute to agricultural development throughout the world in last decades (Albaseer 2019). They can, however, persistently remain in the ecosystem (Nordborg *et al*. 2017). Besides, these chemicals can enter

surface waters via spray drift or surface runoff because of the wide use of them (Ding *et al.* 2019). Previous studies have demonstrated that pesticide residues have been a significant threat to aqueous ecosystem (Yan *et al.* 2016; Bonmatin *et al.* 2019; Chen *et al.* 2020). Although many investigations have evaluated the toxic effects of single pesticides (Souders II *et al.* 2021; Wan *et al.* 2020), aquatic organisms in natural ecosystems are commonly exposed to pesticide (Nowell *et al.* 2014; Ernst *et al.* 2018; Silva *et al.* 2019). Thiamethoxam, [3-(2-chloro-1,3-thiazol-5-methyl) -5-methyl-4-nitroimino-perhydro -1,3,5 oxa-diazine], is one kind of neonicotinoids, which are the most important class of new synthetic insecticides (Thany 2010). Thiamethoxam is among the most profitable pesticides worldwide (Cavusoglu *et al*. 2012; Barganska *et al.* 2013) due to its widespread use to control numerous sucking and biting insect pests to protect crop (Karmakar and Kulshrestha 2009) since introduced into the market in 1991. Nowadays, three kinds of neonicotinoids, including imidacloprid, thiamethoxam, and clothianidin have been seriously accused due to their toxicity to honeybees. They have led to calls for restrictions on their uses in agricultural (EFSA 2013; Van der Sluijs *et al.* 2013) that have recently been carried out across the European Union (European Commission 2013). All we know that the aquatic environment accommodates an amount of external contaminations that have the potential damage to aquatic organisms. Thiamethoxam is a potential contaminant in the surface and underground waters due to its unique properties, such as low soil sorption, high leaching capability, and high solubility in water (Zhao *et al.* 2012). Thiamethoxam is stable to hydrolysis under acidic or neutral pH condition (Morrissey *et al*. 2015). Furthermore, the compound is toxic (Cavusoglu *et al.* 2012; Jones *et al.* 2012; Yang *et al.* 2014), bioaccaumulative (Guillen and Bielza 2013), and difficult to mineralize (Pena *et al.* 2011).

Many scientific experiments have examined the use of insecticides in terrestrial ecosystems and the toxicity of pesticides to animals (Yan *et al.* 2016; Bonmatin *et al.* 2019; Chen *et al.* 2020). However, less is known about their toxicity to aquatic ecosystems, including fish (Cavallaro *et al.* 2017; Finnegan *et al.* 2017; Saraiva *et al.* 2017; Temiz *et al.* 2021). Turan and Ergenler (2022) recently reported that genotoxic effect of acetamipridine in a common carp *Cyprinus carpio*, using the micronucleus test and Comet assay. The presence of pesticides in water is a consequence of weed control in terrestrial ecosystems and water reservoirs. Since there is growing concern over the presence of genotoxins in the aquatic environment, it is important to develop methods for detection of genotoxic effects in aquatic organisms.

The genotoxic effects of environmental pollutants can be monitored using a broad range of both *in vitro* and *in vivo* biomarker assays but the micronucleus test and the Comet assay are gaining popularity over other assays due to their sensitivity for detecting cytogenetic and DNA damage and the short time needed to complete a study (Bogoni *et al.* 2014; Brenerman *et al.* 2014; Turan and Ergenler 2019; Turan *et al.* 2020a, b; Turan and Ergenler 2021a). The

formation of morphological nuclear abnormalities (NAs) was first described in fish erythrocytes by Carrasco *et al.* (1990). NAs, including blebbed nuclei, lobed nuclei and notched nuclei and binucleated cells, have been used by several investigators as possible indicators of genotoxicity (Ayllon *et al.* 2000; Da Silva Souza and Fontanetti 2006; Cavas and Ergene-Gözükara 2005, Cavas and Könen 2007; Turan and Ergenler 2021b, c). Comet testing is widely applied to study the genotoxic effects of contaminants on fish and is a reliable, sensitive, and quick technique used for the detection of DNA strand breakage and alkali-labile regions in cells of the organism (Martins and Costa 2017; Turan *et al.* 2020a).

Advances in technology and frequent use of pesticides have led to pollution of the environment, including aquatic ecosystems (Gibbons *et al.* 2015). Pesticides are known to be the biggest problem for economically and ecologically important non-target aquatic species, including fish living in water (Prusty *et al.* 2015, Rejczak and Tuzimski 2015). Monitoring for toxic effects and screening for different insecticides is vital and crucial for reducing adverse effects on non-target organisms and public health. Therefore, in this study it was aimed to determine genotoxic effect of thiamethoxamin a model fish species, *Cyprinus carpio*, using the micronucleus analysis and Comet assay.

Materials and Methods

Experimental Design

The experiment was carried out with 240 common carp (*C. carpio* L.) (with an average weight of 1.56±0.31 g) at the Aquaculture Research and Development Center, Faculty of Marine Sciences and Technology, Iskenderun Technical University, Turkey in November, 2021, in conjunction with the study presented by Turan and Ergenler (2022). The carp specimens were acclimated for 15 days in a well-aerated 30 L glass aquarium containing dechlorinated water, at room temperature (23±0,5°C) with a constant photoperiod (12:12 light/dark cycle). The specimens were fed with commercial carp feed of 3% of their body weight and feeding was stopped 24 h prior to exposure of the insecticide. After acclimation the specimens were randomly divided into four groups (control and experimental groups with 20 fish per group). Three different concentrations of thiamethoxamine (1.0,1.5 and 2.0 mg L^{-1}) were selected based on previously detected aquatic environmental concentrations, constituting an acute test for 10 days. Each treatment group consisted of triplicates of 60 fish. At the end of the experiment, fish were anaesthetized with 5 mg L^{-1} quinaldine sulphate (Sigma Chemical Company, Germany) (Yanar and Genç 2004). The specimens were manipulated only once they were unresponsive to physical stimuli (approximately 1-2 min), for the removal of tissue (gill and liver) for Comet assay and blood sampling for micronucleus assay.

Micronucleus (Mn) Assay
Blood sampling was performed via cardiac puncture using a heparinized syringe and whole blood was used for subsequent analysis. Blood samples were taken from 15 individuals and the micronucleus test was applied to the erythrocytes and the formation frequencies were calculated. Three blood smears from each individual were prepared immediately after sampling as described in Mitkovska *et al.* (2017). After the prepared preparations are dried in air, they are mixed in 95% ethanol for 20 minutes. has been fixed during. They are stained with 5% Giemsa solution for 20 minutes. Micronucleus evaluation was made by counting 1000 cells from each preparation. Morphological nucleus irregularities by peripheral smear Carrasco *et al.* (1990); They were evaluated under four main groups: notched nucleus, budded nucleus, lobed nucleus and binucleus.

Comet Assay
Comet assay was done according to cellular dissociation technique improved from Cavalcante *et al.* (2008). Firstly, gill and liver tissues of carps were homogenized and centrifuged at 3000 rpm at 4 °C for 5 min for the cell suspension, and then the cell pellet was retained. Singh *et al.* (1990) was followed for performing the single-cell gel electrophoresis. The slides were neutralized with ice-cold 0.4 M Tris buffer (pH 7.5), stained with 80 ml ethidium bromide (20 mg mL^{-1}). The slides were then examined at X400 magnification using a fluorescence microscope Image2M Zeiss). Images of 100 cells from each sample (gill and liver cell) were visually scored as proposed by Collins, 2004 by classifying the nucleoids. For comparison of the data from the comet assay, the damage percentage (%DF), the arbitrary unit values (AU), and genetic damage index (GDI) were calculated as defined by Collins (2004).

Statistical Analysis
Before statistical treatment, all data were tested for normality (Shapiro–Wilk test) and homogeneity (Levene analyze test). One-way ANOVA was performed in order to assess significant difference among treatment groups. Duncan test was used as *post hoc* multiple comparison tests. Differences were regarded as statistically significant at $p < 0.05$ (Norusis 1993).

Results

Means and standard deviations of micronuclei and means of different classes of nuclear abnormalities counted in *C. carpio* from control and three different concentrations of thiamethoxamine are given in Table 1.

In the erythrocytes of the carp, various nuclear abnormalities (micronucleus, binucleus, notched nucleus, lobbed nucleus and bud nucleus) were detected at treatment groups. As shown in Table 1, significant differences were observed ($p<0.001$) in the frequency of micronucleus and other nuclear irregularities

(kidney nucleus, binucleus, notched nucleus, lobed nucleus and budded nucleus) compared with the control group and thiamethoxamine treatment groups during 10 days (Table 1). As result of the study, it is determined that the highest micronucleus frequency and erythrocyte abnormalities was significantly observed in 2.0 mg L^{-1} group ($p<0.001$). Besides, it is observed that the other nuclear abnormalities (kidney nucleus, binucleus, notched nucleus, lobed nucleus and budded nucleus) in peripheral erythrocytes of carps at all treatment groups are significantly higher ($p<0.001$) compared to the control group (Table 1). As can be seen in our results, thiamethoxamine treatment significantly increased the frequencies of nuclear abnormalities ($p< 0.001$). Besides, no fish mortality was observed at thiamethoxamine treatment groups and the control during the experiment.

Table 1. Means (%) and standard deviations of micronuclei and means of different classes of erythrocyte abnormalities counted in *C. carpio* obtained from control and three different concentrations of thiamethoxamine

Group (mg L^{-1})	Micronucleus	Kidney	Binucleus	Notched	Lobed	Budded
Control	3.267 ±0.252[a]	5.167 ±0.153[a]	5.200 ±0.100[a]	7.933 ±0.666[a]	5.233 ±0.208[a]	4.167 ±0.153[a]
1.0	8.500 ±0.300[b]	8.900 ±0.173[b]	12.533 ±0.115[b]	12.333 ±0.152[b]	14.500 ±0.100[b]	20.866 ±0.152[b]
1.5	17.333 ±0.153[c]	12.033 ±0.208[c]	14.700 ±0.100[c]	14.767 ±0.208[c]	17.433 ±0.208 [c]	22.633 ± 0.404[c]
2.0	74.667 ±2.055[d]	60.667 ±3.682[d]	82.333 ±2.055[d]	94.000 ±2.944[d]	110.667 ±4.497[d]	194.667 ±0.943[d]
p	*	*	*	*	*	*

The data are shown as arithmetic mean ± standard deviation. Values with different superscripts in each column indicate significant differences. p indicates significance level between micronucleus frequencies and erythrocyte abnormalities in peripheral erythrocytes of carp obtained from control and three different concentrations of thiamethoxamine (*, $p<0.001$).

Means and standard deviations of the damage frequency (%), arbitrary units values (AU) and genetic damage index (%) in the gill and liver cells of *C. carpio* obtained from the control and three different concentrations of thiamethoxamine are summarized in Tables 2 and 3.

As shown in Tables 2 and 3, significant differences were observed ($p< 0.001$) in the damage frequency and other parameters (AU and GDI) compared with the control and thiamethoxamine treatment groups during the experiment. Thiamethoxamine treatment significantly increased the percentage of DNA damage in gill and liver cells of *C. carpio* ($p < 0.001$). Similarly, AU and GDI values are affected by thiamethoxamine treatment ($p<0.001$). As a result of the study, it was determined that the highest damage frequencies (%) as 74.000±1.732 and 68.000±1.732 were significantly observed in 2.0 mg L^{-1} group at gill and liver cells respectively ($p<0.001$) (Tables 2 and 3).

Table 2. Means and standard deviations of DNA damage in the gill cell of carp obtained from the control and three different concentrations of thiamethoxamine

Groups (mg L^{-1})	Damage Frequency (%)	Arbitrary Unit (AU)	Genetic Damage Index (%)
Control	25.667 ±2.494[a]	48.667 ±2.055[a]	0.487 ±0.021[a]
1.0	45.667 ±2.887[b]	114.667 ±7.024[b]	1.147 ±0.070[b]
1.5	62.000 ±2.000[c]	159.333 ±3.786[c]	1.593 ±0.038[c]
2.0	74.000 ±1.732[d]	210.667 ±2.309[d]	2.107 ±0.023[d]
p	*	*	*

The data are shown as arithmetic mean ± standard deviation. Values with different superscripts in each column indicate significant differences. Indicate significance level between DNA damage in gill tissues of carps obtained from control and three different concentrations of thiamethoxamine (*, $p<0.001$)

Table 3. Means and standard deviations of DNA damage in the liver cells of carp obtained from the control and three different concentrations of thiamethoxamine

Groups (mg L^{-1})	Damage Frequency (%)	Arbitrary Unit (AU)	Genetic Damage Index (%)
Control	21.667 ±1.247[a]	36.333 ±1.886[a]	0.363 ±0.019[a]
1.0	38.667 ±4.509[a]	73.333 ±6.658[b]	0.733 ±0.067[b]
1.5	58.000 ±0.000[b]	108.667 ±5.508[c]	1.087 ±0.055[c]
2.0	68.000 ±1.732[c]	184.667 ±7.371[d]	1.847 ±0.074[d]
p	*	*	*

The data are shown as arithmetic mean ± standard deviation. Values with different superscripts in each column indicate significant differences. Indicate significance level between DNA damage in gill tissues of carps obtained from control and three different concentrations of thiamethoxamine (*, $p<0.001$).

The lowest damage frequencies (%) were 25.667±2.494 and 21.667±1.247 obtained in the gill and liver cells of control group in this study, respectively. Besides, it is observed that other damage parameters (AU and GDI) in the gill and liver samples of 1.0 and 1.5 mg L^{-1} group were significantly higher ($p<0.001$) compared to the control group (Tables 2 and 3). The lowest AU and GDI were significantly obtained in control group in this research. As a result of the study Likewise, it is determined that the highest GDI as 2.107±0.023 % and 1.847±0.074% were significantly observed in 2.0 mg L^{-1} group at gill and liver cells respectively ($p<0.001$). In this study, the DNA damage increased due to the increase in the concentrations of thiamethoxamine.

Discussion

Synthetic organic insecticides, including neonicotinoids etc., are wildly used for agricultural or indoor purposes. Despite the low insecticide concentrations in the environment, insecticide users may be exposed to higher levels. Accessing the insecticide toxicity at higher concentrations could provide references for their potential effects, and draws attention to insecticides application (Liu *et al.* 2018). To date, relatively some assessments of the toxicity of thiamethoxam to aquatic species have been published in the peer-reviewed literature (Georgieva *et al.* 2014; Ugurlu *et al.* 2015; Cavallaro *et al.* 2017; Finnegan *et al*. 2017; Saraiva *et al.* 2017; Temiz *et al.* 2021). Here we compare the lower concentrations of thiamethoxam dataset (as described in the present study) with values reported in the literature to date, and find that there is general agreement in the observed responses. Our study shows that low concentratrions of thiamethoxam is also toxic to *C. carpio*, since chronic exposure to low concentrations caused significant DNA damage and nuclear abnormalities. Our results also showed that blood, gill and liver cells of *C. carpio* can respond differently to DNA damage, reinforcing the importance of using different tissues as complementary tools for detecting genotoxicity in fish. Fish respond to environmental toxic changes with adapting of their metabolite functions. They have been successfully used as a model to study the negative effects of various pesticides to the environment (Georgieva *et al.* 2014).

Synthetic organic insecticides, including neonicotinoids and so forth, are stunningly utilized for rural or indoor purposes. Regardless of the low bug spray fixations in the climate, insect spray clients might be presented to more elevated levels. Getting to the insect poison poisonousness at higher fixations could give references to their likely impacts, and causes to notice insect poisons application (Liu *et al.* 2018). Up to now, generally a few evaluations of the harmfulness of thiamethoxam to marine species have been made (Georgieva *et al.* 2014; Ugurlu *et al.* 2015; Cavallaro *et al.* 2017; Finnegan *et al.* 2017; Sanraiva *et al.* 2017; Temiz *et al.* 2021). Here we consider the lower centralizations of thiamethoxam dataset (as depicted in the current review) with values published to date and examine if there is general arrangement in the noticed reactions. Our review shows that low concentratrions of thiamethoxam is additionally harmful to *C. carpio*, since ongoing openness to low fixations caused altogether DNA harm and atomic anomalies. Our outcomes additionally showed that blood, gill and liver cells of *C. carpio* can react contrastingly to DNA harm, building up the significance of involving various tissues as integral instruments for identifying genotoxicity in fish. Fish react to natural poisonous changes by adjusting their metabolite capacities. They have been effectively utilized as a model to concentrate on the adverse consequences of different pesticides to the climate (Georgieva *et al.* 2014).

Temiz *et al.* (2021) announced that all focuses (50, 100, and, 150 mg L^{-1}) and periods (for 48 h and 15 days) of thiamethoxam expanded 8-OHdG levels in the liver and brain tissues of *Oreochromis niloticus.* As a similar result, thiamethoxam exposure was reported to produce DNA damage determined by Comet assay in zebrafish liver at concentrations of 0.30, 1.25, and 5.00 mgL^{-1} (Yan *et al.* 2016). In the exposure of neonicotinoid insecticide imidacloprid, 8-OHdG levels in the brain tissue of *Gobiocypris raru* were reported to increase with an effect of 2.0 mg L^{-1} (Tian *et al.* 2018). Topal *et al.* (2017) reported that rainbow trout exposed to neonicotinoid imidacloprid (5, 10, and 20 mg L^{-1}) for 21 days caused neurotoxic effects in the brain tissues. Research shows that the increase in 8-OHdG levels in tissues has been reported to be a response to oxidative stress.

On the other hand, Riaz *et al.* (2013) examined toxicity to larvae of the mosquito *Aedes aegypti* and reported a 24-h LC50 of 0.183 (95% CI: 0.162-0.205) mg thiamethoxam/L. Similarly, Stevens *et al*. (2005) determined a 24-h LC50 for *Chironomus tepperi* larvae of 0.121 (95% CI: 0.108-0.136) mg thiamethoxam/L. Finally, Van den Brink *et al.* (2016) assessed toxicity of thiamethoxam to *Cloeon dipterum* nymphs over 96 h and reported a 24-h EC50 (immobility) of 0.092 (95% CI: 0.085-0.099) mg/L.

Previous literature reviews of neonicotinoid toxicity data have identified imidacloprid as the most toxic neonicotinoid active ingredient, or as equally toxic as some other neonicotinoid compounds to aquatic invertebrates, followed by clothianidin and thiamethoxam (Morrissey *et al.* 2015; Cavallaro *et al.* 2017). Whiteside *et al.* (2008) conducted a rank-based risk assessment focusing on the adverse effects of agrochemicals on aquatic communities, including algae, invertebrates, fish, and other aquatic organisms. Of the 206 compounds evaluated, imidacloprid ranked the highest of the three neonicotinoids at number 51, followed by clothianidin at 143 and thiamethoxam at 190. A similar pattern surfaced in a review by Morrissey *et al.* (2015), with imidacloprid, clothianidin, and thiamethoxam displaying similar acute toxicity geometric means (LC50 data from 24-h to 96-h tests), but the lack of clothianidin and thiamethoxam toxicity data in the primary literature preclude accurate comparison across compounds (Cavallaro *et al.* 2017). At this point our results are in agreement with those reported genotoxic potential of commercial formulations of thiamethoxam.

Consequently, the current findings reveal that the thiamethoxam is a genotoxic insecticide inducing micronucleus frequency, erythrocyte abnormalities and DNA damage frequencies in *C. carpio*. Our findings also indicated the suitability of the fish micronucleus test and comet assay in assessment of aquatic genotoxicity of insecticides.

Acknowledgements

Thanks are due to the Scientific Research Projects Office, Iskenderun Technical University for financial support (2019 YP-01), the Scientific & Technological Research Council of Turkey (TUBITAK-2211/C National Ph.D. Scholarship Program for Priority Areas), and the Council of Higher Education for 100/2000 Ph.D. scholarship program for A. Ergenler.

Cyprinus carpio'da thiamethoxamın genotoksik etkisinin mikronükleus ve Comet testleriyle değerlendirilmesi

Öz

Pestisitler, zararlı canlıları öldüren; ayrıca zararlıları bulunduğu ortamdan uzaklaştıran, popülasyon yoğunluğunu azaltan ya da tamamen ortadan kaldıran doğal veya çeşitli kimyasal işlemlerle oluşan bileşikler olarak adlandırılmaktadır. Toksik etkilerin izlenmesi ve farklı insektisitlerin taranması, suda yaşayan organizmalar ve halk sağlığı üzerindeki olumsuz etkilerinin belirlenip azaltılması için çok önemlidir. Bu nedenle bu çalışmada, bir model organizma olan sazan balığı (*Cyprinus carpio*)'da thiamethoxamın genotoksik etkisinin mikronükleus ve Comet testleri ile belirlenmesi amaçlanmıştır. Ortalama ağırlıkları 1,56 ± 0,31 g olan sazanlar, daha önce tespit edilen sucul ortam konsantrasyonlarına dayalı olarak on gün boyunca kontrol grubu ile birlikte thiamethoxamın üç farklı konsantrasyonuna (1,0; 1,5 ve 2,0 mg L^{-1}) maruz bırakıldı. Çalışmanın sonunda Comet testi ile sazanın solungaç ve karaciğer hücrelerinde; DNA hasar yüzdesi (%), Arbitrary unit ve Genetik hasar indeksi (%); mikronükleus testi ile de sazanın eritrosit hücrelerinde mikronükleus frekansları ve eritrosit anormallikleri belirlenmiştir. Çalışma sonucunda en yüksek mikronükleus frekansı ve eritrosit anormalliklerinin 2.0 mg L^{-1} grubunda ($p<0,001$) görüldüğü ve yine aynı grupta en yüksek DNA hasar frekanslarının (%) 74,000±1.732 ve 68,000±1.732 olarak sırasıyla solungaç ve karaciğer hücrelerinde görüldüğü tespit edilmiştir ($p<0,001$). Çalışmada, thiamethoxamin maruziyetin ardından *C. carpio*'daki mikronükleus ve DNA zincir kırılmalarında önemli artışlar olduğu ve böylece bu pestisitin balıklar üzerindeki genotoksik etki gösterdiği sonucuna varılmıştır.

Anahtar kelimeler: DNA hasarı, Thiamethoxam, Micronükleus test, Comet assay, pestisit

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