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Studies on triazophos-induced oxidative stress and genotoxicity in freshwater fish *Cyprinus Carpio* following sublethal exposures

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ABSTRACT

Triazophos (TAP), an organophosphorus insecticide, is widely used in agricultural practice for controlling various insect pests. The present research work aimed to elucidate the impact of TAP on the antioxidant status and DNA content of the freshwater fish *Cyprinus carpio*. The fish were grouped into batches (n=6) and received a sublethal dose of 0.3mg/L for a duration of 1 (E1), 10 (E2), 20 (E3) and 30 days (E4). Another group, devoid of any toxicant, was maintained as the control (C). Changes in the enzymatic threshold of the selected antioxidants and malondialdehyde (MDA) levels suggested the conformation of oxidative stress in the livers of the freshwater fish *C. carpio* due to TAP exposure. Additionally, comet assays and micronucleus tests performed on the peripheral blood of the fish suggested increased damage in the form of the percentage of tail DNA formation and a high frequency of micronucleus as compared to the control. A positive correlation was seen between the decline in antioxidant activity, the elevation in MDA and the comet length and micronucleus frequency. The study thus highlights the impact of TAP on antioxidant levels in the livers and genotoxicity in the blood of the freshwater fish *C. carpio*. The findings of the study confirm that the antioxidant status, along with the comet assay and micronucleus tests could be used as tools in determining the potential genotoxicity due to the TAP impact. It is therefore suggested that extensive use of TAP should be avoided as it may contribute to the decline in the *C. carpio* population in its natural habitats.

Key words: comet assay; erythrocytes; DNA; micronucleus and Triazophos

Introduction

The steady increase in the use of pesticides has attracted special attention in environmental pollution research (SATHYAMOORTHI et al., 2019, KUMARESAN et al., 2019). Pesticide contamination of the aquatic ecosystem has been found to affect the health of fish, either directly by uptake from the water, or indirectly through their diet of vegetation, invertebrates or smaller fish (CORCORAN et al., 2009; DAVID and KARTHEEK, 2014). Since fish are part of the natural diet of both aquatic mammals and birds, as well as providing an increasingly important protein source for humans, their population and health is of major concern (KIME, 1995).

One of the important groups of pesticides are organophosphates (OP) which are known

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to control insect pests through various means (VIDHYASAGAR et al., 2004; DAVID and KARTHEEK, 2015a). Even though the OP's mechanism of action is to bind avidly to acetylcholinesterase molecules, resulting in the over-activation of cholinergic receptors at the neuromuscular junctions and in the autonomic and central nervous systems (PAUDYAL, 2008), their ability to induce antioxidant imbalance is of great importance as an important biomarker in the field of toxicology (CHIAPELLA et al., 2013; PEARSON and PATEL, 2016).

Oxygen as a vital element for survival, plays a supportive role in physiological stability (TAUFFENBERGER and MAGISTRETTI, 2021). However, its generation in the form of reactive oxygen species (ROS) due to biochemical events inside the body has been reported in many studies (SLANINOVA et al., 2009). OP's have been shown to cause overproduction of ROS which, if not adequately neutralized by antioxidant mechanisms, could result in oxidative stress and potential tissue damage (MANSOUR and MOSSA, 2009). The major antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD), are directly involved in the neutralization of ROS (DAVID and KARTHEEK, 2016). With pesticide exposure being linked to antioxidant imbalance, resulting in the generation of ROS, genotoxicity is viewed as a potential resulting process, and has also been reported by several authors (HUTTER et al., 2020; LUSHCHAK et al., 2018).These endogenously synthesized antioxidants act as free radical scavengers, thereby preventing and repairing the damage caused by ROS, thus enhancing the immune defence and lowering the risk of genotoxocity and other degenerative diseases (VALKO et al., 2006; CHATTERJEE et al., 2007). The toxicological mechanism of OP's have been identified as similar in higher animals and fish (DAVID and KARTHEEK, 2015b). Investigations with respect to these events are extremely necessary, as OP's have been found to induce changes in antioxidant status, resulting in oxidative stress and further leading to the possibilities of genotoxicity.

Triazophos (O,O-diethyl-O-(1-phenyl-1H-1,2,4-triazol-3-yl) phosphorothioate (TAP), is an efficient broad-spectrum OP widely used in Asian agriculture for its properties as an insecticide, nematicide and acaricide, to protect various crops such as cotton, rice, wheat, tea, fruits, oil seeds and vegetables (BHANDARI et al. 2019; HONG et al. 2019; KUMARI and JOHN, 2019). However, the widespread application of TAP has presented a risk to human health and to the environment (YANG et al., 2019). As TAP is an OP insecticide, its potential to pose a serious threat to the aquatic fauna has to be investigated. The selected model of fish *Cyprinus carpio* is an important bridge in the food chain and constitutes nearly 10 % of annual freshwater aquaculture production globally (XU et al. 2014). Additionally, *C. carpio* is found to dominate freshwater aquaculture practice in terms of production quantity and demand (FAO 2006; BOSTOCK et al. 2010), making it a potential vector for OP poisoning in humans. Thus the present work aimed to elucidate the toxic effects of TAP on the freshwater fish *C. carpio* following sublethal exposure.

Materials and methods

Toxicant selected and test solutions. Commercial grade Triazophos (TAP) of 40% EC was selected as the toxicant for the present study, and was procured from the local market under the trade name Trizocel. A stock solution at a concentration of 1 g/L was prepared by dissolving 1.0 g of TAP (40% EC) in 1000 ml of double distilled water. The required test concentrations were freshly prepared by diluting the stock solution just before the initiation of the acute toxicity study.

Procurement and maintenance of experimental fish. Healthy *C. carpio* of both sexes, weighing 7.0 ± 2.0 g with a length of 8.0 ± 1.0 cm, were procured from State Fisheries Department, Neersagar, Dharwad (Karnataka, India). The fish were allowed to acclimate in cement tanks of 250 litres under laboratory conditions for 10 days. During acclimation, the water was aerated using a static system, and the photoperiod of 12 h light and 12 h dark was maintained. The water was renewed daily and its physico-chemical characteristics were analysed following standard methods, as mentioned in APHA (2005).

Grouping of the experimental fish. For experimentation, the fish were divided into four groups, namely: Control (C), 1 day exposure (E1), 10 days' exposure (E2), 20 days' exposure (E3) and 30 days' exposure (E4). Each group was maintained in triplicate and consisted of 10 individual fish each, irrespective of their gender.

Exposure to sublethal concentrations and collection of tissue. A single sublethal concentration of 0.3mg/l was selected. Ten healthy *C. carpio* were randomly selected and transferred to each glass aquaria containing 1000 ml water with its respective concentration of TAP solution. The fish remained there for 96 h. However, before the fish were transferred, it ensured that the aquaria were clean enough and free from any kind of toxicant. For each concentration, including the control, triplicates were maintained and their mean values were taken into account for the present study. The blood was collected by puncturing the caudal vein of fish from the control and exposed groups. The blood obtained was transferred into EDTA coated tubes for genotoxicity assay. It was further processed in chilled phosphate buffer, and centrifugation was carried out at 5000 rpm for 10 minutes. Only the pellet was stored at −20 °C in a refrigerator until further analysis. For assessment of the antioxidant status, livers were collected by sacrificing the experimental fish. The tissue was washed in buffered saline, and maintained by freezing at -80 °C until further use.

Antioxidant assay and Lipid peroxidation. Catalase (EC 1.11.1.6) activity was determined by measuring the decrease in hydrogen peroxide concentration at 240 nm, according to Luck (1974). Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by the methodology described by KAKKAR et al., (1984). Lipid peroxidation (LPO) levels were found according to the method of BUEGE and AUST (1978), and estimated by thiobarbituric acid reactive substance (TBARS) assay, performed by an optically measured malondialdehyde (MDA) reaction with TBA.

Comet assay. Comet assay was performed on blood cells, according to the protocol of DEVAUX et al. (1997).

Micronucleus test. The micronucleus test was performed on pheripheral blood according to HAYASHI et al., (1990).

Statistical analysis. The antioxidant activities are reported as the mean ± standard error of the mean (SEM) obtained from the triplicates. The data were subjected to one-way analysis of variance and further subjected to Tukey's test for post hoc analysis by defining the significance level at $p<0.05$.

Ethics statement. The present study was carried out at the Department of PG Studies and Research in Zoology, Karnatak University, Dharwad (Karnataka, India), in accordance with the ethics committee regulations. The test animals used were maintained and subjected to the experimental process and disposed of upon completion of the experiment according to the guidelines issued by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Results

The antioxidant enzyme status in the fish exposed to 0.3 mg/L of TAP for 1 (E1), 10 (E2), 20 (E3) and 30 days (E4) was found to significantly vary ($P<0.05$), unlike control (C). In the experimental groups, the activity of the investigated antioxidant enzymes, CAT and SOD, was found to decline significantly ($P<0.05$) in a constant pattern following exposure to TAP. Changes in CAT activity under TAP stress varied significantly ($P<0.05$) with percentage decreases of −2.97, −32.06, -49.75 and −51.23 % for E1, E2, E3 and E4 respectively (Fig. 1). Changes in the activity of SOD were noted in relation to exposure and recovery durations. The changes in SOD activity noticed were in the order of −12.50, −49.41 −54.82 and -61.94 % for E1, E2, E3 and E4 respectively (Fig. 2). Both the antioxidants showed the highest decline in enzymatic threshold at E4. The oxidative damage determined through MDA levels indicated significant ($P<0.05$) elevation in its levels as compared with group C. A percentage increase of 27.36, 100.49, 134.82 and 272.13 % for E1, E2, E3 and E4 respectively (Fig. 3) was noticed for MDA values, which indicates LPO activity, signifying the membrane damage potential of TAP.

A significant increase in the frequency of micronuclei (MN) was observed in the erythrocytes for the E2, E3 and E4 groups (Table 1; Fig. 4) unlike E1 as compared to the control. On other hand, DNA damage, as indicated by the percentage tail formation of DNA determined by comet assay in the blood cells of *C. carpio,* was also observed at different durations after exposure to TAP. They are shown in Fig. 5. In all the durations of TAP exposure, the fish showed a significant increase in % tail DNA and MN percentage, except , E1, as compared to the control (Fig. 4 and Fig. 5). A significant increase in duration-dependent damage was observed in the form of % tail DNA in the erythrocytes of *C. carpio* compared to the control.

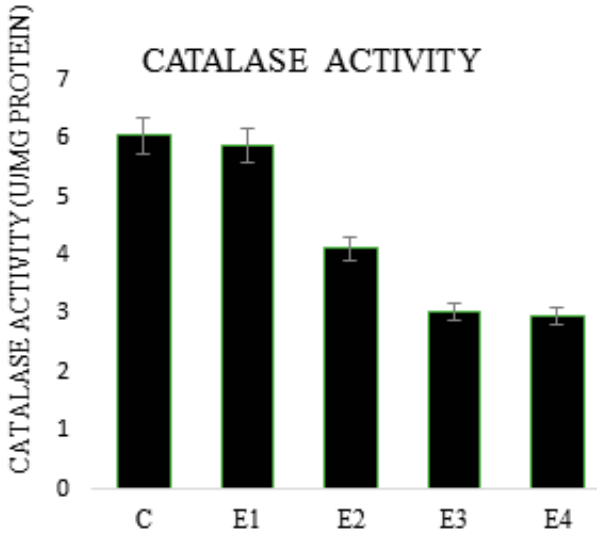

Fig.1. Changes in Catalase activity in the livers of *Cyprinus carpio* fish exposed to a sublethal concentration of Triazophos

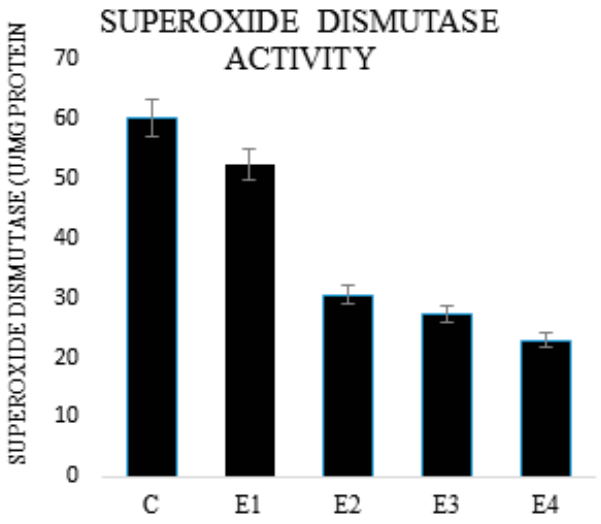

Fig. 2. Changes in Superoxide dismutase activity in the livers of *Cyprinus carpio* fish exposed to a sublethal concentration of Triazophos

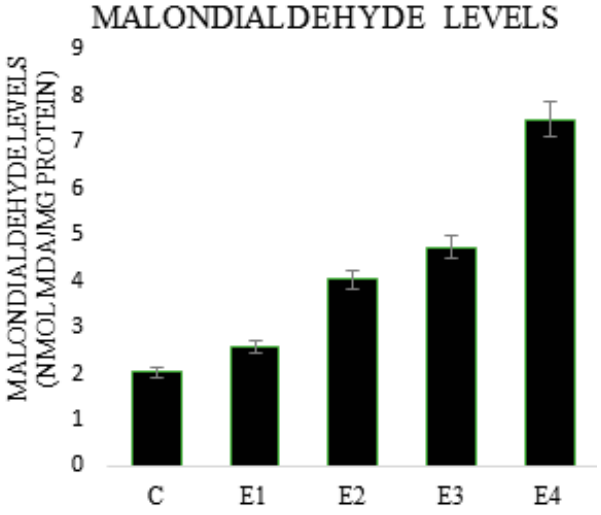

Fig. 3. Changes in Malondialdehyde levels in the livers of *Cyprinus carpio* fish exposed to a sublethal concentration of Triazophos

Table 1: Changes in micronucleus frequency in the erythrocytes of *Cyprinus carpio* exposed to a sublethal concentration of Triazophos

Calculation of MN	Groups				
	Control	Exposure			
		E1	E2	E3	E4
Number of MN Observed	2.2±0.37	2.9±0.44	11.6±0.67*	11.9±0.58*	18.1±0.5*
% Frequency of MN	0.11	0.145	0.58	0.59	0.90

Values expressed as mean ± SEM for micronucleus are significantly different at * $P<0.05$ from the control and their respective frequency is expressed in terms of percentage per 2000 cells counted.

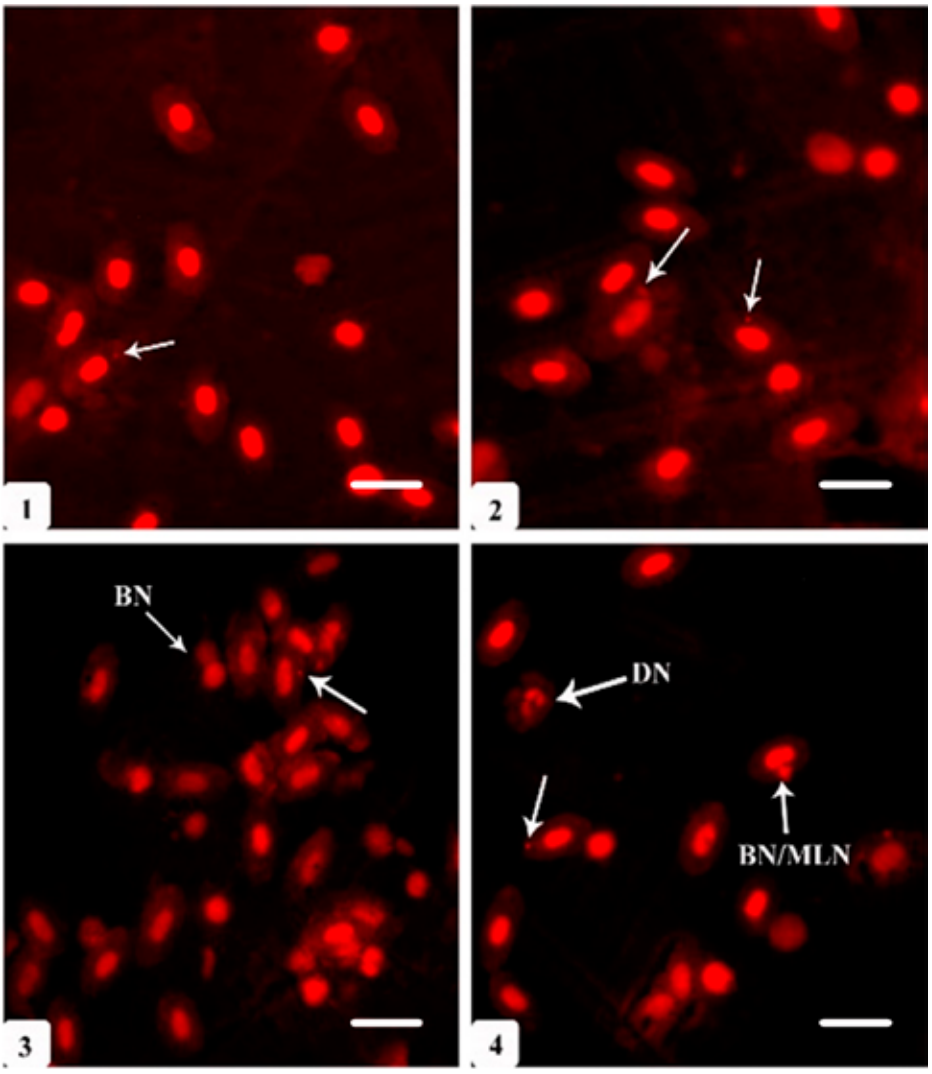

Fig. 4. Showing the occurrence of micronucleus in the erythrocytes of freshwater *C. carpio* fish exposed to triazophos. Notice the findings of micronucleus (arrow), binucleation (BN), multi-lobed nucleus (MLN) and degenerated nucleus (DN) in the different groups.

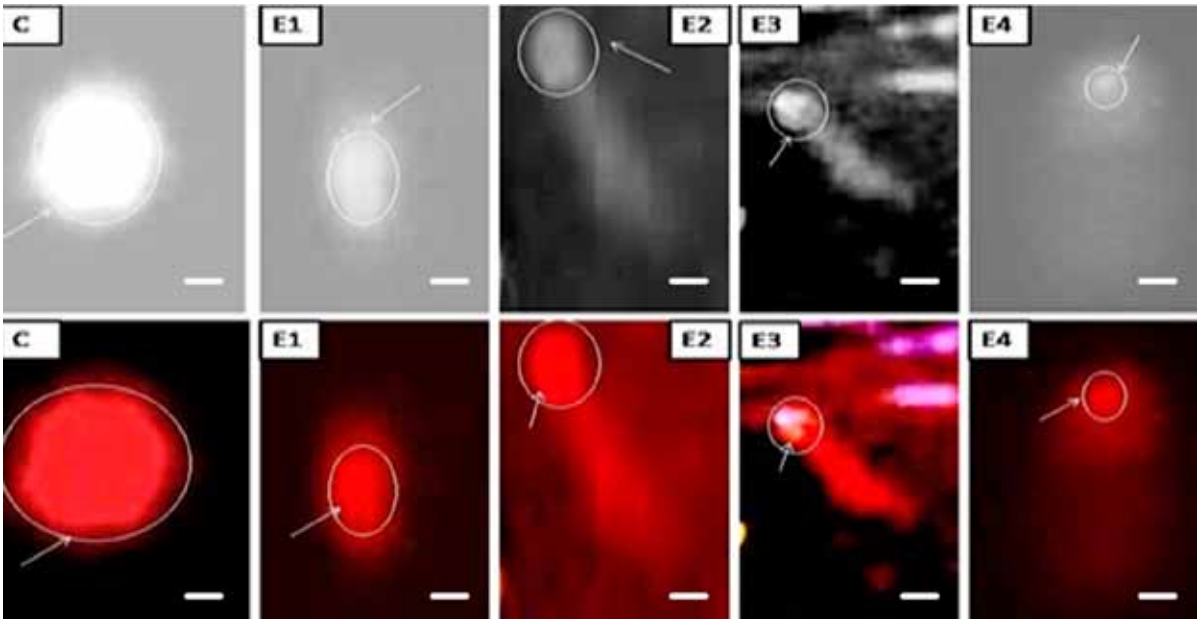

Fig. 5. Showing non-fragmented Control which appears intact with a nuclear boundary; minimum fragmentation at E1, scattered nuclear content and fragmented DNA content in E2, E3 and E4 with a duration-dependent increase in the tail length of comets.

Discussion

Fish are considered to be the sentinels of toxicological studies (TISLER and ZAGORC-KONCAN, 2002). These organisms have an important commercial importance due to their edible nature, and also play a vital role in the food web (DAVID and KARTHEEK, 2016). The receptiveness of fish to the presence of different toxicants and their ultimate response in terms of changes in physiological parameters has led to an understanding of the toxic effect of pesticides (DE ANDRADE et al., 2004). According to HARSHBARGER and CLARK (1990), fish species may be used to estimate the possible effects of toxicants to produce carcinogenic and teratogenic effects in human.

A number of toxicants have been reported to evoke a series of molecular events involved in oxidative stress, leading to lipid metabolism disorders which in turn may be manifest in tissue and DNA damage (SHADNIA et al., 2005; XU et al. 2013; DOBRAKOWSKI et al., 2017; KARTHEEK and DAVID, 2016; KARTHEEK and DAVID, 2018). Various antioxidants are known to attenuate the ROS within the cell, thus avoiding DNA damage as well. Since superoxide is the primary ROS produced from a variety of sources, its dismutation by SOD is of primary importance for each cell. Additionally, H_2O_2 which is synthesized as a result of the action of oxidases, is reduced to water by yet another crucial antioxidant catalase. The resulting falls in SOD and CAT could be due to the fish's loss of the ability to synthesize their increased levels as compared to ROS generated upon TAP toxicity. Similar reports have been published by DAVID and KARTHEEK, (2016) in relation to sodium cyanide toxicity.

An abundance of ROS within a tissue can lead to alterations in the structural configuration of DNA in different aspects that mainly involve the degradation of nitrogenous bases, single- or double-stranded DNA breaks, sugar-bound modifications, mutations, deletions or translocations, and cross-linking with proteins (BIRBEN et al., 2012). Examination of various bodily tissues of fish, which mainly include peripheral blood has been found helpful in evaluating the genotoxic

potential of different chemicals (ZHOU et al., 2006). Among the genotoxicity studies, the MN test remains one of the preferred methods used for determining chromosome breakage (FENECH, 2000). An essential feature of a toxicant that induces genotoxicity is the fact that it should be an alkylating agent that produces gene mutations, micronuclei (MN) and sister chromatid exchanges (ANDERSON et al., 1995). Different pesticides have been reported to induce similar changes causing adverse effects, especially in the hematopoietic system that can produce secondary tumors due to its genotoxicity (SCHUURMAN et al., 2005).

The current investigation suggested an elevation in the formation of micronucleus in fish erythrocytes upon exposure to TAP concentrations at different durations. A significant increase in the frequency of micronuclei (MN) was observed in the erythrocytes for the E2, E3 and E4 groups (Table 1; Fig. 2), unlike E1, as compared to control. In a study reported by PIETRIPIANA et al., (2002) it was shown that that heavy metal pollution could contribute to the formation of a micronucleus within the erythrocytes of fish, resulting in a higher rate of their appearance. The current outcome is in line with these reports as an increased frequency of the presence of a micronucleus was found in fish exposed to the different varieties and concentrations of toxicants. In their study, BUCKER and DA CONCEICAO (2012) exposed *Eingenmannia virescensto* to concentrations of benzene (50 ppm) for different periods. Although no significant results were found relating to the micronucleus, another parameter where there was fragmentation of DNA, as witnessed by the appearance of comets, suggested the genotoxicity of benzene. This was further confirmed by the gradual increase in the number of damaged nucleoids, as evidenced by a dose-dependent response. These results suggest that the comet assay was more sensitive than the micronucleus assay. Thus the current investigation also included comet assay and it also showed the clear impact of TAP on fish erythrocytes.

Genome stability is an extremely vital factor for an organism to have a functional biological organization (MARTINS and COSTA, 2017). Following the disturbance of this stability due to the intervention of any given chemical agent, the genomic organization begins to dysfunction with the further possibility of cell death. The current investigation demonstrated increased DNA damage, as evidenced by the ratio of head and tail formation in the erythrocytes of the fish exposed to TAP. The DNA damage due to strand breaks was measured by the comet assay, which is an important biomarker of genotoxicity in fish (MITCHELMORE and CHIPMAN, 1998). Previous studies have indicated the genotoxicity potential wherein DNA damage was reported in different tissues of fish that were exposed to different kinds of pollutants in the aquatic environment (BAJPAYEE and PARMAR, 2009). In the present investigation, a marked increase in duration-dependent damage was observed in the form of % tail DNA in the erythrocytes of *C. carpio* compared to the control (Fig. 3). The current study showed a time-dependent increase in DNA damage, which is in agreement with the reports by CAVAS and KONEN, (2007) who suggested the increased tail length of the comet obtained from the blood cells of *Carassius auratus* exposed to glyphosate. Similar results were also reported by CAVALCANTE et al., (2008), who demonstrated genotoxicty in the blood and gill cells of *Prochilodus lineatus* following exposure to glyphosate. On other hand, some heavy metals are found to impact the genomic organization of fish in similar pattern, for instance, DNA damage in the blood cells of *Oreochromis mossambicus* increased as the heavy metal concentration increased, as reported by AHMED et al., (2011). In yet another investigation, following exposure to industrial effluents, erythrocytes of *Cobitis elongate* showed large DNA comet tails and this was also supported by the report by KOPJAR et al., (2009). Similar reports have been published for various non-target species, including birds (BAOS et al., 2006), mammals (PARK and CHOI, 2009; GARAJ-VRHOVAC et al., 2009), amphibians (COTELLE and FERARD, 1999; YIN et al., 2009), reptiles (BRONIKOWSKI, 2008) and mollusks (COTELLE and FERARD, 1999; CANTY et al., 2009).

Conclusion

On the basis of the outcome of the present investigation, it is suggested that the insecticide TAP has the potential to induce oxidative stress and genotoxicity in the liver and erythrocytes of the freshwater fish *C. carpio* at the selected concentration and durations. Thus, it is highly recommended that the selected pesticide be used under extreme caution and care. The outcome also suggests that the selected parameters could be useful biomarkers of aquatic toxicity. The current work, however, has limitations and thus further investigations using different molecular levels are necessary to ascertain the mechanism of the genotoxicity of TAP.

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PATTAR, S. RM., M. DAVID: Istraživanje oksidativnog stresa i genotoksičnosti slatkovodnih riba *Cyprinus Carpio* nakon izloženosti subletalnoj dozi triazofosa Vet. arhiv 93, 85-96, 2023.

SAŽETAK

Triazofos (TAP) je organofosforni insekticid koji se široko koristi u poljoprivrednoj praksi za suzbijanje raznih štetnih insekata. Cilj ovog istraživanja bio je procjeniti utjecaj TAP-a na antioksidativni status i DNK slatkovodne ribe *Cyprinus carpio*. Ribe su razvrstane u skupine (n = 6) u kojima su primale subletalnu dozu TAP-a 0.3mg/L u trajanju od 1 (E1), 10 (E2), 20 (E3) i 30 dana (E4). Druga skupina, koja nije izložena TAP-u, smatrana je kontrolnom (C). Promjene enzimskog praga odabranih antioksidansa i razina malondialdehida (MDA) potvrdile su da izloženost TAP-u izaziva oksidativni stres u jetrama slatkovodne ribe *C. carpio*. Osim toga, komet analize i mikronukleusni testovi provedeni na perifernoj krvi riba ukazali su da u eksperimentalnih skupina u odnosu na kontrolu postoji povećani postotak oštećenja u obliku DNK repa i povećane učestalosti mikronukleusa. Uočena je pozitivna korelacija između pada antioksidativne aktivnosti, porasta MDA, duljine kometa i učestalosti mikronukleusa. Uzevši u obzir navedeno, istraživanje naglašava utjecaj TAP-a na razine antioksidansa u jetrama i genotoksičnost u krvi slatkovodne ribe *C. carpio*. Rezultati potvrđuju da se antioksidativni status, zajedno s kometnim testom i mikronukleusnim testovima, može koristiti kao alat za određivanje potencijalne genotoksičnosti TAP-a. Stoga se predlaže izbjegavanje široke uporaba TAP-a koja bi mogla doprinijeti smanjenju populacije *C. carpio* u njezinim prirodnim staništima.

Ključne riječi: kometni test; eritrociti; DNA; mikronukleus i triazofos