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ORIGINAL ARTICLE

TRICLOSAN INDUCED GENOTOXIC EFFECT ON ZEBRA FISH, *Brachydanio rerio* BY USING ALKALINE COMET ASSAY.

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ABSTRACT

Triclosan (TCS) is commonly used as an antibacterial and antifungal agent in household cleaning products. TCS can be bio-accumulated and create endocrine-disrupting and damaging the DNA in some exposed fish. The present work has been proposed to study the effects of sub-lethal concentrations of triclosan on the blood of DNA damage by using comet assay on blood erythrocyte of zebra fish, *Brachydanio rerio* after 7 and 28 days exposure. The present study results shows, the highest erythrocyte tail DNA damage was observed in 28 days of exposure and following low level of tail DNA observed at 7 days of exposure. These genotoxic acessment along with the oxidative stress could be effectively used as potential non-specific biomarkers of peticides toxicity to the freshwater fish in the field of environmental biomonitoring.

Keywords: Triclosan, comet assay, *Brachydanio rerio*

1.INTRODUCTION

Pollution is an unfavorable alteration of our surroundings, largely as a result of our own actions, through direct or indirect effects of changes in every pattern, radiation levels, chemical and physical constitution and abundance of organisms. There exists a number of different pathways for anthropogenic chemicals to enter the aquatic environment. The primary source(s) of water-borne toxicants include municipal and industrial wastewater, non-point source runoff and atmospheric deposition (Campbell *et al*., 2006). Once in the aquatic environment, chemicals may undergo a number of different degradation processes to produce by-products (Lin *et al*., 2006). Water-borne chemicals are widely distributed in surface water around the world and are typically detected in the ng l^{-1} to µg l^{-1} range. Many classes of chemicals, such as pharmaceuticals, pesticides, industrial chemicals and phytochemicals have a ubiquitous presence in most rivers and streams (Kolpin *et al*., 2002).

Triclosan is a commonly used antimicrobial that is incorporated into dish soap, detergent, toothpaste, mouthwash, hand soap, fabric, deodorant and shampoo, in addition to innumerable other personal care and household products (Dann and Hontela 2011). Triclosan (TCS) is a polychloro phenoxy phenol commonly used as an antibacterial and antifungal agent in household cleaning products. The usage amount of TCS was more than 3 million tons per year and it was more than 3.5 million tons per year in Europe (Halden and Paull 2005). TCS is used extensively from more than 30 years. It also used not only for human and veterinary medication, but also for the promotion of growth in livestock and aquaculture species (Daughton and Ternes 1999). TCS can be bio-accumulated and create endocrine-disrupting and damaging the DNA in some exposed fish and mammals (Crofton *et al*., 2007)

The genotoxic chemical becomes more hazardous when it possesses bio-accumulative properties and enters in the food chain of the ecosystem. Fishes as bioindicators of pollutant effects are very sensitive to changes in their environment and play major roles in assessing potential risk associated with contaminations of new chemicals in aquatic environment (Lakra and Nagpure 2009). The effects of genotoxicity are reported to be several folds on fitness traits, genetic patterns and subsequent population dynamics in fish have been highlighted during genotoxicity assessment experiments (Porto *et al*., 2005). The Single Cell Gel Electrophoresis (SCGE) or Comet assay, detects DNA strand breaks and alkali labile sites by measuring the migration of DNA from immobilized nuclear DNA and useful technique for environmental contamination bio-monitoring (Tice *et al*., 1991). This is a rapid and sensitive procedure to measure DNA lesions in any organ regardless of its mitotic activity.

The comet assay is a sensitive, fast and economic test, besides requiring just few cells for its execution (Mitchelmore and Chipman 1998; Tice *et al*., 2000), and it has been indicated as a detection method of small changes in the DNA structure, such as repair activities, its packing mode and its entirety (Koppen *et al*., 1999and Lemos *et al*.,

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2005). According to several authors (Matsumoto et al., 2006; Nacci *et al*., 1996; Belpaeme *et al*., 1996). The comet assay has been successfully applied in erythrocytes of many fish species exposed to the different genotoxic agents, because that assay allows to evaluate the potentiality of DNA strand breaks of such organisms, due to the action of different xenobionts. In this paper, we evaluated mutagenic and genotoxic effects of triclosan pesticides, using zebra fish, *Brachydanio rerio* as test system, by using comet assay.

2.MATERIALS AND METHODS

The fish, Zebra fish, *Brachydanio rerio* having mean weight 3 to 5 g and length 4 to 6 cm were collected from your friend's aquarium, Kolathur, Chennai at acclimatized to laboratory conditions. They were given the treatment of 0.1%KMNO4 solution and then kept in plastic pools for acclimatization for a period of nine days. They were fed on rice bran and oil cake daily. The triclosan was used in this study and stock solutions were prepared. Triclosan LC_{50} values were 32 mg/L taken as sub-lethal concentrations for this study. Thirty fish were selected and divided into 3 groups of 10 each. The first group was maintained in free from triclosan and served as the control. The second and third group was exposed to sub-lethal concentration of triclosan for 7 and 28 days exposure periods. At the end of each exposure period, the fish were sacrificed and the required blood samples were collected for comet assay estimation. The DNA damage was estimated by alkaline single cell gel electrophoresis (Comet assay) according to the method of (Singh *et al*.,1988).

Comet assay technique
Procedure

Prefrosted slides were prepared by pouring 3.0-5.0 ml of 1% normal agarose over clean glass slides. It was allowed to dry at room temperature and placed in hot-air oven at 70-l80 °C for 30 min. A freshly prepared 10 µl of whole blood in 1% low-melting point agarose (LMPA) (1:3 ratio) was cast on to prefrosted microscopic slides, immediately covered with cover slip and kept for 10 min in a refrigerator to solidify. Then the cover slip was removed and a top layer of 100 µL of LMPA was added and the slides were again cooled for 10 min. The cells were then lysed by immersing the slides in the lysis solution for 1 h at 4 °C. After lysis, slides were placed in a horizontal electrophoresis tank. The unit was filled with electrophoresis buffer to a level of 0.25 cm above the slides. The cells were exposed to the alkalilne electrophoresis solution for 20 min to allow DNA unwinding. Electrophoresis was conducted in a cold condition for 20 min at 25 V and 300 mA. After electrophoresis, the slides were placed horizontally and neutralised with tris-HCl buffer. Finally, 50 µl of ethidium bromide was added to each slide and analysed (CASP software) using a fluorescence microscope. To prevent additional DNA damage, all steps were conducted under dimmed light or in the dark. Twenty five images were randomly selected from each sample and were examined at 200 magnification in a fluorescence microscope connected to a personal computer-based image analysis system, CASP. The relative amount of DNA appearing in the tail of the comet (percent tail DNA), tail length and tail moment (% tail DNA × tail length) were linearly related to DNA break frequency.

3.RESULTS

The DNA damage was measured as % tail DNA in the erythrocytes in the control as well as exposed groups as shown in Fig. 1. The percentage of tail DNA was significantly ($p<0.01$) increased with the days of exposure increasing the concentration of triclosan. However, inducting DNA damage was specific and showed significantly ($p<0.01$) higher DNA damage. The highest tail DNA damage was observed in 28 days of exposure (Fig. 1 C) and following low level of tail DNA observed at 7 days of exposure (Fig. 1 B). There was a general increase in the I time.

Table.1. Changes of DNA damage in triclosan exposed in Zebra fish, *Brachydanio rerio* **at different interval in days**

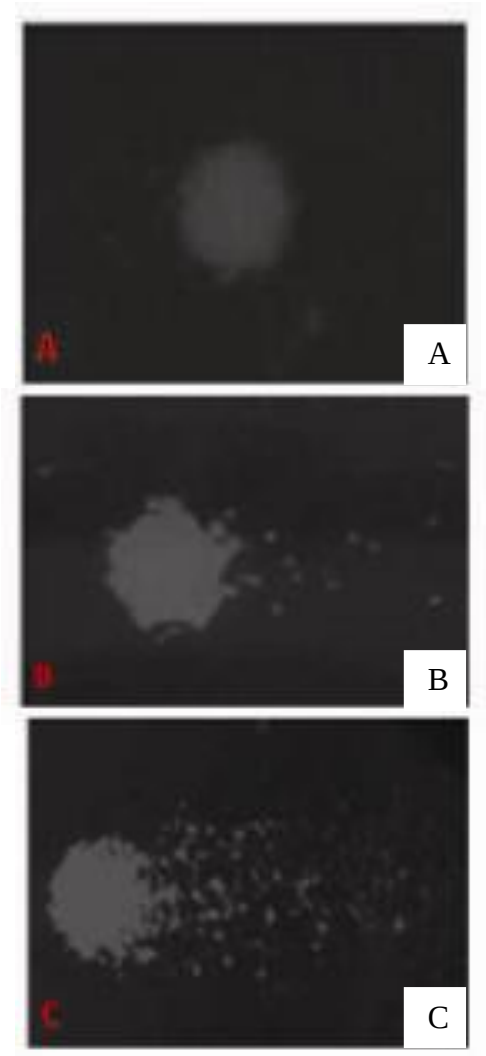

Table.1. Changes of DNA damage in triclosan exposed in Zebra fish, *Brachydanio rerio* **at different interval in days**

Group	Control	7 days exposed	28 days exposed
Head	86.41±0.22	79.06±0.58	67.94±0.46
Tail	0.257±0.05	22.19±0.06	31.07±0.04
Tail movement	0.218±0.07	26.11±0.14	53.16±0.09

4.DISCUSSION

The effects of genotoxicity are reported to be several-folds on fitness traits like reproductive success; genetic

patterns and following population dynamics in fish have been highlighted during genotoxicity assessment experiments (Belfiore and Anderson 2001; Bony *et al*., 2008). The present study brings together information based on *in vivo* systems to evaluate triclosan induced genotoxicity in freshwater Zebra fish, *Brachydanio rerio*. The comet assay performed with blood erythrocytes cells, was shown to be a complementary tool for detecting DNA damage. In addition, data indicate that, the mechanism of action to explain the genotoxicity of triclosan exposed to the test Zebra fish, *Brachydanio rerio*. The might be related to the generation of ROS. The use of *in vivo* approaches was demonstrated as a valuable tool for understanding the effects of triclosan on DNA molecules. Similar results were obtained with arsenic on zebra fish, *Brachydanio rerio* (Oliveria and Francisco, 2005).

Our results corroborate the researchers developed by some authors (Mitchelmore *et al*., 1998; Ateeq *et al*.,2005), which showed that the comet assay with erythrocytes of fishes seems to be efficient to detect the genotoxicity of chemicals. The applicability of comet assay as a sensitive tool for the detection of chemicals genotoxicity also was verified in other research (Nanthawan *et al*., 2002). Our date are still concordant with the ones related by several authors (Lin *et al*., 2006; Matsumoto *et al*., 2005), who affirm that the comet assay is an efficient method for the evaluation of DNA damages induced by chemicals.

The DNA fragmentation or DNA strand breaks are considered a kind of lesion potentially pre-mutagenic (Kammann et al., 2001), the production of breaks in the DNA strands being related to mutagenic and carcinogenic properties of chemicals (Frenzilli et al., 2000). The genotoxicity of the triclosan which was evaluated by the evidence of fragmentation of genetic material of different organisms submitted to that herbicide (Ribas *et al*., 1995; Garaj-Vrhovac *et al*., 2005).

The genotoxicity study of the herbicide glyphosate was published recently (Kier and Kirkland 2013). Although discordant results have been reported, they demonstrate the ability of the herbicide glyphosate and several glyphosate based products to induce DNA single-strand breaks evaluated by the SCGE bioassay in several fish. Positive results have been reported in circulating erythrocytes after laboratory exposure of *Carassius auratus* when not only the MN but also the comet assay was employed as an end point (Cavaş and Könen 2007). Furthermore, it has been reported a high rate of DNA damage revealed by the comet assay in blood and hepatic cells of *Corydoras paleatus* (de Castilhos Ghisi and Cestari 2013), and in erythrocytes and gill cells of *Prochilodus lineatus* (Cavalcante *et al*., 2008). It has been also demonstrated that not only Roundup™, but also its surfactant POEA (polyethoxylated tallow amine) as well as its active ingredient are able introduce DNA primary lesions in erythrocytes (Guilherme *et al*., 2012b), and in addition to gill and liver cells of *Anguilla anguilla* (Guilherme *et al*., 2012a).

Heavy metals can bind to phosphates and a wide variety of organic molecules, including DNA base residues, and can lead to mutations by altering primary and secondary structures of the DNA (Wong, 1988). Genotoxic properties of Cu, Cd and Hg, have also been demonstrated on *X. laevis* and *P. waltl* (Mouchet 2002). The *in vivo* and *in vitro* to evaluate Hg- and H_2O_2-induced genotoxicity in a marine fish species. The comet assay performed with gill kidney and blood cells, besides erythrocytes, was shown to be a complementary tool for detecting DNA damage (Lakra and Nagpure 2009). The use of *in vivo* and *in vitro* approaches was demonstrated as a valuable tool for understanding the effects of Hg on DNA molecules. The antioxidant potential of bioactive molecules of algae in reducing clastogenicity may also be due to the induction phase II enzymes such as SOD, CAT, and interaction of bio-molecules with DNA and mutagenic agents, which need to be confirmed by additional *in vivo* and *in vitro* studies in higher organisms.

An enhancement of DNA damage was observed in erythrocytes cells of *Channa punctatus* after *in vivo* exposure of chlorpyrifos (Ali *et al*., 2002b). High rate of DNA damage revealed by the comet assay in blood and hepatic cells of *Corydoras paleatus* (de Castilhos Ghisi and Cestari 2013), and in erythrocytes and gill cells of *Prochilodus lineatus* (de Castilhos Ghisi and Cestari 2013). It has been also demonstrated that not only Roundup™, but also its surfactant POEA (polyethoxy-lated tall owamine) as well as its active ingredient are able introduce DNA primary lesions in erythrocytes and in addition to gill and liver cells of *Anguilla Anguilla* (Ali *et al*., 2002b). Accordingly, our current results represent the evidence that, the triclosan is able to exert genotoxic damage through inflicting primary DNA-strand breaks evaluated by the SCGE assay.

5.CONCLUSION

The comet assay is sensitive tools for the effective evaluation of genotoxicity biomarkers. The results found in comet assay method are in agreement with each other when compared at 7 and 28 days of exposure**.** At 28 days of triclosan exposure in comet assay test revealed an increase in % tail DNA . The results of this study showed the importance of fish blood as potential biomarker of triclosan toxicity for comet assay. Our experimental data point out that zebra fish, *Brachydanio rerio* could be a suitable monitoring to study the bioavailability of water bound pesticides in freshwater habitats. It is also envisaged that features of oxidative stress could be used in aquatic pollution biomonitoring with varying degrees of specificity. Further studies are in progress to understand the underlying mechanisms involved in long-term toxicity profile of tricloan in freshwater fish, *Brachydanio rerio*. These genotoxic acessment along with the oxidative stress could be effectively used as potential non-specific biomarkers of peticides toxicity to the freshwater fish in the field of environmental biomonitoring.

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