Int. J.Mar.Sci.Eng., 2(4), 233-238, Autumn 2012
ISSN 2251-6743
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Assessment of DNA damage induced by butachlor on *Cyprinus carpio* (L. 1758) using Single Cell Gel Electrophoresis

[1]A. Mashinchian Moradi;[1,*]M. Alidoust Salimi; [2]M. H. Shahhosseini;[1]P. Alidoust Salimi

[1] Department of Marine Biology, Graduate School of Marine Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2] Department of Microbiology, Shahr-e-Qods Branch, Islamic Azad University, Tehran, Iran

Received 19 January 2012; Revised 20 February 2012; Accepted 10 March 2012

ABSTRACT: Butachlor (N-(butoxymethyl)-2-chloro-N-2, 6-diethylacetanillide) is widely used chloroacetanilide herbicide in Asian region, especially in the north of Iran. Several studies have been reported on toxicity of butachlor, but little information is accessible on DNA damaging potential. Thus, the present study was undertaken to investigate of DNA damage by butachlor in *Cyprinus carpio* (Pisces: Cyprinidae) using single cell gel electrophoresis. This study was done according to static method. The specimens were exposed to different concentrations of butachlor (0.07, 0.15 and 0.3 mgl^{-1}) and sampling was carried out after 48 hour (short-time exposed). Based on the results, a significant increase in genetic damage index was observed in treatments compared with control samples ($P<0.01$). Also, the results showed the genotoxicity potential of this herbicide on fish. This study corroborated that comet assay is applied on the fish is a functional method to determine potential genotoxicity pollutants.

Keywords: *Comet assay; Common carp; Genetic Damage Index (GDI); Genotoxicity; Herbicide*

INTRODUCTION

Large amount of pesticides are applied to increase the production in agriculture. Herbicides are one the most important form of pesticides that frequently used in the north of Iran due to rice cultivation (Nasrabadi *et al*., 2011). Butachlor (N-(butoxymethyl)-2-chloro-N-2, 6-diethylacetanillide; C12H26ClNO2) is widely used herbicide in Asia and South America, and it is classified in chloroacetanilide group. Butachlor utilized in rice and barley fields for control grass and broadleaf weeds (Ateeq *et al*., 2005; Willson and Takei, 1999). Studies have explained pollutants such as pesticides directly and indirectly (by runoff, rainfall, floods and etc.) released into aquatic environment (Ateeq *et al*., 2002; Pandrangi *et al*., 1995; Polard *et al*., 2011). Therefore they can affect on target and non-target organisms (Cavas, 2011). Both physiological and biochemical processes induced by pollutants can be caused damage on aquatic organisms (Ali and Kumar, 2008). In addition, several studies showed different pesticides have potential to interact with DNA and resulted DNA damage (Pandrangi *et al*., 1995). Pollutants that have genotoxic potential can causes variety of DNA strand breaks such as single and double strand breaks (Kamer and Rimkevich, 2002). Among different methods for detecting DNA damage, comet assay was selected because neither depends on chromosomal characteristics nor requires mitotic cell division (Pandrangi *et al*., 1995; Simoniello *et al*., 2009). Moreover, several studies were explained that the comet assay is sensitive and widely used *in vitro* and *in vivo* studies on fish for assessment of genotoxic and mutagenic pollutants (Ali *et al*., 2009; Cotelle and Ferard, 1999; Klobučar *et al*., 2003; Sharma *et al*., 2007). For assessment of DNA damage, Fishes were selected because they are appropriate biomonitoring tools for detection of genotoxic pollutants in aquatic ecosystems. Fishes are suitable organisms to carry out comet assay and sensitive to pesticides (Mohanty *et al*., 2011; Polardo *et al*., 2011). Fish blood cells are specially preferred because they comprise 97% erythrocytes, thus ensuring great homogeneity of cells for comet assay (Ateeq *et al*., 2005). In many studies butachlor is mentioned as toxic compounds, but data on their DNA damaging potential is scarce (Abul Farah *et al*., 2003). The main objective of this study is to investigate the genotoxic effect of butachlor using comet assay in erythrocytes of *Cyprinus carpio* (Pisces: Cyprinidae).

*Corresponding Author Email: *m.alidoustsalimi@srbiau.ac.ir*

MATERIALS AND METHODS

1. Fish samples

For this study *Cyprinus carpio* were purchased from local aquaculture in the north of Iran. The fishes were healthy in appearance. The average weight of fish was 19.29±2.82 gr and average length was 11.54±0.56 cm. For adaptation, samples were kept in glassy aquarium for 10 days which filled with dechlorinated tap water. During adaptation, fishes were fed with pellet foods and water change was done every day. 24 hour before starting the experiment the feeding of fishes were stopped.

2. Experiment design and treatment

This study was done according to static method (OECD, 1992). Three non-lethal concentration of Butachlor (0.07, 0.15 and 0.3 mgl^{-1}) were selected (EC 60%, Moshkfam Fars). A set of 10 specimens were exposed to each of the three concentrations. Sampling was done in 48 hour (2 days) at the rate of three fish per each concentration. Blood samples were directly obtained from heart with heparinized syringes (2.5 ml) from each fish and immediately were used for comet assay. The comet assay was conducted with whole blood under dim light to prevent UV induced DNA damage.

3. Single cell gel electrophoresis (Comet assay)

The alkaline comet assay was performed according to the method of Tice et al., (2000) with some modification. First, clean slides were coated by NMA (Normal Melting Agarose). About 15 µl of the blood were mixed with 145 µl of 0.75% LMP (Low-Melting Agarose). A 40 µl of the mixture were layered on slides and immediately covered with cover slips. The slides kept for 10 min in refrigerator ($4^{\circ}c$). After solidification, cover slips were slowly removed from the slides. The slides were immersed in cold lysine solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH=10 with 10% DMSO and 1% Triton X-100 added fresh) for 1 hour in refrigerator ($4^{\circ}c$). After lysis, the slides were placed side by side on a horizontal electrophoresis tank. The tank was smoothly filled by cold electrophoresis solution (1 mM EDTA, 300 mM NaOH, pH=13.4). The slides were stayed in the tank for 1hour in $4^{\circ}c$ to allow DNA unwinding and conversion of alkali-labile sites to single strand breaks then electrophoresis was started for 20-25 min (25 mV, 300 mA). Finally, the slides distained with EtBr (Ethidium Bromide). Then, slides were prepared for comet scoring. Observation slides were done with the CETI fluorescent microscope (Model: 3100.5000- Triton II) that equipped with Sony camera. Two slides per specimen were prepared and 50 cells per slide (300 cells per concentration) were scored randomly.

The DNA Damage was measured by visual classification of cells into 5 level "comets" according to the tail length: n_0 undamaged, n_1 minimum damaged, and n_4 are highly damaged. A Genetic Damage Index (GDI) was calculated for each treatment according to below equation (Cavas, 2011).

$$GDI = (n_1+2n_2+3n_3+4n_4) / (\sum / 100)$$

GDI: Genetic Damage Index (arbitrary units), n_1: Minimum damaged, n_4: Maximum damaged, $\sum$: Total number of the cells (300 per concentrate)

4. Data analysis

One-way analysis of variance (ANOVA) was employed by using SPSS software to compare the mean (±SD) differences in GDI between concentrations with control. The *p* values less than 0.01 was considered statistically significant.

RESULTS AND DISCUSSION

The mean (±SD) of the water temperature, pH values and dissolved oxygen concentration included 22.37±0.62$^{\circ}c$, 8.2±0.36 and 6.5±0.16 mg/l during the experimental period, respectively. No mortality was observed during experimental. Statistical analysis of the results suggested a significant difference in treatment groups compared with control (Table 1; Fig. 1; Fig. 2). The fish exposed to lower concentrate of butachlor (0.07 mgl^{-1}) did not show a significant difference compared to control (P>0.01).

Table 1: Mean (±SD) GDI in erythrocyte cells of *C.carpio* exposed to different concentration of butachlor (mgl^{-1}), (n=300 cells per concentration were scored)

Concentration (mgl^{-1})	Mean % DNA damage			
	Con.	0.07	0.15	0.3
(% GDI)	2±1	3±0	4.66±0.57*	5.33±0.57**

* P<0.005, ** P<0.001

Unmarked value (T_1) is not significant

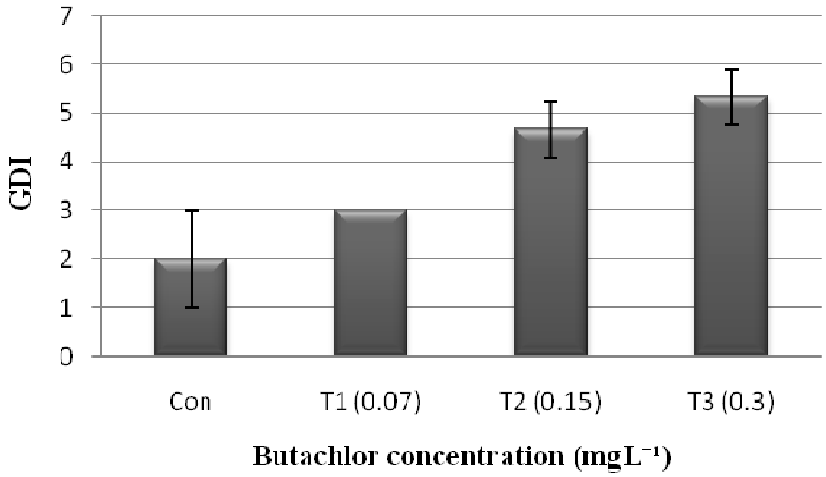

Fig. 1: DNA damage (GDI) in peripheral blood cells of *C. carpio* exposed to Butachlor

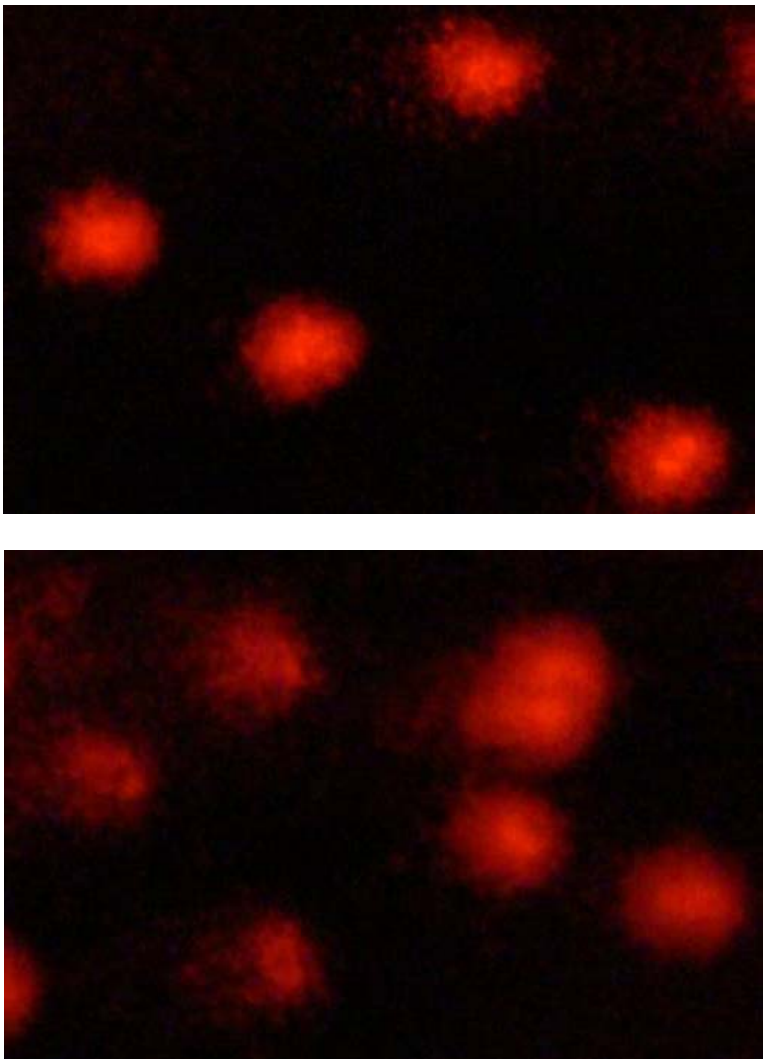

Fig 2: (Left) Control, (Right) Blood cells after exposure to butachlor

Fish are directly exposed to pesticides consequent from agriculture production via surface run-off or indirectly by food chains of ecosystem (Cavas and Ergene-G□zükara, 2005; Nwani *et al*., 2011). They are used as excellent tools for biomonitoring of genotoxic pollutant in environment because they can metabolize, concentrate and respond to low concentration of pollutant (Nwani *et al*., 2010). In addition, fish occupy various ecological niches in aquatic ecosystem therefore fish can be applied as one of the key groups for evaluating the overall health of the aquatic environment (Šrut *et al*., 2011). Butachlor can be detected in aquatic environment such as rivers. The concentration of butachlor has been detected 2.79 ppb in Oshmak River that is one of the most important rivers for agriculture in Guilan province (Khara *et al*., 2009). The herbicide has a moderate persistence in soil, but persists for a long time in water (Bashiri juybari *et al*., 2011). Butachlor can bioconcentrate in fish, clam and shrimp (Wang *et al*., 1992). Several studies indicated that butachlor is toxic, but little information accessible on DNA damage and genotoxicity potential (Baorong *et al*., 2010).

In this study, the results showed that the amount of DNA damage in different concentration were highly significant comparing to control samples, thus indicated the genotoxic potential of butachlor to fish. Our results are in agreement with the finding of Ateeq et al., (2002 and 2006) that reported butachlor is able to induce apoptosis and erythrocyte alternations in *Clarias batrachus*. Also, Ateeq et al., (2005) reported butachlor can be caused DNA damage in *C.batrachus*. The butachlor's DNA damage potential is not limited to aquatic organism. Other study on amphibians has correlated with our results. Liu et al., (2011) found butachlor can be induced DNA damage in erythrocytes of *Limnocharis* tadpole and Yin et al., (2008) observed DNA damage in erythrocytes of Chinese toad (*Bufo bufo gargarizans*) exposed to butachlor, acetachlor and paraquat. Moreover, other studies demonstrated herbicides, such as alachlor, atrazine, maleic hydrazide, paraquat and trifluralin in human peripheral lymphocytes were able to induce DNA damage (Ateeq *et al*., 2005). The DNA damage detected in this study could have originated from DNA single-strand breaks, DNA double-strand breaks, DNA adducts formation and DNA-DNA and DNA-protein cross links (Mitchelmore and Chipman, 1998), resulting from the interaction of pesticide or their metabolites with genetic material (Garaj-Vrhovac and Zeljezic, 2000). The comet assay has several advantages more than the other cytogenetic methods like chromosome aberrations, sister chromatid exchange and micronucleus test used to detection DNA damage inasmuch as it needs a small number of cells and sensitive to low pollutant concentration (Kamer and Rimkevich, 2002; Nwani *et al*., 2010). Furthermore, the assay has been applied on fish cells and other aquatic organisms *in vitro* and *in vivo* (Klobučar *et al*., 2010).

CONCLUSION

The comet assay can be successfully used for detecting DNA damage on fish. Fish that are sensitive to pesticides can be employed for biomonitoring genotoxic pollutants in aquatic ecosystems. Based on the results, butachlor can be induced DNA damage on blood cells of the fish. Several studies demonstrated butachlor and other herbicides can be caused DNA damage. However, further studies are needed to evaluate DNA damaging potential in aquatic organisms after exposure to butachlor.

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How to cite this article: (Harvard style)
Mashinchian Moradi, A.; Alidoust Salimi, M.; Shahhosseini, M. H.; Alidoust Salimi, P., (2012). Assessment of DNA damage induced by butachlor on Cyprinus carpio (L. 1758) using Single Cell Gel Electrophoresis. Int. J. Mar. Sci. Eng., 2 (4), 233-237.