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Cytogenetic damage induced by crude oil in *Anodonta cygnea* (mollusca, bivalvia) assessed by the comet assay and micronucleus test

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ABSTRACT: Crude oil is enriched in polycyclic aromatic hydrocarbons (PAHs). Many PAH analogs have proved to potentially damage DNA. DNA damage can be assessed using various biomarkers to find out the degree of genotoxicity of pollutants following in vitro exposure. In this research the comet assay and micronucleus (MN) test were used to detect DNA damages and cytogenetic changes following crude oil exposure. For this purpose, freshwater bivalve mollusks (*Anodonta cygnea*) were exposed for ten days to 0.25, 0.5 and 1.0 ppm of crude oil. For the comet assay and for the MN test, hemolymph and gill cells of mussels were sampled respectively. Statistically, significant increase of DNA damage and micronuclei were found with 0.25, 0.5 and 1.0 ppm of crude oil. This study was performed to ascertain that *A. cygnea* is a good bioindicator of pollutants in aquatic environments; also identified hemolymph and gill of *A.cygnea* are most effective and practical tissues for genotoxicity studies.

Keywords: *Crude oil; comet assay; Micronucleus test; mussel; haemoplymph*

INTRODUCTION

Thousands of chemical compounds are released into aquatic ecosystems and can cause hazardous effects in marine and freshwater organisms. These substances (heavy metals, oil products, chlorinated pesticides, halogenated aromatic hydrocarbons) have the ability to accumulate in water organisms (Woodhead *et al.*, 1999). Different types of bioassays have been widely utilized for assessing the genotoxic, mutagenic and carcinogenic potencies of a range of devastating substances. Markers of genotoxic effects are at high priority due to reflection of damage to genetic material of organisms (Moore *et al*., 2004).

Oil products among pollutants are widely distributed in the hydrosphere, and hydrocarbons are a well known primary source of persistent toxicity in aquatic environment. Hazardous effects of different PAHs arise mainly as a result of oxidative biotransformation producing to highly DNA reactive metabolites. These metabolites are recognized as carcinogenic and mutagenic compounds (Torres-Bugarin *et al*., 1998; Woodhead *et al.*, 1999; Maria *et al*., 2002). Some studies described an increase in environmental genotoxicity in oil spill zones (Parry *et al*., 1997; Pietrapiana *et al*., 2002; Baršienė *et al*., 2004).

In aquatic environments, PAH toxicity increases with the increase of their molecular weight, and their bioaccumulation is prone to be fast (Eisler, 1987). Indeed, it is known that the hydrophobic characteristic of PAHs causes their accumulation in organisms beyond their concentration levels in the environment (Oliver and Niimi, 1988; Spies *et al*., 1988; Van der Oost *et al*., 1988). The mechanisms of PAH metabolic transformation have been studied and the genotoxic potency of these metabolites was confirmed in the European eel *Anquilla anquilla* (Pacheco and Santos, 1997, 2002; Maria *et al*., 2002a, 2002b; Teles *et al*., 2003).

Little attention has been paid to genotoxic effects in invertebrate aquatic organisms that inhabiting contaminated environments. Invertebrates present over 90% of the species in aquatic communities and have a particularly important role in the ecosystem function (Dixon *et al*., 2002).

Mollusks have been widely used as indicator organisms, because they are ubiquitous. Furthermore, they have highly conserved control and regulatory

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pathways that are often homologous to vertebrate systems, and are extremely sensitive to anthropogenic inputs. A comparatively high bioaccumulation factors for organic pollutants, relatively low metabolic detoxification rates and a sessile filter feeding life style allow using the bivalves as sensitive organisms in biomonitoring studies (Depledge and Fossi, 1994; Bolognesi *et al*., 2004). Mussels have been effectively used as bioindicator organisms in other marine catastrophes, such as the study carried out after Sea Empress spill in Wales in 1996 (Dyrynda *et al*., 1997).

Freshwater mussels store toxicants in their tissues and pseudofaeces and may play a key role in maintaining water quality (James, 1987; Strayer *et al*., 1994). Therefore freshwater mussels have been included in "mussel watch" programs with their marine counterparts (Metcalfe-Smith *et al*., 1996).

Different methods have been developed for detection of both double and single strand breaks of DNA, DNA adducts, micronuclei formation, and chromosome aberrations. The assessment of cytogenetic damage has been presented as a very important assay in identification of pollution effects in marine environments (Dixon *et al*., 2002).

Relative to other genotoxicity tests, such as chromosomal aberrations, sister chromatid exchanges, alkaline elution, and micronucleus assay, the advantages of the Comet assay include its demonstrated sensitivity for detecting low levels of DNA damage (one break per 1010 Da of DNA) (Singh *et al*., 1988, 1999; Cotelle and Ferard, 1999), requirement for small number of cells (~10,000) per sample, flexibility to use proliferating as well as non proliferating cells, low cost, ease of application, and the short time needed to complete a study. It can be conducted on cells that are the first site of contact with mutagenic or carcinogenic substances (e.g., oral and nasal mucosal cells). The data generated at cell level allow for robust types of statistical analysis. The other drawbacks such as single cell data (which may be rate limiting), small cell sample (leading to sample bias), technical variability, and interpretation are some of its disadvantages. (Mitchelmore and Chipman, 1998; Olive and Banath, 2006).

Since its inception, this assay has been modified at various steps (lysis, electrophoresis) to make it suitable for assessing various kinds of damage in different cells (Collins, 2004; Speit and Hartmann, 2005).

It has gained wide acceptance as a valuable tool in fundamental DNA damage, repair studies (Speit and Hartmann, 2005) and genotoxicity testing (Moller, 2005; Moller, 2006).

The Comet assay is widely influenced by laboratory procedures suggesting that standard protocols are required for both fish and mussel cells. Recently, Pavlica *et al*. (2001) have used comet assay on zebra mussel. The comet assay offers considerable advantages over other cytogenetic methods for DNA damage detection, because the cells studied do not need to be mitotically active.

The micronucleus (MN) test, based on the presence of micronuclei that occurs in actively dividing cell populations, has served as an index of cytogenetic damage for over 30 years (Ayllon and Garcia-Vazquez, 2000, 2001). It has been assessed in mollusks as a biological indicator of pollution in situ, and also for genotoxicity evaluation of physical and chemical agents after direct or indirect exposure in vivo (Metcalfe, 1988; Al-Sabti and Metcalfe, 1995; Ayllon *et al*., 2000; Ayllon and Garcia-Vazquez, 2001; Farah *et al*., 2003; Rodriguez-Cea *et al*., 2003). Micronuclei are produced in all cell types after irregular division process when a chromosome fragment or a whole chromosome is not lost during the anaphase, but is delayed with respect to the rest of chromosomes, constituting a small secondary nucleus in an interphase (Heddle et al., 1991). In a study, the zebra mussel (*Dreissena polymorpha*) was used for genotoxicity testing of different chemicals by micronucleus test (Mersch *et al*., 1996; Mersch and Beauvais, 1997; Pavlica *et al*., 2000).

MATERIALS AND METHODS

Animals and treatments

Mussels (*Anodonta cygnea*) of 9.0–11.5 cm in length were collected from Anzali wetland in north of Iran. For the experiment, mussels were transferred into aerated glass tanks containing 80 dm^3 of dechlorinated tap water. The water temperature ranged from 18 to 20°C. Eight animals were used in each exposure study tank. Three concentrations of crude oil (crude oil from the Caspian Sea) were chosen as treatments: 0.25, 0.5 and 1 ppm. Two samples were used as controls (tap water as a negative control and DMSO as a solvent control). The exposure period was 10 days. Haemolymph for comet assay and gill cells for micronucleus t0est were collected at the end of the experiment. Bivalves of similar sizes were subdivided into experimental groups (Table 1).

Every day, the main hydrochemical parameters were measured. The content of dissolved oxygen, water pH and temperature were stable during the experimental period (Table 2).

Table 1: Morphological characteristics of mussels

Oil concentration/ Morphological characteristics	Control 1 (tap water)	Control 2 (water+DMSO)	0.25 ppm	0. 5 ppm	1 ppm	(mean±S.D.)
Length, cm	9.62	9.5	9.75	10	10.5	9.874±0.39
High, cm	5.37	5.5	5.5	5.62	5.62	5.522±0.10
Weight, g	78.5	77.62	83.25	94.25	99.25	86.574±9.69

Table 2: Hydrochemical parameters of the water in the experimental tanks

Treatment / Date Parameters	First day			second day			3th day			4th day			5th day		
	T c°	pH	O2 mg/l	T c°	pH	O2 mg/l	T c°	pH	O2 mg/l	T c°	pH	O2 mg/l	T c°	pH	O2 mg/l
Control 1 (tap water)	20.1	8.1	5.8	20	5.8	5.5	20.5	8	5.8	19.3	7.5	6	21	8.15	6
Control 2 (water+DMSO)	20.5	7.4	6	19.7	6	5.7	21.8	8	5	20.9	8.3	6.3	19.9	8.25	6.1
0.25 ppm	22	7.4	6.3	22.1	6	5	20	8.3	5.6	22.4	8	5	19	8.30	5.8
0. 5 ppm	18.9	8	5.8	21	5.5	6.2	20.3	7.2	6	21.2	8.5	5.5	20.7	8.30	5.5
1 ppm	19	8.1	5.6	20	5.6	6	21	8.1	5.9	17.5	7.8	5.4	18.2	8	5
Treatment / Date Parameters	6th day			7th day			8th day			9th day			10th day		
	T c°	pH	O2 mg/l	T c°	pH	O2 mg/l	T c°	pH	O2 mg/l	T c°	pH	O2 mg/l	T c°	pH	O2 mg/l
Control 1 (tap water)	20	8.15	6.2	8.15	6	5.8	18.9	8.41	5.5	20	8.1	5.8	20.5	8.2	6.1
Control 2 (water+DMSO)	18.9	8.2	6.1	8.25	6.1	5.8	17.8	8	5.6	21.5	7.7	6	21	8.5	5.8
0.25 ppm	19	8.30	5.5	8.30	5.8	5	20	8.35	6.1	22	7.4	6	19.6	8.3	5
0. 5 ppm	20.1	8.25	5.5	8.30	5.5	6	21	8.20	5.9	19.9	8	5.5	21.2	8.2	6.3
1 ppm	20.2	8	5.3	8	5	6.4	20.5	7.68	5	19	8.2	5.6	20	7.9	5.5

Comet assay

For the comet assay, haemoplymph was collected in darkness from both posterior and anterior adductor muscles with a syringe. Comet assay was conducted as described by Singh et al. (1988, 1999) with slight modifications. Frosted slides were precoated with 1% agarose (normal melting point; NMA) in PBS. After agarose was solidified, aliquots of 1 ml of haemolymph were centrifuged in Eppendorf tubes at 1000 rpm for 10 minutes. The supernatant was discarded and 15 µl of pellet was mixed with 65 µl of 0.75% agarose (low melting point; LMA) and placed on the first agarose layer. A coverslip was added and agarose was allowed to solidify for 5 minutes at 4°C. After removing the coverslip, the third layer of 0.75% LMA agarose was added and left to solidify at 4°C. The coverslip was removed and the cells were lysed in freshly made lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris · HCl, 1% Triton X-100 and 10% DMSO, pH = 10.0) for 1 h at 4°C. After lysis, the slides were placed on a horizontal gel box and covered with cold (about 4°C) alkaline buffer (10 N

NaOH and 200 mM EDTA, pH = 12.3). The slides were left in the alkaline buffer for 20 minutes to allow the unwinding of DNA. Slides were electrophoresed at 25 V and 300 mA for 20 minutes. After electrophoresis, slides were neutralized three times for 5 minutes with cold neutralization buffer (0.4 M Tris · HCl, pH = 7.5), then stained with 20 µg/ml ethidium bromide and examined at the same day using a Nikon Optiphot 2 epifluorescence microscope. For each animal, 100 cells per slide (500 cells per treatment) were visually scored at random and given an empirical score depending on the degree of damage. The damage categories were allocated according to the approximate percentage of DNA in tail as described by Wilson *et al.* (1998).

DNA migration was visually determined in randomly select and non overlapping cells per mussel. 100 cells were analyzed per slide, with five slides per animal. DNA damage was classified in four classes, according to the tail length of the comet: 0, undamaged; 1, minimum damage; 2, medium damage; 3, maximum damage, according to Kobayashi *et al.* (1995). The mean number of damaged nucleoids (classes 1–3) was calculated per specimen exposed to crude oil and their respective controls, for exposure period (Fig. 1). The score of damage for each mussel was calculated as the number of nucleoids observed in each damage class multiplied by the value of its respective damage class (0, 1, 2 or 3). Results were expressed as the mean score of damage, for each treatment group, where 0 represents absence of damage and 300 indicates the highest damage score. Cells were scored randomly on the central part of the gel. Slides were coded independently and scored blindly. The percentage of DNA damage was chosen to express damage rate (De Boeck *et al*., 2000).

$DD = (0n_0 + 1n_1 + 2n_2 + 3n_3)/(\Sigma/100)$

DD: DNA damage

n_0 - n_3 : the number of comets from 0 to 3

Σ: total of counted cells

Micronucleus test

Two branches of mussel gills were placed in a big drop of 3:1 ethanol acetic acid (or methanol acetic acid) solution separately on two clean microscopic slides and gently nipped with tweezers for 2–3 minutes (until cells spread within a drop). Then the cell suspension was softly smeared on the whole surface (except label place) of both slides. Dried slides were fixed in methanol for 10 min and stained with 5% Giemsa solution in phosphate buffer pH = 6.8. The stained slides were examined under the light microscope Olympus BX51 at a final magnification of 1000×. The frequencies of MN were expressed per 1000 cells by 25 slides from each sample. Micronuclei were identified according to the following criteria spherical or ovoid shaped extranuclear bodies in the cytoplasm (Baršienė *et al*., 2006).

Data Analysis

All statistical analysis were carried out using the SPSS 14 for Windows. One way analysis of variance (ANOVA) with Tukey test was performed to compare the mean differences at $P \leq 0.05$..

RESULTS AND DISCUSSION

DNA Damage

The current study was the performance of genotoxicity screening assay on freshwater mussel *Anodonta cygnea*, to explore the capacity of this organism to detect the presence of genotoxic compounds. The results of comet assay in hemocyte of *A.cygnea* exposed to the crude oil and its respective negative and solvent controls are presented in Table 3. Mussels exposed to crude oil showed more damaged cells with a significant increase in the number of damaged nucleoids compared to their respective negative control (Table 3).

Comet scores with DNA damage percentage for mussel exposed to crude oil in experimental periods were significantly higher than the respective negative control group (Fig. 2).

cytogenetic change

The results of micronucleus test obtained after 10 days of exposure to crude oil in mussel's gills (Table 4), were congruent with those obtained through comet assay. The presence of micronucleated cells was noticeable mainly in the samples exposed to crude oil. There was significant difference between the micronuclei observed in different experimental groups.

Many recent studies on aquatic environment monitoring have used mussels as sentinel organisms and comet assay as biomarker (Baussant *et al.*, 2011; Frenzilli *et al*., 2009; Steinert *et al*., 1998a, b; Large *et al*., 2002; Rank and Jensen, 2003; Akcha *et al.*, 2004; Haldorson *et al*., 2004), demonstrating the utility and efficiency of these organisms in evaluating the extent of a contamination event in the marine environment, and its possible effects on ecosystem and human activities. Most of these works show that gill cells examination is very suitable for the evaluation of cytogenetic damages. Because gill filters great quantities of contaminant agents present in the water. Moreover, comet assay has been previously used in order to determine hydrocarbon exposure in mussels (Steinert, 1996; Mitchelmore *et al.*, 1998; Pe´rez-Cadahı´a *et al.*, 2004).

The results of this study revealed that exposure of the filter feeding mussel (*Anodonta cygnea*) to 0.25, 0.5 and 1.0 ppm of crude oil increased the induction of DNA damage and nuclear abnormality (MN). Comet assay has been used in previous study in which invertebrates were exposed to crude oil (Hamoutene *et al.*, 2002; Taban *et al.*, 2004). In an investigation, digestive gland cells of mussels exposed to water contaminated with petroleum hydrocarbons showed significantly higher DNA damage compared to control group. Mussels (*Mytilus edulis*) exposed to crude oil (Arabian crude oil 0.2%) also showed an increase in DNA damage on digestive gland cells (Hamoutene *et al.*, 2002). Another study showed a significantly higher percentage of DNA damage in the comet tail of cells from sea urchins and mussels exposed to crude oil compared to untreated control cells (mean values of 24% and 14% DNA in the comet tail in sea urchins coelomocytes and mussels haemocytes, respectively (Taban *et al.*, 2004).

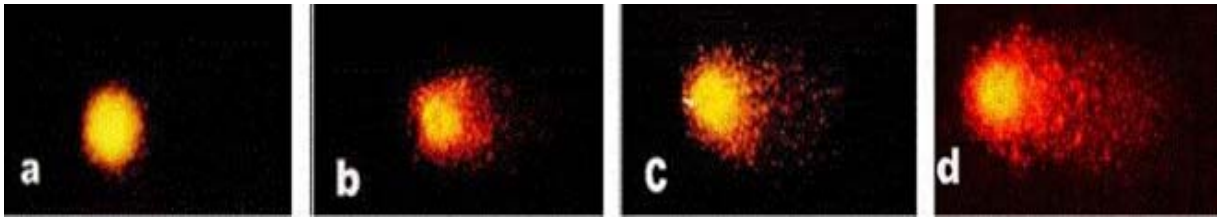

Fig. 1: Examples of rat hemocyte nuclei observations in each class of DNA damage (classes 0–3)
a) class0 b) class1 c) class2 d)class3

Table 3: Number of nucleoids observed in each comet class (0–3) and mean number of damaged nucleoids per mussel in hemocyte of *Anodonta cygnea* exposed to crude oil, taking into account the total number of mussel (N) analyzed for each experimental treatment and their negative and solvent controls (100 cells per specimen were analyzed)

Period of exposure	Treatments	N	Comet classes 0	1	2	3	Number of damaged nucleoids per mussel (mean±S.D.)
10 days	Control 1 (tap water)	5	229	168	54	49	82.6±4.13
	Control 2(water+DMSO)	5	94	221	96	89	136±6.8
	0.25 ppm	5	16	68	193	223	224.6±11.23
	0. 5 ppm	5	8	39	153	300	249±12.45
	1 ppm	5	9	17	114	360	265±13.25

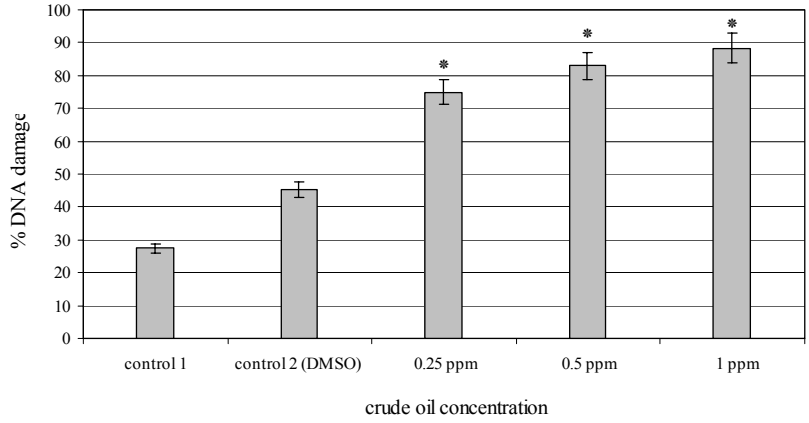

Fig. 2: Results of the comet assay expressed as the percentage of damaged haemocytes of Anodonta cygnea after exposure to crude oil.
(* $p \leq 0.05$ statistically significant difference from control (ANOVA with Tukey test)

Table 4: Results of micronucleus (MN) frequency analysis in *Anodonta cygnea* after exposure to crude oil

Treatments	No. of individuals examined	No. of gill cells examined	Total number of micronuclei (MN)	Frequency [‰] of MN (mean±S.D.)
Control 1 (tap water)	5	5000	0	0
Control 2 (water+DMSO)	5	5000	1	0.2±0.01
0.25 ppm	5	5000	8	1.6±0.08 *
0. 5 ppm	5	5000	13	2.6±0.13 *
1 ppm	5	5000	10	2±0.1 *

* $p \leq 0.05$ statistically significant difference from control (ANOVA with Tukey test)

A dose related increase in DNA damage has been reported in green lipped mussels, *Perna viridis* exposed to 0.3, 3 and 30 µg/l benzo[a]pyrene (Ching *et al.*, 2001). Treatment with 0.3 and 3 µg/l benzo[a]pyrene induced a significant increase in DNA strand breaks in mussel hepatopancreas after one day of exposure, followed by a gradual decrease in strand breaks after 3–6 days. The frequency of DNA strand breaks returned to the control level after 12 days. Different kinds of genotoxic damage were observed after a 30 µg/l benzo[a]pyrene exposure. A significant increase in the damage was observed from day 12 to day 24 (Ching *et al.*, 2001).

Another result of this study, demonstrated the highest induction of micronuclei in mussels after a 10 day treatment with 0.5 ppm of crude oil. It is known that oil contains potentially genotoxic components (Klekowski *et al.*, 1994). Thus, the elevation of genotoxicity in mussels after a 10 day exposure to crude oil is an obvious response to the genotoxic substances of crude oil. On the other hand, exposure to 1 ppm of crude oil showed a lower induction of genotoxicity (micronuclei) in gill cells than exposure to 0.5 ppm of oil. The analysis of chromosome aberrations and aneugenic effects in tissues of the same specimens showed a suppression of cell division in bivalves exposed to the highest concentration of crude oil; thus, the mitostatic effects caused by some of the components present in Caspian sea crude oil is evident.

Crude oil is a complex mixture of various hydrocarbons including nitrogen, oxygen compounds and heavy metals. The content of components differs depending on the areas of oil drilling (Wake, 2005).

Nuclear abnormalities (micronuclei) were analyzed in gill cells of mollusks, which were in direct contact with crude oil dispersed in water. Gills, as a respiratory and filtration organ, present the first barrier of contaminant intake and often have been used as a target tissue in biomonitoring studies (Baršienė *et al.*, 1999; Baršienė snd Bučinskienė, 2001; Bolognesi *et al.*, 2004). It is known that the frequency of micronuclei largely depends on the type of tissue analyzed (Hayashi *et al.*, 1998). Furthermore, a higher level of micronuclei was also observed in blue mussel from Lithuanian coastal areas of the Baltic Sea and from the North Sea compared to fish (Baršienė *et al.*, 2004).

Significant elevation of micronuclei level in mussels after 30 days post oil spill and persistence of the cytogenetic damage up to 100 days later have been described (Parry et al., 1997). Micronucleated cells were on the increase in the gills or hemolymph of marine molluscs treated with benzo[a]pyrene (Burgeot *et al.*, 1995; Venier *et al.*, 1997; Siu *et al.*, 2004) and dimethylbenzol[a]anthracene (Bolognesi *et al.*, 1996).

More frequent cytogenetic damage has been described in mollusks inhabiting the marine port and oil terminal areas in the Baltic Sea (Baršienė and Baršytė-Lovejoy, 2000; Baršienė, 2002), in French Mediterranean coast at Fossur Mer, affected by the oil refinery industry (Burgeot *et al.*, 1996).

An assessment of cytogenetic damage in early life stages and in mature organisms (including somatic and gonad cells) is desirable since the level of cytogenetic damage can vary depending on age, tissue, sex, season, temperature and oxygen factors (Brunetti *et al.*, 1986).

CONCLUSION

The results of the Comet assay and MN test in this research clearly showed dose and time dependent responses to crude oil exposure in bivalves (*Anodonta cygnea*). Consequently, the complex interactions between exposure doses and duration need to be verified.

Future research should focus on identifying both the short and long term consequences of genotoxicity in organisms exposed to crude oil compounds.

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