Bull. Environ. Contam. Toxicol. (2003) 71:263–269

DOI: 10.1007/s00128-003-0159-1

Bulletin of Environmental Contamination and Toxicology

Detection of DNA Strand Breaks in the Liver of *Boleophthalmus pectinirostris* Treated with Benzo(a)pyrene

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Received: 27 March 2001/Accepted: 22 April 2003

Genetic damage is considered to be a useful parameter for assessing the genotoxic properties of environmental pollutants. Many of these pollutants are chemical carcinogens and mutagens with the capacity to cause various types of DNA-damage. Being a representative polycyclic aromatic hydrocarbons (PAHs), benzo[a]pyrene(BaP) deserves serious study because PAHs are now recognized as major environmental pollutants and are strongly suspected of being a causative factor in certain tumors in aquatic species and cancer in humans.

Current research on DNA damages have focused on detecting and quantifying DNA adducts (Willett et al. 1997; Burgeot et al. 1996), oxidized based in DNA (Collins et al. 1996; Loft et al. 1993), DNA strand breaks (Martin and Black 1998; Muller et al. 1998; Vukmirovic et al. 1994), chromosomes aberrations and micronuclei formation (Pacheco and Santos 1996; Santos and Pacheco 1995), etc. The detection of DNA strand breaks that are produced either directly or indirectly is facilitated by the alkaline unwinding assay (Shugart 1988). The feasibility of utilizing this technique on aquatic species as a general biomarker for pollution-related genotoxicity has being evaluated by numerous laboratory and field research (Everaarts 1997, 1995; Everaarts and Sarkar 1996; Di Diulio et al. 1993). Most of the studies indicate that the levels of DNA single strand breaks are related to the levels of contaminants. However, before these techniques can be used reliably in an assessment of the response of DNA integrity of feral fish to environmental pollution, more researches are still needed.

The present study measured dose- and time-effect of DNA strand breaks in *Boleophthalmus pectinirostris* treated with BaP. The main aim is to investigate the possibility of using DNA strand breaks as a biomarker of BaP exposure. The repair of DNA lession existed in this fish after BaP was removed is also explored.

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MATERIALS AND METHODS

All reagents were analytical grade or better. BaP was obtained from Sigma Chemical Co., Sephadex G-50 from Sigma Chemical Co., and Hoechsy dye 33258 from Calbiochem Co.

Boleophthalmus pectinirostris (weight, 12-18 g; length, 11 ± 15 cm) were collected from Fuqing sea areas in Fujian Province. They were acclimated to laboratory conditions in tanks for several days prior to exposure to BaP and were fed algae. The tanks were supplied with clean sea water (40 L, 20±2℃). Fish placed in the tank exhibited no signs of stress or physical damage due to confinement.

The carrier solvent for BaP was acetone. The same volume of acetone was added to the control aquaria. There were two tanks for each concentration. The water was aerated and replaced every day with sea water containing BaP of same concentration.

Table 1. Experimental Design

exposure	BaP concentrations			
time	control	.05 mg/L	0.2 mg/L	0.5 mg/L
6hr	+	+	-	+
12hr	-	+	-	+
24hr	-	+	-	+
36hr	-	+	-	+
3d	+	+	+	+
7d	+	+	+	+
				(+)

+ Samples for determination of DNA strand break.
( )Samples after 3d BaP exposure(0.5mg/L) and 4d BaP-free water.

Fish were exposed to BaP at concentrations of 0, 0.05, 0.2 and 0.5mg/L respectively. Samples (the liver of *Boleophthalmus pectinirostris*) were collected in different times from 6h to a week. Experimental design was shown as Table 1.

Experiment 1. *Boleophthalmus pectinirostris* were exposed to BaP at concentrations of 0, 0.05 and 0.5mg/L. Samples (the liver of *Boleophthalmus pectinirostris*) of both exposure groups were collected at 6hr, 12hr, 24hr, 36hr, 3d and 7d respectively, whereas samples of control group were collected at 6hr, 3d and 7d.

Experiment 2. *Boleophthalmus pectinirostris* were exposed to BaP at concentrations of 0, 0.05, 0.2 and 0.5mg/L for 3d and 7d respectively.

Experiment 3. *Boleophthalmus pectinirostris* were exposed to BaP at concentration of 0.5mg/L for 3d and then transferred to BaP-free water for 4d.

After dissection, the livers of *Boleophthalmus pectinirostris* were frozen in liquid nitrogen for preservation of the activity of DNA. The DNA isolation is accomplished by homogenization of the liver tissue in 0.2%Triton-X in 1N NH_4OH and further purified by extraction with chloroform/isoamyl alcohol/phenol (24/1/25 v/v). After centrifugation on a Beckman J2-MC centrifuge, the supernatant passes through a molecular sieve column (Sephadex G-50).

DNA strand-breaks were determined according to an alkaline unwinding procedure (Shugart 1988) in which three parameters were measured: the percentage of double-stranded and single-stranded DNA, and the fraction of double-stranded DNA after alkaline unwinding. Hoechst dye 33258 combines with dsDNA to form a stable fluorescent product, the intensity of which is reduced upon binding of the dye to ssDNA. Unwinding of DNA takes place at single-strand breaks within the molecule, hence the amount of double-strand DNA remaining after a given period of alkaline unwinding will be inversely proportional to the number of strand breaks present at the initiation of the alkaline exposure provided that renaturation is prevented. The amounts of these two types of DNA present are estimated by measuring the fluorescence due to interaction with Hoechst dye 33258. All the DNA determinations were made on a Hitachi 850 spectrofluorometer.

The F-value which represents the integrity of DNA can be quantified using the following equation (Kanter and Schwartz 1982):

$F=(X_{auDNA}-X_{ssDNA})/X_{dsDNA}-X_{ssDNA})$

Where X is the observed fluorescence.
dsDNA is double-stranded DNA.
ssDNA is single-stranded DNA.
auDNA is DNA treated according to alkaline unwinding procedure.

Mean values and standard deviations were calculated for each test group based on individual liver tissue from six fishes. These values were compared by analysis of variance (ANOVA) and one-tailed Student *t* test. The significance level was set at 0.05.

RESULTS AND DISCUSSION

Results in experiment 1 revealed that compared with the control group, there were no significant changes for the level of dsDNA in the liver of *Boleophthalmus pectinirostris* at 0.05mg/L BaP, whereas it decreased progressively at 0.5mg/L (Table 2). At concentration of 0.5mg/L ($P<0.05$), all the exposure periods except the 6h were significantly different with control group. Additionally, fractions of dsDNA decreased rapidly during the early exposure period of 36hr (nearly 40% of control) at concentration of 0.5mg/L, which indicated that the exposure in this

concentration may cause serious harm to DNA in short period. With prolonged exposure from 36hr to 7d in this concentration, fractions of dsDNA decreased slowly (only 20% of control) although the level of dsDNA decreased continuously.

Table 2. Changes of DNA integrity in the liver of *Boleophthalmus pectinirostris* after exposure to BaP for periods of 6hr to 7d.[a]

exposure time	exposure concentrations		
	control	0.05mg/L	0.5mg/L
6hr	0.78±0.17	0.76±0.19	0.68±0.27
12hr	-	0.64±0.09	0.53±0.06*
24hr	-	0.66±0.20	0.38±0.13*
36hr	-	0.70±0.24	0.46±0.05*
3d	0.87±0.07	0.70±0.11	0.38±0.04*
7d	0.73±0.16	0.77±0.29	0.28±0.13*
			0.35±0.10)*,b

[a]Data expressed as *F*(fraction of dsDNA) present in fish examined. Each point is the average of data from six separate fish (=standard error of the mean)

*Significantly different with corresponding control (data of exposure groups at 12, 24 and 36hr should be compared with data of control group at 6hr), $P<0.05$

[b]Data obtained after 3d BaP exposure(0.5mg/L) and 4d BaP-free water.

- Datas were not be detected.

Results of experiment 2 indicated that at 3d exposure, the level of dsDNA (*F* values) decreased with the increase of BaP concentrations (Figure 1). Analysis of dose-response relationship between fractions of dsDNA and exposure concentrations using the method of regression indicated a linear correlation, of which the regression equation was $y=-0.8733x+0.8112$ ($r^2=0.93$). Similarly with 3d exposure, the level of dsDNA decreased with the increase of BaP concentration at 7d exposure. However analysis of dose-response relationship indicated a conic correlation, of which the regressive equation was $y=3.8512x^2-2.9809x+0.8023$ ($r^2=0.91$). It is worhwhile to notice that fractions of dsDNA in concentrations of 0.2 and 0.5mg/L were similar to each other (44% and 38% of the fraction of dsDNA in the control group respectively). The results indicated that the effect of BaP concentrations on the integrity of DNA would not increase further with the increase of BaP concentrations (>0.2mg/L). The effect will reach saturation when the BaP concentration is larger than 0.2mg/L. The above correlations show that DNA strand breaks in the liver of *Boleophthalmus pectinirostris* and the BaP exposure concentrations have close correlation in the concentration range of our study.

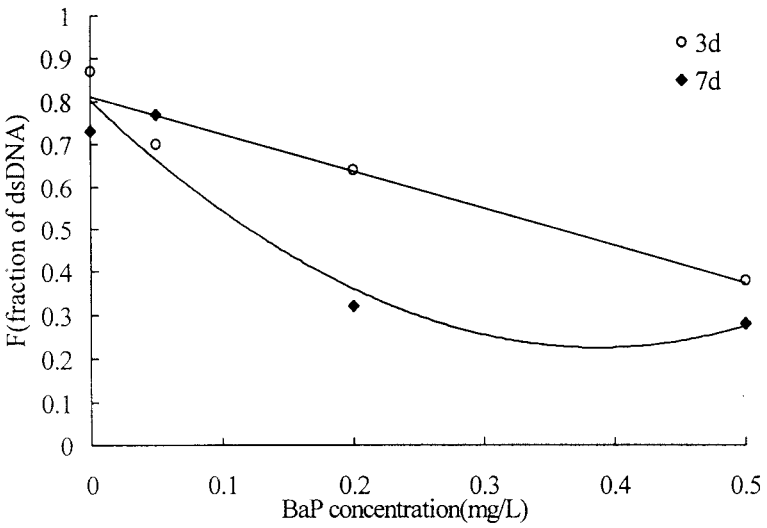

Figure 1. The effects of BaP exposure at different concentrations on DNA integrity in the liver of *Boleophthalmus pectinirostris* in 3d and 7d.

Results in experiment 3 showed that after 3d BaP exposure at 0.5mg/L BaP and 4d recover in BaP-free sea water, fraction of dsDNA neither significantly differed from fraction of dsDNA of 7d exposure, nor of 3d exposure (Table 2). This indicated a weak DNA repair in the liver of *Boleophthalmus pectinirostris* at the BaP concentrations used.

Many researches demonstrated that factors such as species, exposure time and exposure concentration might affect the level of DNA integrity. McCarthy (1991) has detected the integrity of DNA from *Oryzias latipes,* acutely exposed to water containing 200mg/L of diethylnitrosamine (DEN) for 24hr and then transferred to DEN-free water for 6d. The level of dsDNA decreased to half of the control after this exposure protocol was repeated three additional times. In another experiment, Di Giulio (1993) found that fraction of hepatic DNA remaining double-stranded following alkaline unwinding in channel catfish exposed to sediment from either Black Rock Harbor significantly decreased at 2, 14 and 28d than that of reference site. Everaarts et al. (1995,1996,1997) has measured the level of integrity of DNA in seastars (*Asterias rubens*) an Dab (*Limanda limanda*) collected from different areas of the North Sea. Highest DNA integrity was found in specimens from offshore reference sites, whereas lowest integrity was identified in specimens from the coastal zone and certain expected uncontaminated offshore areas. Similarly with former researches, DNA integrity has shown dose-related and time-dependent relationship when *Boleophthalmus pectinirostris* were exposured to BaP at concentrations of 0.05, 0.2 and 0.5mg/L for a week, which indicates that DNA strand breaks are suitable to be a biomarker of BaP exposure.

DNA integrity induced by chemical carcinogens has also been detected in culture cells. For example, Daniel (1985) has studied the genotoxicity of four chemicals (BPDE, (+)-r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro-benzo(a)pyrene; DMS, dimethylsulfate; MNU, N-methyl-N-nitrosourea; ENU, N-ethyl-N-nitrosourea) to human lymphoblastic cell line CCRF-CEM (1h exposure). These studies demonstrate that the dose-effect relationship of the four chemicals was of typical linearity (y=kx+b), only that k values may greatly differ for the different chemicals. Differing with the studies above, the present study, by analysis of dose-response relationship between DNA integrity and exposure concentrations using the method of regression, indicated that they were shown to be linear correlation at 3 days exposure (y=kx+b) and conic correlation at 7 days exposure ($y=ax^2+bx+c$). All of these suggested that studies in the level of cells can't substitute for the studies in the entire level of animals, because studies in vitro will usually be influenced by more unknown factors and will have more complicated results.

The study on repair of DNA damage caused by pollutants is still insufficient in fishes. In a study with *Parophrys vetulus* (Varanasi et al. 1989), no significant change in the level of DNA adducts was observed for up to 60d after a single exposure to BaP. This might indicate the limitation of DNA repair in fishes. But in a study with *Lepomis macrochirus* (Shugart 1988), chronically exposed to BaP in low concentration, DNA single strand breaks can be repaired by itself. All the results indicate that the repair of DNA damage in fishes might be limited by many factors such as species, pollutants, exposure concentration, exposure time. In our study, DNA single strand breaks were not repaired in the liver of *Boleophthalmus pectinirostris*, which might be due to the high BaP exposure concentration.

Acknowledgments This research was supported by Nature Scientific Fund(B0110034) in Fujian Province, P R China and Jimei University Fund. The authors thank Dr. David Klumpp in Australian Institute of Marine Science for his revision to the manuscript.

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