Bull. Environ. Contam. Toxicol. (1991) 47:783–789

Bulletin of Environmental Contamination and Toxicology

Comparative Analysis of Aromatic DNA Adducts in Fish from Polluted and Unpolluted Areas by the ^{32}P-Postlabeling Analysis

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The ^{32}P-postlabeling technique, developed by Randerath and his colleagues (1981), is a highly sensitive assay to detect the damaged DNA as DNA-carcinogen adduct. Consequently, measurement of this DNA-carcinogen adduct concentrations in target tissues of organisms may provide a key biologic end-point of exposure to environmental carcinogens. By using this technique, Dunn et al. (1987) reported that hepatic DNA of brown bullheads (*Ictalurus nebulosus*), sampled from Buffalo and Detroit Rivers polluted by polycyclic aromatic hydrocarbons, exhibited several DNA adducts not present in the hepatic DNA of aquarium-raised fish. On the other hand, Kurelec et al. (1989) found that livers of freshwater fish species such as chub (*Leuciscus cephalus*), barbel (*Barbus barbus*), bream (*Abramis brama*) and carp (*Cyprinus carpio*), as well as a marine fish mugil (*Mugil auratus*), revealed the presence of four to nine qualitatively similar adducts irrespective of whether they were caught from polluted or unpolluted waters.

In this study, we will elucidate the above discrepancy by analyzing the hepatic DNA from a bottom-feeding fish, tilapia (*Tilapia mossambica*), sampled from the down stream Damsui River where fish are exposed to high levels of polycyclic aromatic hydrocarbons (PAH) in sediment and from the upstream Fe-Tsui reservoir where the water has been used as the source of drinking water for Taipei Metropolitan area. In doing so, we will be able to determine whether this ^{32}P-postlabeling analysis of hepatic DNA from the bottom-feeding tilapia can be used to monitor large molecule aromatic carcinogens in our environment.

MATERIALS AND METHODS

Tilapias (*Tilapia mossambica*), a highly pollution-resistant freshwater

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fish, were caught near the Fu-Ho bridge (25° 00' 33" N, 121° 31' 17" E) of the Damsui River. For controls, tilapias caught from the Fe-Tsui reservoir (24° 54' 40" N, 121° 34' 00" E), which is located on the the upstream of the Damsui River and is not polluted, were used. These fish ranged from 10 to 25 cm in length, are approximately 1.0 to 2.0 years old. The fish were kept alive and transported to the laboratory. Fish were killed by a blow to the head. Livers were removed immediately and rinsed using ice-chilled 1.15% KCl. The tissues were homogenized in the KCl solution for the preparation of washed microsomes according to the differential centrifugation method, as described by Lake (1987).

DNA was extracted by the standard phenol method and followed by chloroform purification (Maniatis et al. 1982). The total DNA was then precipitated by cold ethanol. All the above extraction procedures were carried out in Teflon tubes. Five micrograms of DNA were then digested in 10 uL of 20 mM sodium succinate, 10 mM $CaCl_2$, pH 6.0, containing 5 ug of spleen exonuclease, 5 ug of micrococcal endonuclease, at 38°C for 3.5 hr. The digested deoxyribonucleoside 3'-monophosphates were further treated with 6 ug nuclease P1, in 0.25 M sodium acetate (pH 5.0) and 0.3 mM zinc sulfate (pH 5.0) at 37°C for 40 min as described by Reddy and Randerath (1986). In this reaction, the normal nucleotides were dephosphorylated to corressponding nucleosides by nuclease P1, whereas the 3'-phosphates on the carcinogen-attached nucleotides were not cleavaged by nuclease P1. Consequently, only the DNA-carcinogen adducts were labeled with [γ-^{32}P]ATP. The DNA labeling procedure of Gupta et al. (1982) was carried out in a buffer mix (100 mM bicine, 100 mM magnesium chloride, 100 mM dithiothreitol, and 10 mM spermidine, pH 9.5) containing 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol), purchased from Du Pont-NEN (Boston, MA), and 4 units of T4 polynucleotide kinase (Amersham International). The mixture was then incubated at 38°C for 30 min.

The ^{32}P-labeled adducts were then resolved on polyethylene-imine (PEI) cellulose TLC as outlined by Gupta et al. (1982). In brief, the incubation mixture was spotted on PEI-cellulose (Macherey-Nagel, Germany) plate and developed in 5-D TLC system. The D1 development was run in 1M sodium phosphate, pH 6.8. The D2 development, same direction as D1, was in 3.75 M ammonium formate, pH 3.5. The D3 development, at the opposite direction from D1 and D2, was in 5.3 M lithium formate, and 8.5 M urea, pH 3.5. The D4 development, at a right angle to D3, was run in 1.2 M lithium chloride, 0.5 M Tris and 8.5 M urea, pH 8.0. The D5 development, the same direction as D4, was in 1.7 M sodium

phosphate pH 6.0. Adducts were visualized by intensifying screen-enhanced autoradiography using Kodak XAR-5 film. The adduct areas or zones and blank areas were excised from the PEI plate for quantitation by Cerenkov counting (Gupta et al. 1982). DNA modified in vitro by anti-7,8-diol-9,10-oxide of benzo[a]pyrene, kindly provided by Dr. Regina of Columbia University of USA, was used as a standard to calibrate the ^{32}P-postlabeling assay. Adduct levels were calculated from the amounts of radioactivity on the chromatograms, the DNA amount and the specific activity of the [γ-^{32}P]ATP used for labelling (Reddy and Randerath 1986). Quantitative estimation of the adducts was expressed as number of adducts in 10^8 nucleotides.

Hepatic microsomes were prepared using differential centrifugation at 9,000 g and 100,000 g at 4^{o}C (Lake, 1987). The activities of aryl hydrocarbon hydroxylase in hepatic microsomes were determined by the method of Nebert and Gelboin (1968) with modifications described elsewhere (Ueng et al., 1983). The hydroxylase assay for tilapia microsomes was carried out under linear conditions for protein concentration and incubation time. Protein determinations were carried out using the Bio-Rad protein assay kit. Bovine serum albumin was used as a standard.

RESULTS AND DISCUSSION

Our data indicated that hepatic AHH activity was higher in tilapia caught from polluted Damsui River near Fu-Ho bridge than that of the same kind of fish caught from Fe-Tsui reservoir (Table 1). Ueng et al. (1990) had demonstrated that the activities of AHH and other P450-dependent monooxygenases can be induced by 3-methylcholanthrene and PCBs in tilapia. Since the fish of Damsui River contained high concentrations of PCBs (Chou et al. 1986), this significantly higher ($p < 0.05$) hepatic AHH activity of tilapia from contaminated Damsui River may be the result of induction by PAHs and PCBs. On the other hand, Fe-Tsui reservoir is a non- polluted site; consequently, the hepatic AHH activity of tilapia from this area is low which is consistent with the results of Ueng et al. (1990). It should be point out here that many physiological factors including species, sex, maturity, and reproductive cycle may affect the fish AHH activity. It may be of future interest to expand the present study to investigate the significance of the physiological factors in this environmental induction of AHH activity.

The autoradiograms of ^{32}P-labeled hepatic DNA digests from tilapia caught from polluted Damsui River revealed two distinct diagonal radioactive zones (DRZ) and one distinct spot (Fig 1E). The DRZs

Table 1 Comparison of AHH activities and levels of DNA adducts of tilapia caught from the polluted and non-polluted part of Damsui River.

	Polluted Site (Fu-Ho Bridge)	Non-polluted Site (Fe-Tsui Reservoir)
AHH Activity (pmol/min/mg protein)	244.6± 121.9 (6)*	19.0±3.8 (8)
Adduct Level (adduct/10^8 nucleotides)	24.4± 12.1 (6)	< 1 (6)

Values are represented as mean ± SD
Number in parenthesis indicated sample size
* Significantly different ($p < 0.05$) from non-polluted site by Student's t test

radiated from the origin and sometimes showed some very distinct spots distributed within it. However, these DRZs are not exactly the same, some fish with clear DRZ1 and some fish with darker DRZ2 (Fig 1A and 1B). On the other hand, none of the tilapia caught from non-polluted Fe-Tsui reservoir revealed such DRZs, but some tilapia did show a spot which correspond to the spot in autoradiograms from the fish caught from polluted Damsui River (Fig 1C, and 1D). However, we are not sure whether this spot is species specific, natural factor related adduct as reported by Kurelec et al. (1989) or the pollutant-related adduct.

The results of our finding coincided with the report of Dunn et al. (1987) who showed that ^{32}P-labeled hepatic DNA digest exhibited several adducts from fish sampled in polluted area and these adducts were not detectable in hepatic DNA of aquarium raised fish. In addition, the pattern of our DRZs are very similar to the DRZs of ^{32}P-labeled hepatic DNA digest from English sole and winter flounder treated with contaminated sediment extract as reported by Varanasi et al. (1989). Similarly, they did not find DRZs in English sole from non-polluted reference site. On the other hand, by using the same technique, Kurelec et al. (1989) found that adducts from hepatic DNA digest in fish was not related to the pollution level of the river. The averaged AHH activity (22±17 pmol/min/mg protein) in the livers of fish living in polluted Sava River was low compared to the averaged AHH activity (244.6±121.9 pmol/min/mg protein) of

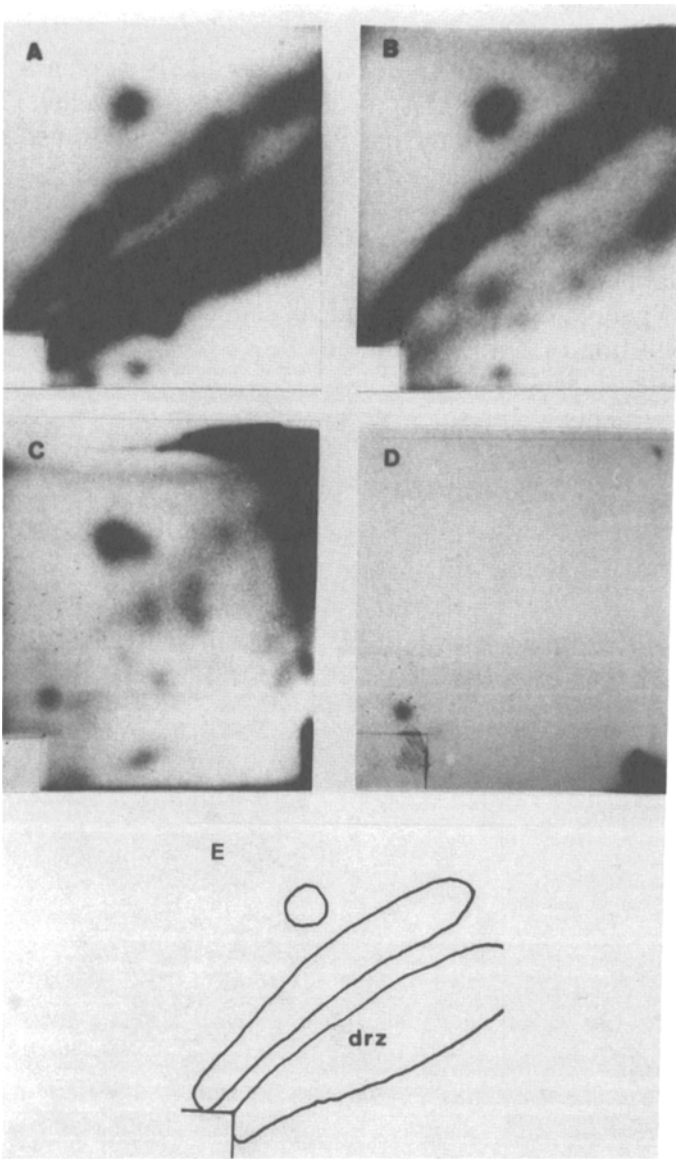

Figure 1 Autoradiograms of PEI-TLC maps of ^{32}P-labeled hepatic DNA digests from tilapia: A and B, tilapia sampled from polluted Damsui River near Fu-Ho bridge; C and D, tilapia sampled from the non-polluted Fe-Tsui reservoir; E, the areas of the TLC maps designated as DRZs. The origin is located at the bottom left-hand corner of each TLC map. The autoradiography was carried out at -70°C for 36 hr.

fish from polluted Damsui River. The most likely reason for this discrepancy in two different fish species may lie in the level of pollution in Sava River which was not severe enough to produce detectable ^{32}P-labeled adducts in fish liver.

In conclusion, ^{32}P-postlabeling analysis of hepatic DNA of bottom-feeding tilapia could be used as a sensitive method in determining the exposure to genotoxic chemicals and subsequently be used to assess the level of pollution of our environment.

Acknowledgments. This study was supported in part by grants (NSC 79-0421-B075-01Z, 80-0421-B075-01Z and 80-0421-B002-07Z) from the National Science Council of the Republic of China.

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Received March 20, 1991; accepted August 7, 1991.