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Alkaline Single-Cell Gel Electrophoresis Assay of *Actinia equina* for Monitoring Seawater Genotoxicity

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This study was suggested by a recent evaluation of marine pollution in the harbour of Genova (*Pane et al., unpublished research*). This evaluation indicated contamination of sea water with various chemicals, especially with polynuclear aromatic hydrocarbons (PAHs) in the zone reserved for oil tankers (Porto Petroli of Genova-Voltri). The six PAHs measured were fluoranthene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, and indeno (1,2,3-cd)pyrene. In the period November 1997 – June 1998 their cumulative concentrations ranged in the sea water from 0.001 to 0.007 ppb, in the seston from 0.105 to 2.733 ng/g, and in the copepods from 0.022 to 2.343 ng/g. The purpose of the study was to examine the possibility of assessing the genotoxic effect of this contamination using the sea anemone *Actinia equina* (Cnidaria: Anthozoa) as the target organism.

Actinia equina (L.) is a common species living in rocky coasts along the tide-line or on other hard substrata, such as docks, where it could be exposed to high concentrations of pollutants. Although its physiological characteristics are still not entirely known, it possesses some interesting activities, such as the production of toxins and the capability of movement more advanced than those of shellfishes usually employed to study aquatic environmental pollution (Ueda et al. 1992; Sasaki et al. 1997). Furthermore, it can be maintained easily in an aquarium. The toxin of this Cnidarian has been extensively studied (Ferlan and Lebez 1974) and three components, Equinatoxin (EqT) I, II and III, were characterized (Macek and Lebez 1988). The toxin of *Actinia equina* has been also the subject of research that demonstrated its hemolytic (Macek and Lebez 1981; Turk and Macek 1986) and cytotoxic properties (Batista et al. 1986, 1987, 1990, Zorec et al. 1990; Mariottini et al. 1998). The toxin of *Actinia equina* seems also to show phospholipase activity, and interacts with membrane phospholipids of injured cells affecting ion exchange and increasing the permeability of the plasmalemma (Batista and Jezernik 1992).

Due to the limited number of studies on genotoxicity biomonitoring using aquatic organisms, we deemed it of interest to investigate whether the presence of genotoxic water pollutants may be revealed by the induction of DNA damage in

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A. equina, as measured with the alkaline single-cell gel electrophoresis assay. This technique, also known as Comet assay, is a highly sensitive method to assess DNA single-strand breakage and/or alkali-labile sites (Fairbairn et al. 1995).

MATERIALS AND METHODS

N-ethyl-N-nitrosourea (ENU) was purchased from Serva Feinbiochemica (Heidelberg, Germany); benzo(a)pyrene (B(a)P), regular (type I B) and low melting point (type VII) agarose, ethidium bromide and dimethyl sulfoxide (DMSO) from Sigma Chimica (Milano, Italy). All the other chemicals were of the purest grade available. Water samples were obtained from the open Ligurian Sea and from the Porto Petroli of Genova-Voltri during 1999; they were stored at 4°C and tested within 10 days. Three specimens of *Actinia equina* of similar size were harvested from the reefs of the Ligurian Riviera. Prior to use, they were housed for at least 10 days in an aquarium containing water from the open sea, maintained at 25°C.

The DNA-damaging activity of ENU, of B(a)P, and of the marine water of Porto Petroli was examined in three independent experiments carried out using three different specimens of *A. equina*. The three anemones were first examined to establish the basal values of DNA migration (negative control). A week later each anemone was exposed to ENU, 15 days later to the marine water of Porto Petroli, and after 15 additional days to B(a)P, using the following procedure. The organism was placed for 4 hr into a beaker-containing 200 ml of water from the open Ligurian Sea (at 25°C) in which the test mutagen was dissolved, or was placed for the same time in the same volume of water sampled from the Porto Petroli. ENU was directly dissolved in water; B(a)P was dissolved in DMSO and then diluted in water. When DMSO was used as the solvent, its final concentration was 0.5% (v/v), as routinely used in DNA damage assays.

At the end of the 4 hr exposure and 1 and 7 days afterwards, a small fragment was cut from the foot of *Actinia equina*; this choice was suggested by both the reduced number of nematocysts in this part of the animal, and its rapid regeneration. The fragment was rapidly minced in Merchant's solution (0.14 M NaCl, 0.075 mM KH_2PO_4, 2.7 mM KCl, 9.5 mM Na_2HPO_4, 0.7 mM Na_2EDTA) and shaken in a magnetic strirrer for 10 min. The suspension was filtered, gently pipetted to disaggregate clusters, and diluted to obtain a concentration of ~5×10^3 cells/µl. Cell viability was measured by the trypan blue-exclusion assay, and the presence of DNA damage was evaluated by the alkaline Comet assay.

The procedure of the Comet assay was essentially that described by Singh et al. (1988). Ten µl of the cellular suspension were mixed with 75 µl of low melting point agarose at 37°C and added to regular melting point agarose-coated microscope slides. The slides were immersed in cold lysing solution (38 mM NaOH, 20 mM Na_2EDTA, 6.82 mM sarcosyl, 2 M NaCl; pH 10.2) for 30 min, and

then placed in an electrophoresis tray with an alkaline solution (300 mM NaOH, 1 mM Na_2EDTA; pH 13) for 15 min to allow DNA to unwind. Electrophoresis was performed at room temperature for 15 min at 25 V and 300 mA (0.83 V/cm). The slides were washed, stained with ethidium bromide, and examined at 400× magnification using a fluorescence microscope. Images of 50 randomly-chosen cells from a single foot fragment were examined for each animal and the extent of DNA migration was quantified by measuring the tail length and the tail moment with computerized image analysis (Sarin System, Firenze, Italy) (Hellmann et al., 1995). The data from each specimen were expressed as mean ± S.D. The statistical analysis for the comparison between control and each time interval after exposure was performed with the Wilcoxon two-sample (two-tailed) test.

RESULTS AND DISCUSSION

A preliminary assay (data not shown) indicated that a 4 hr exposure of a specimen of *A. equina* to 50, 100 and 200 ppm of ENU induced a dose-dependent increase in DNA migration without any reduction in the fraction of viable trypan-blue excluding cells. When the same specimen was exposed to ENU 15 days afterwards, the values of DNA migration were very similar, thus indicating that its sensitivity was unchanged. On the basis of this finding we judged that a 15 day interval was sufficient to remove the DNA lesions produced by ENU and, as a consequence that it was possible to re-expose the same speciments of *Actinia equina* to new mutagens. The correctness of this choice was confirmed by the minimum degree of DNA fragmentation observed 7 days after exposure to 200 ppm of ENU. Table 1 shows the migration of nuclear DNA from *A. equina* at the end of 4 hr exposure to 200 ppm of ENU, and 1 and 7 days afterwards.

The migration of nuclear DNA from *A. equina* after a 4 hr exposure to sea water sampled from the Porto Petroli of Genova-Voltri is presented in Table 2. The data, based on three independent experiments, show that both tail length and tail moment were increased, as compared to controls, to a modest but statistically significant extent. This result indicates the occurrence, in the sample of examined sea water, of chemicals capable to induce DNA damage in *A. equina*. Taking into account the documented presence of PAHs in this water (Pane et al. 2003), it was of interest to verify whether *A. equina* is capable of biotrasforming B(a)P, a representative promutagen of this family of chemicals, into reactive species. This capability is demonstrated by data presented in Table 2, which indicate that a 4 hr exposure to 200 ppm of B(a)P induced in three specimens of *A. equina* a marked and reproducible increase in DNA damage without reducing the fraction of living cells. As compared with ENU, the repair of B(a)P-induced DNA lesions initially took place more slowly.

In all the experiments the fraction of viable cells was higher than 85%; therefore the results of this study indicate that the Comet assay can detect DNA damage induced in *A. equina* by subtoxic concentrations of a direct acting alkylating agent

Table 1. Migration of nuclear DNA from *Actinia equina* cells after 4 hr exposure to ENU and time course of DNA repair. Significance level as determined by the Wilcoxon two-sample (two-tailed) test.

Treatment condition	Specimen no.	Time interval after treatment	Comet assay	
			Tail length (μm) (mean ± SD)	Tail moment (mean ± SD)
Control	1		0.9 ± 0.67	97 ± 74
	2		1.7 ± 1.03	180 ± 111
	3		1.4 ± 0.74	136 ± 70
Pooled data			1.3 ± 0.89	138 ± 93
ENU 200 ppm	1	4 hrs	48.9 ± 8.11	3283 ± 174
	2		50.1 ± 5.34	3428 ± 421
	3		51.7 ± 8.94	3313 ± 694
Pooled data			50.2 ± 7.70†	3341 ± 483†
ENU 200 ppm	1	1 day	26.3 ± 7.60	1948 ± 553
	2		31.9 ± 7.50	2334 ± 514
	3		30.2 ± 8.32	2254 ± 597
Pooled data			29.5 ± 8.16†,‡	2179 ± 580†,‡
ENU 200 ppm	1	7 days	2.9 ± 2.19	230 ± 167
	2		3.4 ± 3.01	278 ± 244
	3		2.5 ± 2.01	224 ± 191
Pooled data			2.9 ± 2.47†,‡	244 ± 205†,‡

† $p<0.05$ versus control; ‡ $p<0.05$ versus ENU 4 hr.

(ENU) and of a promutagen requiring metabolic activation (B(a)P).

Although this is not yet completely known, *Actinia equina*, with respect to shellfish, possesses different functions such as the capability of pollutant biotransformation. A comparison with the results obtained by Sasaki et al. (1997), who measured with the Comet assay the degree of DNA damage induced by four genotoxic agents in two species of shellfish, *Patunopecten yessoensis* and *Tapes japonica*, suggests that *A. equina* might be more sensitive to the DNA damaging activity of pollutants. As a matter of fact, the ratios treated/control of DNA migration obtained by Sasaki et al. (1997) were 1.8 for *P. yessoensis* and 1.5 for *T. japonica* after a 4 hr exposure to 200 ppm of ENU and 1.9 after a 4 hr exposure to 100 ppm of B(a)P of the former and to 50 ppm of B(a)P of the latter. Moreover, at the most effective doses, the four genotoxins tested by Sasaki et al. (1997) never increased DNA migration more than 3-fold, whereas the increases observed in *A. equina* were of 55.8-fold with ENU and 48.7-fold with B(a)P. With respect to the importance of assessing the genotoxicity of contaminated water, our results confirm that the Comet assay might be an excellent tool for this type of investigation.

Table 2. Migration of nuclear DNA from *Actinia equina* after 4 hr exposure to the sea water of Genova-Voltri Porto Petroli or to B(a)P. Significance level as determined by the Wilcoxon two-sample (two-tailed) test.

Treatment condition	Specimen no.	Time interval after treatment	Comet assay	
			Tail length (μm) (mean ± SD)	Tail moment (mean ± SD)
Control	1		0.9 ± 0.67	97 ± 74
	2		1.7 ± 1.03	180 ± 111
	3		1.4 ± 0.74	136 ± 70
Pooled data			1.3 ± 0.89	138 ± 93
Porto Petroli	1	4 hrs	5.1 ± 3.20	366 ± 210
	2		4.2 ± 1.62	300 ± 121
	3		2.7 ± 0.80	197 ± 58
Pooled data			4.0 ± 2.34†	288 ± 160†
B(a)P 300 ppm	1	4 hrs	44.9 ± 9.75	2341 ± 708
	2		41.7 ± 13.84	2143 ± 753
	3		44.7 ± 12.80	2043 ± 601
Pooled data			43.8 ± 12.34†	2176 ± 701†
B(a)P 300 ppm	1	1 day	48.1 ± 20.36	3663 ± 1405
	2		44.1 ± 20.06	3592 ± 1663
	3		34.0 ± 23.83	2465 ± 1680
Pooled data			42.3 ± 22.53†,‡	3240 ± 1680†,‡
B(a)P 300 ppm	1	7 days	3.2 ± 1.86	255 ± 152
	2		2.5 ± 1.09	204 ± 82
	3		2.1 ± 1.57	192 ± 148
Pooled data			2.6 ± 1.61‡	217 ± 134‡

† $p<0.05$ versus control; ‡ $p<0.05$ versus B(a)P 4 hr.

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