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Application of an immunoperoxidase staining method for detection of 7,8-dihydro-8-oxodeoxyguanosine as a biomarker of chemical-induced oxidative stress in marine organisms

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Abstract

7,8-Dihydro-8-oxodeoxyguanosine (8-oxo-dG) is a typical modification of DNA caused by oxygen free radicals and can be an useful biomarker for pollutants inducing oxidative stress. An immunoperoxidase method using monoclonal antibody 1F7 toward 8-oxo-dG was applied to tissues and smeared cells of marine organisms for detection and quantification of oxidative DNA damage in such models. The assay, previously employed on human cells, was assessed for the first time on Mediterranean mussels (*Mytilus galloprovincialis*) and European eels (*Anguilla anguilla*), exposed to model pro-oxidant chemicals, namely benzo[*a*]pyrene (B[*a*]P) and copper. Quantification of 8-oxo-dG was microscopically carried out and expressed as relative nuclear staining intensity. Higher levels of oxidative DNA damage were detected in the digestive glands of treated mussels compared to controls, while the effect was less pronounced in haemocytes, characterized by more elevated basal levels of 8-oxo-dG. The assay was suitable for detection of 8-oxo-dG also in fish liver sections indicating consistent damage after B[*a*]P exposure. The main advantage of the immunohistochemical approach is the elimination of DNA extraction which considerably reduces the processing of biological samples. In addition, the assay requires small amounts of frozen tissues or fixed cells for detection of 8-oxo-dG and is potentially able to discriminate variable susceptibility to oxidative stress in different cell types. Although further investigations are required for the improvement and the validation of the assay in field conditions, laboratory exposures provided useful indications on the consistency of the approach and the efficacy of antibody 1F7 in marine organisms for a rapid assessment of pollutant-induced oxidative DNA damage.

Keywords: 7,8-Dihydro-8-oxodeoxyguanosine; Oxidative DNA damage; Immunoperoxidase staining; Monoclonal antibody 1F7; Mussels; Eels

1. Introduction

DNA is the target of several classes of marine pollutants (López-Barea and Pueyo, 1998) and a number of them, altering the cellular equilibrium between

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pro-oxidant forces and antioxidant defenses may induce oxidative stress in aquatic organisms (Winston and Di Giulio, 1991). 8-Oxo-dG is widely accepted as index of oxidative DNA damage since it is one of the principal oxidative base modifications, selectively formed by hydroxyl radicals. Among DNA bases, guanine has the lowest oxidative potential (Kawanishi et al., 2001) and the eighth position of its imidazol ring is particularly susceptible to the action of oxidizing agents (Kawanishi et al., 2001; Wallace, 2002). In vitro exposure of naked DNA or chromatin to hydroxyl radicals (Arouma et al., 1989; Dizdaroglu et al., 1991) markedly increased both 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G) and 8-oxo-dG, the main products of guanine oxidation which have extremely different biological implications. The open-ring formamidopyrimidine structure destabilizes the DNA, prevents the action of DNA polymerase and replication fork progression, thus representing a potentially lethal mutation. On the other hand, 8-oxo-dG does not interfere with advancement of the replication fork, but, because of mispairing with an adenine residue, it may induce mutations (GC → TA transversions) (Wallace, 2002).

The detection of 8-oxo-dG in marine organisms is of increasing interest in ecotoxicology to investigate the hazards of environmental genotoxins (López-Barea and Pueyo, 1998). Enhanced levels of 8-oxo-dG have been documented within target tissues of aquatic vertebrates and invertebrates, after natural or artificial exposure to environmental pollutants (Di Giulio et al., 1995; Canova et al., 1998; López-Barea and Pueyo, 1998; Ploch et al., 1999; Rodríguez-Ariza et al., 1999; Akcha et al., 2000a). Moreover, the role of oxidative DNA damage including 8-oxo-dG in fish liver neoplasia was documented (Malins and Haimanot, 1990, 1991; Malins and Gunselman, 1994).

In recent years, several methods have been developed for measurement of 8-oxo-dG levels in biologic samples. The most widely used are direct approaches in which DNA is chemically or enzymatically hydrolyzed and analyzed by high performance liquid chromatography coupled with electrochemical detection (HPLC–ECD) (reviewed in Halliwell, 1999), gas chromatography–mass spectrometry (GC–MS) (Malins and Gunselman, 1994) or ^{32}P postlabeling (Schuler et al., 1997). However, remarkable disagreement still remains concerning the real values and the elevated variability obtained within cells and tissues of standard matrix (Halliwell, 1999; ESCODD, 2002).

Alternative methods have also been developed in humans and mammals to detect oxidative DNA damage *in situ* without cell disruption, DNA isolation or hydrolysis; these approaches include the comet assay joined with the use of bacterial endonuclease III and formamidopyrimidine glycosylase (which respectively nicks oxidized pyrimidines and recognizes ring opened purines, reviewed in Collins and Dušinká, 2002), or the use of specific antibodies developed to detect 8-oxo-dG (Yin et al., 1995).

A series of highly specific antibodies have been developed to detect different chemical adducts on DNA and extensively used in cancer epidemiological studies for competitive ELISA and immunohistochemical staining (Santella, 1999). However, their application in non-human models is very limited, with only two examples of B[*a*]P DNA-adduct detection in marine animals (Bourgeot et al., 1996; Mathieu et al., 1997).

The aim of this work was to explore the applicability of the immunohistochemical approach for the rapid detection of chemically-induced oxidative DNA damage in marine organisms commonly used in ecotoxicological applications. In this respect, the monoclonal antibody 1F7 (Yin et al., 1995) was tested for immunoperoxidase staining on haemocytes and digestive gland sections of the Mediterranean mussel (*Mytilus galloprovincialis*) and on liver sections of the European eel (*Anguilla anguilla*). Mussels are sessil bivalve mollusks, filter feeding organisms, able to accumulate in their tissues different classes of pollutants and excellent sentinel species for “Mussel Watch” monitoring programs (O’Connor, 1998). Eels can accumulate several contaminants in their lipid-rich tissues and have been recommended as sentinel species for coastal lagoons where they spend most of the juvenile phase (Regoli et al., 2003).

Based on previous data (Canova et al., 1998; Nigro et al., 2002), laboratory exposures were designed to induce oxidative DNA damage in both mussels and eels. Organisms were treated with different doses of B[*a*]P, a model compound, with oxidative and genotoxic properties. In addition mussels were also exposed to a sub-lethal concentration of copper, a pro-oxidant chemical, impairing the antioxidant

system and lysosomal compartment in marine bivalves (Regoli et al., 1998; Regoli, 2000).

2. Materials and methods

2.1. Samples collection and laboratory exposures

Mediterranean mussels (*M. galloprovincialis*) were collected from Portonovo, an unpolluted coastal site near Ancona, Italy and acclimatized to laboratory conditions for one week in artificial seawater at 18 °C ± 0.5, 37‰ salinity (1 l of seawater per mussel). Organisms were exposed to either B[*a*]P or Cu as copper sulphate. B[*a*]P was first dissolved in dimethylsulfoxide (DMSO) and added to seawater at final concentrations of 100, 500 and 1000 ppb (final DMSO concentration was 0.0001%) while Cu was dosed at 60 μg/l. Water and chemicals were changed and redosed daily; control organisms were maintained in clear artificial sea-water. After 10 days of exposure, six mussels were removed from each treatment group, digestive glands dissected, frozen in liquid nitrogen and stored at −80 °C. Cryostat sections (5 μm) were prepared from frozen digestive glands, air dried, fixed in acetone and Carnoy fixative (methanol/acetic acid 3:1) for 10 min each at −20 °C and maintained at room temperature until use. Haemocytes, collected from posterior adductor muscle, were rapidly smeared on poly-lysine coated slides, and fixed in the same way.

Sexually immature specimens of European eel (*A. anguilla*) were collected in the Orbetello Lagoon (Tuscany, Italy) and acclimatized for one week to laboratory conditions (running filtered seawater at 18 °C ± 0.5 and salinity at 37‰). Eels were exposed by intraperitoneal injection, to B[*a*]P at 0.1, 1, 10 and 50 mg/kg in corn oil. Controls received corn oil alone. After 7 days fishes were sacrificed, livers rapidly dissected, frozen in liquid nitrogen and stored at −80 °C. Cryostat sections (5 μm) were prepared as previously described for mussels.

2.2. Immunoperoxidase staining and microscopic quantification

Immunoperoxidase staining for 8-oxo-dG and microscopic quantification were carried out according to Yarborough et al. (1996). Slides were washed twice with 1X PBS, incubated at 37 °C for 1 h with 100 μg/ml RNase in Tris buffer (10 mM Trizma base, 1 mM EDTA, 0.4 M NaCl, at pH 7.5) to prevent cross-reaction with RNA adducts and washed again twice in PBS. Proteins were removed from DNA by digestion with proteinase K (PK) (10 μg/ml in Tris buffer, at pH 7.5) at room temperature for 5, 7 and 10 min, respectively, for mussel digestive gland, fish liver and mussel haemocytes. To increase the antibody accessibility to the antigen, a denaturation step with 4N HCl for 10 min (fish liver and mussel haemocytes) or 7 min (mussel digestive gland) was followed by neutralization with TRIS base for respectively 5 and 4 min. Non-specific binding was blocked with 10% normal horse serum in blocking buffer (ABC Kit, Vector Laboratories, Burlingame CA) at 37 °C for 1 h. Incubation with the primary antibody 1F7 (dilution of hybridoma supernatant antiserum 1:2 for tissues and 1:5 for cells) was carried out overnight at 4 °C. Slides were washed in PBS and incubated with secondary goat anti-mouse antibody conjugated to biotin (ABC Kit) for 30 min at 37 °C and washed again with PBS. Endogenous peroxidases were quenched in 0.3% H_2O_2 in methyl alcohol at room temperature for 30 min, slides were washed from ABC reagent (Vector Laboratories, Burlingame, CA) and added with avidin conjugated to horseradish peroxidase (30 min at 37 °C). After three washing (5 min each) with PBS, slides were treated (30 s) with 1% Triton X-100 (in PBS), washed with PBS and incubated with diaminobenzidine for 10 min at room temperature. Lastly, slides were washed with tap water, dehydrated in 95%, 100% ethanol, and xylene and mounted with Permount. The relative staining of nuclei was quantitated with a Cell Analysis System 200 microscope (Becton Dickinson, San Jose, CA) using the Cell Measurement Program software package. A total of 50 cells per slide were counted on five different randomly chosen fields, 10 cells per field. Quantification of 8-oxo-dG content was expressed as arbitrary units of relative staining intensity (RSI). The relative damage (RD) was calculated as the ratio between mean staining values of treated and control samples. The specificity of staining reaction for 8-oxo-dG was verified with samples digested with DNase before staining or stained omitting the primary antibody.

According to the method of Yarborough et al. (1996), human cultured cells were processed simulta-

neously with each batch of marine samples to account for the variability of the immunohistochemical procedure. Human lymphoblastoid cells (cultured in RPMI 1640 complete medium, with 10% of fetal calf serum) were incubated (30 min) with or without 5 mM hydrogen peroxide and used as positive and negative human control slides.

The effects of various chemical exposures were statistically tested by analysis of variance followed by the Newman–Keuls post hoc comparison test.

3. Results

The immunoperoxidase staining of 8-oxo-dG was set up for the different tissues of marine organisms prepared both as cryostat sections (liver of eels and digestive gland of mussels) and smeared cells (mussel haemocytes). The original method of Yarborough et al. (1996), based on monoclonal antibody 1F7, required some modification due to the different histological features between our samples and classic murine or human tissues.

RNase digestion, necessary to prevent cross-reaction between the antibody and RNA oxidized bases, was not critical for tissue integrity. On the other hand, the tubular organization of the mussel digestive gland was particularly sensitive to PK digestion that can compromise tissue integrity, leading to failure of the whole immunohistochemical procedure. Different incubation periods were tested to minimize such alterations, while maintaining the effectiveness of PK treatment; the optimal time was fixed at 5 min. Liver sections were less susceptible to PK action and the optimum digestion time was 7 min; haemocytes were incubated with PK for 10 min as originally reported by Yarborough et al. (1996). Compared to the original protocol, the denaturation and washing steps were slightly reduced for the mussel digestive glands to 7 and 4 min, respectively. The primary antibody was used at a higher concentration on tissue sections than smeared cells (with less DNA) while detection of secondary antibody was maintained constant in the different biologic samples. Representative examples of staining for digestive gland and haemocytes of mussels and eels liver are given in Fig. 1. The specificity of the immunohistochemical reaction was further verified for marine samples by incubating slides with DNase before the staining or omitting the primary antibody. All these treatments considerably reduced nuclear staining to a faint gray both in mussel and eel tissues (Fig. 1).

Human smeared cells, treated with or without H_2O_2, were always processed with each batch of marine samples as an additional control on the procedure validity. Immunoperoxidase detection of 8-oxo-dG revealed considerable variation between negative and positive human controls with RSI increasing 2.46-fold after treatment with hydrogen peroxide from 0.372 ± 0.049 to 0.917 ± 0.129. The resulting RD value, expressed as the ratio between the mean gray intensity of treated and control samples, was close to the 2.5, as reported by Yarborough et al. (1996). The CVs for replicate analysis of negative and positive controls were 13.1 and 14.1%, respectively.

Quantification of 8-oxo-dG content clearly revealed oxidative DNA damage in digestive gland of mussels treated with both B[*a*]P and Cu (Table 1). At the lower dose of B[*a*]P (100 ppb) the staining intensity between treated and control organisms increased 1.30-fold. Higher concentrations of B[*a*]P induced a greater level of DNA damage with similar increase of RSI values measured in organisms exposed to either 500 or 1000 ppb (Table 1). Also copper induced a marked increase of 8-oxo-dG in digestive cells of exposed mussels where relative staining intensity was 1.73-fold higher than in controls (Table 1).

Compared to digestive gland, haemocytes revealed a less marked variation in DNA damage after 10 days under various experimental conditions (Table 1). However, these cells were characterized by higher levels of basal staining intensity, with RSI values approximately two-fold greater than those measured in control digestive cells; nonetheless, the induction of oxidative DNA damage was significant also in these cells with a relative damage of approximately 1.5 in treated haemocytes, independently of the chemical and dose of exposure.

Oxidative damage to DNA was clearly detectable also in liver sections of eels exposed to B[*a*]P (Table 1). A considerable increase in the relative damage (2.02) was observed even at the lowest dose of exposure (0.01 mg/kg); a maximum RD value of 2.50 was measured in liver sections of eels treated with the highest dose of B[*a*]P (50 mg/kg, Table 1).

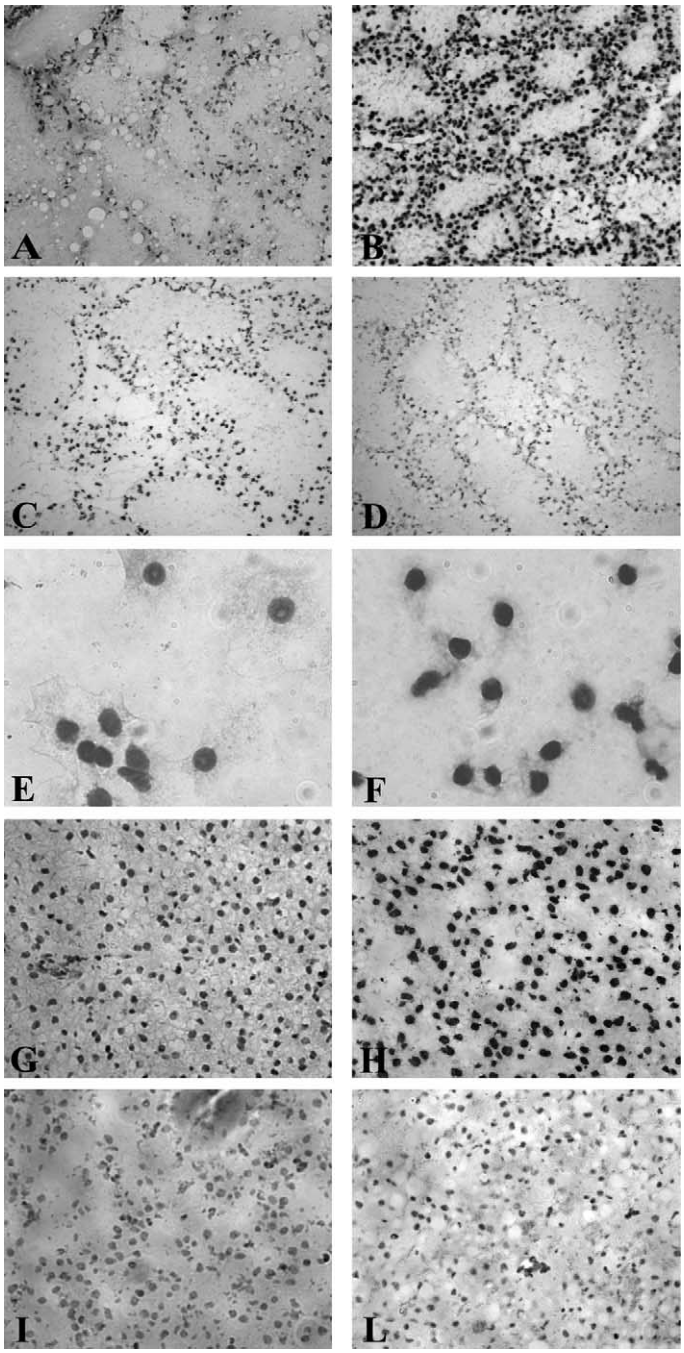

Fig. 1. Immunoperoxidase detection of 8-oxo-dG in digestive glands and heamocytes of mussel *M. galloprovincialis* and in liver of eel *A. anguilla* treated with or without B[*a*]P. (A) Tissue section of control mussel, (B) exposed mussel, (C) exposed mussel stained without the primary antibody and (D) exposed mussel stained after DNase digestion (magnification 200×). (E) Haemocytes of control mussel and (F) exposed mussel (magnification 1000×). (G) Liver sections of control eel, (H) exposed eel, (I) exposed eel stained without the primary antibody and (L) exposed eel stained after DNase digestion (magnification 400×).

Table 1
Relative staining intensity for immunoperoxidase detection of 8-oxo-dG in digestive gland and haemocytes of mussel and in eel liver

Treatment	Digestive gland		Haemocytes		Treatment	Liver	
	Staining intensity[a]	RD[b]	Staining intensity[a]	RD[b]		Staining intensity[a]	RD[b]
Control	0.396 ± 0.154		0.670 ± 0.234		Control	0.305 ± 0.061	
B[*a*]P 100 ppb	0.514 ± 0.270*	1.30	0.969 ± 0.137*	1.45	B[*a*]P 0.1 mg/kg	0.615 ± 0.053*	2.02
B[*a*]P 500 ppb	0.750 ± 0.174*	1.89	0.952 ± 0.060*	1.42	B[*a*]P 1 mg/kg	0.611 ± 0.030*	2.00
B[*a*]P 1000 ppb	0.816 ± 0.132*	2.06	1.002 ± 0.041*	1.50	B[*a*]P 10 mg/kg	0.640 ± 0.022*	2.10
Cu 60 μg/l	0.683 ± 0.017*	1.73	0.946 ± 0.129*	1.41	B[*a*]P 50 mg/kg	0.761 ± 0.150*	2.50

[a] Mean nuclear staining intensity ± S.D.
[b] RD, relative damage compared to control.
* $P < 0.05$ for comparison to control group.

4. Discussion

The development of methods to assess the level of DNA damage in marine organisms is of great interest for ecotoxicological investigations on the effects and the causal mechanisms of damage induced by environmental pollutants. Among the several DNA alterations commonly measured such as strand breaks, micronuclei and adducts (Canova et al., 1998; Lee and Kim, 2002; Akcha et al., 2003; Dixon et al., 2002), detection of 8-oxo-dG is recognized as an useful marker of oxidative-mediated genotoxicity (López-Barea and Pueyo, 1998).

Physico-chemical assays, such as HPLC–ECD or GC–MS have good selectivity and sensitivity and are the most used approaches for monitoring 8-oxo-dG and other modified bases in ecotoxicology. However, some methodological improvements were recently emphasized as crucial for field research (Helbock et al., 1998; Beckman et al., 2000; Huang et al., 2001). For example noticeable discrepancies were obtained in different laboratories, with estimates of 8-oxo-dG varying over almost one order of magnitude using the same method (Halliwell, 1999). More recently, this problem was addressed by the "European Standard Committee on Oxidative DNA Damage" (ESCODD) with an inter laboratory study to define baseline levels of 8-oxo-dG from standard biological matrices. Using almost identical chemical procedures (HPLC–ECD or GS–MS) on the same pig liver and human Hela cell line, quantitative results on 8-oxo-dG were distributed over three orders of magnitude, in different European laboratories, (ESCODD, 2002). The artificial oxidation of guanine to 8-oxo-dG and/or incomplete DNA hydrolysis are considered the main drawbacks that may occur during the analysis (Halliwell, 1999). The protocol for DNA extraction has been found to influence background level of 8-oxo-dG in cultured human cells (Ravanat et al., 2002).

Among the in situ assays already used to detect oxidative DNA damage, some modifications of the comet assay have applied for monitoring levels of 8-oxo-dG (Collins and Dušinká, 2002; ESCODD, 2002). Although a general consensus is still to be reached about exact values of 8-oxo-dG in standard biological samples, the improvement of methodologies to reveal such oxidative DNA damage and their integration into environmental toxicology, would be of utility in understanding the environmental impact of genotoxins.

In the present work, we explored for the first time the applicability of a potential new approach for marine organisms based on the immunohistochemical detection of 8-oxo-dG with monoclonal antibody 1F7. Antibody 1F7 was originally developed to specifically detect 8-oxo-dG in humans (Yin et al., 1995) and has been successfully used to investigate oxidative DNA damage resulting from environmental exposures (Yarborough et al., 1996; Calderon-Carciduenas et al., 1999). Compared to methods measuring 8-oxo-dG in DNA from whole tissue homogenates, the detection of oxidative DNA damage directly on fixed tissues or cells can provide additional indications on the dynamics and morphological distribution of cell responses; in fact, various cell types within a target tissue may exhibit different susceptibility to genetic insult and non homogeneous responses to toxicity mediated by single or multiple chemical stressors.

Additional advantages of the immunohistochemical approach include the limited processing of samples (liquid nitrogen freezing, cryostat sections, brief fixation and staining) and no DNA extraction or nuclear lysis (thus limiting an important source of artificial oxidation). There is no need for freshly collected tissues and the assay can be easily performed on frozen samples maintained in liquid nitrogen. This suggest the assay will be quite helpful under field conditions. Moreover, limited amounts of tissues or cells are required and the method is potentially able to discriminate different susceptibility to genotoxic insult for various cell types within complex tissues. Disadvantages of the antibody approach include the cross-reactivity and the semiquantitative detection of the oxidative DNA damage, microscopically assessed, that does not provide absolute values of 8-oxo-dG. However, because of the rapidity and the relatively low cost, these antibodies are an interesting tool that should be further investigated for environmental toxicological studies.

The biological models utilized in this work were the digestive gland and haemocytes from the common mussels *M. galloprovincialis* and the liver from the European eel *A. anguilla*. These tissues, typical targets for both bioaccumulation and biological effects of pollutants, demonstrated the possibility of immunoperoxidase reaction to detect 8-oxo-dG in marine organisms.

Our findings confirmed the genotoxic properties of B[*a*]P in mussels (Mitchelmore et al., 1998; Canova et al., 1998; Akcha et al., 2000a); genetic lesions were detected in the digestive gland of mussels exposed to 100 ppb B[*a*]P and further increased at higher doses, although similar levels of 8-oxo-dG were measured in organisms exposed to 500 and 1000 ppb. The absence of a dose-effect relationship was observed also by Canova et al. (1998) in *M. galloprovincialis* exposed for 24 and 72 h at 100, 500 and 1000 ppb B[*a*]P. The increased level of 8-oxo-dG is consistent with the enhancement of intracellular ROS production and with the altered efficiency of antioxidant defenses. In this respect, it has been reported that aromatic hydrocarbon quinones, including those derived from B[*a*]P, have the potential to increase ROS formation by microsomes of mussel digestive gland (Martinez and Livingstone, 1995; Sjölin and Livingstone, 1997). Polycyclic aromatic hydrocardons including B[*a*]P have also been shown to mediate oxidative toxicity in mussels through the impairment of cellular antioxidant systems, both in field and laboratory conditions (Solé et al., 1995; Akcha et al., 2000b; Cheung et al., 2001).

A considerable increase in 8-oxo-dG was observed after copper exposure, indicating the oxidative insult as an important pathway of toxicity for this element. Transition metals (like Fe and Cu) can enhance intracellular ROS generation via Fenton-like reactions (Winston and Di Giulio, 1991) and the effect of copper as a pro-oxidant stressor has already been documented in marine mussels (Regoli and Principato, 1995; Regoli et al., 1998; Canesi et al., 1999; Regoli, 2000). Organisms exposed in both field and laboratory conditions exhibited variations in antioxidant defenses and accumulation of oxidative products, confirming the importance of ROS-mediated toxicity for metal contaminants (Regoli et al., 1998; Regoli, 1998).

Oxidative DNA damage was detectable also in mussel haemocytes, but variations between control and exposed organisms were more limited compared to those described for digestive glands. These results seem influenced by the high basal levels of 8-oxo-dG in mussel haemocytes and should not be interpreted as a limited relevance of the genotoxic injury to these cells. Haemocytes contain a wide array of antioxidant enzymes (including catalase, different superoxide-dismutases and glutathione peroxidases, Pipe et al., 1993) which have been reported to counteract enhanced generation of oxygen radicals caused by quinone redox-cycling (Winston et al., 1996). Intracellular levels of these antioxidants can be overwhelmed also without the involvement of pro-oxidant chemicals (Winston et al., 1996), i.e. when blood cells, to fulfill normal immunological functions, release ROS during active phagocytosis. The high oxidative pressure caused by this essential role could possibly explain the naturally elevated DNA damage in these cells under basal conditions.

The immunohistochemical approach also was suitable for detecting 8-oxo-dG in fish liver sections. For fishes, lipid reserves normally accumulate within liver fat-storing cells. Although not necessary, a lipase and/or amylase treatment might further improve antibody accessibility during the immunohistochemical staining.

B[*a*]P induced a remarkable pattern of liver DNA damage, generating a significant increase of 8-oxo-dG although a clear dose-dependent relationship was not observed. These results are consistent

with the enhancement of endogenous ROS, during the rapid metabolism of PAHs through the activity of cytochrome P450 (Pacheco and Santos, 1997; Schlezinger and Stegeman, 2000). The biotransformation pathway for B[*a*]P is also known to produce highly reactive metabolites causing further damage to DNA (Peters et al., 1997; Ploch et al., 1998; van Schanke et al., 2003; Willett et al., 2001; Regoli et al., 2002).

Although these results must be considered preliminary, laboratory exposures were helpful to investigate the consistency of the immunohistochemical approach and the efficacy of antibody 1F7 in marine organisms. Future goals of this work will include the extension of the chemical substances assayed with the immunoperoxidase stainings and the possible improvement of the assay sensitivity (i.e. by use of FITC-conjugated antibodies). Field investigations will be necessary to validate the immunohistochemical approach as a tool for detecting oxidative DNA damage in environmental conditions, however, the presented evidence suggest that the method might have applications in marine ecotoxicology.

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