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Parental exposure to the herbicide diuron results in oxidative DNA damage to germinal cells of the Pacific oyster *Crassostrea gigas*

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ABSTRACT

Chemical pollution by pesticides has been identified as a possible contributing factor to the massive mortality outbreaks observed in *Crassostrea gigas* for several years. A previous study demonstrated the vertical transmission of DNA damage by subjecting oyster genitors to the herbicide diuron at environmental concentrations during gametogenesis. This trans-generational effect occurs through damage to genitor-exposed gametes, as measured by the comet-assay. The presence of DNA damage in gametes could be linked to the formation of DNA damage in other germ cells. In order to explore this question, the levels and cell distribution of the oxidized base lesion 8-oxodGuo were studied in the gonads of exposed genitors. High-performance liquid chromatography coupled with UV and electrochemical detection analysis showed an increase in 8-oxodGuo levels in both male and female gonads after exposure to diuron. Immunohistochemistry analysis showed the presence of 8-oxodGuo at all stages of male germ cells, from early to mature stages. Conversely, the oxidized base was only present in early germ cell stages in female gonads. These results indicate that male and female genitors underwent oxidative stress following exposure to diuron, resulting in DNA oxidation in both early germ cells and gametes, such as spermatozoa, which could explain the transmission of diuron-induced DNA damage to offspring. Furthermore, immunostaining of early germ cells seems indicates that damages caused by exposure to diuron on germ line not only affect the current sexual cycle but also could affect future gametogenesis.

1. Introduction

The living cell is constantly exposed to potentially-damaging free radical species of endogenous origin, such as those arising from normal cellular metabolism, or exogenous origin, resulting from exposure to ultraviolet radiation, ionizing radiation or xenobiotics (Evans et al., 2004). Exposure of aquatic organisms to chemical pollutants can hence promote an increase in the production of reactive oxygen and nitrogen species (ROS/RNS) (Alves de Almeida et al., 2007). When the rate of ROS/RNS production exceeds the efficiency of antioxidant defenses and repair systems, an imbalance occurs in the redox status and oxidative stress can arise, leading, inter alia, to the oxidation of key cell components such as proteins, fatty acids and DNA, thereby contributing to toxicity (Sies, 1993). Among chemicals, certain pesticides are known to induce oxidative stress (Lushchak, 2011). Pesticides are ubiquitous pollutants of aquatic systems. In France, 93% of rivers are contaminated by pesticides (SoeS, 2013). In addition to inputs by rivers, pesticides used mainly for agricultural purposes can be dispersed to coastal waters through various processes, including run-offs, leaching and spray drift. Marine environments and, in particular, coastal ecosystems, are often considered as the end receptacle of chemical pollutants. Coastal areas are usually characterized by high primary production, supporting the development of numerous species, e.g. shellfish farming zones. The Pacific oyster, *Crassostrea gigas* (Thunberg), was introduced into France in 1966 (Grizel and Héral, 1991) and is now the most-cultivated bivalve species. With annual production reaching 82,000 tons in 2012 (FAO, 2014), France is currently Europe's leading country for oyster production. For several years, this species has been facing mass summer mortality events; the causes of these are undefined, but appear to be multifactorial and include physiological stress, infection by pathogenic organisms and environmental conditions (Renault et al., 1994; Samain and McCombie, 2008; Dégremont et al., 2010; Huvet et al., 2010). Chemical pollution by pesticides has been identified as one of the possible factors involved in this mortality phenomenon, due to their toxic

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effects on oysters (His and Seaman, 1993; Gagnaire et al., 2007; Buisson et al., 2008; Wang et al., 2009; Akcha et al., 2012; Mai et al., 2014; Ochoa et al., 2012; Mottier et al., 2014). In the Ebro delta (Spain), Köck et al. (2010) found a correlation between pesticide concentrations in water and shellfish flesh and episodes of mortality. Agrochemical inputs are known to display seasonal variations: in France, high pesticide concentrations have been detected in spring in a major shellfish farming zone, the Marennes Oleron basin, corresponding to the highly-sensitive oyster gametogenesis period (Munaron, 2004; Soletchnik et al., 2005; Burgeot et al., 2008). Pesticides have not only direct toxic effects on adult oysters, but also indirect trans-generational effects on their offspring (Bouilly et al., 2003, 2007; Barranger et al., 2014, 2015). In a previous experiment conducted by our laboratory, oyster genitors in gametogenesis were subjected to short exposures (two 7-day pulses) of environmental concentrations of the herbicide diuron (0.3 $\mu g \cdot L^{-1}$). The genotoxicity of diuron was demonstrated in genitor hemocytes. Moreover, DNA damage was also detected for the first time in genitor spermatozoa (strand breaks) and in offspring (DNA aneuploidy in spat), highlighting the vertical transmission of DNA damage further to parental exposure (Barranger et al., 2014). The genotoxicity of diuron could result from oxidative stress. The oxidation of DNA by ROS/RNS can actually produce strand breaks and a variety of modified DNA bases. Base oxidation is probably the foremost source of DNA damage. Among the four normal nucleobases, guanine (Gua) is the most susceptible to oxidation due to its low oxidation potential. The interaction of HO• (the most reactive oxygen-free radical) with DNA strand nucleobases, such as guanine, leads to the formation of C8-hydroxyguanine (8-OHGua), or its nucleoside form deoxyguanosine (8-hydroxy-2′-deoxyguanosine). 8-oxodGuo is the most abundant oxidized nucleobase found in DNA and is widely used as a marker of DNA damage, carcinogenesis and oxidative stress in humans (Halliwell and Aruoma, 1991). If it is not removed by DNA repair systems, 8-oxodGuo can result in mutagenesis by G:C to T:A transversions during DNA synthesis (Shibutani et al., 1991). Its formation has also been reported in bivalves following exposure to pollutants or exposure in the field. It has been put forward and used in ecotoxicology as an efficient marker of both oxidative stress and genotoxicity (Canova et al., 1998; Akcha et al., 2000a; Aloisio Torres et al., 2002; Charissou et al., 2004; Almeida et al., 2005; Lemiere et al., 2005; Alves de Almeida et al., 2007).

Our previous results suggest that the vertical transmission of DNA damage occurs through damage to genitor-exposed gametes. In order to further our understanding of diuron genotoxicity, gonad tissue of genitors originating from our previous experiment (Barranger et al., 2014) was analyzed for the detection of 8-oxodGuo. Two different methods were used for this study. Firstly, 8-oxodGuo levels in gonad tissue were measured using high-performance liquid chromatography coupled with UV and electrochemical detection (HPLC–UV–ECD). In a second step, immunohistochemical detection of 8-oxodGuo was performed to locate base damage to the various types of cells present in gonadal tubules and, particularly, in germ line. Our results should contribute to improving understanding of diuron genotoxicity in oysters and how genitor exposure can significantly impact the DNA integrity of the following generation, with potential effects on oyster physiology at a population level.

2. Materials and methods

2.1. Genitor origin and diuron exposure

The adult Pacific oysters (*C. gigas*) used for this experiment were progenies of wild oysters sampled in the Marennes-Oléron Bay (France). Oyster husbandry/broodstock conditioning and diuron exposure were performed as described by Barranger et al. (2014). Briefly, the oysters were acclimatized for one month at the hatchery. Next, sea water temperature (8 °C ± 1 °C) was gradually raised by 2° per day for 1 week, to reach 19.8 °C (±0.3 °C). Once gonad development had begun, the oysters were divided into three experimental groups: a seawater control, a solvent control and a diuron-exposed group. Three 250-L tanks were used for each experimental group, each containing 240 oysters. Two 7-day exposure periods took place at the start and mid-course of gametogenesis. Diuron – the pesticide selected for our study – is a substituted urea herbicide used in agriculture for on-land weed control. This herbicide is also used as an antifouling biocide (Thomas et al., 2001). In France, its use as a phytosanitary product has been banned since 2008 (The Official Journal of the French Republic, 2007), and as a biocide used in antifouling paints since 2009 (The Official Journal of the French Republic - bylaw, 2008). However, diuron is still the fourth most commonly-found pesticide in French rivers (SOeS, 2013) and recent studies have reported its presence in various French coastal waters (Atlantic bays, estuaries and Mediterranean Sea) (Buisson et al., 2008; Munaron et al., 2012; Caquet et al., 2013). The oysters were exposed to nominal diuron concentrations of 0.4 and 0.6 $\mu g\ L^{-1}$, respectively. However, the analysis of passive samplers (polar organic chemical integrative samplers, POCIS) used in the previous experiment showed oyster exposure to integrated concentrations as low as 0.2 and 0.3 $\mu g\ L^{-1}$.

2.2. Sampling program

Various gonad samples were collected after completion of genitor exposure. For HPLC–UV–ECD analysis, the gonads were sampled and stored in liquid nitrogen prior to analysis. 10 males and 10 females were analyzed in each experimental group. For histological analysis, transverse sections (5 mm) cut in the vicinity of gill–palp junction were fixed in Davidson's solution (48 h; 10% glycerol, 20% formaldehyde, 30% ethanol (95%), 30% sterile sea water, 10% acetic acid), then stored in 70% ethanol. 5 males and 5 females were analyzed in each experimental group. For HPLC–UV–ECD and histological analyses, each sample/individual was realized in duplicate.

2.3. Quantification and location of oxidative DNA damage

2.3.1. Measurement of 8-oxodGuo levels in genitor gonads using HPLC/UV–ECD

2.3.1.1. DNA extraction. For each gonad sample, DNA was extracted from 100–150 mg of gonad tissue using the chaotropic NaI method derived from Helbock et al. (1998), slightly modified by Akcha et al. (2000b). The samples were centrifuged at 1500 *g* for 10 min at 4 °C. Supernatants were discarded and the pellets were suspended in 2 mL of Buffer A (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris–HCl, 0.1 mM deferoxamine mesylate, 1% Triton X-100, pH 7.5). Following centrifugation (1500 *g*, 10 min, 4 °C), the pellets were recovered and resuspended in 600 μL of Buffer B (5 mM EDTA-Na_2, 10 mM Tris–HCl, 0.15 mM deferoxamine mesylate, pH 8). After addition of 35 μL of 10% SDS, RNA digestion was performed by incubation with 120 μg of RNase A and 20 U of RNase T1 for 15 min at 50 °C. Protein digestion was performed by incubation with 600 μg of protease for 1 h at 37 °C. The samples were then centrifuged at 5000 *g* for 15 min at 4 °C, and supernatants were recovered in 15 mL sterile tubes. After the addition of 1.2 mL of sodium iodide solution (20 mM EDTA-Na_2, 7.6 M NaI, 40 mM Tris–HCl, 0.3 mM deferoxamine mesylate, pH 8) and 2 mL isopropanol, the tubes were centrifuged for 15 min at 5000 *g*. The pellets were then recovered and resuspended in 2 mL 40% isopropanol. After centrifugation (5000 *g*, 15 min, 4 °C), the pellets were washed in 2 mL of 70% glacial ethanol and centrifuged at 5000 *g* for 5 min at 4 °C. Ethanol was then discarded using a pipette, and the pellets were left to dry for 1 h at room temperature. DNA was finally resuspended in 100 μL of deferoxamine mesylate 0.1 mM and left to dissolve overnight at 37 °C. DNA quantification was performed by spectrophotometry at 280, 260, and 230 nm wavelengths using a ND1000 NanoDrop (NanoDrop Technologies, Inc.). After quantification, the DNA samples were stored at −20 °C prior to digestion.