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Genotoxic effects of polychlorinated biphenyls (PCB 153, 138, 101, 118) in a fish cell line (RTG-2)

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ABSTRACT

Polychlorinated biphenyls (PCBs) are persistent pollutants in aquatic environments, often causing the decline or disappearance of wild populations. The primary aim of this study was to investigate the genotoxic effects of some PCBs (PCB153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) and 138 (2,2′,3,4,4′,5′-hexachlorobiphenyl), both non-dioxin-like compounds, and the pentachlorobiphenyls PCB118 (2,3′,4,4′,5-) and 101 (2,2′,4′,5,5′-), the former an ortho-substituted, low-affinity dioxin-like compound and the latter a non-coplanar congener classified as non-dioxin-like) in fish cells (RTG-2). These congeners are mostly present in surface waters and in edible aquatic organisms and the loss of DNA integrity *in vitro* serves as a sensitive biomarker of cytogenetic alterations and is considered as an initial step for the identification of genotoxic effects.

The alkaline comet assay and the micronucleus test show clear genotoxic damage after short and longer exposure (2 and 24 h) to maximum soluble, non-cytotoxic doses, evident sooner with PCBs 101 and 118. Oxidative stress situations involving ROS release, reduction in total GSH, lipid peroxidation and alteration to superoxide dismutase, seen after exposure with all the congeners, though with different kinetics, seem the most likely explanation for the genotoxic damage. This appears to be confirmed by the modified comet assay (pH 10) for detection of oxidized bases using endonuclease III. The increased generation of intracellular ROS might explain the apoptosis seen after treatment with the single PCBs and evaluated on the basis of the rise in 3–7 caspase activity. Therefore both the non-coplanar, non-dioxin-like PCBs (153, 138, 101) and the low-affinity dioxin-like compound PCB118 cause evident genotoxic damage, probably as a consequence of oxidative stress.

1. Introduction

The continuous presence of genotoxic chemicals in the aquatic environment is of major concern as regards its effects on the health status of biota. DNA integrity is one of the biomarkers of pollution (Sarkar et al., 2006) so much effort is aimed at finding speedy, sensitive *in vitro* and *in vivo* methods giving information on potential genotoxicity. Single-cell gel electrophoresis (the "comet" assay) and the micronucleus test seem sensitive enough to detect the genotoxic responses of aquatic organisms to environmental pollution (Jha, 2008; Sánchez et al., 2000).

Some of the most persistent, ubiquitous contaminants are the polychlorinated biphenyl compounds (PCBs), though found only at low concentrations (Domingo and Bocio, 2007; Brown et al., 2006; Ross, 2004). In this study we used PCB 101 (2,2′,4′,5,5′-pentachlorobiphenyl), 138 (2,2′,3,4,4′,5′-hexachlorobiphenyl) and 153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) as non-dioxin-like (NDL) compounds, and PCB 118 (2,3′,4,4′,5-pentachlorobiphenyl) as a dioxin-like (DL) compound; these are considered sentinels indicating the levels of contamination of marine environments (Garritano et al., 2006; Danis et al., 2006).

The expert panel of the European Food Safety Authority (EFSA) concluded that we need to improve the understanding of the environmental and human risks associated with NDL PCBs, because of their abundance in food and human tissues (Stenberg and Andersson, 2008).

Better documented are the toxic effects of PCB mixtures in different parts of the world and on the autochthonous aquatic species in the various zones (Ohe et al., 2004; Bordajandi et al., 2006; Perugini et al., 2006; Ohyama et al., 2004; Howell et al., 2008; Davis et al., 2007; Jorgensen et al., 2006). Although generally 90% of the PCBs found in animal samples or sediments are NDL, the specific risk does not appear to have been assessed for these congeners. In addition, the experimental data employed to assess their

Abbreviations: MN, micronuclei; ROS, reactive oxygen species; LPO, lipid peroxidation; SOD, superoxide dismutase activity; MMC, mitomycin-C; B(α)P, benzo(α)pyrene.

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toxicity are often complicated by the fact that the technical mixtures used contain DL and NDL PCBs, plus toxicologically important impurities (Knerr and Schrenk, 2006).

The genotoxicological problems of these mixtures have generally not been studied much. Oxidative DNA damage assessed on the basis of high levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the liver and kidneys of female cetaceans has been reported (Li et al., 2005) *In vitro*, PCBs were not genotoxic in *Salmonella typhimurium* or V79 Chinese hamster cells; only in rat liver did lower chlorinated congeners lead to covalently modified macromolecules including proteins and DNA (Knerr and Schrenk, 2006).

An *in vitro* test using cells from homeothermic species is a powerful alternative to the use of live animals in the first tier of the genotoxicity testing required for chemicals (OECD guidelines). Besides not needing vertebrates, the advantages of *in vitro* assays include cost, versatility, volume of waste, and laboratory facilities required. The established fish cell line RTG-2, from the rainbow trout (*Oncorhynchus mykiss*) has been successfully used in cytotoxicity research as an alternative to acute fish bioassays and provides extremely useful results in cytotoxicity and genotoxicity studies, because this line maintains some ability to metabolize xenobiotics without the need for an exogenous metabolic system (Zurita et al., 2007; Ferrero et al., 1998).

In this study we investigated the genotoxic damage caused by PCBs, as predicted by two tests with different endpoints: DNA strand breaks and alkali-labile sites, using single-cell gel electrophoresis (comet assay and modified comet assay with lesion-specific bacterial repair enzymes, endonuclease III), and cytogenetic damage using the micronucleus assay for clastogenic and aneugenic effects.

Although we know little about the metabolism of these compounds, which are not classically planar hence, not CYP1A1 substrates, it is conceivable that they form hydroxylated metabolites through a CYP2B-dependent mechanism, and these might be the cause of various aspects of the toxicity. Also worth investigating is the potential for oxidative damage at different exposure times, on the basis of ROS release, lipid peroxidation, and changes in cell antioxidant systems.

Therefore the present study was designed: (1) to see whether the single PCBs were genotoxic at doses causing no cytotoxicity in the RTG-2 cell line; (2) to evaluate a situation of oxidative damage correlatable with the genotoxicity and apoptotic response to the single PCBs.

2. Materials and methods

2.1. Chemicals

PCBs were obtained from Dr. Ehrenstorfer (Labor. Dr. Ehrenstorfer–Shafers, Augsburg, Germany) and dissolved in dimethylsulfoxide (DMSO, Fluka, Italy); the incubation solutions contained a final concentration of 0.1% DMSO. Mitomycin C (MMC) (Sigma–Aldrich, Italy), benzo-α-pyrene (B(α)P) (Sigma–Aldrich, Italy) and 30% hydrogen peroxide (H_2O_2) (Sigma–Aldrich, Italy) were used as positive controls respectively in the micronucleus (MN) test, the alkaline comet assay and the modified alkaline comet assay (pH 10). 5(6) Carboxy-2′,7′dichlorofluorescein diacetate was from Fluka-Italy and endonuclease III (Endo-III) from *Escherichia coli* was supplied by Sigma–Aldrich, Italy.

2.2. Cell culture

The RTG-2 cells, an established fibroblast-like line from gonadal tissue of the rainbow trout (*O. mykiss*), were obtained from the American Type Cell Culture collection (Istituto Zooprofilattico, Brescia, Italy). The cells were maintained in Minimal Essential Medium with Earle salt (MEM-Earle) (Sigma–Aldrich, Italy), supplemented with 10% fetal bovine serum (Biochrom, Spa Italy), penicillin (100 IU/mL), streptomycin (100 μg/mL), amphotericin B (25 μg/mL) and glutamine (2 mM) (Sigma–Aldrich, Italy) at an optimal growth temperature of 20 ± 1 °C without CO_2. When they reached the exponential growth phase cells were trypsinized and allowed to adhere for 24 h before exposure to the PCBs.

2.3. Cytotoxicity assay

The viability of the RTG-2 cells, seeded on 96-well plates and exposed to the different concentrations of PCBs for 24 h, was examined using the widely used and validated microculture 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cell viability was expressed as a percentage of the negative control (Caminada et al., 2006).

2.4. Micronucleus test

RTG-2 cells seeded in 25-cm^2 culture flasks in MEM were treated with the different PCBs and MMC (1 μM in DMSO) for 24 h. The medium from each well was removed and cells were trypsinized and centrifuged at 2000 rpm for 5 min. The number of cells in each sample was adjusted to 10^6.

We then employed the two-step procedure developed by Sánchez et al. (2000) adding 1 mL of solution I (584 mg/L NaCl, 1000 mg/L sodium citrate, 25 mg/L ethidium bromide, 10 mg/L RNase and 0.3 mg/L Nonidet P-40) to the resuspended cell pellet to disrupt the cell membranes. After 15 min at room temperature 1 mL of solution II (1.5% citric acid, 0.25 M sucrose and 40 mg/L ethidium bromide) was added and the suspension was agitated slightly. At this stage the suspension contained only cell nuclei and micronuclei (MN) and other unspecific particles (debris).

Intensities of MN and cell nuclei (N) were measured simultaneously in list mode using a FACTStar + Flow cytometer (Becton Dickinson, FACSCalibur). Ethidium bromide was excited by the 488 nm line of an argon laser. MN formation was calculated as the fold-increase over the negative control; 20,000 events were measured. (Kohlpoth, 1999).

2.5. Comet assay

2.5.1. Alkaline single-cell gel electrophoresis

The alkaline single-cell gel electrophoresis assay (comet assay) was done according to Singh et al. (1988) with some modifications. Cells were exposed to PCBs (at maximum soluble, non-cytotoxic doses) for 2 h and 24 h before preparation for analysis of DNA damage at pH > 13. We examined the slides under a fluorescent microscope (Axioplan 2, Zeiss), to visualize ethidium bromide-DNA fluorescence, with a filter combination of 485/530 nm (excitation/emission beam splitter). For each sample, 30–50 randomly selected nucleoids were acquired. Images of the fluorescently stained cell nuclei were analyzed using Metamorph 6.0 Imaging software (Universal Imaging Corp. USA).

The tail moment TM was chosen as the effect parameter, calculated according to the equation: TM = TL × Tail DNA% where TL is the tail length, i.e. the distance (μm) between the center of the comet head and the end of the comet tail. The Tail DNA is expressed as a percentage of the total DNA content based on the total fluorescence intensity.

2.5.2. Modified comet assay for oxidized bases

The comet assay modified with an extra step after cell lysis, in which DNA is digested with the repair enzymes, was done according to Collins et al. (1996). After cell lysis, the slides were washed