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Usefulness of RTL-W1 and OLCAB-e3 fish cell lines and multiple endpoint measurements for toxicity evaluation of unknown or complex mixture of chemicals

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ABSTRACT

Fish are currently used for the assessment of chemical toxicity. The REACh regulation and the European directive on the protection of animals used for scientific purposes both recommend the use of methods other than animal testing. In view of this, fish cell lines are increasingly used to provide fast and reliable toxic and ecotoxic data on new chemicals. The sensitivity of the Rainbow trout liver cell line RTL-W1 and Japanese medaka embryos cell line OLCAB-e3 were used with different toxicity endpoints, namely cytotoxicity, EROD activity, ROS production and DNA damage for various classes of pollutants displaying different modes of action but also with complex environmental mixtures. Toxicity tests were coupled with chemical analysis to quantify the chemical concentrations in cell cultures. Differences in sensitivity were found between fish cell lines. MTT reduction assay revealed that OLCAB-e3 cells were more sensitive than RTL-W1 cells. On the contrary, RTL-W1 gave higher response levels for the Fpg-modified comet assay and ROS assay. The OLCAB-e3 cell line did not express EROD activity unlike RTL-W1. This study highlights the capacity of the two different fish cell lines to measure the toxicity of individual toxicants but also environmental mixtures. Then, results obtained here illustrate the interest of using different cell lines and toxicity endpoints to assess the toxicity of complex or unknown mixture of chemicals.

1. Introduction

Pollution of aquatic ecosystems by an increasing number of chemical compounds requires the development of methods and tools to accurately evaluate the effects of pollutants on aquatic organisms (Castaño et al., 2003). Indeed, numerous and various chemical compounds are discharged in the environment and represent a potential threat for the biota particularly when they are present in mixture. A major concern of the EU is to assess the toxicity of new substances before they are put on sale (REACh) and to monitor and control the release of toxic substances in aquatic environments (WFD (European union, 2000), DCSMM (European union, 2008)) while respecting the protection of animals used for scientific purposes (Directive 2010/63/EU (European Union, 2010)). Today, there are a number of economic, scientific and ethical reasons for supporting efforts to develop and apply in vitro assays in aquatic ecotoxicology as alternative tools to animal testing (Castaño et al., 2003). These assays aim to quickly obtain reliable data on the toxic and ecotoxic properties of chemicals, while considering ethical concerns about animal testing. They must meet the 3R principle by replacing animal models whenever possible, reducing the number of animals used in experiments, and refining the procedure and breeding conditions applied to animals. The application of fish cell cultures is being considered as an alternative to whole fish assays in the testing of aquatic pollutants or in the monitoring of aquatic environment quality. They have the advantage of providing a specific screening tool, easy, fast, and cost-effective using a small volume of test substance. Fish cell lines can also be useful as a model to study molecular mechanisms of toxicity. RTL-W1 (Lee et al., 1993), cell line from rainbow trout liver and OLCAB-e3 (Hirayama et al., 2006), derived from Japanese medaka embryos are part of fish cell lines developed in recent years. RTL-W1 cell line has been widely used for the measurement of different toxicity endpoints including cytotoxicity (Dayeh et al., 2005), genotoxicity (Brinkmann et al., 2014; Kienzler et al., 2012, 2013), 7-ethoxyresorufin-O-deethylase (EROD) activity (Behrens et al., 1998; Hinger et al., 2011) and reactive oxygen species (ROS) production (Pietsch et al., 2011). On the contrary, very few studies have been

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carried out to date on fish embryonic cell lines. These cell lines have been isolated from a limited number of fish species including Japanese flounder (Chen et al., 2004), Marine medaka (Lee et al., 2015), Zebrafish (Pereira et al., 2012; Šrut et al., 2015) or Japanese medaka (Hirayama et al., 2006). The OLCAB-e3 cell line, along most of the medaka cell lines, has yet to have its response to chemicals examined in depth. However, the use of embryonic cell line could be suitable for studying toxicity of chemicals due to their low xenobiotic biotransformation capacities. In addition, results obtained could be compared or coupled with data generated with embryo-larval assay, another model accepted as alternative tools by EU's legislation.

Numerous bioassays have been developed over the last years based on cell lines. Among assays currently used to determine the toxicity of environmental contaminants the MTT viability assay is based on the mitochondrial metabolic function in living cells by measure of dehydrogenase enzyme activity (Mosmann, 1983). In numerous ecotoxicological studies, the induction of EROD activity has been accepted as a valuable exposure biomarker to aryl hydrocarbon receptor (AhR) inducers e.g. dioxin-like and polycyclic aromatic hydrocarbons (PAH) compounds, in particular in fish (Behrens et al., 2001; Kienzler et al., 2012; Laville et al., 2004). The genotoxicity of environmental pollutants has previously been monitored in fish cells (Devaux et al., 1997; Kienzler et al., 2012). The classic comet assay in alkaline conditions detects breaks in DNA strands (Singh et al., 1988; Morin et al., 2011). This test has received considerable attention in toxicology and ecotoxicology, as it is a sensitive, fast and cheap method. The comet assay sensitivity can be significantly increased by the use of a DNA repair enzyme, the formamidopyrimidine DNA-glycosylase (Fpg). The use of this enzyme, recommended by the European Standards Committee on Oxidative DNA Damage (ESCODD) improves the sensitivity of the comet assay through the detection of oxidative damage (Collins et al., 2003). It was successfully applied to various environmental pollutants on RTL-W1 cells, and made it possible to lower the detection limit of DNA damage compare to the standard assay (Kienzler et al., 2013). During their aerobic metabolism, cells generate naturally reactive oxygen species (ROS) which can generate oxidative damage. ROS production can be increased by the exposure to certain pollutants such as polychlorobiphenyls (PCBs) (Coteur et al., 2001), copper, paraquat or benzo(a)pyrene B(a)P (Gomez-Mendikute and Cajaraville, 2003). One of the most widely used techniques to measure ROS production uses permeable fluorescent and chemiluminescent probes: H2DCFDA, 2′-7′-dichlordihydrofluorescein diacetate (Eglon et al., 2010). In this study, a chloromethyl derivative of these probes, CM-H2DCFDA, was used. This indicator shows a much better retention in living cells than H2DCFDA. CM-H2DCFDA diffuses passively into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chlorormethyl group reacts with intracellular glutathione and with other thiols. The resulting oxidation produces a fluorescent adduct, which is trapped inside the cell, facilitating long-term studies.

In this context, our study aimed to compare the cytotoxicity, genotoxicity, ROS production and 7-ethoxyresorufin-*O*-deethylase (EROD) activity cause by model pollutants and environmental mixture using two cell lines (RTL-W1 and OLCAB-e3). RTL-W1 cell line was selected because it is widely used in ecotoxicology and very well-known cell line. The embryonic medaka cell line was selected because Japanese Medaka is increasingly used for toxicity testing and is recommended in various OECD guidelines (OECD 210, 212, 240) (OECD, 1998, 2013, 2015). The embryonic stem cells are also of interest because they are pluripotent and are thought to be more sensitive to toxicants because of partially or totally inefficiency detoxification and repair systems. Another point of importance of the embryonic cell line is its shorter generation time compared to adult cells, allowing reduced experimental time. To date, embryonic cell lines are still unexploited and limited in applications (Pereira et al., 2013; Šrut et al., 2015). Therefore, embryo cell lines complement the experimental tools that are already available for assessing the toxicity of chemicals. We choose model pollutant with different modes of action: methyl methane sulfonate (MMS) is an alkylating agent not naturally found in the environment, cadmium chloride ($CdCl_2$), an oxidative stress generator, benzo[*a*]pyrene (B(a)P), a pro-genotoxic PAH, and the polychlorinated biphenyl 126 (PCB126), a dioxin-like compound. Two environmental mixtures of PAHs from light or heavy oil were also tested on the same fish cell lines.

2. Material and methods

2.1. Cell lines

Two cell lines were used for this study. RTL-W1 is a fibroblast-like non transformed permanent cell line taken from the liver of adult rainbow trout (*Oncorhynchus mykiss*) (Lee et al., 1993). It is a sensitive tool for assessing the toxic potential of chemicals due to its capacity to biotransform lipophilic xenobiotics (Lee et al., 1993). OLCAB-e3 is an embryonic medaka cell line established by Mitani et al. in 2006 (Hirayama et al., 2006). Cells were cultured routinely in 75 cm^2 culture flasks (Cell start ® cell culture Flask Greiner) at 20 °C for RTL-W1 and at 28 °C for OLCAB-e3 in CO_2 free incubators. Cell lines were cultivated in Leibovitz's (L-15) medium supplemented with 5% fetal bovine serum (FBS) for RTL-W1 or with 20% FBS and 10 mM HEPES for OLCAB-e3. Penicillin (100 IU/mL) and streptomycin (100 lU/mL) were added to the medium. These mediums allowed optimal growth of both cell lines. Experiments were carried out with cells aged from passage 62–75 for RTL-W1 and from 10 to 25 for OLCAB-e3.

2.2. Chemicals

2.2.1. Model compounds

Cadmium chloride (CdCl2, CAS Number: 10108-64-2) and methyl methane sulfonate (MMS, CAS Number 66-27-3) stock solutions were prepared in ultrapure water while benzo(a)pyrene (B(a)P, CAS Number: 50-32-8) and polychlorinated biphenyl 126 (PCB126, CAS Number: 57465-28-8) stock solutions were made in dimethylsulfoxide (DMSO).

2.2.2. Oil samples

Two kinds of oils provided by the Center of Documentation, Research and Experimentation on Accidental Water Pollution (CEDRE) were used in this study: Arabian Light Crude Oil (BAL 110; LO) and Erika Heavy Oil (HFO n°2; HO) (Guyomarch'h et al., 2001). These oils contain a large number of PAHs and alkylated PAHs (Le Bihanic et al., 2014b). Both oils were diluted at 10% in acetone after 10 min of sonication by ultrasound to fluidized and homogenized the oils. Concentration of diluted solution was 87 mg/mL for LO and 50 mg/mL for HO.

2.2.3. Exposure conditions

Comet assay was performed in 24-well plates, ROS measurement in 48-well plates and MTT assay or EROD activity were performed in 96-well plates. RTL-W1 or OLCAB-e3 were seeded 24 h prior to chemical exposure. Cell density was 200 000 cells/mL for RTL-W1 and 500 000 cells/mL for OLCAB-e3. After removing the medium, cells were exposed in triplicate to a large range of concentrations of MMS, $CdCl_2$, B(a)P and PCB126 or hydrocarbon mixture in total medium. For MTT assay and EROD activity, cell lines were contaminated from 22.00 mg/L of PCB126, 25.23 mg/L of B(a)P, 550.65 mg/L of MMS, 36.64 mg/L of $CdCl_2$ and from 1% of diluted solution of LO and HO (10% in acetone) with 1:1 (v:v) dilution. For Comet assay and ROS measurement, concentrations tested were 5.5, 25.5 and 55 mg/L for MMS, 0.13, 0.25 and 1.26 for B(a)P, 3×10^{-4}, 3.3×10^{-3} and 0.33 mg/L for PCB126, 0.02, 0.18, 0.92 and 1.83 mg/L for $CdCl_2$ and 0.001%, 0.01% and 0.1% of diluted solution of LO and HO. ROS measurement was realized only for model compounds. Cells were exposed to $CdCl_2$, B(a)P, PCB126, LO and HO for 24 h and 15 min for MMS. For chemicals dissolved in DMSO, two controls were performed: one with the maximum DMSO