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Alterations in gene expression levels provide early indicators of chemical stress during *Xenopus laevis* embryo development: A case study with perfluorooctane sulfonate (PFOS)

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ABSTRACT

In the present study, *Xenopus laevis* embryos were exposed to a range of perfluorooctane sulfonate (PFOS) concentrations (0, 0.5, 6, 12, 24, 48 and 96 mg/L) for 96 h in laboratorial conditions to establish toxicity along with possible gene expression changes. Mortality and deformities were monitored daily and head-tail length was measured at the end of the assay as an indicator of growth. At 24 and 96 h post-exposure (hpe), the mRNA expression levels of the genetic markers involved in general stress responses (*hsp70*, *hsp47*, *crh-a* and *ucn1*), oxidative stress (*cat.2* and *sod*), lipid metabolism (*ppard*) and apoptosis (*tp53* and *bax)* were analyzed by RT-qPCR. Malformations were significantly higher in the embryos exposed to the highest PFOS concentration (41.8% to 56.4%) compared to controls (5.5%) at 48, 72 and 96 hpe. Growth inhibition was observed in the embryos exposed to PFOS concentrations $\geq$ 48 mg/L. At 24 hpe, a statistically significant up-regulation of genes *hsp70*, *hsp47*, *ppard*, *tp53* and *bax* in relation to controls was found. Similar responses were found for genes *hsp70*, *hsp47*, *crh-a*, *ucn1*, *sod* and *ppard* at 96 hpe. Alterations in the mRNA expression levels indicated both a stress response to PFOS exposure during *X. laevis* embryo development, and alterations in the regulation of oxidative stress, apoptosis, and differentiation. These molecular alterations were detected at an earlier exposure time or at lower concentrations than those producing developmental toxicity. Therefore, these sensitive warning signals could be used together with other biomarkers to supplement alternative methods (i.e. the frog embryo test) for developmental toxicity safety evaluations, and as tools in amphibian risk assessments for PFOS and its potential substitutes.

1. Introduction

Perfluorooctane sulfonate anion (PFOS) is a synthetic compound that consists of a hydrophobic alkyl chain of eight fluorinated carbon atoms, with sulfonate as a hydrophilic end group. The term PFOS-related substance is used to refer to perfluorooctane sulfonic acid and some of its commercially important salts, which can be broken down in the environment to give PFOS. The chemical structure of PFOS confers it unique properties, such as an amphiphilic character, stability, and low surface energy. Due to these physicochemical properties, this substance has been widely used in industrial activities and consumer products, including stain-resistant coatings for fabrics and carpets, and lipophobic coatings for suitable paper products to be used with food, fire-fighting foams, mining and oil surfactants, floor polishes, insecticide formulations, mist suppressants for hard chromium plating, and hydraulic fluids for aviation (3M Company, 2003). PFOS has been manufactured and used in the last 60 years, which has led to its dispersal in the environment. This compound has been detected worldwide in numerous environmental samples up to nanograms per liter (ng/L) levels (Zareitalabad et al., 2013). However, higher levels could be found; for instance, a mean value as high as 1021 μg/L PFOS has been reported in effluents from the WWT pond of a manufacturing facility located in China (Wang

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et al., 2010). PFOS has been detected in various aquatic species (Houde et al., 2011). For example, concentrations from 36.8 to 125.9 μg/kg wet weight (ww) were found in the mussel, *Mytilus galloprovincialis*, (Cunha et al., 2005) and 35 to 290 μg/kg ww in the amphibian, *Lithobates clamitans*, (Giesy and Kannan, 2001).

According to the PFOS hazard assessment made by the Organization for Economic Cooperation and Development (OECD) in 2002, this compound is considered a pollutant of concern because of its persistence, bioaccumulation and potential toxic effects (OECD, 2002). The effect of aggregate carbon-fluorine bonds confers PFOS good stability and, consequently, this compound is resistant to degradation (Sáez et al., 2008). Hence, it persists in the environment for a long time. Since 2010, the use of PFOS has been restricted in the European Union (EU) after its inclusion in the Annex B of the Stockholm Convention on Persistent Organic Pollutants in 2009, meanwhile the continued production and use of PFOS were allowed in other territories, particularly in China (Wang et al., 2009).

Previous studies have shown that PFOS exposure caused acute and chronic toxicity in a wide range of aquatic organisms (reviewed in Giesy et al., 2010). Developmental toxicity of PFOS have also been studied, for instance in zebrafish; PFOS exposure to concentrations of 1 mg/L or above for 128 h was associated with numerous teratogenic effects, including epiboly deformities, hypopigmentation, yolk sac edema, tail and heart malformations, and spinal curvature (Shi et al., 2008). Furthermore, effects of PFOS at cellular and molecular levels have been studied during embryo development in some species of mammals, birds and fish. Among others effects, they include peroxisome proliferation in mouse embryos (Rosen et al., 2009), and oxidative stress and apoptosis in zebrafish embryos (Shi et al., 2008). Laboratory studies in amphibians using Frog Embryo Teratogenesis Assay-Xenopus (FETAX) have revealed a correlation between PFOS exposure and malformations (Palmer and Krueger, 2001; Rongguo et al., 2012). However, the biological effects of PFOS in developing amphibian embryos, compared to other vertebrate groups, have been poorly described at the molecular level. To our knowledge, no previous studies have focused on the potential effects of PFOS on gene expression during *Xenopus laevis* embryo development.

In response to ethical and regulatory demands to replace, reduce or refine animal usage for toxicity testing (i.e., the EU ban on animal testing for the Registration, Evaluation and Authorisation of Chemicals, REACH program), alternative methods need to be developed and implemented urgently. The amphibian embryo model may be a potential alternative to animal testing because, according to EU Directive 2010/63/EU on the protection of animals used for scientific purposes, the early-stage embryos of these animals are not considered to be protected. In addition, the integration of functional genomics into ecotoxicity tests can contribute to the 3Rs objectives. Understanding the molecular response to chemical exposure will improve our knowledge about the potential risk of pollutants in the environment. Particularly, the *Xenopus* embryo model has become an attractive system used to provide toxicological information at the molecular and genomic levels in a complex cellular context because the genome and transcriptome sequence data for this amphibian model are available, and this information continues to grow (Karpinka et al., 2014). This animal model has also been used as experimental systems in which to assess early developmental toxicity of environmental pollutants (ASTM, 2004).

Environmentally exposing living organisms to specific chemical concentrations produces a stress response, which has been associated with the differential expression of genes (Roelofs et al., 2008). It is known that certain chemical stressors activate a set of genes to synthesize a group of proteins called heat shock proteins (HSPs) (Gupta et al., 2010). These proteins protect cells from stress-induced damage by assisting the correct folding of other proteins. In vertebrates, corticotropin-releasing hormone (CRH), and related peptides such as urocortins (UCNs), are neuropeptides involved in stress response. Exposure of *X. laevis* tail explants to abiotic stress conditions leads to increases in both *crh-a* and *urocortin 1* (*ucn1*) mRNAs (Boorse et al., 2006). Chemical stress can also lead to reactive oxygen species (ROS) overproduction. Excessive ROS can cause damage to cellular structures, but can also modulate redox-sensitive transcription factors by up-regulating antioxidant enzymes, such as superoxide dismutases (SODs) (Trachootham et al., 2008). Some xenobiotics can also induce fatty acid catabolism, which may contribute to increase ROS. It is known that ROS production in response to pollutants is related with apoptotic cell death. Ultimately, severe stress may result in cell death via necrosis or apoptosis (Kultz, 2005). In the light of the foregoing considerations, the purposes of the present study were (1) to determine the potential effects of PFOS on mRNA expression levels of the genetic markers involved in general stress responses (*hsp70*, *hsp47*, *crh-a* and *ucn1*), oxidative stress (*cat.2* and *sod*), lipid metabolism (*ppard*) and apoptosis (*tp53* and *bax*) during *X. laevis* embryo development; and (2) to evaluate these potential molecular changes as response biomarkers of early effects of PFOS in *X. laevis* embryos.

2. Materials and methods

2.1. Chemical and analytical measurement of PFOS

Perfluorooctane sulfonate potassium salt (PFOS; purity > 99%, CAS 2795-39-3) was purchased from Sun Chemical Technology (Shanghai. Co. Ltd, China). In the present study, PFOS was dissolved in boiling distilled water following the procedure described by Wolf et al., 2008; briefly, 45 mg of PFOS was dissolved in 150 ml of boiling distilled water to obtain a stock solution of 300 mg/L. Testing solutions of PFOS (0, 0.5, 6, 12, 24, 48 and 96 mg/L) were prepared in Frog Embryo Teratogenesis Assay-Xenopus (FETAX) solution (Dawson and Bantle, 1987).

PFOS concentrations within the 0.5–96 mg/L range were analyzed by high-performance liquid chromatography (HP 1200 Series, Agilent Technologies, Santa Clara, CA, USA) coupled with triple quadrupole mass spectrometry (G6410A QQQ, Agilent Technologies) (HPLC/MS/MS/MS) according to the procedure described by Hu et al., 2011. Samples from the expected PFOS concentrations were injected directly into the HPLC system at 0 and 24 h of incubation under the exposure conditions (see below) in triplicate. Quantification was performed by an external standard procedure using a six-point calibration curve built by diluting a PFOS stock standard solution in a serial two fold steps in FETAX solution within the 3–96 mg/L range.

2.2. Animals and exposure conditions

Xenopus laevis embryos were obtained from the broodstock at the National Institute for Agricultural and Food Research and Technology in Madrid, Spain. Frog housing and husbandry procedures were carried out as described by Martini et al., 2010. *X. laevis* mating and breeding were induced by two intra-lymphatic injections of human chorionic gonadotropin (hCG) (Veterin Corion®, Divisa Farmavic S.A., Spain). Sexually mature males were injected with 400 International Units (IU) of hCG, and breeder females with 250 IU; both adults were placed separately. Eight hours after the first injection, males and females were administered 400 and 800 IU hCG, respectively. Adults were subsequently transferred together to a spawning tank that was half filled with FETAX solution. Eggs were collected the next day and dejellied in a 2% w/v L-