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Texture analysis in liver of common carp (*Cyprinus carpio*) sub-chronically exposed to perfluorooctanoic acid

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ABSTRACT

An operator-neutral, objective method was implemented to comparatively assess liver pathology in 30 specimens of common carp (*Cyprinus carpio*): 20 after experimental flow-through exposure to two perfluorooctanoic acid (PFOA) dosages (10 fish exposed to 200 ng l^{-1} and 10 fish exposed to 2 mg l^{-1}) for 56 days and 10 unexposed (negative control). The method relies on texture analysis as a complementary approach to traditional histopathology and chemical dosage analysis performed previously on the same experimental material. Texture features data were analyzed by means of Redundancy Analysis (RDA), Linear Discriminant Analysis (LDA) and Canonical Variates Analysis (CVA). LDA resulted in the correct classification of 80% of cases (24 out of 30 cases) with a sensitivity of 83.3% and a specificity of 83.3. In particular, four male samples from the low dosage group (200 ng l^{-1}) were misclassified as unexposed fish and two female samples from the unexposed group were misclassified as low dosage exposed. Nevertheless, PFOA liver chemical dosage analysis results were the same both in unexposed and in low dosage group fish, all below the limit of detection. No sample from the high dosage group (2 mg l^{-1}) has ever been misclassified. Interestingly, texture features correlated with the PFOA concentrations detected in the liver of each sampled fish. In the present study the technique of texture analysis was combined with techniques of multivariate exploratory data analysis (RDA, LDA/CVA). This approach resulted in a robust, sufficiently sensitive and specific means to study PFOA-induced liver pathology. The new method can discriminate between unexposed and two PFOA exposed groups with better confidence and in a more affordable way, compared to chemical quantification of liver PFOA. The texture features correlated well with liver PFOA concentrations and objectively quantified degenerative liver morphology. In conclusion the overall approach may be a suitable candidate as a reliable and broad-ranging method for biomarker analysis of exposure and effect.

1. Introduction

Perfluorooctanoic acid (PFOA) is an emerging pollutant belonging to the class of perfluorinated alkylated substances (PFASs) (Post et al., 2012). Due to its oil- and water-repellency and resistance to heat and chemical reactions, PFOA has been and is still used as an emulsifier or surfactant in a broad spectrum of industrial and commercial applications including fluoropolymer production, textile, household and personal care products, food-packaging, and fire-fighting foams (Ahrens and Bundschuh, 2014; Post et al., 2012; Suja et al., 2009). The presence of PFOA and its precursors in a large number of widely distributed consumer products, increases the likelihood of equally numerous and widely scattered sources of this pollutant in the environment. Risks to the environment are compounded further as PFOAs are released during the entire lifetime of these products, either as aqueous or gaseous emissions (Vierke et al., 2012). The primary discharges are due to industrial and municipal wastewater treatment plants (Vierke et al., 2012). PFOA is ubiquitously detected in ground and surface waters, humans and wildlife, and also in remote regions (Armitage et al., 2006; Kannan et al., 2002; La Rocca et al., 2012). The worldwide concern for PFOA arises from its wide distribution and persistence in the environment, bioaccumulation in food webs, long

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half-life in organisms, and potential toxic, carcinogenic and endocrine disrupting effects on animals (ECHA, 2012; EFSA, 2008; La Rocca et al., 2012; Liu et al., 2007; Post, 2012; Suja et al., 2009; Vierke et al., 2012).

Fish are the major member of the vertebrate classes and are thus excellent candidates in aquatic biomonitoring programs (Saleh and Marie, 2016; van der Oost et al., 2003), especially for PFASs which are continuously introduced in waterways (Giari et al., 2015; Nania et al., 2009; Sinclair et al., 2006; Squadrone et al., 2014; Ye et al., 2008). Ecotoxicological studies frequently focus on liver since this organ is pivotal for the health of the whole organism and highly sensitive to contaminants, including PFASs (EFSA, 2008; Myers et al., 1998; Thophon et al., 2004). Indeed, it is well known that both acute and chronic exposure to PFOA primarily affects the liver as it is the principal target organ (EFSA, 2008). Most of the hepatic effects of PFOA reported in fish are alterations of enzyme activity and gene expression with variable results depending on the species studied (*i.e. Cyprinus carpio*, *Gobiocypris rarus, Oryzias latipes*, *Oreochromis niloticus*, *Pimephales promelas*) and the treatment regimen employed (Kim et al., 2010; Liu et al., 2007; Oakes et al., 2004; Wei et al., 2007, 2009; Yang, 2010). Depletion of liver glycogen was documented in zebrafish exposed to waterborne PFOA for 4 and 28 days (Hagenaars et al., 2013) and hepatic tumor promotion resulted from a PFOA enriched diet in *Onchorhynchus mykiss* (Tilton et al., 2008). Recent research suggests that altered mitochondrial membrane permeability, increased levels of pro-inflammatory cytokines, perturbations of proteins involved in carbohydrate and lipid metabolism, and induction of estrogen-responsive genes could be the modes of action of PFOA toxicity in teleost tissues (Hagenaars et al., 2013; Tilton et al., 2008; Wei et al., 2007; Yang, 2010).

Histopathology has proven its usefulness in evaluating toxicological and ecotoxicological effects on fish health (Al-Zaidan et al., 2015; Manera, 2013a; Samanta et al., 2015; van der Oost et al., 2003). Nevertheless, histopathology is a descriptive, mainly qualitative diagnostic discipline relying on trained personnel and operator-dependent errors can arise (Manera et al., 2016a,b; Wolf et al., 2015). Pathologists are continually seeking objective, replicable diagnostic tools, free from operator-dependent bias (Al-Janabi et al., 2012; Belsare and Mushrif, 2012; Gurcan et al., 2009; Madabhushi, 2009; Manera et al., 2016a). Among them, texture analysis is a reliable image analysis tool widely used in biomedical imaging (Amin and Mahmoud-Ghoneim, 2011; Castellano et al., 2004; Diamond et al., 2004; Herlidou-Même et al., 2003; Loukas and Linney, 2004; Manera and Borreca, 2012, 2014; Manera, 2013b,c; Sertel et al., 2009). Until now image texture analysis has not been extensively utilized by fish biologists, although some examples of application to environmental and experimental fish pathology have been reported by Przytulska et al. (2016), Manera et al. (2015, 2016a,b). Also, colour texture features have been used to discriminate cells categories in histological images of fish ovary by González-Rufino et al. (2013). The paucity of such studies certainly stresses the need to expand the knowledge of this image analysis method among fish biologists.

The aim of the present research was to implement an operator-neutral, objective method to comparatively assess common carp liver pathology after experimental exposure to two PFOA concentrations for 56 days. The results obtained with texture analysis are compared to those obtained with traditional histopathology and with chemical dosage of PFOA in tissues, which were previously performed on the same experimental material (Giari et al., 2016). Furthermore, misclassification, sensitivity and specificity, both with original and cross-validated cases, were computed. To our knowledge, this is the first application of texture analysis to evaluate the effects of an endocrine disrupter on fish.

2. Materials and methods

The present study was based on image analysis of photomicrographs taken from paraffin-embedded sections obtained in a previous experimental trial (Giari et al., 2016). The experimental design is therefore only briefly summarized here and readers are referred to the previously cited reference.

2.1. Experimental fish and acute exposure

Thirty two-year-old carp (mean ± SD; total length: 19.3 ± 2.5 cm; body mass: 104.8 ± 27.8 g) were obtained from a local fish farm, randomly divided into 3 groups/tanks (2 PFOA treated tanks and 1 control, unexposed tank) of 10 fish each. Fish were acclimated for four weeks before starting the experiment and treated according to test guidelines of the Organisation for Economic Co-operation and Development (OECD, 2012a). The carp were fed with a commercial pellet food (Tetra Pond Pellets Mini, Tetra, Melle, Germany) three times per week at 2% of their total body weight. Waste and uneaten food were removed regularly. A flow-through exposure test was conducted for 56 days by a system that continuously delivered PFOA to the test tanks to maintain concentrations of 200 ng l^{-1} or 2 mg l^{-1}.Test tanks were 120 l glass aquaria filled with a continuous supply of tap water at a flow-through rate of 500 ml/min. The tested concentrations were selected, respectively, on the basis of environmental reports (Loos et al., 2008, 2009) and experimental data from the literature (Oakes et al., 2004; Road et al., 2007; Wei et al., 2007). The stock solutions were prepared by dissolving PFOA (PFOA standard, chemical purity 96%, Sigma-Aldrich, Milan, Italy) in distilled water and delivered into the treatments tanks by a peristaltic pump at a flow rate of 0.42 ml/min. At time 0 of exposure, an initial volume of the stock solution was added to the treatments tanks to immediately achieve the desired exposure concentration. Water parameters were monitored and recorded three times weekly for temperature (10–15 °C), pH (6.70–8.00), and oxygen saturation (> 80%). At the end of the 56-day exposure, the fish were anesthetized with tricainemethanesulfonate (MS-222), pithed, dissected and sexed. In each group of carp the sex ratio was approximately 1:1 (unexposed 5♀:5♂; 200 ng l^{-1} 4♀:6♂; 2 mg l^{-1} 5♀:5♂). Biometric parameters (total length, body mass, liver mass) and PFOA accumulation in liver were obtained for each fish as has been reported elsewhere (Giari et al., 2016).

2.2. PFOA analysis

PFOA were extracted using an ion-pairing extraction procedure and measured using high performance liquid chromatography with electrospray ionization tandem mass spectrometry, following a widely used method (Giari et al., 2015, 2016; Guerranti et al., 2013). The limit of detection (LOD), determined as three times the signal-to-noise ratio, was 0.4 ng g^{-1} wet weight. Data quality assurance and quality control protocols included matrix spikes, laboratory blanks, and continuing calibration verification. Blanks were analyzed with each set of five tissue samples as a check for possible laboratory contamination and interferences. Recoveries were assessed using a 5 ng g^{-1} spiked matrix and were over 89%. All other analytical detail is available in (Giari et al., 2016; Guerranti et al., 2013).

2.3. Tissue processing and histological observation

Sampled livers were cut along section lines parallel to the major axis in order to obtain slices of approximately 3 mm in thickness so as to evaluate gross morphology and exclude gross pathology. Particular care was taken to obtain representative samples of liver, including the organ periphery and its inner core. Subsequently, the samples of liver were fixed in 10% neutral buffered formalin for 24 h, then stored in 70% ethanol, dehydrated through an alcohol series, clarified in xylene and paraffin wax embedded. Particular attention was paid to ensure the same sampling volume and fixation procedure among experimental groups. Five-micrometer sections were cut from each tissue block