SEABREAM (*SPARUS AURATA* L.) SPECIMENS EXPOSED TO TCDD: CHEMICAL ANALISIS, CYP1A-INDUCTION AND HISTOPATHOLOGICAL EFFECTS IN LIVER

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Introduction

Several human activities pollute the environments, particularly littoral areas were chemicals from industry, mining, agriculture and urban waste reach the sea. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the by-product of industrial processes and pyrolytic reactions[1]. TCDD has been shown to accumulate into the tissues of fish[2]. Some fish species, particularly salmonids, are sensitive to the effects of TCDD (Walker and Peterson, 1994). From mammalian studies it has been reported that the exposure to the 2,3,7,8-chlorine substituted PCDDs and PCDFs results in a combination of biological effects including strong and prolonged P4501A1 and 1A2 induction, immuno- and hepatotoxic effects, porphyria and body weight loss mediated by the cytosolic Ah-receptor. In vertebrates, exposure to diverse xenobiotics results in induction of one or more forms of enzymes involved in xenobiotic biotransformation[3]. Cytochrome P4501A (CYP1A) forms are the mayor oxidative enzymes induced in fish and other vertebrates by polycyclic aromatic hydrocarbons (PAHs) and polyhalogenated aromatic hydrocarbons (PHAHs). CYP1A responds to environmental levels of these compounds in a dose-dependent manner and is commonly used in field and laboratory studies to evaluate exposure and effects. The inducibility of CYP1A by xenobiotic exposure has led to suggest its use as biomarker in pollution monitoring studies of the aquatic environment[4]. All steps of the CYP1A induction cascade, i.e. mRNA, protein and catalytic activity, can be used to measure CYP1A in fish. In the present study we investigated the expression, cellular distribution and inducibility of CYP1A in liver and muscle or the teleost fish, *Sparus aurata*, following waterborne exposure to TCDD. Basal expression and xenobiotic induction of CYP1A was measured at the levels of catalytic activity (7-ethoxyresorufin-o-deethylase, EROD), protein content (Western blotting, ELISA), and mRNA (RT-PCR); the cellular distribution was examined using immunohistochemistry. *Sparus aurata* was selected as an experimental species because of its economical importance. As organs for quantification and CYP1A induction, the liver (which represent a metabolically active organ) and the muscle (because the problems derivated of the consumption of the fish) were studied.

Methods and Materials

Fish

Immature male specimens of seabream, *Sparus aurata* (average weight: 100-250 g) from a commercial fish farm (CUPIMAR, S.A. San Fernando. Cádiz , Spain) were kept in tanks during 2 weeks for acclimation. The tanks were supplied with continuously flowing seawater at constant temperature.

Xenobiotic exposure

After the acclimation period, fish were randomly distributed to the experimental tanks (10 fish per each 60-l experimental tank) and were subjected to the following treatments: (a) control (only vehicle

added (toluene)), (b) exposure to 4 pg TCDD l^{-1}. Each treatment was done in duplicate with 10 fish per each tank. The fish were exposed to TCDD for 10 days under semi-static conditions. The water in the experimental tanks was exchanged every 24 h; afterwards, fresh TCDD solution was added. No mortalities were recording during the experimental period. Fish samples were taking at 0, 5 and 10 days during experimental period. At sampling, specimens were anaesthetized with 2-phenoxyethanol (Sigma, St. Louis, MO). Then the liver and muscle, were dissected and were either immediately shock-frozen in liquid nitrogen for biochemical analysis, lyophilized for contaminant quantification or immersed in Bouin´s fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, 0.2 % picric acid) for immunohistochemical purposes.

EROD activity

EROD activities were determined in a microplate format assay using a fluorometer plate reader (Fluostat, SLT-Tecan). The assay was run with 47 mM NADPH and 0.4 mM ethoxyresorufin in phosphate buffered saline. The rate of resorufin formation in the assay was followed at an excitation wavelength of 344 nm and an emission wavelength of 590 nm.

Semi-quantitative CYP1A determination by ELISA

An indirect ELISA method was performed according to Goksøyr (1991). The primary antibody used for ELISA (C10-7, monoclonal antibody) was diluted at 1:500. The primary reaction was followed by a horseradish-peroxidase-conjugated goat IgG anti-mouse (Dako) as secondary antibody. Staining was performed using 3,3´-diaminobenzidine as substrate. The peroxidase reaction product was measured as optical density in a spectrophotometer at 405 nm.

Immunohistochemical analysis of CYP1A

Sections of 5 mm thickness were prepared from paraffin-embedded tissues. The sections were incubated overnight in a humid chamber at room temperature with the primary monoclonal C10-7 anti-CYP1A antibody at a dilution of 1:250. For further staining the ABC kit (Vectastain, USA) included a biotinilated anti mouse IgG secondary antibody was applied.

RT-PCR

For RNA extraction 50-100 mg of tissue samples were immediately mixed with 1ml TRIZOL and homogenized. The RNA was extracted using chloroform and precipitated using isopropanol. The RNA content was quantified using an UV spectrophotometer. First-strand cDNA was produced following a protocol of the SuperScript II RT kit, using 1 mg of total RNA. Two microliter of the resulting cDNA were amplified in the PCR reaction. The PCR products were electrophoresed on 1.6% agarose gel and visualized with ethidium bromide staining.

TCDD quantification

The extraction of PCDDs involved a solid phase matrix dispersion (SPMD) procedure. Final clean up and fractionation among the TCDD and other possible interferences was achieved by using SupelcleanTM Supelco ENVITM-Carb tubes as described elsewhere[6].

Resolution and quantification of PCDDs, PCDFs and co-planar PCBs were performed by HRGC-HRMS using a VG AutoSpec Ultima (VG Analytical, Manchester, UK) coupled to a Fisons Series 8000 (8060) Gas Chromatograph. A minimum resolution of 10,000 was used when operating with the HRMS instrument. Methods blanks were routinely analysed, and low contributions were detected. A fused silica capillary DB-5 column (60m, 0.25 mm id., 0.25mm film thickness, J&W Scientific, USA) and a DB-DIOXIN column were used. The carrier gas was helium at a column head pressure of 175 Kpa.

Results and Discussion

CYP1A catalytic activity

EROD activity was analyzed in microsomes from individual portions of liver and muscle from different specimens. In control and TCDD-exposed fish, no muscular EROD activity could be detected although liver samples from both control and exposed fish showed well detectable EROD levels (figure 1).

ELISA

For detection of CYP1A protein we used the C10-7 monoclonal antibody directed against cod CYP1A (Biosense AS, Bergen, Norway). In control and exposed fish detectable levels of CYP1A were found only with microsomes from liver but not from muscle samples. The levels of CYP1A protein detected by ELISA gave similar results as those detected in EROD activity (Figure 2).

Immnuhistochemical demonstration of cellularCYP1A distribution in Sparus aurata specimens

In the liver of seabream control specimens, moderate to strong CYP1A staining was located at the endothelium of the vascular system. No staining was detected in the cytoplasm of hepatocytes (figure 3A). In the liver from TCDD exposed seabream specimens, CYP1A induction was detected in cytoplasmic and nuclear membranes, and positive granules were also evident throughout the cytoplasm of the hepatocytes, increasing the number of these inmunostained granules during TCDD exposure-time

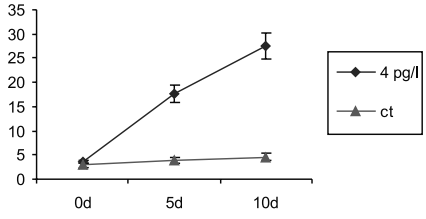

Figure 1. Catalytic activity in liver samples from *Sparus aurata* specimens exposed to 4 pg TCDD l^{-1} during 10 days of exposure.

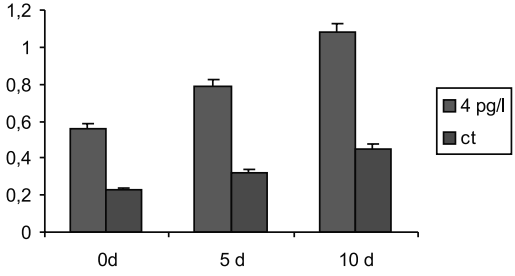

Figure 2. Changes of CYP1A protein levels in liver microsomes in relation to duration of TCDD treatment. CYP1A levels are expressed in arbitrary optical density (OD_{450nm}) units.

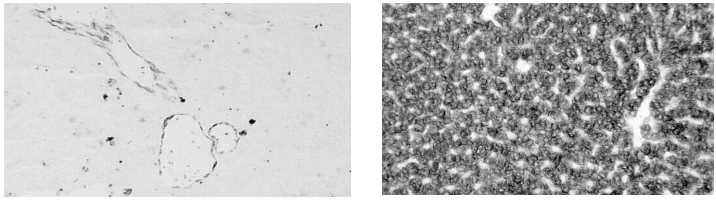

Figure 3. (A) moderate CYP1A immunoreactivity in vascular system in liver from control specimens; (B) strong CYP1A immunoreactivity in hepatocytes from TCDD (10 days) exposed specimens.

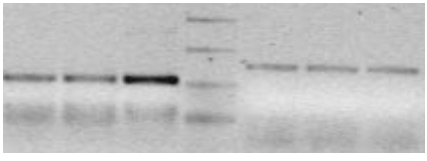

Figure 4. Changes in CYP1A levels by mean of RT-PCR in liver from TCDD exposed Sparus aurata specimens.

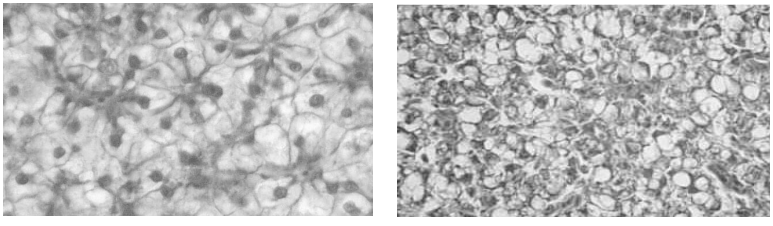

Figure 5. (A) Liver from control seabrean, Sparus aurata, specimens. (B) Histological section of liver from TCDD exposed specimen showing cellular disorganization and lipid droplets deposits within the cytoplasm of hepatocytes.

(figure 3B). No CYP1A staining was observed in muscle from control or exposed specimens. The prominent expression and induction of CYP1A in the liver is consistent with the role of this organ in xenobiotic metabolism, detoxification and excretion. In fish, the liver is mainly composed of hepatocytes, which account for up to 80% of the total liver volume[7]. Contrary to mammals where CYP1A shows a heterogeneous distribution throughout the liver parenchyma, no zonation was observed in fish liver [8].

CYP1A mRNA

The levels of CYP1A mRNA were measured by means of RT-PCR. Expression of CYP1A mRNA in non-exposed animals was detectable in the liver tissue only. After exposure to TCDD the expression of CYP1A increased in liver during the exposure time while muscle tissue presented negative results (figure 4).

Histopathology

Our results show that exposition to TCDD in seabream causes significant alterations in the liver (Figure 5). Basically, an augmentation of glycogen and lipid droplets. However**,** Van der Weiden *et al.*, (1992) reporterd that in rainbow trout following a single injection of TCDD (3.06 ìg /Kg) the liver contained less glycogen compared to unexposed fish. This discrepancy could be related to interespecific metabolic/physiological differences between both species.

Hepatic alterations, such as variation in nuclear size and chromatin pattern, mild variation in hepatocytes size and/or increased number of mitotic figures and lipid vacuoles have been detected in rainbow trout exposed to dietary (90ngTCDD/Kg food)[10]**.** The degree of alteration in organs/tissues (liver, gills, digestive tract, vascular endothelium) may be related to different toxicants, their concentration and route of incorporation of contaminants. In rainbow trout exposed to dietary (90 ng TCDD/Kg), lesions most frequently noted were: focal chronic granulomas, occasional blunting, fusion of secondary lamellae and lymphocytic infiltrates[10]**.** Nonetheless, no histological alteration was detected in gills, liver, digestive tract, etc from European flounder exposed to 500 µg TCDD /Kg[11].

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