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Genotoxicity of gemfibrozil in the gilthead seabream (*Sparus aurata*)

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ABSTRACT

Widespread use of pharmaceuticals and suboptimal wastewater treatment have led to increased levels of these substances in aquatic ecosystems. Lipid-lowering drugs such as gemfibrozil, which are among the most abundant human pharmaceuticals in the environment, may have deleterious effects on aquatic organisms. We examined the genotoxicity of gemfibrozil in a fish species, the gilthead seabream (*Sparus aurata*), which is commercially important in southern Europe. Following 96-h waterborne exposure, molecular (erythrocyte DNA strand breaks) and cytogenetic (micronuclei and other nuclear abnormalities in cells) endpoints were measured. Gemfibrozil was positive in both endpoints, at environmentally relevant concentrations, a result that raises concerns about the potential genotoxic effects of the drug in recipient waters.

1. Introduction

The presence of human and veterinary pharmaceuticals in the environment is of increasing concern [1–4]. The environmental release of these substances and their metabolites, their persistence, and their bioactivities have led to their classification as emerging environmental contaminants of concern [2–4]. Lipid regulators, a group of human pharmaceuticals, are frequently reported in wastewater and surface waters, due to their increased use in recent years [2,5–8,4]. The lipid regulator gemfibrozil (GEM) has been found in wastewater treatment plant effluents at levels as high as 2.1 μg/L in Canada [6] and 4.76 μg/L in Europe [2]. In surface waters, the highest concentrations of GEM were detected in North America and Europe, around 0.75 and 1.5 μg/L, respectively [5].

The risks to aquatic organisms associated with the presence of pharmaceuticals in the environment include behavioural alterations, genotoxicity, reduced pathogen resistance, and endocrine disruption [9–11]. Studies with GEM have shown that it affects feeding and attachment of the cnidarian *Hydra attenuata* [12]; growth of the alga *Chlorella vulgaris* [13]; decreases plasma testosterone levels in the goldfish (*Carassius auratus*) [14]; and activates antioxidant enzymes and interferes with metallothionein expression in the blue mussel (*Mytilus edulis*) [4] and zebra mussel (*Dreissena polymorpha*) [15]. Henriques et al. [16] showed that exposure to GEM affects the development and locomotor activity of zebrafish (*Danio rerio*) larvae. Only a few studies have evaluated its aquatic genotoxicity [17]. GEM can damage DNA in the zebrafish after 7-d exposure; [17] and in marine (*Mytilus* spp.) and freshwater (*Dreissena polymorpha*) mussels after 96-h exposure; [15,4]. However, to our knowledge, no study has reported GEM effects on an estuarine/marine top-predator fish species.

Contaminants may interact with DNA directly or they may disrupt normal cellular processes, e.g. inducing oxidative stress [18]. Elevated levels of reactive oxygen species (ROS) and/or depressed antioxidant defences may result in DNA oxidation and increased steady-state levels of unrepaired DNA leading to genotoxicity [19–21].

The comet assay is widely used in environmental toxicology for assessing DNA damage [22–25] it combines the simplicity of biochemical techniques for detecting DNA single-strand breaks (strand breaks and incomplete excision-repair sites), alkali-labile sites, and cross-linking, by measuring the migration of DNA from immobilized nuclear DNA, using the single-cell approach typical of cytogenetic assays [26–30]. The micronucleus (MN) assay, one of the most popular tests of environmental genotoxicity, is based on chromatid/chromosome fragments or whole chromatids/chromosomes resulting from DNA strand damage, which are not reincorporated into the daughter nucleus and are transformed into a MN [31–35]. MN may be induced by oxidative stress, by exposure to clastogens or aneugens, or by defects in cell-cycle checkpoints or DNA repair [33]. The simultaneous expression of other morphological nuclear abnormalities in addition to MN has proven to be a valuable tool in detecting genotoxicity of several

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contaminants at low concentrations [36–42]. We have tested the genotoxicity of waterborne GEM to a predatory fish species, *Sparus aurata*, following a 96-h exposure, by assessing damage with the comet assay and erythrocytic nuclear abnormalities (ENAs) assay.

2. Material and methods

2.1. Chemicals

All reagents used were analytical grade and acquired from Sigma-Aldrich. Gemfibrozil (GEM) (≥98%) was purchased from TCI and isotopically labelled d_6-GEM was purchased from Santa Cruz Biotechnology (Dallas, USA). A stock solution (50 g/L) was prepared in dimethyl sulfoxide (DMSO). Exposure solutions of GEM (1.5; 15; 150; 1500; and 15,000 μg/L) were prepared by serial dilutions in artificial seawater.

2.2. Test animals and experimental design

All experimental procedures were carried out following the Portuguese and European legislation (authorization N421/2013 of the Portuguese legal authorities). Animal handling was performed by an accredited researcher.

Juvenile gilthead seabream (*Sparus aurata*), length 9 ± 0.9 cm, acquired from an aquaculture facility (Spain), were acclimated for 4 weeks in 220-L aquaria containing aerated and filtered artificial seawater (salinity, 35), under a controlled room temperature (20 °C) and natural photoperiod. During this period, the experimental fish (n = 55) were fed daily with commercial fish food (Sorgal, Portugal) at a ratio of 1 g per 100 g of fish and the water in the aquarium was renewed daily.

The procedures generally followed the OECD guidelines for fish acute bioassays [43]. The experiment was carried out in 80-L aquaria, under the conditions described for the acclimation period. Following acclimation, fish were randomly distributed into seven aquaria, with seven fish per aquarium. The experimental design included a negative control (seawater only), a solvent control (0.03% DMSO, the DMSO concentration used for the highest concentration of GEM) and five GEM concentrations: 1.5; 15; 150; 1500; and 15,000 μg/L. Fish were exposed for 96 h as recommended by the OECD guideline for fish acute toxicity testing (203), without feeding, with 80% medium renewal every 24 h, to prevent significant GEM degradation and to reduce the build-up of metabolic residues. Fish mortality, behavioural alterations, and water parameters (such as temperature, salinity, conductivity, pH, and dissolved oxygen) were monitored daily.

After 96 h exposure, the animals were anesthetized with tricaine methanesulfonate (MS-222) and a blood sample was collected from the posterior cardinal vein of each fish. For the comet assay, blood samples were diluted with saline phosphate buffer (2:2000, v/v) and used immediately. Blood smears were prepared for the assessment of MN and other erythrocytic nuclear abnormalities.

2.3. Quantitation of GEM in the test media

Water samples (10 mL) were collected each day (at 0 and 24 h) from each aquarium, and GEM was analysed by solid-phase extraction (SPE). Briefly, Strata X cartridges (200 mg, 3 mL) (Phenomenex, USA) were conditioned with 5 mL methanol and 5 mL ultra-pure water. Then, the water sample (10 mL) was percolated through the cartridge at a flow rate of 3–5 mL/min; the cartridge was rinsed with ultra-pure water (5 mL), and dried under vacuum for 20 min. Finally, GEM was eluted with methanol, 10 mL. Extracts were evaporated until dryness under a gentle stream of nitrogen and reconstituted with acetonitrile/ultra-pure water (30:70, v/v, 1 mL). An aliquot (10 μL) of gemfibrozil-d_6 (5 mg/L) was added to the extract as internal standard before UHPLC–MS/MS analysis. GEM analysis was performed on a Nexera UHPLC system with a triple-quadrupole mass spectrometer detector LCMS-8030 (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was achieved on a Kinetex C18 column (2.1 × 150 mm i.d., 1.7 μm particle size) from Phenomenex (USA); column temperature, 30 °C; autosampler temperature, 4 °C; injection volume, 5 μL. Elution conditions were: solvent A, 5 mM ammonium acetate/ammonia buffer (pH 8); solvent B, acetonitrile; flow rate, 0.22 mL/min. Gradient program was as follows: initial conditions: 30% B; 0–2.0 min, 30%–100% B; 2.0–4.5 min, maintained at 100% B; 4.5–5.5 min, return to initial conditions; from 5.5–9.5 min, re-equilibration of the column.

GEM was analysed in the negative ionization mode and quantitation was performed in multiple reaction monitoring mode (MRM) using two transitions between the precursor ion and the most abundant fragment ions. A summary of individual MS/MS parameters is shown in Table S1 (Supplementary Material). Quantitation was performed by the internal standard calibration method. The method detection limit (MDL) for GEM in water was 4.0 ng/L. Detailed QA/QC information is given in the Supplementary Material (Table S2).

2.4. Evaluation of genetic damage

2.4.1. Comet assay

The alkaline comet assay was conducted according to the method of Singh et al. (1988) with some modifications. To prevent UV-induced DNA damage, the procedure was conducted under yellow light. Briefly, diluted blood samples (20 μL) were added to 1% (w/v) low-melting-point agarose, 140 μL (at 40 °C) and the mixtures applied to microscope slides pre-coated with 1% (w/v) normal-melting-point agarose. A coverslip was added to each slide, which was then placed on ice for agarose solidification; then, the coverslips were carefully removed and the slides immersed, for 1 h at 4 °C, in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0), containing freshly added 1% Triton X-100. Slides were incubated in alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 10 min for DNA denaturation and unwinding. Electrophoresis was performed using the same buffer, for 30 min at 300 mA and 20 V. Note: State the field strength in 0.83 V/cm. After electrophoresis, slides were neutralized in 400 mM Tris buffer (pH 7.5), dehydrated with absolute ethanol for 10 s, and left to dry for 1 d in the dark. Slides were stained with ethidium bromide (20 μL/mL, 100 μL), covered with a coverslip, and analysed using a fluorescence microscope (Olympus BX41TF) at 400X magnification. To verify that the electrophoresis conditions were adequate, negative (blood from fish maintained in an aquarium with seawater only) and positive (blood from fish treated with 25 μM H_2O_2 for 10 min) controls were included in each electrophoresis run. H_2O_2 was used as a model genotoxic agent since it produces both single-strand breaks and oxidative DNA damage [80] and has been used routinely as a positive control in the comet assay [44,45]. To avoid bias, slides were randomly analysed, counting one hundred randomly selected cells from each slide. Cells were scored visually, according to tail length, into five classes: class 0 – undamaged, without a tail; class 1 – with a tail shorter than the diameter of the head (nucleus); class 2 – with a tail length 1–2 times the diameter of the head; class 3 − with a tail longer than twice the diameter of the head; class 4 – comets with no heads [19]. A damage index (DI) expressed in arbitrary units was assigned to each replicate (for 100 cells) and consequently for each treatment, using the formula:

$$DI = (0 \times n0) + (1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4)$$

where: n = number of cells in each class.

DI can range from 0 to 400 [46]. The percentage of DNA damage relative to the control was calculated.

2.4.2. Erythrocytic nuclear abnormalities (ENAs) assay

This assay was carried out in mature peripheral erythrocytes according to the procedure of Pacheco and Santos [47]. Blood smears were fixed in methanol during 10 min and stained with Giemsa (5%) for 30 min. The nuclear abnormalities were randomly scored under a light