Effects of endocrine disruptors chemicals on the immune response of gilthead seabream (*Sparus aurata* L.)

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ABSTRACT

Endocrine disruptors chemicals (EDCs) exert their effects via agonistic/antagonistic interactions with hormone receptors or by interfering with the normal synthesis, transport, metabolism, and secretion of endogenous hormones [1]. EDCs, from the pharmaceutical industry, such as 17α-ethinylestradiol (EE_2) and tamoxifen (Tmx,), have been founded in the aquatic environment. EE_2 is used in most oral contraceptive pills and in hormone replacement therapy, while Tmx is an estrogen receptor ligand used in cancer therapy. This study aims to investigate the effects of the dietary intake of EE_2 (5 µg/g food) or Tmx (100 µg/g food) on the immune response of gilthead seabream species, a marine protandrous teleost, with a great commercial value in the Mediterranean area.

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are natural or synthetic compounds that have the ability within the body to alter endocrine functions often through mimicking or blocking endogenous hormones (reviewed by [2]). Among EDCs, the most studied are the compounds that interfere with estrogen receptors (ER). Some of these compounds are pharmaceutical products and chemicals used in manufacturing of polycarbonate plastics released in waste waters which reach the aquatic environment through sewage treatment effluents [3]. 17α-ethinylestradiol (EE_2) is the major constituents of contraceptive pills [4] and is one of the most potent compounds in the aquatic environment. Another EDC is tamoxifen (Tmx) which is widely used as a drug in cancer therapy.

However, the effect of these compounds on the immune system of fish and their ability to recover from estrogen exposure has drawn little attention. The gilthead seabream (*Sparus aurata* L.) is a marine, protandrous hermaphrodite teleost with a great commercial value and, therefore, the impact of EDCs on its immune system is an important concern.

The aims of this work was first to determine if EE_2 or Tmx might act as an EDC in gilthead seabream and secondly to evaluate their possible effects on the immune response of gilthead seabream and, more importantly, the capacity of the immune system to recover its functionality after the treatments ceased, in both adults and weaned larvae gilthead seabream specimens.

MATERIALS AND METHODS

***In vivo* treatments**

Specimens of gilthead seabream were maintained at the Oceanographic Centre of Murcia. Adults and juveniles specimens were fed with pellet diet supplemented with EE_2 (5 µg/g food) or Tmx (100 µg/g food). After that, they were fed with the commercial food (recovery period) in order to analyze the capacity of the immune system to recover its functionality after this period. In order to evaluate the effect of these compounds on any induced adaptive immune response, the specimens were intraperitoneally injected with phosphate buffered saline (PBS) (control fish) or hemocyanin (200 µg/fish) and Imject Alum Adjuvant (4 mg/fish) (vaccinated fish).

To evaluate the effect of the *in vivo* EE_2 or Tmx treatments, the body weight (BW), hepatosomatic index (HSI), and splenosomatic index (SSI) were calculated during the treatments and during the recovery period. HSI is the 100 x (ML/MB)(%) and SSI is 100 x (MS/MB)(%), where ML is liver mass, MS is spleen mass and MB is body mass (all in grams).

Analysis of gene expression

Total RNA was extracted from liver and head kidney with TRIzol Reagent (Invitrogen), and quantified with a spectrophotometer (NanoDrop, ND-1000). The RNA was then treated with DNase I, amplification grade (1 U/µg RNA; Invitrogen) to remove genomic DNA traces that might interfere with the PCR reactions, and the SuperScript III RNase H Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligo-dT18 primer from 0.5-1µg of total RNA, at 50ºC for 50 min. The expression of the genes coding for vitellogenin (VTG), the pro-inflammatory cytokine, IL-1b, and the anti-inflammatory IL-10, was analyzed by real-time PCR (qPCR) performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95˚C, followed by 40 cycles of 15 s at 95˚C, 1 min at 60˚C, and finally 15 s at 95˚C, 1 min at 60˚C, and 15 s at 95˚C.

Determination of IgM specific titer

The hemocyanin specific IgM titer was determined by an ELISA kit (Aquatic Diagnostic). Serial dilutions of serum of all specimens groups were added to hemocyanin-precoated 96-well ELISA plates, followed by a monoclonal antibody (Ab) specific to seabream IgM and an anti-rabbit IgG (whole molecule)-peroxidase Ab produced in goat (Sigma-Aldrich). Finally, the chromogen tetramethylbenzidine was added, and the absorbance was read at 450 nm using a FLUOstart luminometer (BGM; LabTechnologies).

ROS production assay

The production of reactive oxygen intermediate (ROS) was measured as the luminol-dependent chemiluminescence produced by 0.5 x 10^6 leukocytes (control, borh vaccinated + EE_2 or Tmx-treated groups and vaccinated control) during the treatments and in the recovery period in the presence or absence of 50 ug/ml of phenol-extracted genomic DNA from *Vibrio anguillarum* cells (VaDNA) for 48 h. This was achieved by adding 100 µM luminol (Sigma-Aldrich) and 1 µg/ml phorbol myristate acetate (PMA, Sigma-Aldrich), while

the chemiluminescence was recorded every 127 s during 1 h in a FLUOstart luminometer (BGM; LabTechnologies). The values re-ported are the average of triple readings from 3 different samples, expressed as the maximum and slope of the reaction curve from 127 to 1016 s, from which the apparatus background was subtracted.

Determination of IgM positive cells

The percentage of IgM positive cells was measured in aliquots of 0.5×10^6 leukocytes from head kidney. In some experiments, the leukocytes were incubated in the presence or absence of hemocyanin and/or *Vibrio anguillarum* DNA genomic (VaDNA), for 48 h, in sRPMI containing 10% charcoal/dextran-treated hormone-free fetal bovine serum (hf-FBS, Hyclone). After that, the cells were washed in PBS containing 2% FCS and 0.05% sodium azide (FACS buffer) (Life technologies). Cells were then incubated with 0 and 2 µg/ml (0, 1:100) IgM Ab (Aquatic Diagnostic) in PBS containing 2% FCS during 30 min at 4ºC. After washing, cells were incubated with a 1:1000 dilution of a Fluorescein isotiocyanate (FITC)-conjugated rabbit during 30 min at 4ºC, washed again, and analyzed by flow cytometry using a flow cytometer (BD Biosciences).

Statistical analysis

ANOVA and Tukey on Bonferroni multiple range tests were applied to determine differences among groups. T test and an Unpaired tests were applied to determine differences between two groups. The critical value for statistical significance was taken as $p \leq 0.05$. The asterisks *, ** and *** refer to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. All statistical analyses were carried out using the GraphPad Prism 5 program.

RESULTS

EE_2 and Tmx promoted a transient alteration in the hepatic *vtg* gene expression analyzed by qPCR, in both adult and larvae gilthead seabream specimens.

We showed an alteration of the BW in EE_2 and Tmx-treated fish. While EE_2 promoted a decrease, Tmx increased the BW of treated adults and young gilthead seabream. In this line, EE_2 exposure significantly increased HSI and SSI. This alteration remains in the recovery period. However, Tmx showed any effect on these indexes.

The expression of genes encoding for two key cytokines, *il1b* and *il10*, was analyzed by qPCR in head kidney during the treatments and in the recovery period. Vaccination resulted in an increased *il1b* expression, while Tmx reversed this increase in adult specimens. However, during the recovery period, no differences in *il1b* expression were observed between the different groups analyzed. Neither the vaccination nor Tmx treatment affected *il10* gene expression in head kidney.

Adult vaccinated and Tmx-treated fish showed a higher IgM titer than vaccinated and untreated fish, respectively. Moreover, we observed an amplification of this effect in larvae during the recovery period.

When we analyzed the production of ROS triggered by PMA in naïve and VaDNA-stimulated head kidney leukocytes from untreated and EE_2 or Tmx-treated (vaccinated or not) gilthead seabream adults resulted in increased ROS production while in juveniles, no significant differences were found among the groups.

An increase in the percentage of head kidney IgM-positive cells in adult and larvae specimens was observed after the immunization in the recovery period which correlated with the modulation in the hemocyanin-specific IgM serum level.

DISCUSSION AND CONCLUSIONS

Estrogens and androgens are regulators of fish immunity. A wide variety of compounds, acting as EDCs, seem to exhibit immunotoxicological actions [5]. Despite the impact of estrogenic EDCs on fish immune system is an important concern, none one has studied the effect of these compounds during fish ontogeny and in adult seabream and more interestingly, on the possible reversibility of their effects.

In this study we have used 17α-ethynylestradiol (EE_2), a synthetic estrogen used in oral contraceptives and hormone replacement therapy. Also we have administrated a cytostatic agent, Tmx. We have selected these compounds due to they are present in waste water and are generally released directly to the sewage network without any pre-treatment.

We have demonstrated that EE_2 and Tmx act as an EDC in gilthead seabream, since increased hepatic *vtg* mRNA levels, a yolk precursor protein produced by hepatocytes and generally accepted as a biomarker of estrogenic effects [6].

EE_2 and Tmx are able to inhibit the vaccination-induced *il1b* gene expression as was previously described in EE_2-bath exposed fish [7]. However, neither EE_2 or Tmx exposures nor immunization affected the gene expression profile of the anti-inflammatory cytokine, il10, during treatments or during the recovery period.

Interestingly, both treatments resulted in increased ROS production in both unstimulated and VaDNA-stimulated head kidney leukocytes from vaccinated fish compared with untreated, vaccinated fish. However, this effect was not observed in the recovery period. Nevertheless, in naïve and VaDNA-stimulated head kidney leukocytes from un-treated and EE_2 or Tmx-treated (vaccinated or not) gilthead seabream juveniles during the treatment, no significant differences were found among the groups. The dietary intake of EE_2 and Tmx increased the IgM serum levels of immunized fish at the end of the treatments although only Tmx increased the IgM titer, accompanied by an increase in the IgM B cells, during the recovery period. Moreover, EE_2 and Tmx significantly increased the antibody titer of vaccinated fish during the recovery period. However, further studies are needed to clarify the mechanism of action of this compound.

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