Comparative Biochemistry and Physiology, Part C 179 (2016) 94–106

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Tamoxifen disrupts the reproductive process in gilthead seabream males and modulates the effects promoted by 17α-ethynylestradiol☆

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ARTICLE INFO

Article history:
Received 25 May 2015
Received in revised form 22 July 2015
Accepted 10 September 2015
Available online 25 September 2015

Keywords:
Tamoxifen
17α-Ethynylestradiol
Endocrine disruption reversibility
Spermatogenesis
Steroidogenesis
Gilthead seabream

ABSTRACT

17α-Ethynylestradiol (EE_2), which is used in oral contraceptives and hormone replacement therapy, is a well documented estrogenic endocrine disruptor and an aquatic contaminant. In the present study, adult male specimens of the marine hermaphrodite teleost gilthead (*Sparus aurata* L.) were fed a diet containing tamoxifen (Tmx), an estrogen receptor ligand used in cancer therapy, alone or combined with EE_2, for 25 days and then fed a commercial diet for a further 25 days (recovery period). The effects of short (5 days) and long (25 days) treatments on several reproductive and gonad immune parameters and the reversibility of the disruptive effects after the recovery period were examined. Our data showed that Tmx acted as an estrogenic endocrine disruptor as revealed by the increase in the hepatic transcription of the *vitellogenin* gene in males, the serum levels of 17β-estradiol and the gonad expression levels of the estrogen receptor α and G protein-coupled estrogen receptor genes, and the recruitment of leukocytes into the gonad, a well known estrogenic-dependent process in gilthead seabream males. On the other hand, Tmx also increased sperm concentration and motility as well as the serum levels of androgens and the expression levels of genes that codify for androgenic enzymes, while decreasing the expression levels of the gene that code for gonadal aromatase. When applied simultaneously, Tmx and EE_2 could act in synergy or counteract, each other, depending on the parameter measured. The disruptive effect of EE_2 and/or Tmx was not reversible after a 25 day recovery period.

1. Summary statement

In gilthead seabream males, Tmx disrupts the reproductive process including the gonad immune response and counteracts or enhances the effects of EE_2. A 25-day recovery period did not reverse these effects in adult males.

2. Introduction

Endocrine disrupting 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 (Segner et al., 2006). Among EDCs, the most studied are the compounds that interfere with estrogen receptors (ERs), which have hazardous and estrogenic effects on fish reproduction (Folmar et al., 1996; Jobling et al., 1998, 2002; Hassanin et al., 2002; Penáz et al., 2005). Some of these compounds are pharmaceutical products released in waste waters which reach the aquatic environment through sewage treatment effluents (Mills and Chichester, 2005).

In the group of estrogenic EDCs, 17α-ethynylestradiol (EE_2), a major constituent of contraceptive pills (Owen and Jobling, 2012), has a higher binding affinity to ERs than natural 17β-estradiol (E_2) (Blair et al., 2000) and is one of the most potent compounds in the aquatic environment. Low concentrations of EE_2 (3–17 ng/L) are sufficient to induce vitellogenin (Vtg) production in male fish (Holbech et al., 2001; Rose et al., 2002; Andersen et al., 2003), to modify sexual behavior (Coe et al., 2010; Reyhanian et al., 2011; Filby et al., 2012) and to disrupt the reproductive capacities of fish (Nash et al., 2004; Pawlowski et al., 2004; Fenske et al., 2005; Schäfers et al., 2007). However, the ability of fish to recover from estrogen exposure has drawn little attention, apart from some studies on sexual differentiation and reproductive capacity (Hill and Janz, 2003; Nash et al., 2004; Schäfers et al., 2007; Larsen et al., 2009; Baumann et al., 2014).

Another EDC is tamoxifen (Tmx) which is widely used as a drug in cancer therapy. Studies in humans have shown that approximately 65% of administered Tmx is excreted with feces, while its active metabolite OH-Tmx is excreted with bile and urine. Tmx is a nuclear ER ligand which in mammals, acts as estrogen agonist on some cell types but as an antagonist or partial agonist on others, which reflects the diversity of

☆ The genetic nomenclature used in this manuscript follows the guidelines of the Zebrafish Nomenclature Committee (ZNC) for fish genes and proteins and the HUGO Gene Nomenclature Committee for mammalian genes and proteins.

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http://dx.doi.org/10.1016/j.cbpc.2015.09.005
1532-0456/

the mechanisms that mediate ER actions in different tissues (Fitts et al., 2011). In addition, Tmx acts as an agonist on the G protein-coupled estrogen receptor (GPER), a transmembrane receptor that mediates rapid responses of estrogen and is widely expressed in estrogen target tissues (Revankar et al., 2005), including fish testis and ovary (Liu et al., 2009; Pang and Thomas, 2010). However, the effects of Tmx and its action mechanisms in fish are just beginning to be understood, in part because of the interest that binary mixtures of EDCs have attracted in the recent years (Sun et al., 2009, 2011a, 2011b). Such studies have been performed in gonochoristic fish and showed that the estrogenic or anti-estrogenic effects of Tmx depend on the gender, concentration and tissue analyzed (Leaños-Castañeda et al., 2002; Chikae et al., 2004; Sun et al., 2011a, 2011b). Tmx treatment leads to the masculinization of genetic female fish (Kitano et al., 2007; Hulak et al., 2010; Liu et al., 2010). Moreover and although the effects of Tmx mask or neutralize many signs of estrogen exposure, the impairment of the fish reproductive process is not restored (Santos et al., 2006; Elias et al., 2007; van der Ven et al., 2007; Sun et al., 2009). To the best of our knowledge, no such studies have been studied in hermaphrodite fish such as gilthead seabream.

The gilthead seabream (*Sparus aurata* L.) is a marine, seasonally breeding, protandrous teleost that develop a functional testicular area nearby an immature previtellogenic ovary during the first two reproductive cycles. We have recently reported that EE_2 dietary intake increases the hepatic expression levels of *vtg*, disrupts spermatogenesis and promotes leukocyte infiltration in the gonad (Cabas et al., 2011, 2013), a physiological process needed for gonad renewal after spawning (Chaves-Pozo et al., 2005a, 2005b; Liarte et al., 2007). Moreover, most of these effects vary with the reproductive stage of the specimens (Cabas et al., 2011, 2013). On the other hand, the dietary intake of Tmx has been shown to be a suitable approach for studying its potentially endocrine disruptive effects (Benninghoff and Williams, 2008; Singh et al., 2014).

In the present study, we investigate the effect of the dietary intake of Tmx alone or in combination with EE_2 on some reproductive events in gilthead seabream and the possible reversibility of these effects after a recovery period of 25 days, during which fish were again fed with a commercial diet. This approach, as a way to unbalance the endocrine status of the fish, would improve our understanding of the complex network acting on the regulation of the reproductive function in this species, which has a great commercial interest in the Mediterranean area.

3. Material and methods

Healthy specimens of gilthead seabream (Actinopterygii, Perciformes, Sparidae) were bred and kept at the Centro Oceanográfico de Murcia (Instituto Español de Oceanografía, Mazarrón, Murcia, Spain).

The experiment was performed using 80 male specimens of gilthead seabream, all in the spermatogenesis stage, with a mean body weight of 215 ± 6.5 g. Fish were kept in 2 m^3 tanks with a flow-through circuit, suitable aeration and filtration system and natural photoperiod. The water temperature ranged from 14.6 to 17.8 °C. Environmental parameters, mortality and food intake were recorded daily. The EE_2 (98% purity; Sigma) and Tmx (Sigma) were incorporated in the commercial feed (44% protein, 22% lipids, Skretting, Spain) at doses of 0 (control), 5 μg EE_2/g food, 100 μg Tmx/g food or 5 μg EE_2 + 100 μg Tmx/g food, using the ethanol evaporation method (0.3 L ethanol/kg of food) as described elsewhere (Shved et al., 2007). The concentration of EE_2 used in this study was previously assayed and shown to be the lowest concentrations producing an effect on some reproductive events of gilthead seabream (Cabas et al., 2011, 2013), while the concentration of Tmx used was twenty-fold greater than the concentration of EE_2 in order to guarantee a Tmx–ER interaction, considering that Tmx has a lower affinity than EE_2 to bind ER (Denny et al., 2005). In any case, the Tmx concentration used in this study is similar to, or lower than, those tested in previous investigations (Chikae et al., 2004; Hulak et al., 2010).

The specimens were fed with EE_2 and/or Tmx supplemented feed for 25 day, after which they were fed with the commercial food for a further 25 days (recovery period). The specimens were fed ad libitum three times a day and fasted for 24 h before sampling, which was carried out after 5 and 25 days of the EE_2 and/or Tmx exposure and after the recovery period ($n = 6$ fish/group and time). Specimens were anesthetized with 40 μL/L of clove oil and the urogenital pore was dried before collecting sperm as described below. The specimens were then weighed, decapitated, and the gonads removed and weighed. Fragments of liver and gonad were processed for gene analysis and light microscopy, as described below. Serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at −80 °C until use.

The experiments comply with the Guidelines of the European Union Council (2010/63/UE) and the Bioethical Committee of the University of Murcia (Spain) and that of the "Instituto Español de Oceanografía" (Spain) for the use of laboratory animals.

3.1. Analysis of gene expression

Total RNA was extracted from liver and gonad fragments with TRIzol Reagent (Invitrogen, Barcelona, Spain) following the manufacturer's instructions, and quantified with a spectrophotometer (NanoDrop, ND-1000). The RNA of five fish per group was independently treated with DNase I (amplification grade, 1 unit/μg RNA, Invitrogen, Barcelona, Spain) to remove genomic DNA traces that might interfere with the PCR reactions, and the SuperScript III RNase H-Reverse Transcriptase (Invitrogen, Barcelona, Spain) was used to synthesize first strand cDNA with oligo-dT18 primer from 1 μg of total RNA, at 50 °C for 50 min.

Real-time PCR was performed with an ABI PRISM 7500 (Applied Biosystems, Madrid, Spain) using SYBR Green PCR Core Reagents (Applied Biosystems, Madrid, Spain) and used to analyze the expression of the genes coding for (i) hepatic vitellogenin (*vtg*); (ii) steroidogenesis-related molecules: steroidogenic acute regulatory protein (*star*), cholesterol side chain cleavage cytochrome P450 (*cyp11a1*), steroid 11-beta-hydroxylase (*cyp11b1*), 11β-hydroxysteroid deshydrogenase (*hsd11b*), aromatase (*cyp19a1a*), 5α reductase (*srd5a*) and 3β-hydroxysteroid deshydrogenase (*hsd3b*); (iii) testicular specific protein, double sex-and mab3-related transcription factor 1 (*dmrt1*); (iv) hormone receptors: follicle stimulating hormone (FSH) receptor (*fshr*), luteinizing hormone (LH) receptor (*lhr*) and estrogen receptor α (*era*), G protein-coupled estrogen receptor (*gper*); and (v) immune-relevant molecules: interleukin 1β (*il1b*), tumor necrosis factor α (*tnfa*), transforming growth factor β1 (*tgfb1*), matrix metalloproteinase (*mmp*) 9 and 13 (*mmp13*), major histocompatibility complex I α protein (*mhc1a*) and toll-like receptor 9 (*tlr9*). For each mRNA, gene expression was normalized to the ribosomal protein S18 gene (*rsp18*) content in each sample using the comparative Ct method ($2^{-\Delta\Delta Ct}$) (where Ct is a cycle threshold). The gilthead seabream specific primers used are shown in Table 1. In all cases, each PCR was performed in triplicate.

3.2. Analytical techniques

Serum levels of testosterone (T), 11-ketotestosterone (11KT) and E_2 were quantified by ELISA following the method described by Rodríguez et al. (2000) and previously used in gilthead seabream (Chaves-Pozo et al., 2008). Steroids were extracted from 20 μL of serum in 0.6 mL of methanol (Panreac). The methanol was then evaporated at 37 °C and the steroids were resuspended in 400 μL of reaction buffer [0.1 M phosphate buffer with 1 mM EDTA (Sigma), 0.4 M NaCl (Sigma), 1.5 mM NaN_3 (Sigma) and 0.1% albumin from bovine serum (Sigma)]. By using 50 μL in each well, 2.5 μL of serum was used in each well for all the assays. The T, 11KT and E_2 standards, mouse anti-rabbit IgG monoclonal antibody (mAb), and specific anti-steroid antibodies