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Assessment of estrogenic and thyrogenic activities in fish feeds

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ABSTRACT

Normal functioning of the endocrine system is essential for the proper development and reproduction of animals. Substances interfering with its homeostasis are called endocrine disruptors (EDs) and may represent a risk for the health of the organism. One of the mechanisms of endocrine disruption that has attracted great attention in recent years concerns alterations in the normal functioning of the estrogen receptor (ER), but far less attention has been paid to those substances interfering with the thyroid axis, which, in fish, plays several critical roles in a variety of biological functions. In aquaculture, feedstuffs can be a source of hormones or persistent pollutants which act as potential EDs. In this study, the main purpose was to assess the possible estrogenic and thyrogenic activities of 32 commercial fish feeds. For the assessment of estrogenicity, a new estrogen receptor specific reporter gene assay using sea bass ERα (sbER α) was developed and validated. Potential thyroidal disruption was screened with a cell line permanently transfected with luciferase as reporter gene under the control of avian thyroid receptor α (THRα). The results obtained showed that 11 and 18 out of 32 assayed feeds were able to activate the sbERα or the avTHRα1, respectively. The present study is pioneer in demonstrating thyrogenic activity in fish diets commercially available and widely used in aquaculture. Given that maintaining the homeostasis in the endocrine system is critical for the proper development and reproduction of fish, any estrogenic or thyrogenic activity caused by the feedstuffs should be taken into account with regards to its potential impact on farmed fish.

1. Introduction

Endocrine disruptors (EDs), as defined by the World Health Organization (WHO), are "exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effect in an intact organism, its progeny, or (sub) populations" (WHO/IPCS, 2002). The impact of EDs is of particular concern in teleost fish since these animals are exposed to waterborne contaminants during their whole life span. Intensive fish culture makes feedstuffs an alternative vehicle for the incorporation of persistent EDs. The principal potential sources of feed contamination are ingredients of animal origin, and fish aquaculture diets are not an exception (Mantovani et al., 2009; Pelissero and Sumpter, 1992). Long-term sustainability of intensive aquaculture requires the replacement of fish meal and fish oils in aquafeeds by vegetable equivalents (Drew et al., 2007; Gatlin et al., 2007; Glencross et al., 2007). However, plant meals used as substitutes also contain substantial quantities of EDs that negatively affect fish physiology (Beresford et al., 2011; Matsumoto et al., 2004; Pelissero and Sumpter, 1992). Soybean meal is the main source of vegetable protein present in animal diets, although a large number of studies have shown that a high dietary percentage of soybean meal may result in decreased growth and reproductive changes in fish (Drew et al., 2007; Pelissero and Sumpter, 1992). The poor growth rates exhibited by fish fed diets rich in soy flour have been attributed to the presence of estrogenic isoflavones, e.g. daidzein and genistein, in the bile of these fish (Kaushik et al., 1995). In fact, estrogenicity of commercial fish feeds has already been assessed using yeast estrogen-screen assays (Matsumoto et al., 2004) or in vivo experiments (Beresford et al., 2011).

Estrogenic substances can emulate the action of the endogenous estrogen via activation of the estrogen receptors ERs which work as ligand-activated transcription factors. Following agonist binding, the receptor undergoes a conformational change which enhances its affinity for DNA, where it interacts with specific sequences called estrogen responsive elements (ERE), inducing the expression of estrogen-dependent genes (Beato and Klug, 2000). These genes are mainly related to reproduction, differentiation and growth. However in teleosts,

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estrogens are involved in immune system regulation and several studies have related estrogen-like disruptors with immunosuppression (Milla et al., 2011). For all the above, anti/estrogenic compounds have received substantial attention in recent years (Hotchkiss et al., 2008).

Unfortunately, far less attention has been paid to the detection of substances that may disrupt the hypothalamus–pituitary–thyroid axis (HPT). The thyroid of fish secretes L-thyroxine (T4) into the circulation. T4 enters target cells, where it undergoes monodeiodination to 3,3′,5-triiodo-L-thyronine (T3). Thyroid hormones (THs) play critical roles in growth, metabolism and development in all vertebrates (Yen, 2001). But, in fish and amphibians, the thyroid axis also plays a key function in normal development and metamorphosis, larval stages being particularly sensitive to the disruption of the HPT axis (Blanton and Specker, 2007; Carr and Patiño, 2011). There is also evidence that TH may be involved in gonadal sex differentiation, probably via its action on aromatase activity (Mukhi et al., 2007), but also in the proliferation of Sertoli and Leidyg cells and, by extension, in the testis development and function (Matta et al., 2002).

In recent years, many chemicals have been suspected of acting as thyroid disruptors including some polychlorinated biphenyls (PCBs), tetrabromobisphenol A (TBBPA) and polybrominated diphenyl ethers (PBDEs) (Boas et al., 2006). These substances may compete with the endogenous hormones for binding to transport proteins (transthyretin) and/or to TH receptors (TR), acting as either agonists or antagonists and disrupting TH homeostasis (Boas et al., 2006; Kashiwagi et al., 2009). TRs, together with the steroid receptors, belong to the nuclear receptor family and act as ligand-dependent transcription factors which bind to a specific region of the DNA named TRE (thyroid hormone responsive element). Previous studies have found considerable levels of PCBs and dioxin-like substances in fish feeds (Berntssen et al., 2010), where they could mimic endogenous TH and potentially lead to thyroid disruption. To the best of our knowledge, the presence of thyroidal disruptors in fish diets has never been tested. The aim of this study was to simultaneously assess the potential estrogenic and thyrogenic activity of 32 commercial fish diets using hormone receptor-mediated reporter gene activation. For the assessment of estrogenicity, a new estrogen receptor specific reporter gene assay, using sea bass ERα (sbERα) was developed (Muriach et al., 2008). The assay was validated using 17β-estradiol (E2) analogs and ER antagonists and through the screening of sewage effluent samples, previously reported as containing considerable estrogen loads (Carbonell et al., 2011). Potential thyroid disruption was screened with a reporter gene under the control of avian (av) THRα1 (Jugan et al., 2007). We demonstrate that extracts from 11 of the 32 assayed fish diets activated sbERα, while, 18 diets activated avTHRα1.

2. Materials and methods

2.1. Chemicals

17β-Estradiol (≥98% purity), tamoxifen (≥99% purity), 17-α-estradiol (≥ 98% purity), 17-α-methyltestosterone (≥ 97% purity), 3′, 5-Triiodo-l-thyronine (T3, ≥98% purity), etnylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), methanol (≥99.9% purity), tricineKOH, bovine serum albumina (BSA), $MgCl_2$, isobutylmethylxanthine (IBMX), dithiothreitol (DTT), ATP, Coenzyme A hydrate (CoA) and luciferin were purchased from Sigma-Aldrich (Madrid, Spain). Fetal bovine and horse serum (FBS and FHS), ultraglutamine, penicillin–streptomycin (10.000 U/ml), hygromycin, tripsin, geneticin, ultraglutamine 1, and cell culture Dulbecco's Minimal Essential Medium (DMEM) were obtained from Lonza (Barcelona, Spain). Phenol red-free DMEM was from PanBiotech (Zaragoza, Spain). The stock solutions of E2, 17-α-estradiol, tamoxifen and T3 were prepared in DMSO; 17α-methyltestosterone was dissolved in ethanol.

2.2. Extraction of EDs present in fish food

Thirty two commercially available fish feeds were tested for estrogenicity and thyroidal activity. The extraction of estrogenic and thyrogenic substances was carried out with methanol as previously described (Cerdá-Reverter et al., 1996; Matsumoto et al., 2004; Rodríguez et al., 2000) with minor modifications: 0.5 g of each diet were sonicated in 2.5 ml of methanol using Vibra Cell™ ultrasonic probe (Sonic & Materials Inc., Newtown, CT, USA) at 18 kHz in three pulses of 15 s (70% amplitude). Homogenates were then centrifuged at 1700×*g* for 10 min. Supernatants were vacuum-dried and resuspended in 300 μl of methanol. The extracts were maintained at −20 °C until their assessment in the cellular assays.

Recovery tests were designed to evaluate the efficiency of the extraction method. Briefly, 300 μl of a solution of, either 100 μM E2 or 100 nM T3 in methanol, were added to 0.5 g of a diet showing no estrogenic or thyrogenic activity and extracted as above. The spike solution was applied directly onto the grinded feedstuff allowing the interaction of the hormones with the matrix for 30 min. The resulting extracts were named as E2-feed and T3-feed. In order to ensure the proper recovery of the hormones in the methanol and their stability during the extraction process, hormone solutions of E2 and T3 were submitted to the same extraction process. These extracts were named E2–MeOH, and T3–MeOH and contained a concentration of either 100 μM E2 or 100 nM T3, respectively.

Additionally, in a set of fish feeds, this process was performed using hexane as described by Ramos et al. (2004) in order to extract non-polar substances.

2.3. Generation of HER-LUC cell line

The HEK-293 cell line, stably expressing sbERα (Muriach et al., 2008) was cotransfected, in proportion 50:1, with a construct (ERE-TK-LUC) containing the luciferase gene under the control of tandem repetitions of the estrogen responsive element (ERE; Muriach et al., 2008) and a construct carrying resistance to puromycin (Muñoz et al., 2005). Cells were grown in 96-well plates and selected with DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), penicillin (100 units/ml), streptomycin (100 μg/ml) and puromycin (8 μg/ml; Invitrogen) in a humidified atmosphere of 5% CO_2 at 37 °C. Luciferase activity was tested after incubating resistant clones in 96-well plates (15,000 cells/well) with assay medium (DMEM medium + 0.1 mg/ml bovine serum albumin, BSA + 0.1 mM, IBMX) containing 10^{-6}–10^{-12} M estradiol. Forty eight hours post-treatment cells were washed twice with saline phosphate buffer, resuspended into 100 μl of reporter lysis buffer (Promega) and stored at −80 °C until luciferase activity determinations. Lysed cells were pelleted by centrifugation for 30 s at 15,000×*g*, and 20 μl of the supernatant was mixed with 200 μl of luciferin reagent (20 mM TricineKOH, pH 7.8, 0.1 mM EDTA, 8 mM $MgCl_2$, 33.3 mM DTT, 270 μM CoA, 530 μM ATP, 400 μM luciferin). The light emitted was measured in a luminometer (Junior, EG&G, Berthold). The most sensitive cell clone was named as HER-LUC and was selected for subsequent studies. To evaluate unspecific responses due to any basal luciferase transcription activity, HEK 293 cells were transiently transfected only with ERE-TK-LUC construct and exposed to equivalent E2 concentrations.

2.4. Cell culture

HER-LUC cells were grown in 75 cm^2 flasks under 5% CO_2 humidified atmosphere at 37 °C in DMEM supplemented with 10% FBS, 1% antibiotic mixture (Penicillin/Streptomycine) and 2% Ultraglutamine.

The PC-DR-LUC cell line derived from PC-12 cells stably expressing the avTRα1 (Muñoz et al., 1993) and the luciferase reporter gene (Jugan et al., 2007) was used to assess the thyroidal activity of the fish feeds. PC-DR-LUC cell line was a kind gift from Juan Bernal