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Cytotoxicity against fish and mammalian cell lines and endocrine activity of the mycotoxins beauvericin, deoxynivalenol and ochratoxin-A

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ABSTRACT

The toxicity of mycotoxins is well recognized in mammals, but their effects on fish have received less attention. Moreover, in the last years several studies have reported that some mycotoxins may act as endocrine disruptors. The aim of this study was to determine the cytotoxic effects and endocrine activities of three mycotoxins: beauvericin, deoxynivalenol and ochratoxin-A. Cytotoxicity in two fish hepatoma and one mammalian hepatoma cell lines was determined by the AlamarBlue, Neutral Red Uptake and CFDA-AM assays. For the assessment of androgenic, estrogenic and thyroidal agonistic/antagonistic effects three cell lines stably expressing luciferase as reporter gene under the control of hormone receptors were used. Results showed that both fish and mammalian cell lines were very sensitive to the mycotoxins tested. OTA was the least toxic mycotoxin and DON and BEA showed similar acute toxicity. None of the three mycotoxins tested presented agonistic effects at the receptors studied, but all of them showed strong antagonistic effect at the thyroid receptor. BEA showed a weak antagonistic effect at the androgen receptor and OTA produced a biphasic dose-response curve at the estrogen receptor. The data obtained in this work are of high interest for aquaculture industries and for regulators.

1. Introduction

Several fungal species belonging to different fungi genera are capable of producing toxic secondary metabolites known as mycotoxins. Mycotoxins are a large group of organic molecules which show a high chemical structure diversity and a wide variety of biological effects. Under favourable environmental conditions of humidity and temperature these fungi may colonize crops at field level (preharvest stages) or grow on grains during storage, transport and processing (postharvest stages) becoming a source of staple food and/or feed contamination. Moreover, mycotoxins are generally stable compounds during the majority of food/feed processing operations (Bullerman and Bianchini, 2007). Thereby, mycotoxins can be present in food and feed (Bhat et al., 2010; Placinta et al., 1999) representing a serious risk to human and animal health. In addition, in preharvest stages mycotoxins may be run-off from crop fields and reach water bodies affecting aquatic organism and human being, especially through drinking water sources (Hageskal et al., 2009; Pereira et al., 2009), since most mycotoxins are relatively stable in aquatic systems (Pietsch et al., 2011). Several studies have been carried out on cytotoxicity and adverse effects of mycotoxins, especially in mammals and poultry (reviewed in: Escrivá et al., 2015; Hussein and Brasel, 2001). Effects of mycotoxins on fish species have received less attention although both wild and aquaculture farming populations may be potentially affected by waterborne (Schwartz et al., 2010) and/or feed-borne (Adeyemo et al., 2018; Hooft et al., 2011; Moldal et al., 2018; Pietsch et al., 2015, 2014; Tola et al., 2015) mycotoxins. Cereals, as alternative protein sources, are being introduced progressively in fish feed production in the last decade, leading to an increasing cereal-based mycotoxin concentration in fish feed (Pietsch et al., 2011). In this sense, recent studies have reported the presence of different mycotoxins in fish feed or in ingredients used in its elaboration (Fallah et al., 2014; Nácher-Mestre et al., 2015, 2013; Pietsch et al., 2013; Tolosa et al., 2014). For this reason, some toxicity studies have been conducted in fish exposed to mycotoxins. In the reviews of Anater et al. (2016) and Matejova et al. (2017) it is indicated that deoxynivalenol (DON), ochratoxin A (OTA), zearalenone (ZEN) and aflatoxin B1 (AFB1) are responsible of the induction of different disorders in fish growth and physiology. These studies reported reduced feed conversion efficiency, weight loss, increased susceptibility to secondary infection diseases, induced cell and organ alterations, functional and morphological disorders and even mortality in more severe cases. DON is very toxic to rainbow trout (*Oncorhynchus mykiss*) and its effects in this species are related to a reduction of feed intake and metabolic effects (Hooft et al., 2011). AFB1 (El-Sayed and Khalil, 2009) and OTA

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(El-Sayed et al., 2009) affect to behavioral responses (nervous and respiratory manifestations) in marine sea bass (*Dicentrarchus labrax L.*). Moreover, El-Sayed and Khalil (2009) reported that sea bass presented clinical toxic signs and alterations in biochemical parameters after AFB1 exposure. ZEN impairs ovarian development and reproduction in fish (Schwartz et al., 2010; Woźny et al., 2015). However, any data are available about toxicity in fish for emergent Fusarium-mycotoxins such as beauvericin (BEA) or enniatins.

One of the most interesting effects of mycotoxins is their role as potential endocrine disruptors (EDs), as evidenced by the existence of several recent studies on this subject (Fernández-Blanco et al., 2016; Frizzell et al., 2013a, 2013b; 2011; Ndossi et al., 2012). EDs can act at different molecular levels: activating or antagonizing nuclear hormone receptors, affecting the transcriptional activity of nuclear receptors, acting as hormone sensitizers, generating genome-wide effects on DNA metilation status or modulating lipid metabolism and adipogenesis (Tabb and Blumberg, 2006). *In vitro* bioassays are very useful tools for detecting and studying ED actions, measuring effects on nuclear receptors or hormone production (Connolly et al., 2011).

Among the most important mycotoxins, in terms of world-wide occurrence and ubiquity, with potential or confirmed endocrine activity are BEA (Fernández-Blanco et al., 2016), DON (Ndossi et al., 2012) and OTA (Frizzell et al., 2013b). BEA is mainly found in grains, especially in wheat (Serrano et al., 2012). Previous studies (Fernández-Blanco et al., 2016) have assessed BEA effects on estrogen, androgen, glucocorticoid and progestagen receptors, reporting that it induces antagonist responses at glucocorticoid and progestagen receptors level. BEA presents a variety of effects besides acting as endocrine disrupter, such as insecticidal, antitumor, antifungal, antibacterial or antiviral activities (Wang and Xu, 2012) and it is the most potential specific inhibitor of cholesterol acyltransferase (Tomoda et al., 1992). BEA is an ionophoric molecule which transports cations, forming a lipophilic complex with mono or divalent essential cations (K^+, Na^+ or Ca^{2+}) across membranes (Jestoi, 2008). This fact increases ion permeability of biological membranes and thereby affects normal physiological concentrations of these ions (Kouri et al., 2003). Moreover, BEA can disturb the normal cell cycle, induce apoptosis by the mitochondrial pathway, generate reactive oxygen species (ROS) and membrane lipid peroxidation (LPO), modulate the transcriptional factor NF-κB, and generate genotoxic effect (Mallebrera et al., 2018).

DON, like the rest of trichothecenes, is a potent inhibitor of eukaryotic protein synthesis binding to the ribosomal peptidyltransferase site, which results in decreased cell proliferation according to the review by Pestka (2010). Moreover, the same review showed that DON not only alters intracellular signalling via mitogen-activated protein kinases (MAPK) activation but it also can dysregulate the growth hormone, and the neuroendocrine and immune system signalling. In addition, immunomodulatory DON effects vary from induction of the expression of proinflammatory and early response genes at mRNA and protein levels (low DON concentrations) to promotion of leukocyte apoptosis (high DON concentrations) (Pestka, 2010). In relation to its role as ED, previous works (Demaegdt et al., 2016; Ndossi et al., 2012) indicated that DON shows an antagonistic effect on androgen, estrogen and thyroid human hormone receptors and on peroxisome proliferator-activated receptor type 2.

OTA shows a broad toxicological profile, being renal toxicity and carcinogenesis the key adverse effects (Heussner and Bingle, 2015; Pfohl-Leszkowicz and Manderville, 2007). Accordingly, OTA has been associated with several animal and human diseases such as porcine nephropathy, poultry ochratoxicosis, human endemic nephropathies (Balkan endemic nephropathy, BEN) and urinary tract tumours in humans (el Khoury and Atoui, 2010; Heussner and Bingle, 2015; Pfohl-Leszkowicz and Manderville, 2007). Mechanisms of OTA toxicity are not clear yet and seem to be very complex. Between them we can find oxidative stress, inhibition of protein synthesis, mitochondrial dysfunction, genotoxicity, DNA adduct formation, apoptosis, necrosis or cell cycle arrest (Kőszegi and Poór, 2016). However, what does seem clear is that OTA toxicity is associated with its isocoumarin moiety and most likely with the lactone carbonyl group (Heussner and Bingle, 2015). In regard to OTA endocrine disrupting activity, the studies of Frizzel et al. (2013b) and Demaegdt et al. (2016) found that OTA acts as an antagonist for the androgen, estrogen, glucocorticoid, progestogen and thyroid receptors and for the peroxisome proliferator-activated receptor type 2.

The aim of the present study was to assess the cytotoxic effects of mycotoxins on piscine versus mammalian cell lines and to evaluate their endocrine disruption activities at receptor level. Three mycotoxins (BEA, DON and OTA) reported as common food and feed contaminants and susceptible to act as EDs have been selected.

2. Materials and methods

2.1. Reagents/chemicals

Dihydrotestosterone (DHT) (≥97.7% purity), 17β-estradiol (E2) (≥98% purity), triiodothyronine (T3) (≥98% purity), tamoxifen (≥99% purity), dimethyl sulfoxide (DMSO), BEA (MW: 784 g/mol), DON (MW: 296.32 g/mol), OTA (MW: 403.81 g/mol), methanol (≥99.9% purity), sodium dodecyl sulfate (SDS), glacial acetic acid, 3-amino-7-dimethylamino-2-methylphenanzine hydrochloride solution (0.33%), neutral red (NR), sodium pyruvate (100 mM), dithiothreitol (DTT), coenzyme A hydrate (CoA), and luciferin were obtained from Sigma-Aldrich (Madrid, Spain). Foetal bovine and horse sera (FBS and HS), ethylenediaminetetraacetic acid (EDTA), 0.5% trypsin/0.02% EDTA, L-glutamine (200 mM), ultraglutamine (200 mM), penicillin-streptomycin (P/S, 10,000 units penicillin and 10 mg streptomycin/mL), geneticin (G-418, 100 mg/mL), non-essential amino acids (NEAA), cell culture Dulbecco's Minimum Essential Medium (DMEM) and Eagle's Minimum Essential Medium (EMEM) were purchased from Lonza (Barcelona, Spain). Minimum Essential Medium (MEM) and DMEM:F12 were from Gibco (Madrid, Spain). Phenol red-free DMEM was from PanBiotech (Zaragoza, Spain). FBS-Charcoal was from IBIAN Technologies (Zaragoza, Spain). Hygromycin B (100 mg/mL) and zeocin (100 mg/mL) were from Invivogen (Toulouse, France). Ethanol was from Panreac (Barcelona, Spain). AlamarBlue (7-Hydroxy-3H-phenoxazin-3-one-10-oxide) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) were from Invitrogen (Madrid, Spain). The stock solutions of E2, DHT, T3 and tamoxifen were prepared in DMSO; BEA, DON and OTA were dissolved in methanol.

2.2. Cytotoxicity studies

2.2.1. Cell culture

Two piscine cell lines from fish species of cold and warm water (RTH-149 and PLHC-1, respectively) and a mammalian cell line (H4IIE), all from hepatic tissues, were used to evaluate the cytotoxic effects associated to BEA, DON and OTA. Piscine cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and mammalian cell line was from the European Collection of Authenticated Cell Cultures (ECACC). All of them were cultured in 75-cm^2 flasks under a 5% CO_2 humidified atmosphere. PLHC-1 (*Poeciliopsis lucida* hepatocellular carcinoma) cell line was grown in EMEM (Cat. No: BE12-662F, Lonza) supplemented with 5% FBS, 1% antibiotic mixture P/S and 1% L-glutamine at 30 °C. RTH-149 (rainbow trout hepatoma) cell line was maintained at 20 °C in EMEM (Cat. No: BE12-125F, Lonza) containing 10% FBS, 1% antibiotic mixture P/S, 1% NEAA, 1% L-glutamine and 1% sodium pyruvate. Finally, the rat hepatoma cell line H4IIE (derived from *Rattus norvegicus* and used as representative of mammal species) was cultured at 37 °C in the same supplemented EMEM medium used for RTH-149 cells but without sodium pyruvate. Cell lines were split twice a week using PBS/EDTA and trypsin/EDTA.

2.2.2. AlamarBlue, CFDA-AM and Neutral Red Uptake (NRU) assays (triple assay)

A Triple assay consisting in AlamarBlue (metabolic activity), CFDA-AM (plasma membrane integrity) and NRU (lysosomal membrane integrity) assays was performed on the PLHC-1 (cell passages number range 44–55), RTH-149 (cell passages number range 182–189) and H4IIE (cell passages number range 4–12) cell lines. Three assays were conducted to identify the most sensitive endpoint of toxicity avoiding, in this way, sub-estimates of toxicity and to identify possible mechanisms of toxicity. Cells were seeded into transparent, flat-bottom 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) by adding 100 µL of cell suspension ($5{\cdot}10^5$ cells/mL to PLHC-1 and $2.5{\cdot}10^5$ cells/mL to RTH-149 and H4IIE) to each well and incubated overnight to allow them to attach to the plates (reaching at least 80% confluence). Then, cells were exposed for 24 h to serial 1/2 dilutions of mycotoxins resulting in the following concentration ranges: BEA from 0.00076 µM to 25 µM (0.0006–19.6 µg/mL); DON from 0.003 µM to 100 µM (0.0009–29.6 µg/mL) and OTA from 0.003 µM to 100 µM (0.001–40.4 µg/mL). These ranges were chosen as preliminary assays indicated a high toxicity of BEA at 25 µM. In addition, as a positive control for cytotoxicity, six concentrations of SDS were included in each plate (dilution factor of 2/3 from a maximal concentration of 250 µM (72 µg/mL)). Cells receiving only medium were used as negative controls. In addition, cells exposed to the vehicle at the maximal concentration present in the treatments (methanol 0.14% for DON and 0.35% for BEA and OTA) served as the assay controls. The assays were conducted as described originally by Dayeh et al. (2005) with modifications described in detail in Lammel et al. (2013). At least three independent experiments were carried out. Each mycotoxin concentration and negative and assay controls were tested in triplicate in each plate. Briefly, following exposure, the medium was removed and the cells washed once with 200 µl PBS. Then, 100 µl MEM containing 1% NEAA, 1.25% (v/v) AlamarBlue and 4 µM CFDA-AM were added to each well and incubated for 30 min in the dark at the incubation conditions for each cell line. Subsequently the fluorescence intensity was measured at excitation/emission wavelengths of 532/590 nm (AlamarBlue assay) and 485/535 nm (CFDA-AM assay) using a Tecan GENios microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Afterwards, the cells were washed with PBS and incubated for 1 h after adding 100 µL of NR solution (0.03 mg/mL). The cells were washed with PBS and the retained NR extracted with 100 µL of acidified 50% ethanol (ethanol:water:acetic acid 50:49:1, respectively). NR fluorescence was measured at 532/680 nm (excitation/emission) in the Tecan GENios microplate reader. Fluorescence readouts were corrected by subtracting the fluorescence of the cell-free control and normalized with respect to the vehicle control.

2.3. Endocrine effects

2.3.1. Cell culture

In order to evaluate the androgenic, estrogenic and thyroidal activity of the three mycotoxins under study three cell lines were used. The cells AR-EcoScreen™ (Otsuka Pharmaceutical, Tokyo, Japan) were used to assess androgenic activity. These cells are derived from a Chinese Hamster Ovary (CHO) cell line stably transfected expressing the human androgen receptor (AR) and luciferase as reporter gene. The HER-LUC cells (Quesada-García et al., 2012) are derived from HEK-293 cells stably transfected with the marine sea bass estrogen receptor (ER) α (Muriach et al., 2008) and luciferase as reporter gene. This cell line was employed to assess estrogenic activity. Finally, the PC-DR-LUC cell line (kindly donated by Juan Bernal, CSIC, Madrid, Spain) derived from PC-12 cells stably expressing the avian thyroid receptor (TR) α1 (Muñoz et al., 1993) and the luciferase reporter gene (Jugan et al., 2007) was used to evaluate the thyroidal activity of the mycotoxins. All of them were grown in 75-cm^2 flasks under a 5% CO_2 humidified atmosphere at 37 °C. HER-LUC and PC-DR-LUC cell lines were cultured in DMEM (Cat. No: BE12-614F, Lonza) supplemented with 10% FBS, 1% antibiotic mixture P/S and 2% ultraglutamine for HER-LUC and, with 15% serum (10% HS + 5% FBS), 1% antibiotic mixture P/S, 0.5% ultraglutamine, 0.8% geneticin and 0.8% hygromycin B for PC-DR-LUC. AR-EcoScreen™ cells were maintained in DMEM:F-12 medium, supplemented with 10% FBS, 1% antibiotic mixture P/S, 0.025% hygromycin B, 0.05% zeocin and 1.2% L-glutamine. The cells were split twice weekly after detaching them with trypsin/EDTA.

2.3.2. Screening of (anti)-androgenic, (anti)-estrogenic and (anti)-thyroidal activities

Exposure experiments were performed in 96-well, white, opaque, culture plates (Perkin Elmer, Groningen, Netherlands). Cells were seeded by adding 100 µL of cell suspension ($1{\cdot}10^5$ cells/mL to AR-EcoScreen™ and $2.5{\cdot}10^5$ cells/mL to HER-LUC and PC-DR-LUC) and incubated overnight to allow them to attach to the plates and reach at least 80% confluence (cell passages number ranges were 9–20 to AR-EcoScreen™ and 19–29 to HER-LUC cells and PC-DR-LUC cells). After that, the three cell lines were exposed to serial 1/2 dilutions of each mycotoxin for 24 h. BEA and DON concentrations ranged from 0.2 nM to 25 µM whereas the maximal concentration used for OTA could reach 100 µM as no decrease on viability was observed at this concentration. The final volume for each well in the plate was 100 µL. Positive and negative controls were included in each plate. Hormones activating each of the receptors were used as positive controls (serial 1/2 dilutions from 0.04 to 5 nM DHT for the AR, 0.008–1 µM E2 for the ER or 0.008–1 nM T3 for the TR). Cells exposed only to medium served as negative controls. Assay controls were cells treated with the maximum concentrations of the respective vehicle treatments (methanol 0.14% for DON and 0.35% for BEA and OTA; and DMSO $5{\cdot}10^{-5}$% for DHT, $1.25{\cdot}10^{-5}$% for T3 and $1{\cdot}10^{-2}$% for E2). At least three independent experiments were carried out and controls and each concentration of the different mycotoxins were assayed in duplicate in each plate.

In order to evaluate a possible antagonistic effect by the tested mycotoxins anti-androgenic, anti-estrogenic and anti-thyroidal activity assays were also carried out. In these assays cells were co-treated with a constant concentration corresponding to the effective concentration of each hormone causing a 50% of the maximal effect as observed in the positive controls (EC_{50}) (0.1 nM for DHT, 0.1 µM for E2, and 0.1 nM for T3) and with the previously described concentration range for each mycotoxin. Procedures and experimental design were the same in both agonistic and antagonistic effect assays.

Following to the exposure to the mycotoxins, a cell viability assay was conducted to determine the cytotoxic concentrations. This is needed to confirm that luminescence decrease is due to mycotoxin endocrine effects. Viability was determined by measuring the cellular metabolism by means of the resazurin method (O'Brien et al., 2000). Afterwards, in the same plate, the production of luminescence by interaction of the mycotoxins with the receptors was measured as described in Quesada-García et al. (2012). For that, the exposure medium was removed and the cells washed once with 200 µl PBS. Then, 100 µl MEM containing 1% NEAA and 1.25% (v/v) AlamarBlue were added to each well. The 96-well plates were incubated for 30 min in the dark under 5% CO_2 humidified atmosphere at 37 °C. Subsequently the fluorescence intensity was measured at excitation/emission wavelengths of 532/590 nm respectively, using a Tecan GENios microplate reader (Tecan Group Ltd.). After the viability assay, the luminescence assay was performed. For that the medium containing the AlamarBlue was removed and the cells were rinsed once with PBS. 50 µl of Glow mix solution (Besselink et al., 2004) were added per well and the plates incubated for 4 min in the dark at room conditions. Bioluminescence was measured using the microplate reader. Luminescence readouts were corrected by subtracting the luminescence of the methanol vehicle control, normalized with respect to cells treated with the lowest concentration of mycotoxin where any effect was observed, and expressed as percentage of luminescence.

2.3.3. Assessment of OTA effect at ER level

OTA effect at ER level was analyzed to evaluate if the luciferase signal increase could be mediated by a factor other than ER activation. Therefore, HER-LUC cells were pretreated with the ER antagonist tamoxifen at three different concentrations (1, 10 and 20 μM) for 2 h. Then, all the medium was discarded and replaced by fresh medium and tamoxifen pretreated cells were then treated with OTA (0.01–100 μM) and a constant concentration of E2 (EC_{50} = 0.1 μM) like in the antagonist assay. In addition, an anti-estrogenic activity assay as previously described, was repeated in parallel to each assay. Two positive controls were used. The first one consisted in serial 1/2 dilutions of 1 μM E2 to verify the ER response to E2. The second one was the same but with tamoxifen addition (1, 10 or 20 μM) to check the tamoxifen effect as ER blocking compound. Negative controls were cells treated with tamoxifen (1, 10 or 20 μM). Cells treated with the highest methanol (0.35%) and DMSO ($1{\cdot}10^{-2}$%) concentrations for OTA and E2 treatments, respectively, served as vehicle controls. Every exposure was assayed in duplicate in each plate.

2.4. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). All the statistical analyses were performed using Sigma Plot version 12.5 (Jandel Scientific, CA, USA). Normality of the distributions was verified with the Shapiro-Wilk test. Sigma Plot checks automatically for homogeneity of variance. The viability of piscine and mammalian cells after mycotoxin exposure related to the control group (cells treated with the vehicle) was evaluated by a one-way repeated measures analysis of variance (RM-ANOVA, $p < 0.05$) followed by the Holm-Sidak test. To calculate the concentration that causes a 10 or a 50% inhibition (IC_{10} and IC_{50}, respectively), with respect to the control of the three endpoints of cytotoxicity, the results obtained were fitted to a regression equation for a four-parameter logistic curve: $y = \min + \max/(1+(x/IC_{50})^b$ (where max and min are the maximal and minimal response observed, respectively, and b is the slope of the curve). To assess the influence of the intracellular endpoint and cell line parameters in the toxicity of each mycotoxin, a two-way analysis of variance (ANOVA, $p < 0.05$) was run followed by the Holm-Sidak test. Calculation of the EC_{50} values for each hormone in the antagonistic effect assays was done by applying the equation previously described to estimate the IC_{10} and IC_{50} values. To evaluate the statistical significance of luciferase induction within treatments a RM-ANOVA ($p < 0.05$) was used followed by a Dunnett's test to compare means of treatments with respect to the value of the control group (the lowest concentration of mycotoxin and hormone EC_{50} mix in the endocrine activities study and OTA and E2 EC_{50} mix in the assessment of OTA effect at E2 receptor level). Mycotoxin concentrations inducing a significant cell viability decrease (RM-ANOVA followed by Dunnett's test), in the AlamarBlue assay previous to the bioluminescence assay, were considered cytotoxic and excluded from the data analysis.

3. Results

3.1. AlamarBlue, CFDA-AM and Neutral Red Uptake (NRU) assays

As explained above, two piscine cell lines (PLHC-1 and RTH-149) and a mammalian cell line (H4IIE) were employed to assess the cytotoxic effects associated to BEA, DON and OTA. IC_{10} and IC_{50} were calculated regarding to the three different endpoints cellular metabolism at mitochondria level (AlamarBlue), plasma membrane integrity (CFDA-AM) and lysosome membrane integrity (NRU). Table 1 shows the influence of cell line and intracellular endpoints parameters in the cytotoxicity of each mycotoxin.

Regarding BEA IC_{10} values for the different endpoints of cytotoxicity within the same cell line, significant differences were found (ANOVA $F_{2,21}$ = 13.786, $P < 0.001$), as well as within the different cell lines for a same endpoint (ANOVA $F_{2,21}$ = 39.237, $P < 0.001$). As shown in Table 1, BEA resulted to be more toxic for the mitochondria and lysosomes than for the plasma membrane in the PLHC-1 fish cell line. In the other fish cells RTH-149, BEA was more toxic to the mitochondria than to the plasma and lysosomes membranes and it was equally toxic at the three intracellular levels in the rat cells H4IIE. The three cell lines were equally sensible to BEA at mitochondria level, whereas the rat cell line was more sensible than fish cell lines at membrane level. In relation with cytotoxicity at lysosomal level, PLHC-1 and H4IIE cells showed same sensitivities and higher than for RTH-149. From these results it can be concluded that fish cell lines are as sensible as the mammalian cell line to BEA.

The same results were obtained for the IC_{50} values in relation to the endpoints of toxicity within each cell line (ANOVA $F_{2,21}$ = 28.733, $P < 0.001$), except for the RTH-149 cells. Regarding BEA IC_{50} values for each endpoint in the three cell lines (ANOVA $F_{2,21}$ = 199.480, $P < 0.001$), a similar pattern in comparison with the IC_{10} values was only observed with the CFDA assay. At plasma and lysosomal membrane levels the rat cells are more sensible than fish cells whereas at mitochondrial level the H4IIE and PLHC-1 cell lines showed no significantly different EC_{50}s.

In relation to DON IC_{10} values for each endpoint within the same cell line, this mycotoxin was similarly toxic in PLHC-1 and H4IIE cell lines (ANOVA $F_{2,20}$ = 73.067, $P < 0.001$). In both cases, the most sensible endpoints of toxicity were the mitochondria and the lysosome. However in the RTH-149 cells, the cellular metabolism was highly disturbed followed by the lysosome and membrane integrities. With respect to IC_{10} values obtained for each cell line within the same endpoint (ANOVA $F_{2,20}$ = 30.223, $P < 0.001$), no significant differences were found at the mitochondria level between the three cell lines, but the RTH-149 cells were less sensitive than the other two cell lines at lysosome and plasma membrane levels. Looking at DON IC_{50} values within the same cell line for the three endpoints (ANOVA $F_{2,20}$ = 23.374, $P < 0.001$), only similar results were obtained in the PLHC-1 cell line. No IC_{50} could be calculated in the RTH-149 cell line. From DON IC_{50} values within the same endpoint for the three cell lines (ANOVA $F_{2,20}$ = 74.352, $P < 0.001$) it could be observed that DON was similarly toxic to PLHC-1 and H4IIE and less toxic to RTH-149, except for AlamarBlue assay.

Concerning OTA IC_{10} values within the same cell line for the three endpoints (ANOVA $F_{2,21}$ = 30.160, $P < 0.001$) very different results were obtained in each cell line. Different patterns were also observed for each endpoint within the three cell lines (ANOVA $F_{2,21}$ = 18.213, $P < 0.001$). In relation to the OTA IC_{50} values, the most of them were over the concentrations range evaluated in this study. Considering the cell line and the three endpoints (ANOVA $F_{2,19}$ = 50.626, $P < 0.001$), just the NRU assay showed IC_{50} values under the maximum concentration tested in PLHC-1 and H4IIE cell lines. In RTH-149, despite the fact that IC_{50} values were over the maximum OTA concentration tested, the cell viability curves (Fig. 1) followed the same trend than IC_{10} values, presenting a higher effect at metabolic level (cell viability decrease to 66.6% respect to the control) than at plasma membrane and lysosomal levels. Within the same endpoint for the three cell lines (ANOVA $F_{2,19}$ = 21.045, $P < 0.001$), PLHC-1 cells presented the lowest value in NRU assay followed by H4IIE and finally RTH-149 cells (over range value).

Cells can be damaged differently at subcellular level, depending on the toxic mechanism of action of the substance. This is the reason to conduct different cytotoxicity assays informing of different cell targets, to identify the most sensitive endpoint and to avoid sub-estimates of toxicity. Looking at the lowest IC_{10} values from Table 1, OTA was the least toxic mycotoxin followed by BEA and DON for the three cell lines. However, considering the lowest IC_{50}s BEA and DON were more toxic than OTA for PLHC-1 and H4IIE whereas only BEA allowed to calculate an IC_{50} value in RTH-149 cells.

Table 1
Mycotoxin cytotoxic effects at the three different endpoints tested in the Triple assay. Values of inhibitory concentrations (IC) are expressed in μg/mL as mean ± SEM.

IC ± SEM (μg/mL)		IC_{10}			IC_{50}		
		PLHC-1	RTH-149	H4IIE	PLHC-1	RTH-149	H4IIE
BEA	AlamarBlue	1.13 ± 0.07^{A}	1.93 ± 0.31^{A}	1.10 ± 0.16	$2.89 \pm 0.51^{A,a}$	$5.96 \pm 0.63^{A,b}$	1.80 ± 0.12^{a}
	CFDA-AM	$2.95 \pm 0.77^{B,b}$	$5.64 \pm 0.61^{B,c}$	1.10 ± 0.20^{a}	$4.68 \pm 0.64^{B,b}$	$13.61 \pm 0.70^{C,c}$	2.04 ± 0.29^{a}
	NRU	$1.99 \pm 0.25^{AB,a}$	$7.61 \pm 0.36^{B,b}$	1.29 ± 0.13^{a}	$3.63 \pm 0.63^{AB,b}$	$11.30 \pm 0.16^{B,c}$	1.68 ± 0.13^{a}
DON	AlamarBlue	0.09 ± 0.05^{A}	0.20 ± 0.04^{A}	0.29 ± 0.26^{A}	$2.65 \pm 1.28^{A,a}$	$>29.60^{b}$	$>29.60^{B,b}$
	CFDA-AM	$2.07 \pm 0.96^{B,a}$	$13.58 \pm 2.07^{C,b}$	$1.88 \pm 0.69^{B,a}$	$10.51 \pm 4.28^{B,a}$	$>29.60^{b}$	$16.31 \pm 1.74^{B,a}$
	NRU	$0.45 \pm 0.18^{A,a}$	$1.48 \pm 0.27^{B,b}$	$0.02 \pm 0.004^{A,a}$	$1.52 \pm 0.26^{A,a}$	$>29.60^{b}$	$1.20 \pm 0.10^{A,a}$
OTA	AlamarBlue	$2.57 \pm 0.13^{A,a}$	$3.21 \pm 0.29^{A,a}$	$>40.40^{B,b}$	$>40.40^{B}$	>40.40	$>40.40^{B}$
	CFDA-AM	$>40.40^{B,b}$	$20.70 \pm 1.58^{B,a}$	$>40.40^{B,b}$	$>40.40^{B}$	>40.40	$>40.40^{B}$
	NRU	$3.61 \pm 0.48^{A,a}$	$27.00 \pm 1.37^{B,b}$	$9.75 \pm 0.61^{A,ab}$	$5.47 \pm 0.8^{A,a}$	$>40.40^{c}$	$21.65 \pm 2.05^{A,b}$

Upper case letters indicate significant differences between the three cellular endpoints regarding the same cell line for each mycotoxin (vertical direction). Lower case letters indicate significant differences between the three different cell lines within the same endpoint in relation to IC_{10} and IC_{50} (horizontal direction).

3.2. Androgenic, estrogenic and thyroidal activities of BEA, DON and OTA

As previously mentioned, three cell lines permanently transfected with luciferase as reporter gene under the control of hormone receptors were used for the assessment of endocrine activities. None of the three mycotoxins tested activated any of the three types of receptors under study (data not shown). However, all of them showed anti-hormonal activity, thus being able to decrease the response caused by the EC_{50} of the corresponding hormone. These anti-hormonal activities were observed at non-toxic doses as measured by the AlamarBlue assay (data not shown). BEA did not induce any antagonistic effect at ER level in the cell line HER-LUC (data not shown), however it showed anti-androgenic and anti-thyroidal activities (Fig. 2A and B respectively) provoking a reduction of the luminescence induced by DHT EC_{50} and T3 EC_{50} in AR-EcoScreenTM and PC-DR-LUC cells, respectively. In the anti-androgenic activity assay just the highest non-toxic concentration (3.125 μM) showed a significant antagonistic effect (Fig. 2A). On the other hand, in PC-DR-LUC cells BEA showed anti-thyroidal activity at 0.78 μM and 1.56 μM concentrations (Fig. 2B). The mycotoxin DON only showed antagonistic effect at TR level, provoking a significant bioluminescence decrease in PC-DR-LUC cells at 0.39 μM and 0.78 μM concentrations (Fig. 2B). Finally, OTA was able to induce anti-thyroidal and anti-estrogenic responses in PC-DR-LUC and HER-LUC cells respectively. OTA anti-thyroidal effect occurred at the concentration

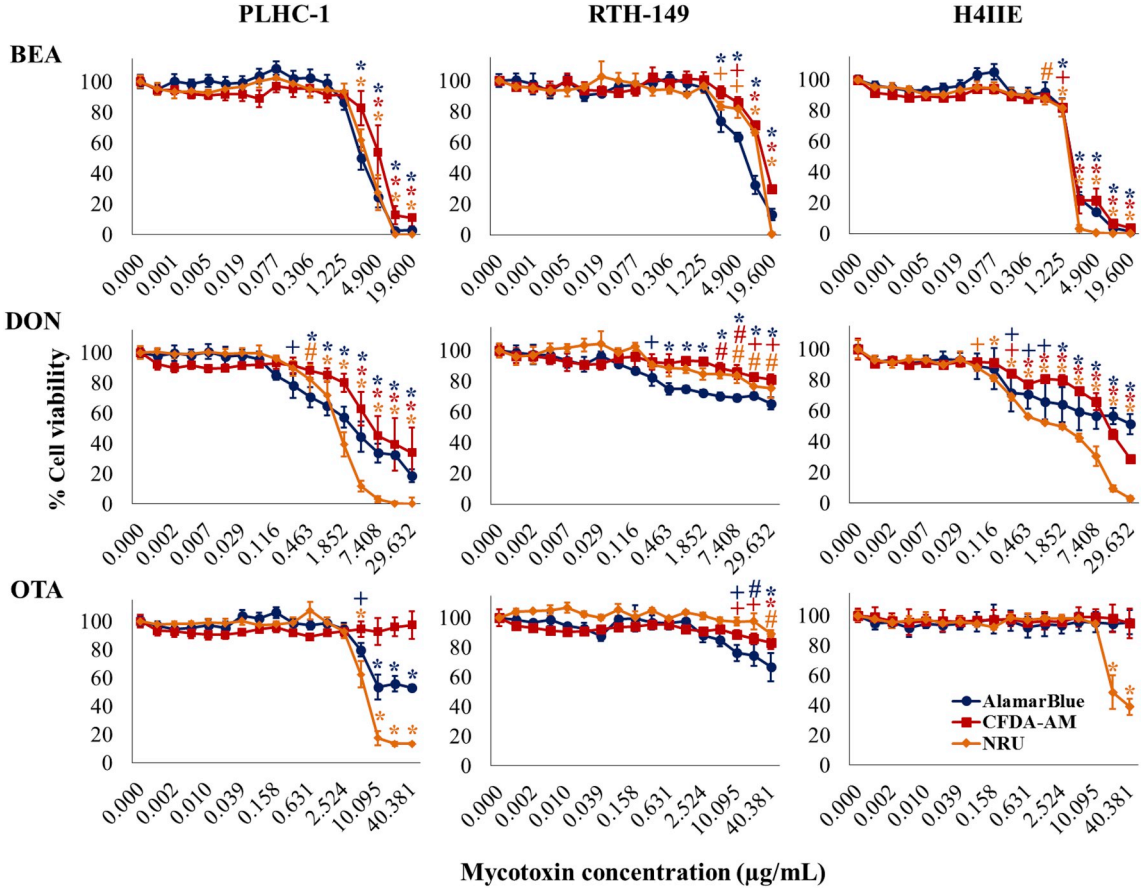

Fig. 1. Viability of piscine and mammalian cells after mycotoxin exposure. Significant differences respect to the control: #p < 0.05, + p < 0.01 and * p < 0.001.

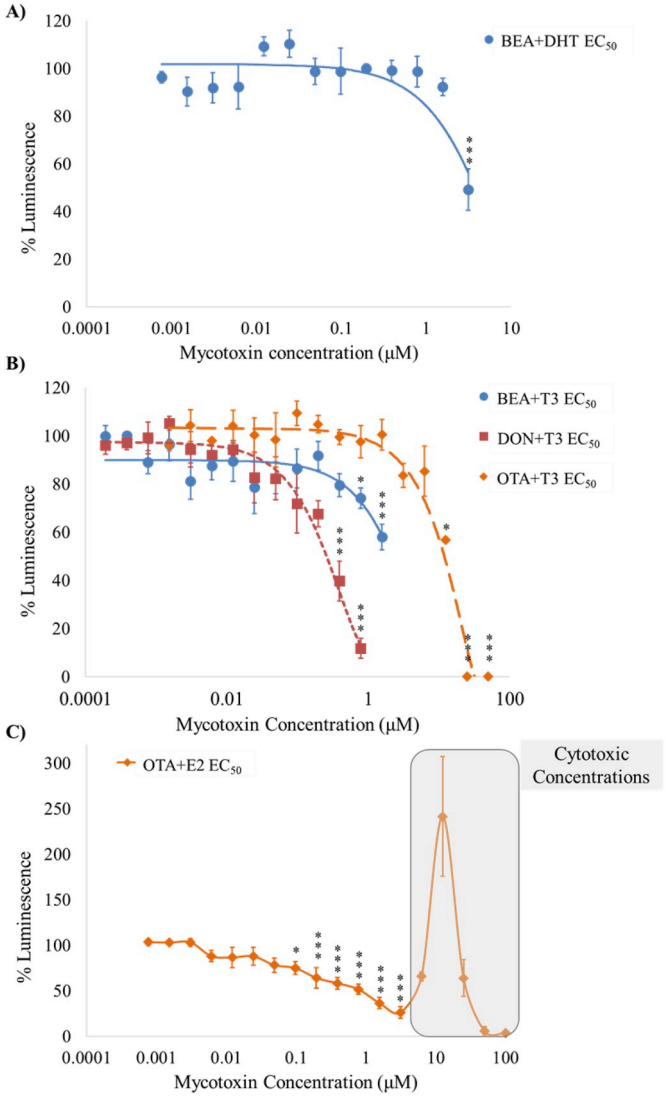

Fig. 2. Endocrine activities determination: A) Anti-androgenic (AR-EcoScreen™ cells), B) Anti-thyroidal (PC-DR-LUC cells) C) Anti-estrogenic activities (HER-LUC cells); DHT: dihydrotestosterone, T3: triiodothyronine, E2: 17β-estradiol. *significant differences respect to the control, *p < 0.05; ***p < 0.001.

range between 12.5 and 50 μM (Fig. 2B). Regarding to the OTA anti-estrogenic activity assay, the treatment with E2 at its EC_{50} and OTA generated a double response by the HER-LUC cells. At non cytotoxic concentrations, OTA triggered an antagonistic response provoking a bioluminescence decrease in a dose-dependent manner in a wide range of concentrations (0.1–3.125 μM). However, at higher doses ranging from 6.25 to 100 μM, which fall within the cytotoxic concentrations range, OTA induced a strong luminescence increase, with a maximum value two times higher than the control at the concentration of 12.5 μM (Fig. 2C). The cytotoxicity provoked at these five concentrations was very low; the cell viability ranged from 82.6 ± 2.14 to 79.6 ± 3.35% at 6.25 μM and 100 μM, respectively.

3.3. Assessment of OTA effect at E2 receptor level

To elucidate if the increase in luminescence emission was direct consequence of the ER activation by the mixture OTA and E2, an anti-estrogenic activity assay with the ER blocking compound tamoxifen was also performed (Fig. 3). The resulting profile was very similar to the previous result obtained in the OTA antagonistic effect assay without tamoxifen. Again, a dose-dependent manner decrease was followed by a very high bioluminescence increase (around six times higher than initial concentration) at the toxic concentrations range indicated previously. Tamoxifen was able to reduce the luminescence emission at each concentration tested following a dose-dependent reduction. At the maximum luminescence emission value (corresponding to 25 μM mycotoxin concentration), the addition of tamoxifen (1, 10 and 20 μM) resulted in a reduction of bioluminescence of approximately 50, 80 and 100% respectively (Fig. 3).

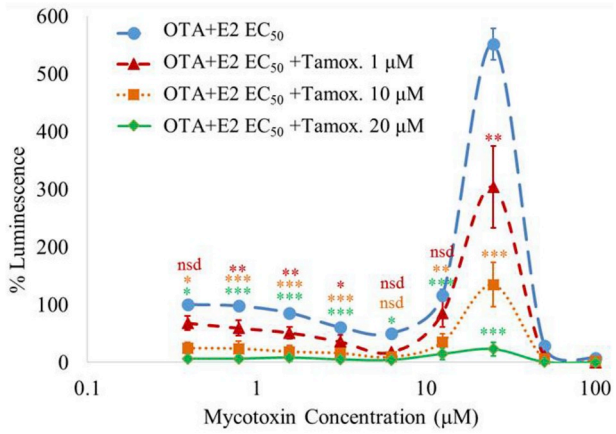

Fig. 3. OTA + E2 EC_{50} response evaluation by addition of a receptor-blocker. *significant differences respect to OTA + E2 EC_{50} at each concentration level analyzed by RM-ANOVA followed by Dunnett's test, *p < 0.05; **p < 0.01; ***p < 0.001; nsd: no significant differences. The upper, intermediate and lower significances (* or nsd) in the graph corresponds to those calculated for tamoxifen at 1 μM, 10 μM and 20 μM, respectively.

4. Discussion

In the two piscine cell lines BEA exerted its cytotoxic effects principally at metabolic level. The results from Table 1 indicated that BEA was similarly toxic to the PLHC-1 fish and the H4IIE mammalian cell line. To our best knowledge, this is the first work about BEA cytotoxic effects on fish cell lines. BEA exhibited high toxicity in the three cell lines under study, with IC values between 1.10 and 13.61 μg/mL depending on the endpoint of cytotoxicity and the cell line. This was specifically true for H4IIE where metabolic activity, lysosome and plasma membranes where similarly and highly affected. Ruiz et al. (2011) also obtained very close IC_{50} values between metabolic (10.02 μM equivalent to 7.85 μg/mL) and lysosomal levels (11.08 μM equivalent to 8.68 μg/mL) in mammalian kidney cells after 24 h BEA exposure. Moreover, the IC_{50} values presented by BEA in the present study at cellular metabolism level in H4IIE are very similar to the ones obtained by Wätjen et al. (2014) in this cell line (IC_{50} for 24 h = 1.9 μM equivalent to 1.49 μg/mL). Previous studies (Prosperini et al., 2013; Tonshin et al., 2010) have reported that BEA affects mitochondrial and lysosomal functionality in mammalian cells. Lysosomal membrane permeabilization has been connected with mitochondrial outer membrane permeabilization (Boya and Kroemer, 2008). This could explain BEA cytotoxic effect at these two levels. In addition, it was well-established that BEA increases the cytosolic calcium concentration forming a lipophilic complex with this divalent essential cation and decreasing the mitochondrial transmembrane potential among other effects (Jow et al., 2004). However, these ionophoric properties of BEA are not only limited to act as a carrier, but it also presents the ability to create pores in mammalian cell membranes, forming a cation-selective channel (Kouri et al., 2003). According to Kouri et al. (2003) and to the obtained results in this work, it may be possible that, in high toxicity conditions (or late phases of apoptotic mechanism), a massive pores creation, compromising the plasma membrane integrity, exists. This hypothesis could explain the low IC_{50} values obtained for BEA at

plasma membrane level. In view of the results obtained in this study, it is very possible that the primary toxic action of BEA is related to its ionophoric properties (Jestoi, 2008) and has a clear concentration-dependent effect.

Regarding DON cytotoxicity, PLHC-1 cells showed low IC_{50} values at mitochondrial and lysosomal levels whereas H4IIE cells were principally affected at the lysosomal level (Table 1). There are several previous works about *in vitro* cytotoxicity of DON in mammalian cell lines (Alassane-Kpembi et al., 2015; Cano-Sancho et al., 2015; Döll et al., 2009; Fernández-Blanco et al., 2018; Ruiz et al., 2011; Smith et al., 2018; Sobral et al., 2018; Wentzel et al., 2017; Widestrand et al., 1999) but only Ruiz et al. (2011) and Döll et al. (2009) evaluated DON cytotoxicity at metabolic and lysosomal levels. The results obtained by these authors showed a high toxicity for both endpoints in the tested cells. However, most of these previous studies (Alassane-Kpembi et al., 2015; Fernández-Blanco et al., 2018; Ruiz et al., 2011; Wentzel et al., 2017) obtained quite lower DON IC_{50} values at mitochondrial level than the obtained results in the present work in H4IIE cells. Similarly to our findings, Widestrand et al. (1999) did not obtain an IC_{50} value at their DON tested concentrations in 3T3 cells. Few studies have been conducted in fish cell lines (Mayer et al., 2017; Pietsch et al., 2011). In Mayer et al. (2017), after 48 h at the highest concentration tested (40 μM equivalent to 11.85 μg/mL), the viability in RTgill W-1 cells was reduced by 52% according to the NRU assay. In Pietsch et al. (2011), five fish cell lines of rainbow trout, salmon and carp were exposed to a DON concentration range from 0.025 to 3 μg/mL and the mycotoxin cytotoxicity was evaluated at mitochondrial, plasma membrane and lysosomal levels after 24 h of exposure. Only the trout cell lines RTL-W1 and RTgill-W1 were sensitive to DON at the three endpoints under study, although its effects on cell viability was not very high (reduction by 10–20% at the highest concentration) except for RTL-W1 cells in the metabolic activity assay (reduction by 30% at 1 μg/mL DON). In the rainbow trout RT EQ clone 8 cells, metabolic function did not seem to be affected by DON at any concentration tested. According to DON IC values obtained in this and previous works, DON seems to exert a minor effect at plasma membrane level. All mentioned results reveal that DON cytotoxicity shows distinct endpoint effects depending on the species and also on the type of tissue from which the exposed cells were derived. Again, as occurred with BEA cytotoxicity, PLHC-1 cells response to DON seemed to be more similar to H4IIE than to RTH-149 cells response.

There are several studies about OTA *in vitro* cytotoxicity in mammalian cell lines but to the best of our knowledge this work is the first in fish cell lines. Some of these studies confirm the results obtained in this work at the plasma membrane level (IC_{50} values > 40.40 μg/mL), since OTA produced no effect (Klarić et al., 2008) or presented a very high IC_{50} for this endpoint (Bouaziz et al., 2008). Dietrich et al. (2001) obtained a very similar response in the porcine cells PKC to the obtained one in the present work in PLHC-1 and H4IIE cells, with a main effect at lysosomal level (IC_{50} = 40 μM equivalent to 16.15 μg/mL) and a IC_{50} metabolic value over 100 μM (40.40 μg/mL) OTA concentration. However, in the same work no differences were found between metabolic and lysosomal levels (IC_{50} values = > 100 μM equivalent to 40.40 μg/mL) in the porcine cell line LLC-PK1. On the other hand, in the human cell line HepG2, the inverse response to PKC cells was found with an OTA IC_{50} value of 35.27 μM (14.24 μg/mL) at metabolic level (Zheng et al., 2013) and over 100 μM (40.40 μg/mL) at lysosomal level (Hundhausen et al., 2005). In accordance to the previous data and the calculated EC_{50} values by other authors (Heussner et al., 2006) it seems that OTA cytotoxicity in mammal cells lies within the micromolar range and, as well as to DON, its mechanism of toxicity at cellular level seems to depend on the species and tissues from which the cell lines were derived.

Most of the times PLHC-1 cell line showed closer IC values to H4IIE cells than to RTH-149 cell line, which normally presented the higher IC values independent of which mycotoxin was being tested. Considering that H4IIE (37 °C) and PLHC-1 (30 °C) cells, due to their origin (mammal and warm water fish species, respectively), are maintained at higher temperature than RTH-149 (20 °C) cells, and that temperature plays a key role in metabolism through its effects on rates of biochemical reactions (Gillooly et al., 2001), it is possible that their similar responses could be explained by the influence of this factor. OTA presented the lowest toxic effect in all the cell lines used in this work. Previous studies, where the individual cytotoxic effect of OTA and DON (Cano-Sancho et al., 2015; Demaegdt et al., 2016) and OTA and BEA (Klarić et al., 2008) among other mycotoxins have been evaluated, confirmed these results.

Acute toxicity *in vivo* studies with OTA in different species also showed that this mycotoxin presents a high acute toxicity to fish after intraperitoneal administration (LD50s of 0.29 mg/kg bw in marine sea bass and 4.67 mg/kg bw in rainbow trout) (Anater et al., 2016). OTA was also very toxic to mammals with oral LD_{50}s ranging from 0.2 mg/kg bw in dogs to 30.3 mg/kg in male rats (Pfohl-Leszkowicz and Manderville, 2007). To our knowledge, the *in vivo* acute toxicity of DON and BEA in fish species has not been investigated.

Few previous studies indicated that the mycotoxins BEA, DON and OTA present endocrine disruption effects at receptor level (Demaegdt et al., 2016; Fernández-Blanco et al., 2016; Frizzell et al., 2013b; Ndossi et al., 2012). In the present work, an antagonistic response in AR-EcoScreen™ cell line was observed following exposure to 3.125 μM BEA. In Fernández-Blanco et al. (2016), this mycotoxin also presented a strong antagonistic response at AR level at 10 μM BEA in the human mammary gland TARM-Luc cell line. Nevertheless this response was due to the cytotoxicity of BEA at this concentration. Considering that 3.125 μM is the last concentration before the BEA cytotoxic effect in AR-EcoScreen™ cells, it could be that the antagonism observed in our study is due to prelethal toxicity and not a true ER response. In PC-DR-LUC cell line, BEA also provoked an antagonistic effect at 0.78 and 1.56 μM BEA concentrations. To our knowledge no previous data exist about BEA effects at thyroid receptor level and according to the results obtained in the present work, BEA could present an antagonistic effect over this receptor.

DON triggered a strong antagonistic response in PC-DR-LUC cell line after 0.39 and 0.78 μM concentrations exposure of this mycotoxin, decreasing the luminescence induced by T3 EC_{50} to approximately 40% and 12% respectively. The first value was in accordance with the results presented by Demaegdt et al. (2016) for the DON TRβ antagonism assay in the human osteoblast U-2 OS cell line. These authors observed that 0.33 μM DON concentration decreased the luminescence induced by T3 EC_{50} to 50%. Demaegdt et al. (2016) also observed an effect of DON at AR and ER level as a low potency antagonist. However, none response was observed at AR or ER level after DON exposure in the present work, which is according to results obtained by Ndossi et al. (2012).

OTA showed an anti-thyroidal effect at TR level in PC-DR-LUC cell line. This response occurred at the concentrations 12.5, 25 and 50 μM OTA with a high reduction of luminescence. Demaegdt et al. (2016) also observed an antagonistic effect of OTA at TR level (IC_{50} = 1.2 μM) stronger than the one produced in PC-DR-LUC cell line (IC_{50} = 12.8 μM). This could be explained by the fact that they used a cell line with a TR from another organism (human) and the isoform TRβ which probably present a different sensitivity than the avian TRα1. In the present study, OTA also induced a marked anti-estrogenic effect in a wide range of non-cytotoxic concentrations (0.098–3.125 μM OTA). Previous studies have reported OTA anti-estrogenic activity at ER level (Demaegdt et al., 2016; Frizzell et al., 2013b), although Frizzel et al. (2013b) did not consider the response as a true antagonistic effect and related it to a change in cell morphology. In any case, the results obtained in the present work suggest that there is a true OTA antagonistic effect at ER level in non-cytotoxic concentrations range. In addition, in our study OTA triggered a luminescence superinduction, at a concentration range of 6.25–100 μM OTA, which induced a low toxicity to the HER-LUC cells (cell viability around 80%). This agonistic effect did

not occur when HER-LUC cells were exposed only to the mycotoxin (data not shown), so OTA seemed to have an E2 enhancer effect at cytotoxic concentrations. The tamoxifen pre-treatment in the anti-estrogenic activity assay confirmed that this response was mediated by the interaction between OTA and the E2 EC_{50} with the ER, as a luminescence emission decrease was observed with the increasing blocking compound concentration. The co-exposure to OTA and E2 EC_{50} provoked a dose-response curve characterized by a low-dose reduction and high-dose luminescence stimulation. The form of this dose-response curve is similar to a J-shape that has been described as a possible hormesis model (Calabrese, 2005). The luminescence superinduction could be due to the fact that probably OTA could work as a concentration depending-selective estrogen receptor modulator (SERM) (Demaegdt et al., 2016), interacting with different subsets of corepressors and/or coactivators which regulate ER function (Dutertre and Smith, 2000). In a previous work, Sotoca et al. (2010) studied a phenomenon of superinduction of estrogen receptor *in vitro* luciferase based reporter gene bioassays for testing estrogenicity. According to these authors, it could be or the presence of an OTA metabolite the responsible of activating the ER or the superinduction of the estrogen receptor by a post-transcriptional mechanism specific for the luciferase based reporter assay. In any case, these statements are only hypotheses and it will be necessary further investigation to clarify this response.

5. Conclusion

Our results indicate a high toxicity of the three mycotoxins to fish cell lines, which show a similar sensitivity as the mammal cell lines. OTA was the less toxic followed by BEA and DON similarly toxic. It seems very probable that BEA cytotoxicity in the fish cell lines is related to its ionophoric properties. However, the mechanism of toxicity at cellular level of DON and OTA seems to depend on species and tissue from which the cell lines employed in the cytotoxic assessment were derived, the plasma membrane being the less affected.

All the mycotoxins evaluated in this work showed endocrine disrupting antagonistic effects at receptor level. BEA showed some antagonist effect at the AR receptor. DON and OTA presented a high antagonistic effect at the TR receptor at very low concentrations whereas the effect of BEA was less pronounced but still considerable and it is the first time reported. OTA presented a biphasic dose-response curve at the ER, provoking antagonism at non-toxic concentrations but a high induction of the receptor at very low toxicity concentrations. Our study is the first one reporting this effect of OTA at the ER receptor and further studies are needed to clarify the mechanism of induction.

These results highlighted that mycotoxins can be as toxic to fish as to mammals presenting a high acute toxicity and that they present strong effects at hormone receptors. All this findings are of high relevance for the aquaculture industries and for regulators. Effectively, the big lack of data on the effects of these mycotoxins in fish avoids the setting of maximum residue levels for these mycotoxins in fish feed. Our data contribute to the advance of the knowledge in this field.

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