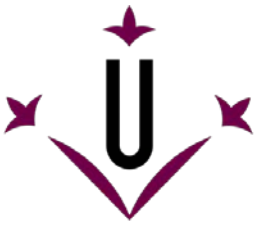

Modulation of the xenobiotic transformation system and inflammatory response by ochratoxin A exposure using a co-culture system of Caco-2 and HepG2 cells.

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Abstract

Cytotoxicity of ochratoxin A (OTA) was evaluated using the MTS assay, and membrane integrity was measured using transepithelial electrical resistance (TEER). A transwell system was used to investigate the effect of OTA on the expression of the *CYP450* (*1A1*, *2A6*, *2B6*, *3A4* and *3A5*), *NAT2*, *COX-2*, *LOX-5*, and *MRP2* genes in Caco-2 and HepG2 cells. TEER decreased by a mean of 63.2% after 24 h in Caco-2 differentiated cells without inducing cell detachment; revealing damage to the intestinal epithelial cell tight junction proteins and an increase in cell permeability. Gene expression analysis showed that modulation of gene expression by OTA was higher in Caco-2 cells than in HepG2 cells, and generally, the duration of exposure to OTA had a more significant effect than the OTA dose. A general OTA down-regulation effect was observed in Caco-2 cells, in contrast with the down- and up-regulation observed in HepG2 cells. In Caco-2 cells, *CYP1A1* was the gene with the highest regulation, followed by *CYP3A4* and *CYP3A5*. Conversely, in HepG2 cells, *CYP2B6* was highly regulated at 3 and 12 h compared to the other cytochromes; *CYP1A1* was slightly modulated during the first 12 hours, but an overexpression was observed at 24 hours.

Our data support the involvement of the *COX-2* and *5-LOX* genes in liver metabolism of OTA. On the basis of the gene expression analysis, the results suggest a possible impairment in OTA secretion at the intestinal and hepatic level due to *MRP2* repression. In addition, we provide evidence of the effect of OTA on *NAT2* gene expression, which had not been reported before.

Keywords: *CYP450*, *NAT2*, *COX*-2, *LOX-5*, *MRP2*, TEER measurement.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by some species of two ubiquitous genera of fungi, *Aspergillus* and *Penicillium*. This mycotoxin is frequently found in a wide variety of raw materials (such as cereals and fruits) and foodstuffs (coffee, beer, wine, cocoa products, and grape juice) (EFSA, 2006). It can also be found in foods of an animal origin due to its bioaccumulation in tissues, even in the case of animals sub-chronically exposed to OTA (Perši et al., 2014). The presence of OTA in feed has been linked to the development of nephrotoxicity, which, in rats, has been associated with renal adenomas and kidney tumours (Boorman, 1989; IARC, 1993). Several mechanisms of OTA toxicity have been described, including competition with phenylalanine for protein synthesis, inhibition of mitochondrial ATP production, production of free radicals, promotion of lipid peroxidation, and direct and indirect damage to DNA (El Khoury and Atoui, 2010; González-Arias et al., 2014; Ringot et al., 2006).

OTA is absorbed in the intestine, where the multi-drug resistance protein 2 (*MRP2* gene) plays an important role acting as a xenobiotic outward transporter to reduce the oral bioavailability and the toxin load to organs and, thereby, OTA toxicity (Berger et al., 2003; Dietrich et al., 2003). Once OTA reaches the bloodstream, it can reach other organs such as the liver, and the MRP2 transporter is again a key primary active transporter involved in anionic conjugate and drug/xenobiotic extrusion into the extracellular space, which contributes to bile formation and the subsequent elimination of the toxin (Dietrich et al., 2003; Jedlitschky et al., 2006).

The inactivation or bioactivation of the OTA molecule via the cytochrome P450 system has been described as a key factor in relation to the *in vitro* and *in vivo* OTA toxicity.

For example, Zepnil et al. (2001) showed that OTA oxidation was mediated by the CYP450 enzymes in *in vitro* reactions with microsomes of liver and kidney from Wistar rats. On the other hand, Pfohl-Leszkowicz et al. (2012) have demonstrated the formation of OTA-derived DNA adducts and mutations and the involvement of the CYP450 enzymes in the bioactivation of OTA. The presence of DNA double-strand breaks and the micronucleus formation observed in *in vivo* or *in vitro* assays with cells capable of expressing cytochrome P450 (CYP450) enzymes support the OTA genotoxicity (González-Arias et al., 2014; Groene et al., 1996; Hibi et al., 2013).

Little is known about the involvement of human N-acetyltransferase 2 (NAT2), a phase II enzyme encoded by the *NAT2* gene. NAT2 shows a restricted tissue distribution, and high levels are found in the human liver and intestine. Lack of NAT2 function is associated with higher incidences of cancer and drug toxicity (Husain et al., 2007). To our knowledge, the study of Lebrun et al. (2002) is the only one that has analysed the involvement of *NAT2* in OTA genotoxicity. The authors observed a positive correlation between DNA damage and *NAT2* polymorphisms in human urothelial cells exposed to OTA.

Regarding the cyclooxygenase-2 (*COX-2*) and lipoxygenase-5 (*5-LOX*) genes, some *in vitro* and *in vivo* assays indicate that the over expression of these genes is related to cancer initiation or chronic inflammation processes in a variety of cancers in humans. For this reason, both genes have been described as inflammatory mediators (González-

Périz and Clàira, 2007; Terzic et al., 2010), but the modulation of these genes by OTA is still poorly studied (Ferrante et al., 2006; Kumar et al., 2013; Ramyaa et al., 2014).

The present study aimed to investigate the effect of OTA on expression of five genes from the CYP450 family (*CYP1A1*, *CYP2A6*, *CYP2B6*, *CYP3A4* and *CYP3A5*), as well as expression of the *NAT2*, *COX-2*, *5-LOX* and *MRP2* genes. Although the involvement of the CYP450 enzymes is well known in OTA biotransformation, much less is known about the role of *COX-2*, *5-LOX* and *NAT2* and the effect of OTA in the modulation of expression of these genes. Considering that OTA is a mycotoxin that mainly enters the human body via ingestion, we have used an *in vitro* cell co-culture system to mimic initial passage through the intestine and hepatic metabolism, using two human cell lines. Caco-2 cell line is a well established *in vitro* model especially indicated to study intestinal absorption, metabolism, and bioavailability of drugs and xenobiotics (Artursson et al., 2001; Natoli et al., 2011). On the other hand, the use of human liver cells, as the HepG2 cell line, in the co-culture system tries to simulate the place where the main xenobiotic metabolism occurs in humans.

2. Methodology

2.1 Reagents and instrumentations

The following products were obtained from Sigma-Aldrich (Steinheim, Germany): OTA standard (98% purity) (ref. O1877), non-essential amino acids (ref. M7145), L-glutamine (ref. G7513), antibiotic mixture (penicillin and streptomycin) (ref. P4333, dimethyl sulfoxide (DMSO) (ref. D8418), and oligonucleotide primers (Table 1). Foetal bovine serum (ref. FB1090500) was supplied by BioSera (Nauille, France). TRIzol® (ref. 15596)

reagent, ethidium bromide (EtBr) (ref. 15585011), TURBOTM DNase (Ambion) (ref. AM2238), SuperScriptTM III reverse transcriptase (ref. 18080) and Oligo(dT)12-18 primer (ref. 18418-012) were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA). Dulbecco's modified Eagle medium (DMEM) (ref. SH30022) and a solution of trypsin-EDTA (0.25%, 0.2 g/L) (ref. SH30042) were obtained from Thermo Fisher (Madrid, Spain). iTaq™ Universal SYBR® Green Supermix (ref. 172-5124) and the Hard-Shell® 96-Well Thin-Wall PCR (ref.6340589) Plates were obtained from Bio-Rad (Hercules, CA, USA). 75 cm^2 flasks (ref. 156499) and 96-well plates (ref. 267313) were supplied by Nunc (Roskilde, Denmark). 6-transwell systems (4.67 cm^2 and 0.4 µm polycarbonate pore size insert) and 6-well plates were obtained from Corning (ref. 3526) (New York, USA). The CellTiter 96® Aqueous One Solution Cell Proliferation Assay (ref. G3582) was obtained from Promega (Madison WI, USA).

2.2 Cell culture

The human colon cell line Caco-2 (HTB-37) and the hepatic cell line HepG2 (HB-8065) were obtained from the American Type Culture Collection (ATCC). Cells were grown as monolayer cultures in 75 cm^2 flasks and maintained with DMEM in an atmosphere at 95% relative humidity and 5% CO_2 at 37 °C. DMEM was supplemented with foetal bovine serum (15% v/v for Caco-2 or 10% v/v for HepG2 cells), 1% (v/v) of non-essential amino acids, 1% (v/v) of L-glutamine and 1% (v/v) of an antibiotic mixture (penicillin and streptomycin). Cellular monolayers (80% confluence) were detached with a solution of trypsin-EDTA, and the cells were reseeded to carry out the cytotoxicity assay or the co-

culture system. Caco-2 and HepG2 cells were used at passages from 33 to 42 and from 15 to 25, respectively.

2.3 Cytotoxic assays

2.3.1 MTS assay

HepG2 cells were reseeded in 96-well plates at a density of 1.5 x 10^5 cells/cm^2, and Caco-2 cells were reseeded at a density of 10^5 cells/cm^2, allowing 24 h for cell adherence. Cell viability was determined using a stable solution of MTS from the CellTiter 96® Aqueous One Solution Cell Proliferation Assay. OTA doses tested were 1, 5, 15, 45, 60, 75, 90 and 180 µM (in DMSO) for 3, 12 and 24 h. The treatments were conducted in cell culture medium, and the percentage of DMSO in the treatments was 0.05% of the final volume. At the end of the treatment, cells were washed once with PBS. Immediately, 100 µL of culture medium with 317 µg/mL MTS-one solution was added per well. Cells were incubated for 2.5 h (Caco-2) or 2 h (HepG2). Optical density was determined using a 96-plate reader (DAS-A3, Roma, Italy) with a 490 nm filter (FGF2, TSP1). Relative cell viability was expressed as a percentage with respect to the cells in the solvent control treatment.

2.3.2 Transepithelial electrical resistance (TEER) measurement

The maintenance of cell monolayers was performed as described in the co-culture section. The TEER value of all of the Caco-2 cell monolayers was recorded at day 21 (before treatments) to determine the effect of OTA on membrane integrity as a

cytotoxicity parameter. TEER recordings were made using a Millicell-ERS electrical resistance system (Millipore Ibérica, Spain) with a STX01 electrode (Millipore Ibérica, Spain), according to the manufacturer´s instructions. Values are expressed as Ω•cm^2, according to the equation 1:

$TEER_{monolayer}$ = ($Resistance_{monolayer}$-$Resistance_{blank}$)·(filter growth area in cm^2) equation 1

$Resistance_{blank}$ is considered as the value of the resistance of a filter without cells.

2.4 Caco-2/HepG2 co-culture system

2.4.1 Caco-2 differentiation

Caco-2 cells were reseeded at 10^5 cells/cm^2 on polycarbonate membrane inserts in 6-transwell plates and maintained for 21 days with complete medium. The TEER increment was monitored at 9, 15 and 20 days (3 wells for each experiment) as a signal of progression of cell differentiation. The TEER increase (mean ± DS) was in the range of 240 ± 20 to 500 ± 35 Ω•cm^2.

2.4.2 Co-culture system and OTA treatments

The transwell permeable supports were used for transepithelial electrical resistance (TEER) and the gene expression studies. HepG2 cells were seeded in 6-well plates at a density of 1.5 x 10^5 cells/cm^2 (at day 20 of the Caco-2 cells differentiated monolayers). Twenty-four hours after the seeding of HepG2 cells, the co-cultures were performed (Day 21). Non-cytotoxic doses (5, 15 and 45 µM) of OTA were selected for 3, 12 and 24 h treatments in transwell plates. The treatments were carried out in culture medium, and

the percentage of solvent (DMSO) in the treatments was 0.05% of the final volume in the transwell system for all of the treatments.

2.5 Gene expression

2.5.1. RNA extraction and cDNA synthesis

Total RNA was prepared from freshly isolated cells using the Trizol® reagent, according to the manufacturer´s protocol. RNA integrity was checked on an agarose gel stained with ethidium bromide (EtBr). The concentration and purity of total RNA were determined by measuring the ratio of absorbance at 260/280 nm and 260/230 nm in a NanoDrop ND-1000 (Thermo Scientific, USA), respectively. 5 µg of total RNA was treated with TURBO™ DNase to remove contaminating genomic DNA. The SuperScript™ III reverse transcriptase kit and a Oligo(dT) primer were used to synthesize first-strand cDNA.

2.5.2. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Primers used in this study are listed in Table 1. The primer set used for amplifying the *CYP2A6* gene was designed with OLIGO Primer Analysis Software V.7. Real-time RT-qPCR reactions were performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) monitoring cDNA amplification with iTaqTM Universal SYBR® green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The program included one cycle at 95 ºC for 2 minutes followed by 40 cycles of 15 s at 95 ºC and 30 s at 58 ºC and, finally, a dissociation and amplification curve step to check the PCR reaction quality.

To validate the RT-qPCR, a standard curve was generated with 6 set points of two-fold serial cDNA dilutions. A pool of cDNA from Caco-2 or HepG2 cells was used to run the standard curve. The standard curve was generated by plotting the quantification cycle (Cq) versus the logarithmic value of the cDNA concentration. The real-time PCR efficiency (*E*) of one cycle in the exponential phase was calculated for each primer pair (equation 2), where the slope belongs to the standard curve (Rasmussen, 2001).

$$E = 10^{[-1/slope]} \qquad \text{equation 2}$$

Expression level was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The relative expression ratio (r) of the genes was calculated according to the $2^{-\Delta\Delta Cp}$ mathematical model, based on *E* and the *Cq* deviation (equation 3) (Pfaffl, 2001).

$$r = (E_{target})^{\Delta CP target} \; / \; (E_{reference})^{\Delta CP reference} \qquad \text{equation 3}$$

ΔCq_{target} is the subtraction of $Cq_{control}$ from the Cq_{sample} for the target gene, and $\Delta Cq_{reference}$ is the subtraction of $Cq_{control}$ from the Cq_{sample} for the reference gene.

[Insert Table 1 in this place, if possible]

2.7 Statistics

Three experiments were run in triplicate, and each sample was run twice (technical replicates) for the gene expression analysis. Each value represents the mean ± SE. Statistical analyses were conducted using Statgraphics plus. Cell viability, membrane integrity and gene expression were analysed using ANOVA and the Bonferroni post-test. *p*-values < 0.05 were considered to be statistically significant.

3. Results

3.1 Cytotoxic assays

3.1.1 MTS assay

The increase of OTA concentration (from 1 to 180 µM) resulted in a significant decrease of cell viability in both cell types, at the three exposure times assayed (Table 2), measured by the metabolic reduction of MTS tetrazolium compound. This became more evident in the 24 h treatment, where cell viability was reduced from 80.6 to 53.8% (Caco-2 cells) and from 87.8 to 66.3% (HepG2 cells) between the two extreme values of OTA concentration tested. Based on the results of the viability assay, three non-cytotoxic OTA concentrations (5, 15 and 45 µM) were chosen for the membrane integrity and gene expression assays (> 70 % viability for both cell types).

[Insert Table 2 in this place, if possible]

3.1.2 Membrane integrity

TEER measurement is useful to detect changes in physiological barrier function caused by the assayed compounds. In particular, TEER reflects the transepithelial permeability

of water-soluble ions. The integrity of the Caco-2 cell monolayer was markedly and significantly affected after OTA treatments (5, 15 and 45 OTA µM) with the longest exposition time. Table 3 shows the TEER values at 3, 12 and 24 h. The 3 or 12 h treatments showed a slight, but significant, decrease in TEER values. The observed decreases in TEER, expressed as a percentage of the value at 24 h of treatment, were 63.6%, 61.5% and 64.6% (at 5, 15 and 45 µM OTA treatments, respectively), which means an increase in permeability without cell detachment. The solvent (DMSO) had no significant effect on the TEER-values ($p > 0.05$) compared to the monolayer exposed to culture medium alone (data not shown).

[Insert Table 3 in this place, if possible]

3.2 Gene expression

To elucidate OTA metabolism, gene expression of the *CYP450* family (*CYP1A1*, *CYP2A6*, *CYP2B6*, *CYP3A4* and *CYP3A5*), the inflammatory markers *COX-2* and *5-LOX,* the xenobiotic metabolizer *NAT2*, and the *MRP2* transporter was analysed in a co-culture system of Caco-2 and HepG2 cells. It should be firstly noted that gene expression was not affected by the added solvent (DMSO) as there were no statistically significant differences between the blank (medium alone) and DMSO treatments, with a *p*-value of 0.6882 for the Caco-2 cells and 0.3128 for the HepG2 cells. Hence, all relative gene expression ratios were calculated by referring to the Cp-values from the control treatments that were exposed to the same DMSO concentration as the OTA treatments.

CYP2A6 expression was not detected in mRNA samples from HepG2 cells. Details of the statistical analysis of treatment (OTA concentration and time exposure) effects on gene expression in both Caco-2 and HepG2 cells are shown in Figures 1 and 2, respectively.

The gene expression analysis shows that when Caco-2 cells were treated with increasing amounts of OTA, expression of most of the analysed genes was down-regulated (Figure 1). Among the *CYP450* family, *CYP1A1* was the gene with the highest down-regulation, with statistical significance for the three treatments and different exposure times ($p < 0.0001$). *CYP1A1* expression was greatly reduced by OTA within the first 3 h; however, the highest effect of OTA was observed after 12 h, with a 52-fold decrease in *CYP1A1* gene expression. Interestingly, cells exposed for 24 h showed a much lower repression of *CYP1A1*, compared with the mean-value of cells treated for 12 h.

According to our results, OTA had an effect on the expression level within the first 3 hours, but we did not detect large changes in the expression level of the *CYP2A6* and *CYP2B6* genes. It is known that *CYP3A4* is a very active gene in the presence of endogenous substrates and xenobiotics. Under our conditions, we obtained an unexpected result for the level of *CYP3A4* expression, showing a very stable level during the 3 first hours and poor modulation by OTA compared to the results with *CYP1A1* and *NAT2*. Increasing OTA concentrations did not cause a significant difference in the expression of *CYP3A4* in cells treated for 3 and 12 h. At 24 h, only the treatment with 45 µM caused a significant difference ($p = 0.0396$) with regard to 5 and 15 µM OTA. A

similar expression pattern was observed in *CYP3A5*, but the down-regulation was slightly higher.

Interestingly, *NAT2*, of which there is limited information regarding its interaction with OTA, was the gene with the second largest down-regulation during 12 and 24 h of exposure ($p < 0.0001$). Expression levels of the inflammation markers, *COX-2* and *5-LOX*, were also decreased. The presence of OTA caused a larger regulation of *COX-2* than *5-LOX*. Additionally, the higher effect on *COX-2* (45 µM for 24 h) shows a greater statistical significance ($p = 0.0003$). Finally, in general, the down-regulation was more pronounced in Caco-2 cells at 24 h and 45 µM OTA.

[Insert Figure 1 in this place, if possible]

Regarding the results of the hepatic cells, OTA induced both up- and down-regulation (Figure 2). Unlike the results obtained in Caco-2 cells, *CYP3A4* expression increased significantly after 12 h exposure to OTA ($p < 0.05$), regarding the results at 3 h of exposure. In this case, *CYP3A5* was down-regulated by OTA at 45 µM, but after 12 h only showed a slight response to OTA exposure at 5 and 15 µM.

The *CYP1A1* expression level results obtained with HepG2 cells showed a significant change with respect to other CYPs analysed, which is very different to the strong down-regulation observed in Caco-2 cells. *CYP1A1* was slightly down-regulated by OTA at 12 h, but a significant increase was detected in the cells exposed for 24 h ($p = 0.00258$ compared to 12 h). The *CYP2B6* gene was down-regulated within the first 3 h, whereas an up-regulation effect was observed after 12 h exposure with a statistically significant

difference (p = 0.002). However, after 24 h, *CYP2B6* expression once again decreased in cells exposed to 15 and 45 µM OTA.

The *NAT2* gene showed a different response at 24 h compared to 3 and 12 h. This gene increased its expression after a longer OTA exposure in the HepG2 cells, in contrast to what happens in Caco-2 cells. With respect to the inflammation markers, these genes were slightly modulated by OTA at 3 h of treatment; both *COX-2* and *5-LOX* genes appeared to be up-regulated within the first 12 h. However, at 24 h, *COX-2* maintained its expression level, but *5-LOX* was down-regulated. Unlike Caco-2 cells, in HepG2 cells, the *MRP2* transporter showed a slight but significant down-regulated response ($p < 0.05$) after treatments at 15 and 45 µM OTA for 12 and 24 h.

[Insert Figure 2 in this place, if possible]

4. Discussion

The first OTA evaluation conducted by the IARC (IARC, 1983) concluded that the evidence for nephrotoxicity, teratogenicity and carcinogenicity was insufficient to classify OTA as a carcinogenic compound to humans. Later, the toxic potential of OTA was greatly supported. However, the toxicological mechanism of this mycotoxin is still unclear, and a controversy exists about its genotoxic or epigenetic mode of action (IARC, 1993; Marin-Kuan et al., 2006). Kinetics studies in animal species showed that OTA is slowly distributed to peripheral compartments (Galtier et al. 1979, Sreemannarayana et al., 1988; Li et al., 1997), and that the molecule could be reabsorbed and redistributed (Hagelberg et al., 1989; Fuchs and Hult, 1992; Sreemannarayana et al., 1988). Several

studies have reported a prolonged half-life of the toxin and a secondary reintroduction to the intestine through the enterohepatic circulation, since the biliary excretion of OTA is very efficient, making the presence of OTA in the intestine prolonged in time (Li et al., 1997; Ringot et al., 2006). OTA toxicity has been attributed to its isocoumarin moiety, and it is well known that OTA is inactivated/bioactivated by cytochrome P450 enzymes (Faucet-Marquis et al., 2006; Malaveille et al., 1991; Ringot et al., 2006; Tozlovanu et al., 2012).

In order to observe the change induced *in vitro* by OTA over time, and taking into account the high half-life and the enterohepatic circulation of OTA observed in *in vivo* studies, in the present study the toxicity and the effect on gene expression of OTA were assayed at three times of exposure (3, 12 and 24 h). To carry out our study we have used a co-culture system with Caco-2 and HepG2 cells that mimics the absorption in the intestine (through of the porous membrane) and the hepatic metabolism.

In this system, the effect of OTA on the expression of cytochrome genes, the *NAT2* gene (involved in phase II of metabolism), two inflammation markers (*COX-2* and *5-LOX*) and the *MRP2* gene (encoding for a transporter of xenobiotics outward during absorption and in liver metabolism) was evaluated.

The cell viability results from the MTS assay showed that only 24 h of OTA exposure caused a decrease in viability. Our data agree with other studies which also described high viability percentages (low cytotoxicity values) in treatments with <100 µM OTA for 24 h or less (Berger et al., 2003; Maresca et al., 2001; McLaughlin et al., 2004; Sergent et al., 2005; Ramyaa et al., 2013). Similarly, other authors obtained viabilities of 75% (HT-

29-D4) and 80% (in Caco-2 cells) at 48 h (Maresca et al., 2001; McLaughlin et al., 2004), using low OTA concentrations (20 µM and 30 µM) in the case of Caco-2 cells. Different results were reported by Zheng et al. (2013), who found that 37.27 µM OTA for 24 h caused 50% cytotoxicity in HepG2 cells.

In addition, OTA cytotoxicity was also determined by evaluating membrane integrity in Caco-2 cells. Our results showed that OTA caused damage in physiological barrier functions through a paracellular transport that decreases TEER. This mechanism involves substances up to 10 but not 20 kDa, as OTA, and the elimination of tight junction proteins (Claudin 3 and 4). Conversely, good membrane homeostasis is related to high TEER and the presence of tight junctions (Kiatsurayanon et al., 2014; Yuki et al., 2007).

In Caco-2 cells, membrane integrity (measured by TEER) and cell viability (from the MTS assay) were closely related. In this study, we found a decrease of 60% in TEER (disruption of the paracellular barrier) and a significant loss of viability when Caco-2 cells were treated with 45 µM OTA for 24 h. Maresca et al. (2001) recorded a decrease of 50% in TEER after a treatment of 100 µM OTA for 48 h that was related to a decrease in the whole cellular protein content, the inhibition of growth cell and morphological changes. In our assay (5-45 µM for 24 h) as well as Maresca et al. (2001) (0.001-1 µM for 48 h), we did not record a decrease of TEER in a time-dependent manner, possibly due to the low range of OTA doses assayed. Conversely, McLaughlin et al. (2004), with higher OTA doses (20-160 µM for 5 to 25 h), recorded a time-dependent reduction in TEER. Studies evaluating TEER during short-term exposures and at low OTA

concentrations, did not detect a significant decrease of TEER in Caco-2 cells, as found by Berger et al. (2003), using 10 µM OTA for 3 h, or in our study during the 3 h treatment. To further investigate the effect of OTA on the xenobiotic transporter gene (*MRP2*), the metabolic enzymes (cytochrome P450: *CYP1A1*, *CYP2A6*, *CYP2B6*, *CYP3A4* and *CYP3A5; and NAT2*), and the inflammatory response genes (*COX-2* and *5-LOX*), a gene expression analysis was performed using Caco-2 and HepG2 cells. Our data showed a down-regulation of *MRP2* expression, indicating an impairment of the secretion of OTA. The MRP2 transporter is a key primary active transporter involved in anionic conjugate and drug extrusion from the human liver but which is also present in the apical membranes of enterocytes, kidney-proximal tubules and other cells (Jedlitschky et al., 2006). In rat, it has been observed that OTA was excreted 15% less in the proximal tubules of the kidney, while the proximal tubular transport of amino acids was not impaired by OTA (Gekle and Silbernagl, 1994). Therefore, the down-regulation of *MRP2* and the TEER decrease found in this study could be the mechanism through which OTA reaches high percentages of bioavailability *in vivo*. In this way, the OTA exposure in hepatic cells would be magnified, contributing to the hepatotoxicity. Considering the nephrotoxic potential of OTA, the down-regulation of the *MRP2* gene may also have a major impact on the proximal tubule, leading to a decreased capacity to eliminate OTA. However, further studies are needed on the OTA transporter mechanism to corroborate this hypothesis.

Concerning the expression of the *CYP450* genes, these genes were down-regulated and up-regulated by OTA in Caco-2 and HepG2 cells, respectively. The induction of *CYP450* genes in the xenobiotic response has been previously studied, and the mechanism is

now largely understood. Nevertheless, the mechanism involved in *CYP450* suppression still remains to be clarified. The *CYP450* genes show a similar response because they often involve activation of common cytosolic or nuclear receptors, including the aromatic hydrocarbon receptor, the constitutive androstane receptor, the pregnane X receptor, and the peroxisome proliferator-activated receptor-alpha (Honkakoski and Negishi, 2000). The effect of polycyclic and halogenated aromatic hydrocarbons is used as model for understanding the mechanisms of *CYP450* suppression, and the results of OTA-modulation gene expression in the present study show both effects, up- and down regulation mainly on HepG2 cells. The literature suggests that the down-regulation is a response to physiological and pathological signals: a) stress signals caused by toxicants and inflammation, b) an adaptive response, allowing controlled generation of reactive oxygen species (ROS), nitric oxide, or arachidonic acid metabolism, or c) a collateral response after controlling a physiological pathway (Morgan, 2001).

In agreement with our results with OTA-treated hepatocytes, in which there was an increase in gene expression (mainly 1A1, 2B6 and 3A4 in HepG2 cells), Zepnik et al. (2001) reported an increase of OTA hydrolysis by microsomal enzymes from rat liver, specifically for P450 3A1/2, 3A4 and P450 1A1/2. In terms of gene expression, Ayed-Boussema et al. (2012) described an up-regulation of expression levels in all cytochromes assayed (*CYP3A4, 2B6, 3A5,* and *2C9*) in a primary human hepatocyte culture. *CYP3A4* mRNA did significantly increase after OTA treatment at 0.1 µM (from 3.1- to 16.7-fold induction, respective of each donor). In contrast, Marin-Kuan et al. (2006) showed a slight but predominant effect of down-regulation (> 56% of the genes) in the liver and kidney from male Fisher-344 rats. In Dark Agouti rats of both sexes, only

CYP3A4 expression was detected in male livers after OTA treatment, and *CYP1A* expression was higher than *CYP2A* (Pfohl-Leszkowicz et al., 1998), as in our *in vitro* assay.

With regard to *CYP2B6*, our results at 5-45 µM OTA for 3 h showed a down-regulation up to 8-fold in both cell types, but an increase was detected at 12 h in HepG2 cells. In contrast with the up-regulation of *CYP2B6* in primary human hepatocytes, the level of *CYP2B6* mRNA was higher (22.5-6.8-fold induction) than the mRNA level of other cytochromes after treatment at 10 µM (Ayed-Boussema et al., 2012).

NAT2 gene, encoding for an inducible and xenobiotic metabolizing enzyme of phase II, had lower expression in Caco-2 cells than in HepG2 cells, in line with results described in the literature (Husain et al., 2007). In our study, the highest effect on *NAT-2* expression was detected after 24 h of OTA exposure, where the *NAT2* gene was strongly modulated by OTA. It is noteworthy that expression of the *NAT2* gene regarding OTA exposure has not been studied previously, although Lebrun et al. (2002) reported a high correlation between *NAT2* polymorphism and DNA damage in an *in vitro* culture of OTA-exposed human urothelial cells. OTA intake has been linked to a chronic tubulointerstitial renal disease (chronic nephropathy) frequently accompanied by urothelial cell carcinomas of the urinary tract, although this relationship has not been proven completely (Batuman, 2006; Grollman and Jelacovic, 2007; Reddy and Bhoola, 2010). Besides, a very strong epidemiological association has been established between the *NAT2* genotypes and a variable risk of urinary bladder cancer caused by arylamines, possibly due to an impaired detoxification of carcinogenic metabolites in the liver (Hein et al., 2000; Husain et al., 2007).

To investigate modulation of the inflammatory response induced by OTA, the expression of *COX-2* and *5-LOX* genes was determined. We have detected a rapid change from 3 to 12 h in cellular *5-LOX* expression in Caco-2 cells, while in HepG2 cells, the changes were over time. The *COX-2* gene was down- and up-regulated in Caco-2 and HepG2 cells, respectively. Changes in the regulation pattern could be due to an adaptive response (Chung et al., 2008; Morgan, 2001).

Changes in the regulation pattern could be due to an adaptive response for controlling the generation of ROS, as observed by Chung et al. (2008) in A549 human lung carcinoma cells treated with acenaphthenequinone. In this sense, the oxidative stress caused by OTA has been widely described in the literature as ROS generation (Ramyaa et al. 2013), DNA damage (González-Arias et al., 2014; Ramyaa et al., 2013) and histopathological changes in the liver and kidney (Aydin et al., 2003). Recently, Kumar et al. (2013) and Ramyaa et al. (2013) investigated the relationship between OTA exposure and *COX-2* expression and obtained similar results as our study. Thus, in mouse (with a single dermal application of OTA), an early inflammatory response, demonstrated by *COX-2* expression, increased after 6 h of OTA exposure and a maximum was observed at 18 h (Kumar et al., 2013). Ramyaa et al. (2013) observed that *COX-2* expression increased after 15 h exposure to OTA in HepG2 cells, but, curiously, it was inhibited after 24 h. In the present study, we have not studied *NF-κB* expression, but several studies indicate that the expression of *COX-2* and *5-LOX* is related to *NF-κB*, the main transcription factor that regulates the proinflammatory response (González-Périz and Clàira, 2007; Chung et al., 2008). Nowadays, few studies have investigated the effect of OTA on the COX-2 and 5-LOX genes, or on their proteins (Ramyaa et al., 2014; Kumar

et al., 2013; Ferrante et al., 2006 and 2008), and a possible mechanism of down-regulation has not been established. Some authors have observed an inhibition of nuclear factor-kappa B (NF-κB) expression and an increase in heat shock protein 72 expression, both in *in vivo* and *in vitro* studies, after OTA exposure, leading the down-regulation of proinflammatory mediators (Ferrante et al., 2006, Ramyaa et al., 2014). Our results support previous studies which described a pro-inflammatory role of OTA but further studies are necessary.

5. Conclusion

The use of cell lines is an alternative to overcome the disadvantages of the primary cell lines, as their scarce availability, limited growth potential, short lifespan, differences between batches, and their susceptibility to undergo early and variable phenotypic alterations. Additionally, basal gene expression in freshly isolated primary human cells is also different between culture passages (Hart et al., 2010). The proper use of data published in literature can support the results and the focus of our study. However, depending on the human cell line or primary cell line used, and its ability to express or induce the genes, comparison of results can be difficult. In addition, an increase in the mRNA level in cells or liver tissues does not necessarily mean that similar results occur at protein level (Hart et al., 2010; Sun et al., 2002). It is known that different processes are implicated in OTA metabolism and toxicity. Direct or indirect DNA damage, as well as epigenetic mechanisms, are responsible for the tumour promotion phase during carcinogenesis, and all of these mechanisms have been related to OTA toxicity at non-cytotoxic doses during long-term exposure (Gonzalez-Arias et al., 2014; Horvath et al.,

2002; Qi et al., 2014). Conversely, histological changes in the liver, kidney and skin have been detected after high doses of OTA exposure (Kamp et al., 2005; Kumar et al., 2013; Palabiyik et al., 2013; Qi et al., 2014). The co-culture system that mimics the passage through the intestine as a first barrier allowed us to obtain results that could be more similar than those recorded in *in vivo* studies. The data generated from the gene expression analysis in enterocytes and hepatocytes exposed to OTA suggest a different mechanism of action in intestinal epithelium during absorption than in OTA metabolism in the liver.

Acknowledgements

The authors are grateful to the Spanish (Projects AGL2011-24862 and AGL2014-52648-REDT) and Catalonian (XaRTA-Reference Network on Food Technology) Governments for their financial support. C.A. González-Arias thanks the Secretaria de Universitats i Recerca del Departament de Economia i Coneixement of the Generalitat de Catalunya for the pre-doctoral grant.

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Table 1. Primers used for the analysis of gene expression by RT-qPCR

Target gen	Forward (F) Reverse (R)	Primer sequence (5´-3´)	Length (bp)	Primer reference
CYP1A1	F	TTCCGACACTCTTCCTTCGT	20	Ayed-Boussema et al. (2012)
	R	ATGGTTAGCCCATAGATGGG	20	
CYP2A6	F	ACCCCAGTTTCTTCTCCAACCC	22	This study (*)
	R	CCGAAACAGTTCCGCTTTCCGAT	23	
CYP2B6	F	GGCCATCGGGAGCCCTTG	18	Ayed-Boussema et al. (2012)
	R	AGGGCCCCCTTGGATTTCCG	20	
CYP3A4	F	GCCTGGTGCTCCTCTATCTA	20	Ayed-Boussema et al. (2012)
	R	GGCTGTTGACCATCATAAAAG	21	
CYP3A5	F	TGACCCAAAGTACTGGACAG	20	Ayed-Boussema et al. (2012)
	R	TGAAGAAGTCCTTGCGTGTC	20	
COX-2	F	CAAATCCTTGCTGTTCCCACCCAT	24	Rasheed and Haqqi, (2012)
	R	GTGCACTGTGTTTGGAGTGGGTTT	24	
5-LOX	F	ACCACGGAGATGGTAGAGTGCAG	23	Zhou et al. (2007)
	R	GCAGCTCAAAGTCCACGATGAA	22	
NAT2	F	ACGTCTCCAACATCTTCATTTATAACC	27	Kocabas et al. (2004)
	R	TCAACCTCTTCCTCAGTGAGAGTTTTA	27	
MRP2	F	ACAGAGGCTGGTGGCAACC	19	Pascolo et al. (2003)
	R	ACCATTACCTTGTCACTGTCCATGA	25	
GAPDH	F	TGCACCACCAACTGCTTAGG	20	Vreeburg et al. (2011)
	R	GGCATGGACTGTGGTCATGAG	21	

(*):sequence designed with with OLIGO Primer Analysis Software V.7. (accession number NM_000762.5).

Table 2. Relative viability (%) determined by the MTS test in cells treated with OTA (1-180 µM) for 3, 12 and 24 hours (n=9).

Cells	OTA (µM)	Relative viability (%)		
		3 hours	**12 hours**	**24 hours**
Caco-2	1	106.78 ± 1.75^{a}	100.43 ± 5.14^{a}	80.57 ± 3.55^{a}
	5	106.87 ± 3.75^{a}	97.96 ± 3.01^{a}	78.37 ± 3.34^{a}
	15	102.60 ± 2.98^{a}	96.80 ± 2.46^{a}	72.17 ± 3.50^{ab}
	45	107.08 ± 2.37^{a}	86.18 ± 1.21^{a}	76.14 ± 8.23^{bc}
	60	99.18 ± 3.01^{a}	90.99 ± 2.70^{ab}	74.00 ± 6.34^{bc}
	75	104.36 ± 2.61^{a}	95.50 ± 6.78^{b}	66.26 ± 3.57^{bc}
	90	99.55 ± 3.75^{a}	91.15 ± 2.47^{b}	60.44 ± 4.03^{c}*
	180	87.01 ± 3.76^{b}	84.98 ± 4.72^{b}	53.81 ± 4.35^{d}
p-value		0.0072	0.0012	<0.0001
HepG2	1	98.53 ± 0.48^{a}	97.48 ± 2.65^{a}	87.75 ± 1.66^{ab}
	5	97.34 ± 2.83^{a}	101.38 ± 2.71^{a}	91.06 ± 1.50^{a}
	15	91.81 ± 2.02^{ab}	101.73 ± 3.42^{a}	85.58 ± 1.30^{ab}
	45	98.44± 2.24^{a}	101.86 ± 3.43^{a}	91.53 ± 2.04^{a}
	60	95.97 ± 2.54^{ab}	100.87 ± 2.16^{a}	90.66 ± 2.04^{ab}
	75	95.55 ± 2.81^{ab}	103.97 ± 3.84^{a}	88.43 ± 2.06^{ab}
	90	88.98 ± 2.06^{ab}	98.34 ± 2.75^{a}	88.64 ± 1.51^{ab}
	180	81.58 ± 2.37^{c}	83.74 ± 2.20^{c}	66.28 ± 2.21^{c}
p-value		0.0001	0.0001	<0.0001

Relative viability was calculated regarding solvent control (0.05% DMSO). Mean ± SE values followed by the same letter within an exposure time are not significantly different ($p > 0.05$). Statistical analysis was performed for each kind of cells separately. *(n=7).

Table 3. Effect of OTA on the transepithelial electrical resistance (TEER) in differentiated monolayers of Caco-2 cells.

	TEER Ω•cm2 (Mean ± SE)			
OTA (μM)	**3 hours**	**12 hours**	**24 hours**	***p*-value**
5	503.50 ± 5.68[a]	475.16 ± 4.88[b]	137.74 ± 3.00b[c]	0.0008
15	480.82 ± 2.19[a]	496.67 ± 1.68[b]	179.06 ± 2.27[c]	0.0001
45	595.56 ± 3.17[a]	557.75 ± 3.65[a]	170.89 ± 4.25[c]	< 0.0001

SD ± SE: mean ± standard error of TEER-values of the Caco-2 monolayer exposed to OTA. Different letters (a, b, c) mean significant differences within the same exposure time.