UPLC-MS/MS analysis of ochratoxin A metabolites produced by Caco-2 and HepG2 cells in a co-culture system.

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Running Head (short title):
Ochratoxin A metabolites generated in an *in vitro* co-culture.

Highlights

- Caco-2 cells were more sensitive to ochratoxin A than HepG2 cells, regardless of time.
- TEER decreased up to 72% after 24 h of exposure to OTA (5, 15 and 45 µm).
- OTA-methyl ester was the major metabolite found in a co-culture system with Caco-2 and HepG2 cells.

Abstract

Ochatoxin A (OTA) is one of the most important mycotoxins, based on its nephrotoxicity, teratogenicity, genotoxicity, neurotoxicity, and immunotoxicity, as well as due to its capacity to produce oxidative stress and cancer development. The oral route is the main gateway of entry of OTA into the human body, and specialized epithelial cells constitute the first barrier. The present study investigated the *in vitro* cytotoxic effect of OTA (5, 15 and 45 μM) and the production of OTA metabolites in Caco-2 and HepG2 cells, using a co-culture Transwell System to mimic the pass through the intestinal epithelium and the hepatic metabolism. Results derived from MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] cell viability assay and the transepithelial electrical resistance (TEER) measurement showed that OTA was slightly cytotoxic at low concentration, but a significant toxicity was observed at all concentrations at 24 h. OTA metabolites generated in this co-culture were detected using a UPLC-MS/MS system and ochratoxin B (OTB), OTA methyl ester, OTA ethyl ester and the OTA glutathione conjugate (OTA-GSH) were identified. OTA methyl ester was the major metabolite found in both Caco-2 and HepG2 cells after all treatments. Our results showed that OTA is able to cause cell damage by means of several mechanics of action, and that OTA exposure time is more important that the doses in *in vitro* studies. On the other hand, OTA methyl ester is proposed as an OTA exposure biomarker, although future studies should be conducted to evaluate it on biological samples.

Keywords: transwell system, *in vitro* cytotoxicity, MTS assay, TEER measurement.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by various species of the *Aspergillus* and *Penicillium* genus. OTA shows potent renal toxicity and carcinogenicity in rodents, with tendency to develop renal tumor. Due to this fact, the International Agency for Research on Cancer (IARC) has classified OTA as probably carcinogenic to humans (group 2B) (IARC, 1993). The accumulated evidence from more recent studies demonstrate the OTA ability to interact with DNA through different mechanisms proved in *in vitro* studies, such as single-strand DNA cleavage, DNA adducts and micronucleus formation (González-Arias et al., 2014; Pfohl-Leszkowicz and Manderville, 2007; Xia et al., 2014). Humans and animals can be exposed to OTA through foods and feeds contaminated with this toxin, respectively. Contamination of several agricultural commodities by OTA has been widely documented in literature over the past years, including cereals as barley (Duarte et al., 2010; Ibáñez-Vea et al., 2012), maize (Saleemi et al., 2012; Serrano et al., 2012), oat (Nguyen and Ryu, 2014; Vidal et al., 2013), rice (Iqbal et al., 2016; Nazari et al., 2014; Nguyen and Ryu, 2014), and other products as coffee (Chen et al., 2016; Drunday and Pacin, 2013; Galarce-Bustos et al., 2014) and cocoa (Brera et al., 2011; Copetti et al., 2010; Kedjebo et al., 2016).

Oral intake is the main via of OTA exposure (Coronel et al., 2012) and it has been described that OTA is absorbed in the gastrointestinal tract by a mechanism of passive diffusion across cellular membranes (Kumagai, 1988). In this way, the oral bioavailability of OTA in *in vivo* models reaches values up to 92%, 61%, 65% and 55%, in multiple mammalian species such as mice, rats, pigs and rabbits, respectively (Galtier

et al., 1981; Hagelberg et al., 1989). At intestinal level, OTA is hydrolysed and converted to ochratoxin-alpha (OTα), or it is metabolized to its hydroxylated metabolites, 4R-4-OH-OTA, 4S-4-OH-OTA and 10-OH-OTA by liver microsomes (Stormer et al., 1981; Stormer and Pedersen, 1980; Syvertsen and Størmer, 1983). Conjugated metabolites include OTA methyl and ethyl esters, OTα-glucuronide, amino-, phenol- and acyl-glucuronides, OTA conjugated with glutathione (OTA-GSH), hydroquinone analogue of OTA (OTHQ), hydroquinone analogue of OTA conjugated with glutathione (OTHQ-GSH) and hydroquinone analogue of OTA conjugated with N-acetylcysteine (OTHQ-NAC). Conjugated metabolites have been detected in kidney and liver samples from rats, as well as from liver microsomes and cell cultures (Faucet-Marquis et al., 2006; Han et al., 2013a; Li et al., 1998; Tozlovanu et al., 2012).

It is known that the liver is the major organ responsible for OTA biotranformation, however extrahepatic OTA biotransformation could take part in tissues such as kidney, lungs, liver and gastrointestinal tract (Nishimura et al., 2003; Renaud et al., 2011). Anatomic and physiologically, the human gastrointestinal tract is a barrier that protects from external agents, as OTA, and could be partially responsible for the first-pass effect (Li et al., 2007; McLaughlin et al., 2004; Versantvoort et al., 2005). In this sense, a novel approach using a Caco-2/HepG2 cells co-culture system has been previously used by our group to estimate the effect of OTA on gene expression using this human cells system (González-Arias et al., 2015). The Transwell co-culture *in vitro* system used retains the main phenotypic characteristics, and could be an alternative for *ex* and *in vivo* models.

The Caco-2 differentiated monolayer represents an optimal *in vitro* model of absorptive human enterocytes. On the other hand, HepG2 cell line is a useful monolayer model to investigate drugs-induced hepatotoxicity and the hepatic metabolism (Donato et al., 2008). In the present study, we used the hybrid Caco-2/HepG2 co-culture system with the aim of assessing cytotoxicity parameters and biotransformation of OTA, in a system that mimics the pass through the intestinal epithelium and the subsequent hepatic metabolism.

2. Materials and methods

2.1 Chemicals and materials

Ochratoxin A (OTA) (CAS: 303-47-9) (purity 98%) was supplied by Sigma-Aldrich (St. Louis, MO, USA), ochratoxin alpha (OTα) (CAS: 16281-39-3) was from Romer Labs Diagnostic (Tulln, Austria). Ochratoxin B (OTB) was a gift from Fredrik C. Størmer (Norwegian Institute of Public Health). The methylated and ethylated metabolites of OTA, OTB and OTα (OTA-ethyl ester, OTA-methyl ester, OTB-ethyl ester, OTB-methyl ester, OTα-ethyl ester, OTα-methyl ester) were prepared in presence of methanol or ethanol, respectively, and a strong acid (HCl) according to Li et al. (1998). The glutathione conjugate of OTA (OTA-GSH) was prepared according to Tozlovanu et al. (2012).

Non-essential amino acids (ref. M7145), L-glutamine (ref. G7513), antibiotic mixture (penicillin and streptomycin) (ref. P4333), dimethyl sulfoxide (DMSO) (ref. D8418) and phenol red (P5530) were supplied by Sigma-Aldrich. Foetal bovine serum (FBS) (ref. FB1090500) was supplied by BioSera (Nauille, France). 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). Flasks of 75 cm^2 (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, ref. 3412) and 6-well plates (ref. 3526) were obtained from Corning (New York, USA). The CellTiter 96® Aqueous One Solution Cell Proliferation Assay (ref. G3582) was obtained from Promega (Madison WI, USA). ExtraBond® QuEChERS extraction kid-EN (QUEXTENAK1 and QUDISENAK2) was obtained from Scharlau (Barcelona, Spain).

2.2 Cell culture

The human colon Caco-2 (HTB-37) and hepatic HepG2 (HB-8065) cell lines were obtained from the American Type Culture Collection (ATCC®). The cells were grown as monolayer cultures in 75 cm^2 flasks, incubated at 37 ºC in a humidified atmosphere of 5% CO_2 and maintained in DMEM. DMEM was supplemented with fetal bovine serum (15% for Caco-2 and 10% for HepG2 cells), 1% nonessential amino acids, 1% glutamine and 1% antibiotic mixture (penicillin and streptomycin). The monolayer was detached with a solution of trypsin and reseeded in 96-well plates for cytotoxicity assays or in 6-traswell plates for the evaluation of permeability.

To perform the different assays, monolayers of Caco-2 cells were used, which we named only as *Caco-2 cells*. The term *differentiated Caco-2 cells* refers to those mature monolayers (21 days in culture) that have developed enterocyte characteristics and have been used in the transwell system. The treatments were conducted in cell culture

medium without serum, and the percentage of DMSO in the treatments was 0.05% of the final volume.

2.3 Cytotoxicity

2.3.1 MTS tetrazolium assay

Metabolic activity was determined by MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay). The tetrazolium reagent ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS), in the presence of phenazine methosulfate (PMS) can be reduced by viable cells to its formazan product, that is soluble in cell culture medium. For the MTS tetrazolium assay (performed in a 96-well plate), the Caco-2 cells were reseeded at a density of $1.0x10^5$ cells/cm^2, whereas the HepG2 cells were reseeded at a density of $1.5x10^5$ cells/cm^2.

Doses assayed to evaluate OTA cytotoxicity were 5, 15 and 45 µM for 3 and 24 hours. At the end of treatment, the cells were washed once with PBS, and 100 µL culture medium with 317 µg/mL MTS-one solution was added per well. Plates were incubated at 37 ºC for 2.5 h (Caco-2 cells) and 2 h (HepG2 cells). Optical density (OD) was determined using a 96-plate reader (DAS-A3, Roma, Italy) with a 490 nm filter (FGF2, TSP1). The percentage of cytotoxicity was calculated using the following formula (Eq. 1):

$$cytotoxicity\ (\%) = 100 - \left(\frac{OD\ sample - OD\ blank}{OD\ control - OD\ blank}\ X\ 100\right) \qquad \textit{Equation 1}$$

2.3.2 Assessment of permeability in Caco-2 cells differentiated in the transwell system

Transwell system is a chamber of cell culture with a permeable support. The apical to basolateral transport (A-B) takes place from the upper to the lower compartment. Caco-2 cells were seeded on the permeable support to perform the differentiation process mentioned in section 2.2.

a) Phenol red apparent permeability

The differentiated monolayers and the basolateral compartment were washed three times with PBS. Later, 1 mM phenol red (in PBS with Ca^{+2} and Mg^{+2} at 0.1 mM each one of them) was added in the apical compartment and PBS with Ca^{+2} and Mg^{+2}, but without phenol red, in the basolateral compartment. The differentiated monolayers were incubated for 1 h under the same conditions described above (2.2). For the spectrophotometric quantification of phenol red, 1 mL PBS solution of the basolateral compartment was removed and mixed with 0.1 mL NaOH (110 mM). The sodium phenoxide product generated was determined at 560 nm.

The transepithelial flux of phenol red to the basolateral compartment was expressed as the apparent permeability coefficient (P_{app}) using equation 2 (Tavelin et al., 1999):

$$P_{app} = \frac{K \cdot Vr}{A} \qquad \textit{Equation 2}$$

Where K is the steady-state rate of change in the ratio between phenol red concentration in the receiver compartment (C_t) and the initial concentration in the apical compartment (C_0) after 1 h, V_r is the volume of the receiver chamber (mL), and A is the surface area of the filter membrane (cm^2).

b) Transepithelial electrical resistance (TEER)

TEER measurement was recorded at beginning and end point of the experiment. A Millicell-ERS electrical resistance system (Millipore, Iberia, Spain) was employed for the measurements, following the manufacturer´s instructions. Values are expressed as $\Omega \cdot cm^2$, according to the equation 3:

$$TEER_{monolayer} = (Resistance_{monolayer} - Resistance_{blank}) \cdot (\text{filter growth area in } cm^2) \qquad \textit{Equation 3}$$

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

2.4 Caco-2/HepG2 co-culture system

Caco-2 cells were seeded at a density of 10^5 cells/cm^2 in a Corning 6-transwell system and incubated at 37 °C for 20 days. HepG2 cells were seeded at a density of $1.5x10^5$ cells/cm^2. Twenty four hours later, the co-culture assays were performed. The differentiated monolayer was washed with PBS and apical-basolateral (A-B) transport direction was tested. OTA concentrations (5, 15 and 45 µM) were assayed during 3, 12 and 24 hours in culture medium without FBS. The percentage of solvent (DMSO) was 0.05% of the final volume for all treatments.

2.5 Metabolites extraction by QuEChERS method

All culture medium from apical or basolateral compartments were removed at the end of OTA treatments. OTA and its metabolites were extracted by the QuEChERS method: 1)

5 mL of 1% formic acid (in acetonitrile) was added to the sample and vortexed for 60 s. Separation was carried out by adding anhydrous magnesium sulphate (4 g), sodium chloride (1 g), di-sodium hydrogen citrate (0.5 g) and tri-sodium citrate dihydrate (1 g). Immediately, the tube was vigorously shaken and vortexed for 60 s. The organic phase was separated by centrifugation at 5000 rpm/5 minutes. To carry out the dispersative-SPE clean-up, the organic phase was transferred into a 15 mL centrifuge tube that contained anhydrous magnesium sulphate (1.2 g) and primary and secondary amine mix (0.1 g). The tube was vigorously shaken and vortexed for 60 s. The clean extracts were recovered and transferred into a glass tube and evaporated to dryness at 40 ºC under a stream of N_2.

2.6 UPLC-MS/MS analysis

Chromatographic analysis of OTA and its metabolites was performed with an Acquity-UPLC system (Waters, Milford MA, USA). The toxin and its metabolites were separated using an Acquity UPLC HSS T3 column (1.8 µm particle size, 2.1 x 100 mm) with an Acquity HSS T3 VanGuard pre-column (1.8 µm particle size, 2.1 x 5 mm). The flow rate was 0.4 mL/min and the injection volume 2.5 µL (partial loop with needle overfill). UPLC conditions were a modification of those described by Jennings-Gee et al. (2010). The mobile phase consisted of a gradient achieved with the following mobile phases: phase A) methanol/acetonitrile/6 mM ammonium formate (20/20/60) adjusted to pH 3.2 with formic acid, and phase B) methanol/acetonitrile/6 mM ammonium formate (35/35/30) adjusted to pH 3.2 with formic acid. The gradient was as follows: 0-1 min 70% B, 1-1.5 min 70 to 100% B, 1.5-6.5 min 100% B, 6.5-7.5 min 100 to 70% B, 7.5-9 min 70% B.

Both the column and guard column were maintained at 30 °C. Detection of mycotoxins was performed in a Waters 2475 Multi λ fluorescence detector (Milford, MA.USA), at 340 nm excitation and 460 nm emission wavelengths.

The mass spectrometry analysis was performed on a Xevo triple-quadrupole (Xevo TQ-S) mass spectrometer (MS) detector (Waters Corporation, Milford, MA, USA), using the positive electrospray ionization mode (ESI^+). The following parameters were employed: capillary voltage of 3 kV, cone voltage of 30 V, source temperature of 150 ºC and desolvation temperature of 350 ºC. Nitrogen was used as desolvation and cone gas with a flow rate of 1000 L/h. Full scan mode was employed in the mass range of 200-700 Da. The dwell time established for the transitions was in a range from 20-50 ms (Table 1). Two transitions from protonated molecules were monitored for each mycotoxin, the most abundant transition was used for quantification. MassLynx (V4.1) software (Waters Corporation) was used for the data acquisition.

Table 1. Precursor ions of mycotoxins, product ions and the parameters and collision energies.

Mycotoxin	m/z	m/z of transition ion	Dwell (ms)	Collision energy (eV)
OTA	404.08	238.94	20	20
OTA-methyl ester	418.07	238.96	20	25
OTA-ethyl ester	432.09	238.97	50	25
OTA-GSH	675.20	190.99	20	30
OTB	370.09	204.97	30	20
OTB-methyl ester	383.99	204.95	20	20
OTB-ethyl ester	398.02	204.92	20	25
OTα	256.99	238.94	50	10
OTα-methyl ester	271.10	238.86	20	10
OTα-ethyl ester	285.01	238.95	20	20

m/z (mass to charge ratio). OTA-GSH: ochratoxin-glutathione conjugated.

Retention time (in min) of the standards used were: 0.7 (OTA-GSH), 0.93 (Otα), 1.84 (OTB), 2.55 (OTA), 2.66 (Otα-methyl ester), 3.17 (OTB-methyl ester), 3.65 (Otα-ethyl ester), 3.91 (OTA-methyl ester), 4.09 (OTB-ethyl ester), and 5.04 (OTA-ethyl ester).

2.7 Method validation

The detection (LOD) and quantification (LOQ) limits for the OTA, OTB and OTα toxins were determined from the calibration curve of a set of two-fold serial dilutions of the toxin standards (from 25 to 0.006 ng/mL), which was linear in the range of 0.012 to 25 ng/mL (R^2=0.999 and 0.998). The LOD and LOQ determined were 0.012 and 0.036 ng/mL for OTA, whereas 0.024 and 0.072 ng/mL for OTα and OTB.

As mentioned above, cells were treated with 5-45 μM OTA and, in order to validate the extraction method, aliquots of cell culture medium were spiked with 1 μM OTA and the extraction by QuEChERS was carried out. OTA recoveries from the cell culture were usually greater than 92% with 8% of RSD_r and 14.2% of RSD_R.

2.8 Statistical analysis

The cytotoxicity, apparent permeability and transepithelial electrical resistance results were analysed using the analysis of variance (ANOVA) and the Bonferroni post-test. *p*-values of $p < 0.05$ were considered to be statistically significant. Statistical analyses were conducted using the GraphPad Prism 5.01 program (GraphPad Software, C.A., USA).

3. Results and discussion

3.1 Cytotoxicity studies

The effect of OTA treatment on HepG2 and Caco-2 cell viability was assessed through different methodologies.

3.1.1 MTS assay

The relative cytotoxicity of OTA for both HepG2 and Caco-2 cells was measured as the mitochondrial function by the metabolic reduction of MTS (Figure 1). Data were analysed in relation to time of exposure and OTA concentration. The cytotoxic effect of OTA on HepG2 and Caco-2 cells was statistically significant, (p=0.02 and p=<0.0001, respectively). A slight cytotoxic effect was observed at 3 h, but it was much evident at 24 h. As expected, the treatments with the higher OTA concentration (45 µM) were the most cytotoxic, causing in Caco-2 cells a 33% of cell death. In general, our data shown that Caco-2 cells were more sensible than HepG2 cells to OTA. Our group previously reported a decrease of 25% in cell viability using 40 µM OTA for 24 h in Caco-2 (Cano-Sancho et al., 2015). Similar conditions were performed by Romero et al. (2016), and the authors reported a cytotoxicity from 20 to 30% (24 h exposure) with 1-30 µM OTA treatments using the MTT reagent. Regarding OTA cytotoxicity on HepG2 cells, our data agree with data reported in literature that showed a low cytotoxicity on primary rat hepatocytes (0.5-12.5 µM for 24 h) (Essid et al., 2012), while a strong toxicity (approximately 40%) was observed on Hep3B cell line treated with 50 µM for 24 h (Anninou et al., 2014).

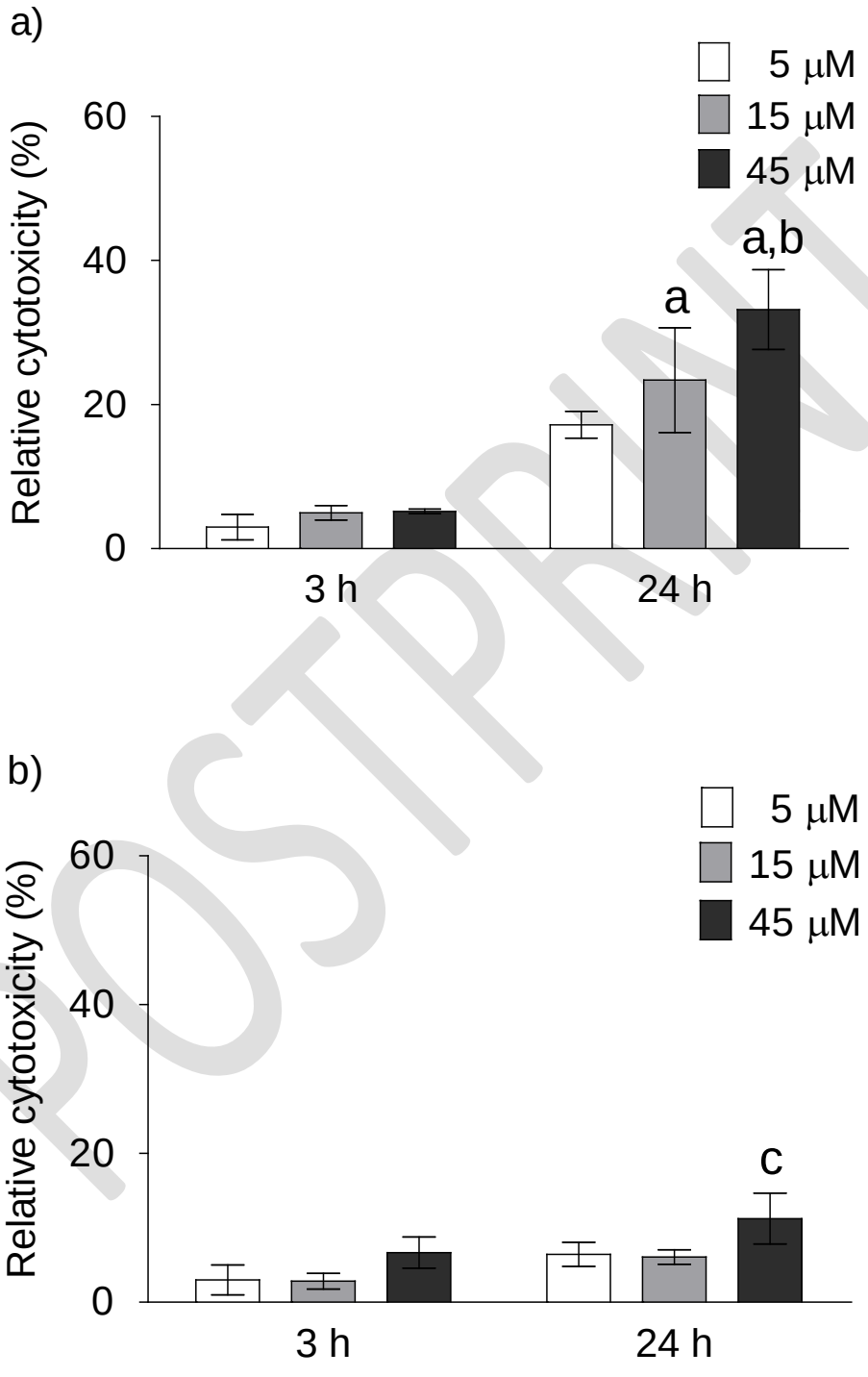

a)
5 μM
15 μM
45 μM
Relative cytotoxicity (%)
60
40
20
0
a
a,b
3 h
24 h
b)
5 μM
15 μM
45 μM
Relative cytotoxicity (%)
60
40
20
0
c
3 h
24 h

Figure 1. Relative cytotoxicity caused by OTA treatment on Caco-2 (a) and HepG2 (b) cells. Relative cytotoxicity was calculated regarding to solvent control (<0.05% DMSO). [a]*p* <0.05 is significantly different regarding to 5 µM, [b]*p* <0.001 is significantly different regarding to 15 µM and [c]*p* <0.05 is significantly different regarding to 5 and 15 µM.

Our results are also consistent with several studies performed previously to to evaluate the toxicity of OTA in different cell lines, studying both biochemical changes and cytotoxic parameters (neutral red uptake, mitochondrial activity, lactate dehydrogenase activity, ATP level, reactive oxygen species), the mRNA´s expression pattern and the genotoxicity (Costa et al., 2016; Cano-Sancho et al., 2015; González-Arias et al., 2015; Simarro-Doorten et al., 2004; Zhao et al., 2016), concluding that OTA toxicity depends on concentration, time of exposure and cell culture type.

3.1.2 Assessment of permeability

In order to evaluate the effect of OTA treatment on the membrane integrity of the differentiated Caco-2 cell monolayer, the apparent permeability (P_{app}) of phenol red and the TEER were assayed. Phenol red P_{app} describes the transcellular transport that confers selectivity to the membrane permeability. It refers to the passage of substances through the cell. On the other hand, the TEER depicts the presence of complex of tight junction proteins. Both, P_{app} and TEER are indicators of the integrity and epithelial confluence, and the modulation of the passage of substances through the membrane, respectively. Phenol red was quantified after 1 h in the basolateral chamber at the beginning of each experiment, and the passage of phenol red across the epithelial cell

layer was of $9.3x10^{-8}$ to $1.1x10^{-7}$ cm/s. Our results of phenol red P_{app} indicated the selective functions of the cell membrane of our monolayers. With the same purpose, other authors have used mannitol (known for its low cellular permeability), instead of phenol red, and the standard protocols for differentiation of human intestinal Caco-2 cells reported typical P_{app} values for mannitol in the range of $1x10^{-5}$ to $1x10^{-6}$ cm/s (van Breemen and Li, 2005; Ferruzza et al., 2012). It is important to note that the P_{app} values are not constant, as the P_{app} results can vary according to the assay conditions and to batch-to-batch characteristics of the monolayers (van Breemen and Li, 2005).

Results of OTA P_{app} shown that OTA did not cause an adverse effect on the Caco-2 cell permeability over first twelve hours (Table 2). However, a significant increase in the transcellular transport was detected at 24 h. Several studies have evaluated the effect of OTA on the transcellular transport of extracellular markers, such as mannitol or FITC-dextran. In both cases the P_{app} increased due to the OTA presence, using 100 µM OTA for 24 or 48 h, respectively (McLaughlin et al., 2004; Ranaldi et al., 2007; Schrickx et al., 2006). In our study, OTA concentrations were less than 45 µM and the time was only up to 24 h.

Table 2. Transcellular transport of OTA in Caco-2 cell monolayers.

	P_{app} (cm/s)			
µM OTA	**3 h**	**12 h**	**24 h**	***p*-value**
5	$1.09X10^{-7}$	$2.50X10^{-7}$	$3.61X10^{-7}$	0.01
15	$1.82X10^{-7}$	$2.29X10^{-7}$	$4.09X10^{-7}$	0.05
45	$1.63X10^{-7}$	$2.91X10^{-7}$	$4.44X10^{-7}$	0.01

P_{app}: apparent permeability coefficient. Values represent the mean ± standard deviation of three monolayers of three independent experiments.

Regarding TEER measurements, it has been described that TEER can be affected by temperature, cell culture and diameter of monolayer. For example, TEER-values have been reported in a range of 1000 to 4000 Ω•cm^2 using a 1.12 cm^2 filter and a pore size 0.4 µm (Ferruzza et al., 2012; McLaughlin et al., 2004).

In our conditions, data of TEER in the monolayers-control were 415.1 ± 8.5, 423.0 ± 2.1, 436.9 ± 14.1 Ω•cm^2, each value representing the mean ± DS of three monolayers of three independent experiments.

TEER was measured at the beginning and at the end of OTA treatment. After OTA treatments the TEER decreased 62% to 72% at 24 h (Figure 2) and the statistical analysis showed that the OTA had a significant effect on the cell membrane, the *p-values* were 0.0008 (5 µM), <0.0001 (15 µM) and 0.0001 (45 µM).

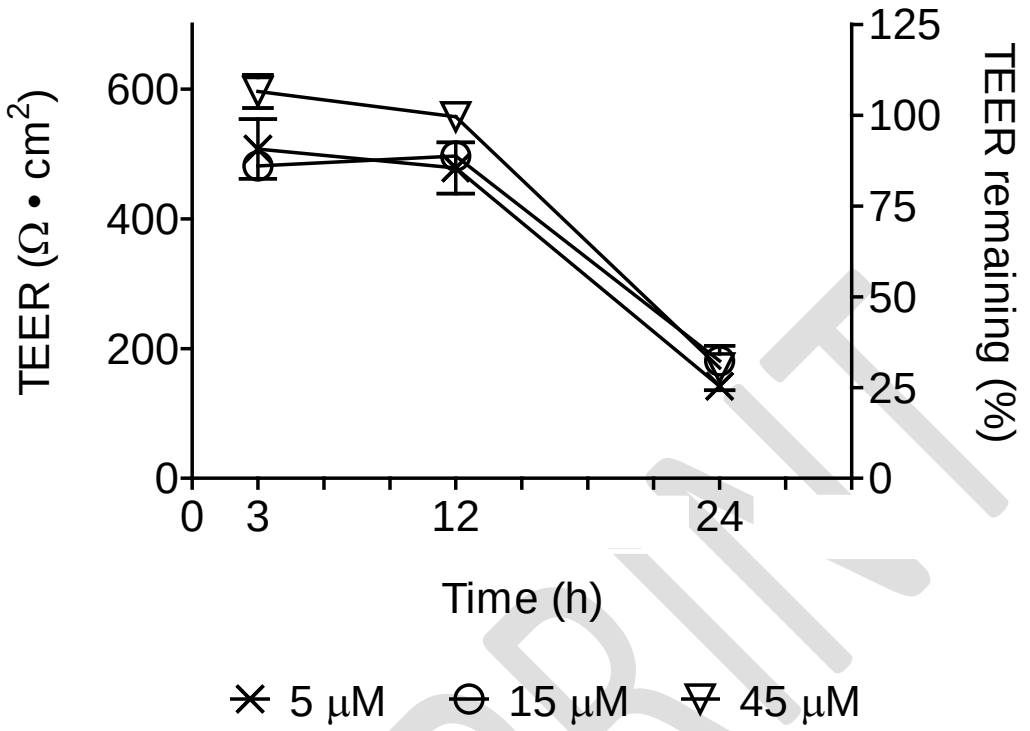


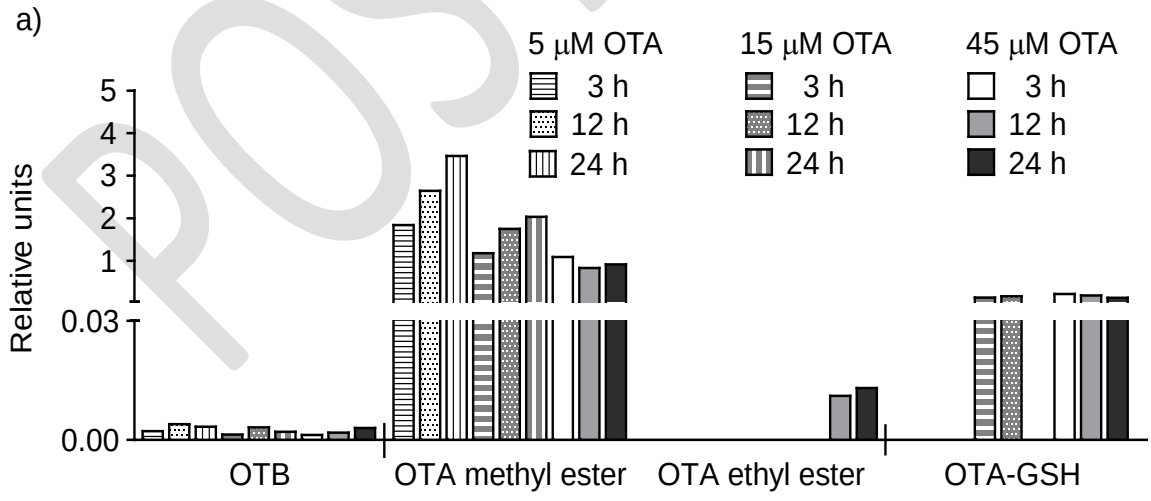
Figure 2. Transepithelial electrical resistance (TEER, in Ω•cm^2) in differentiated monolayers of Caco-2 cells treated with OTA. Data represent mean ± standard deviation of TEER-values of the Caco-2 monolayer treated with OTA.

3.2 Metabolite formation

Abundance of ions was used to evaluate the amount of OTA metabolites formed. A higher number of metabolites was detected in samples from apical chamber (Caco-2 cells) than from the basolateral chamber (HepG2 cells). Figure 3 shows the metabolites detected in cell cultures treated with OTA (3 and 24 h). OTA-methyl ester was the major metabolite formed during the first three hours by Caco-2 cells, as well as the most abundant. The formation ratio of OTA-methyl ester ranged from 1.8 to 3.45-fold regarding to OTA (Figure 3a). In HepG2 cells, OTA-methyl ester was detected in culture

medium from cells treated for over 12 h, but at very low concentration, and the ratio of formation was less than one (Figure 3b). Considering these results, we propose the methylated metabolite as a novel biomarker of OTA in this kind of studies.

Regarding the OTB, its abundance was very low in the cell culture medium. Previously, OTA standard solution was analysed, and OTB was not present in the standard, so a problem of impurities in the standard solution was discarded. Other minor metabolites, OTA-ethyl ester (OTC) and OTA-GSH were detected at a very small amount in both locations of the chamber. The formation ratio was less than 1-fold for both molecules in samples from cells treated for over 12 h or with the highest concentrations of OTA (15 and 45 μM). OTA-GSH was not detected in HepG2 cells. Jennings-Gee et al. (2010) reported also a low ratio of OTC production regarding the OTA initial concentration using a mice model treated by intravenous route.



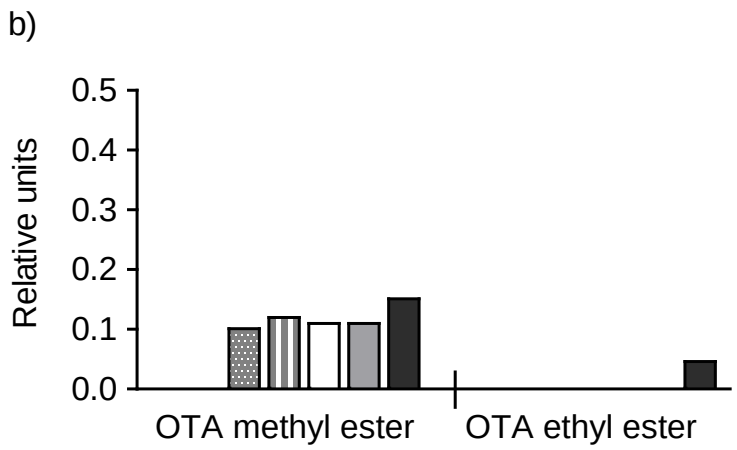


Figure 3. Metabolites detected in the apical compartment (a) and basolateral compartment (b) after 3, 12 and 24 h of OTA treatments in a Transwell system using Caco-2 and HepG2 cells. The initial concentration of OTA represents an arbitrary value of 10 in order to calculate the abundance rates of metabolite formation.

Several studies have been conducted using modulators to increase or inhibit different biochemical processes in cell lines or liver microsomes treated with OTA. For example, dexamethasone, isosafrole, 3-methylcolcanthrene, N-acetylcysteine, buthionine sulfoximine, clofibrate, alpha amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid and phenobarbital (Faucet-Marquis et al., 2006; Omar et al., 1996; Stormer et al., 1980). After treatment with these modulators, changes in the metabolism of OTA have been observed. In our case, modulator compounds have not been used.

3.3 Analysis of OTA and its metabolites by UPLC-MS/MS chromatography

In the present study, we evaluated the transport of OTA using a two-compartments transwell system to perform a hybrid culture with Caco-2 and HepG2 cells and to

assess OTA metabolites formation. As reported in the methods section, the culture medium recovered from the apical and basolateral chamber was analyzed to measure the metabolites production. Metabolite extraction was carried out using the QuEChERS method, a technique that has been previously used to clean-up a wide variety of compounds and matrices (Anzillotti et al., 2014; Fontana and Bottini, 2014; Madureira et al., 2014; Sartori et al., 2015). Finally, extracts were analyzed by UPLC-MS/MS. Those extracts of cell culture medium from cells without treatment (blank), or from cells treated with solvent (DMSO, solvent control), showed not interference signals with the chromatographic analysis of OTA or its metabolites.

In this study, five compounds were identified in the samples after OTA treatments. The chromatographic peaks of these compounds and its spectra were compared with the commercial or in-house reference standards, respectively. The signals in the samples corresponded to OTA (404.08 m/z), OTB (370.09 m/z), OTA-ethyl ester (432.09 m/z), OTA-methyl ester (418.07 m/z), and OTA-GSH (675.20 m/z). The main chromatographic peaks found were those that coincide with the elution time and m/z of OTA and OTA-methyl ester.

Han et al. (2013b) also reported m/z 404.0880 $[M+H]^+$ for OTA, being the transition ion at m/z 426.0705 $[M+Na]^+$, analyzed by means of LC-TOF-MS. The m/z of transition ion in negative mode was reported at m/z 402 $[M+H]^-$ and 358 $[M+H]^-$ (Han et al., 2013a). We also identified the transition ion at m/z 358 $[M+H]^+$ (15 eV), corresponding to the loss of a carboxylic group in CO_2 form (Han et al., 2010, 2013a).

A less-toxic but frequently found OTA metabolite present in mouldy products is OTB, the dechlorinated analogue of OTA (Faucet et al., 2004; Li et al., 1998). OTB was

identified and quantified through a precursor ion of 370.09 m/z and the transition ion was at m/z 204.97 $[M+H]^+$. OTB was present in the culture medium from Caco-2 cells treated with OTA. In culture medium from HepG2 treated cells, OTB was detected only in samples of monolayers treated with 45 µM OTA for 24 h, however the amount detected was lower than 2.06 ng/mL. In agreement with our results, other studies have reported very low biotransformation ratios of OTA to OTB in *in vitro* conditions (Faucet-Marquis et al., 2006; Grosse et al., 1995) or microsomes (El Adlouni et al., 2000; Han et al., 2013a; Yang et al., 2015). Jennings-Gee et al. (2010) and Størmer et al. (1985) reported the presence of both OTB and OTβ in rodents tissue, although OTβ was only detected in urine and liver samples at a negligible amount. Similarly, Han et al. (2013b) also detected OTβ and proposed a possible metabolic pathway of OTA in kidney. They suggested that OTA was transformed to OTB, and then this metabolite led to the formation of OTβ. In the present study, OTβ and other OTA metabolites such as OTα, OTα-methyl ester and OTα-ethyl ester have not been detected.

With regard to ester metabolites of OTA, ethyl ester and methyl ester have been identified in the present study. The precursor ions of both metabolites were identified at the m/z of synthetic standards, 432.09 m/z and 418.07 m/z, respectively. The transition ions of ethyl ester (OTC) and methyl ester of OTA identified were the following: m/z 238.97 and 238.96 $[M+H]^+$, respectively. The amount of OTA-ethyl ester detected was very low. Nevertheless, to our knowledge, there is not information regarding the MS-fragmentation of OTA-ethyl ester neither in animals nor in cell cultures. This metabolite was detected by HPLC at small concentrations with respect to OTA, such as other

metabolites reported (decarboxylated hydroquinone of OTA, quinone of OTA, and glutathione conjugates of ochratoxin A) (Jennings-Gee et al., 2010).

As mentioned above, OTA-methyl ester was the main metabolite produced after OTA exposure, both in Caco-2 and in HepG2 cells. This metabolite has been frequently described in literature, but it has not been reported as the common major metabolite. Han et al. (2013b) assayed the *in vitro* biotransformation of OTA using rat liver microsomes, and identified the presence of OTA-methyl ester after an incubation of 1.5 h. The transformation rate of OTA to OTA-methyl ester was almost 95%, being the main product, in agreement with our data. According to Han et al. (2013a), OTA-methyl ester could be an important metabolite in plasma or urine samples, where the studies are focused on OTα. In contrast, in a study conducted by Faucet-Marquis et al. (2006) formation of OTA-methyl ester was of 5 to 8-fold lower compared with a dechlorinated metabolite of OTA (C_{20},$H_{18}N_2O_5$, 366 mw) using OK cells pre-treated with modulators to increase biotransformation. As reported in literature, the *in vitro* and *in vivo* studies have reported a wide variety of profiles of metabolites, using different OTA doses and times, as described in this paper. Our results agree with those described by Han et al. (2013b), where OTα was found in very low concentration after OTA incubation in rat liver microsomes. In the same way, Han et al. (2013a), in an *in vivo* study with rats, did not found OTα in any of the bio-matrices studied. In the present study, the analysis of a standard solution of OTα was conducted in negative electrospray ionization mode $(ESI)^-$ and showed the precursor ion at m/z 256.99 and the transition ion in negative mode at m/z 238.94 $[M+H]^-$ nevertheless both structures were not identified in the samples. In this context, it is known that OTA is hydrolysed by the action of carboxypeptidase A and

chymotrypsin in gut, whence, the absence of hydrolase enzymes can led to the no formation of OTα in our *in vitro* system. In addition, it is well known that OTα is the major metabolite in plasma and urine, and therefore it is used as biomarker in monitoring studies.

4. Conclusion

In summary, the hybrid Caco-2/HepG2 co-culture system is a promising tool for assessing the cytotoxicity parameters and the biotransformation of OTA. Both cells, Caco-2 and HepG2 cells metabolized OTA to OTA methyl ester at the concentrations where this mycotoxin shows not cytotoxic effect, neither during MTS nor in the TEER assays. In this sense, the use of the methylated metabolite of OTA, reported as the major metabolite in our study, could be a novel biomarker of OTA in biological samples. However, little is known about the specific pathways of OTA biotransformation metabolites that take particular importance in cytotoxicity and in the half-lives/elimination assays. Therefore, this approach should be supported on the future with more *in vitro* and *in vivo* studies in order to compare our finding with known results on biological samples.

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