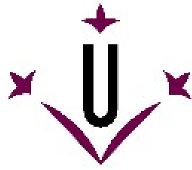

In vitro bioaccessibility of ochratoxin A and assessment of its cytotoxicity and genotoxicity in human cell cultures

Cyndia Azucena González Arias

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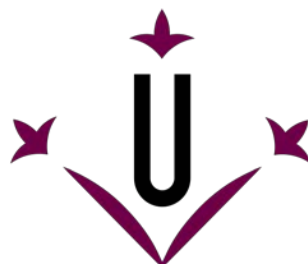

Universitat de Lleida

Escola Tècnica Superior d'Enginyeria Agrària

Departament de Tecnologia d'Aliments

Doctoral thesis

In vitro bioaccessibility of ochratoxin A and assessment of its cytotoxicity and genotoxicity in human cell cultures

Cyndia Azucena González Arias

Thesis supervisors:

PhD. Antonio J. Ramos Girona

PhD. Vicente Sanchis Almenar

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This research work was carried out in the Applied Mycology Unit of the Food Technology Deparmet of the University of Lleida, Spain. This work was developed as a part of the research line "*Integral approximation to the human exposure, simultaneously of deoxynivalenol and ochratoxin A. Bioaccessibility, exposure and food process effect*", and was supported by the Spanish (Projects AGL2011-24862 and AGL2014-52648-REDT) and Catalonian (XaRTA-Reference Network on Food Technology) Government.

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LIST OF ABBREVIATIONS

ALS	Alkali-labile sites
ANOVA	Analysis of variance
APS	American Phytopathological Society
ATP	Adenosine triphosphate
B	Bioaccessibility
BARGE	Bioaccessibility Research Group of Europe
BEN	Balkan endemic nephropathy
BME	Base Modified Eagle
C8 dG-OTA	C8-guanine adduct of OTA
CBMN	Cytokinesis-blocked micronucleus
CBPI	Cytokinesis block proliferation index
CDK4	Cyclin-dependent kinase 4
cDNA	Complementary DNA
COX-2	Cyclooxygenase-2
Cq	Quantification cycle
CYP450	Cytochrome P450 system
Cyt-B	Cytochalasin-B
DCFH-DA	Chloramine-T and 2′,7′-dichlorofluorescein diacetate
DCF	Dichlorofluorescein
DMEM	Dulbecco′s modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DSBs	Double-strand breaks
dT	Deoxy-thymine nucleotides
E	Efficiency
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay

EPA	Environmental Protection Agency
EtBr	Ethidium bromide
EU	European Union
F	Bioavailability
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization, statistics division of
F_B	Bioaccessible fraction
Fig	Figure
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
hPBMC	Human peripheral blood mononuclear cells
HPLC	High performance liquid chromatography
HR	Homologous recombination
IARC	International Agency for Research on Cancer
IC	Inhibitory concentrations
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LOQ	Limit of quantitation
LOX -5	Lipoxygenase-5
MAP	Mitogen-activated protein
MEM	Minimum essential medium
MN	Micronucleus
MRP2	Multi-drug resistance protein 2
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n.d.	Not determined
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT2	N-acetyltransferase 2,
NEAA	Non-essential amino acids
NF-κB	Nuclear factor-kappa B
NHU	N-hydroxyurea

NI	Nuclear index
NOEC	Non observed effect concentration
OECD	Organisation for Economic Co-operation and Development
OTA	Ochratoxin A
Otα	Ochratoxin alpha
OTB	Ochratoxin B
OTβ	Ochratoxin beta
OTC	Ochratoxin C
PBS	Phosphate buffer solution
PHA	Phytohemagglutinin
pKa	Dissociation constant
PMS	Phenazinemethosulfate
ppb	Parts-per-billion, 10^{-9}
qPCR	Quantitative polymerase chain reaction
r	Relative expression ratio
RES	Resveratrol
RIVM	Netherlands National Institute for Public Health and the Environment (Dutch: Rijksinstituut voor Volksgezondheid en Milieu)
ROS	Reactive oxygen species
RSDr	Relative standard deviation
SCE	Sister chromatid exchange
SD	Standard deviation
SHE	Syrian hamster embryo
SOCs	Store-operated Ca^{2+} channels
SSB	Single-strand breaks
TEER	Transepithelial electrical resistance
TGF-b1	Transforming growth factor
NR	Neutral red
TIM-1	TNO (gastro-intestinal model-1)
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization

Summary

Ochratoxin A (OTA) is a mycotoxin produced by certain fungal species of the genera *Aspergillus* and *Penicillium*. OTA is well known as a contaminant of a large variety of foods, feeds and beverages worldwide. Cereal-based foodstuffs are the most important OTA-contaminated foods for human consumption. Wine has been shown to be frequently contaminated with OTA and thus contributes significantly to human exposure to this mycotoxin. Studies in rodents show that exposure to OTA promotes the development of kidney and liver tumours. The International Agency for Research on Cancer (IARC) classified OTA in the group 2B (possibly carcinogenic to humans), because there are insufficient data about its carcinogenicity to humans.

This PhD thesis had two general objectives: i) to assess the bioaccessibility of OTA in red wine, and ii) to assess OTA cytotoxicity, genotoxicity and gene expression modulation.

With regard to OTA bioaccessibility, a study was conducted with red wine at three contamination levels (1.0-4.0 µg/L) using an *in vitro* dynamic digestion model. The results showed that OTA is highly bioaccessible in the gastric chyme (103-128%) but much less bioaccessible in the intestinal chyme (<26%). The digestion system was also used to evaluate the transformation of OTA to OTα; the amount of OTα generated in gastric digestion was found to range from 5.1% to 19.1%, while the amount generated in intestinal digestion did not exceed 5%.

OTA cytotoxicity was evaluated by means of different *in vitro* assays with two human cell lines (Caco-2 and HepG2 cell lines) and primary human lymphocytes. Viability assays showed that low doses of OTA (0.075-45 µM) did not cause viabilities below 65%. At the same doses, significant damage was observed in the membrane integrity of differentiated Caco-2 cells and a cytostatic effect in lymphocytes treated with 15 µM OTA.

In Caco-2 cells, the effect of exposure to low OTA doses, alone and in combination with the mycotoxin deoxynivalenol (DON), was evaluated, as was the apparent protective effect of the antioxidant resveratrol (RES), a natural compound found in red wine. Co-exposure to both mycotoxins significantly increased the cytotoxicity of both compounds in

Caco-2 cells, without an increase in the generation of reactive oxygen species (ROS). Co-exposure to OTA or DON with RES did not diminish the cytotoxicity; on the contrary, an increase in the cytotoxicity was observed that was not associated with an increase in ROS production.

OTA doses lower than 45 μM were used to assess the gene expression modulation of the xenobiotic transformation system (*CYP P450* system genes), as well as of the *COX-2*, *LOX-5*, and *MRP2* genes. To this end, the gene expression level was investigated in a co-culture system of Caco-2 and HepG2 (transwell culture plates) to simulate intestinal absorption and hepatic metabolism. The results suggest that OTA was able to strongly modulate gene expression over time, and that the time of exposure to OTA had a greater effect on expression gene and membrane integrity (as measured by transepithelial electrical resistance) than the dose of toxin used in the treatments.

With regard to the OTA genotoxicity assessment, OTA was shown to be a genotoxic mycotoxin, because it was able to induce micronucleus (MN) formation in human lymphocytes. Moreover, in a comet assay, OTA increased the percentage of DNA in the comets and caused an accumulation of DNA in the comets' tails by delaying the DNA repair pathway.

Our results show that further research is needed to understand the mode of action of OTA, especially considering its possible association with human cancer development. Our findings on OTA cytotoxicity and genotoxicity at low doses support the theory that OTA should be considered a non-threshold carcinogen.

Resumen

La ocratoxina A (OTA) es una micotoxina producida por varias especies de mohos de los géneros *Aspergillus* y *Penicillium*. Es bien conocido que la OTA es una de las micotoxinas más ubicuas, ya que se ha encontrado a nivel mundial en una gran variedad de alimentos tanto de origen vegetal como animal. Los productos para consumo humano elaborados a base de cereales son el grupo de alimentos que presenta una mayor contaminación por OTA. El vino es un alimento que se encuentra contaminado con frecuencia con OTA y que puede tener una importante contribución a la exposición humana a esta micotoxina. Estudios en roedores han demostrado que la OTA es capaz de promover el desarrollo de tumores renales y hepáticos. La Agencia Internacional para la Investigación del Cáncer (IARC) ha clasificado a la OTA en el grupo 2B (posible compuesto carcinógeno para el hombre), porque los datos que demuestran la carcinogenicidad de la OTA en humanos aún son insuficientes.

Esta tesis doctoral tuvo dos objetivos generales: i) evaluar la bioaccesibilidad de la OTA en vino tinto, y ii) evaluar la citotoxicidad, genotoxicidad y la modulación de la expresión génica por la OTA.

En el caso de la bioaccesibilidad de la OTA, se llevó a cabo un estudio en vino tinto con tres niveles de contaminación de OTA (1,0-4,0 µg/L) en un sistema *in vitro* de digestión dinámica simulada. Los resultados de este estudio mostraron que la OTA es altamente bioaccesible en jugo gástrico (103-128%), pero es poco bioaccesible en el jugo intestinal (<26%). Adicionalmente, en este sistema se evaluó también la transformación de la OTA a OTα, encontrando que la cantidad de OTα generada en la digestión gástrica varió entre el 5,1-19,1%, mientras que en el compartimento intestinal no excedió del 5%.

La evaluación de la citotoxicidad de la OTA fue llevada a cabo usando varios ensayos *in vitro*, y tres tipos de cultivos celulares (linfocitos humanos, y las líneas celulares Caco-2 y HepG2). Los ensayos de viabilidad mostraron que bajas dosis de OTA (0,075-45 µM) no causan viabilidades por debajo del 65%. Aplicando estas mismas dosis, tras 24 h de exposición se detectó un daño significativo en la integridad de la membrana celular de las células Caco-2 diferenciadas y un efecto citostático en linfocitos humanos tratados con 15 µM de OTA.

En el caso de las células Caco-2, se estudió el efecto de la exposición a bajas dosis de OTA, sola o de forma conjunta con la micotoxina deoxinivalenol (DON), y se evaluó el presunto efecto protector del antioxidante resveratrol (RES), compuesto naturalmente presente en el vino tinto. La co-exposición a ambas micotoxinas aumentó significativamente la citotoxicidad de ambos compuestos en las células Caco-2, sin aumentar por ello la producción de especies reactivas del oxígeno (ERO). La co-exposición de OTA o DON con RES no disminuyó la citotoxicidad, más bien al contrario, se observó un aumento de la citotoxicidad no asociado con un aumento en la producción de ERO.

Se usaron dosis bajas de OTA, inferiores a 45 µM, para evaluar la expresión de genes del sistema biotransformador de xenobióticos (genes del grupo *CYP P450*), así como la de los genes *COX-2*, *LOX-5*, y *MRP2.* Para ello se empleó un sistema de co-cultivo de células Caco-2 y HepG2 (placas de cultivo *transwell*) para simular la absorción intestinal y el metabolismo hepático. Los resultados indican que la OTA es capaz de modular fuertemente la expresión de los genes estudiados a lo largo del tiempo, teniendo, por lo general, el tiempo de exposición a la OTA un mayor efecto sobre la modulación de los genes y la integridad de la membrana (medida por la resistencia eléctrica transepitelial) que las diferentes dosis de toxina utilizadas en los tratamientos.

En cuanto a la evaluación de la genotoxicidad de la OTA, se ha demostrado que la OTA es un micotoxina genotóxica capaz de inducir formación de micronúcleos en linfocitos humanos, así como de, en un ensayo cometa, incrementar el porcentaje de ADN en el cometa, y causar la acumulación del mismo en las colas de los cometas mediante un retraso en el sistema de reparación del ADN.

Los resultados obtenidos indican que se deben realizar mayores esfuerzos para entender el modo de acción de la OTA, especialmente teniendo en cuenta su posible relación con el desarrollo del cáncer en los seres humanos. Los hallazgos sobre la citotoxicidad y genotoxicidad de la OTA a bajas dosis apoyan la teoría de que la OTA debería de ser considerada un carcinógeno sin umbral.

Resum

L'ocratoxina A (OTA) és una micotoxina produïda per vàries espècies de fongs del gènere *Aspergillus* i *Penicillium*. L'OTA és una de les micotoxines més ubiques, ja que a nivell mundial s'ha trobat en una gran varietat d'aliments tant d'origen vegetal com d'animal. Els productes per a consum humà elaborats a base de cereals són el grup d'aliments que presenta una major contaminació d'OTA. El vi és un altre aliment que freqüentment es troba contaminat amb OTA i que pot tenir una important contribució en quan a l'exposició humana d'aquesta micotoxina. Estudis realitzats en rosegadors han demostrat que l'OTA és capaç de promoure el desenvolupament de tumors renals i hepàtics. L'Agència Internacional per a la Investigació del Càncer (IARC) ha classificat l'OTA dins el grup 2B (possible compost carcinogen per als humans), ja que les dades que demostren la carcinogenicitat de l'OTA en humans encara són insuficients.

Aquesta tesi doctoral consta de dos objectius generals: i) avaluar la bioaccessibilitat de l'OTA en vi negre i, ii) avaluar la citotoxicitat, genotoxicitat i la modulació de l'expressió genètica per a l'OTA.

En el cas de la bioaccessibilitat de l'OTA, es va dur a terme un estudi en vi negre amb tres nivells de contaminació d'OTA (1,0-4,0 μg/L) en un sistema *in vitro* de digestió dinàmica simulada. Els resultats d'aquest estudi van demostrar que l'OTA és altament bioaccessible en suc gàstric (103-128%) però és poc bioaccessible en el suc intestinal (<26%). Addicionalment, en aquest mateix sistema es va avaluar la transformació de l'OTA a OTα i es va observar que la quantitat de OTα generada durant la digestió gàstrica va variar entre el 5,1-19,1%, mentre que en el compartiment intestinal no va excedir el 5%.

L'avaluació de la citotoxicitat de l'OTA es va dur a terme mitjançant diversos assajos *in vitro* i tres tipus de cultius cel·lulars (els limfòcits humans i les línies cel·lulars Caco-2 i HepG2). Els assajos de viabilitat van demostrar que baixes dosis d'OTA (0,075-45 μM) no causen viabilitats per sota del 65%. Aplicant aquestes mateixes dosis, després de 24 hores d'exposició, es va detectar un dany significatiu en la integritat de la membrana cel·lular de les cèl·lules Caco-2 diferenciades i un efecte citostàtic en els limfòcits humans tractats amb 15 μM d'OTA.

En el cas de les cèl·lules Caco-2, es va estudiar l'efecte de l'exposició a baixes dosis d'OTA, sola o de forma conjunta amb la micotoxina deoxinivalenol (DON), i es va avaluar el presumpte efecte protector de l'antioxidant resveratrol (RES), el qual es troba naturalment present en el vi negre. La co-exposició a ambdues micotoxines va augmentar significativament la citotoxicitat de tots dos compostos en les cèl·lules Caco-2, sense produir un augment de les espècies reactives de l'oxigen (ERO). La co-exposició de l'OTA o el DON amb el ROS no va disminuir la citotoxicitat, al contrari, es va observar un augment de la citotoxicitat no associat a un augment en la producció de les ERO.

Es van utilitzar dosis baixes d'OTA, inferiors a 45 μM, per avaluar l'expressió dels gens del sistema biotransformador de xenobiòtics (gens del grup *CYP P450*), així com la dels gens *COX-2*, *LOX-5* i *MRP2*. Amb aquest objectiu es va utilitzar un sistema de co-cultiu de cèl·lules Caco-2 i HepG2 (plaques de cultiu *transwell*) per a simular l'absorció intestinal i el metabolisme hepàtic. Els resultats indiquen que l'OTA té gran capacitat per modular l'expressió dels gens estudiats al llarg del temps. En general es va observar que el temps d'exposició a l'OTA tenia un major efecte sobra la modulació dels gens i la integritat de la membrana (mesurada mitjançant la resistència elèctrica transepitelial) que no pas les diferents dosis de toxina utilitzades en els tractaments.

En relació a l'avaluació de la genotoxicitat de l'OTA, s'ha demostrat que l'OTA és una micotoxina genotòxica capaç d'induir la formació de micronuclis en els limfòcits humans, així com, en un assaig cometa, incrementar el percentatge d'ADN en el cometa i provocar la seva acumulació en les cues dels cometes mitjançant un retràs en el sistema de reparació de l'ADN.

Els resultats obtinguts indiquen que s'han d'invertir més esforços per tal d'entendre el mode d'acció de l'OTA, especialment tenint en compte la seva possible relació amb el desenvolupament del càncer en humans. Les troballes en referència a la citotoxicitat i la genotoxicitat de l'OTA a baixes dosis recolzen la teoria que l'OTA hauria de ser considerada carcinogènica sense llindar.

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1. Introduction

1.1 Introduction to mycotoxins

Mycotoxins are secondary metabolites produced by filamentous fungi that are capable of causing disease and death in humans and animals. The term "*mycotoxin*" derives from the Greek word *mycos*, meaning mould, and the Latin word *toxicum*, meaning poison. Of natural origin, limited taxonomic distribution and, usually, relatively low molecular weight, mycotoxins are not considered to have an obvious function in the producer species, although it is generally accepted that their production could provide an ecological advantage. Mycotoxin production often occurs under stressful conditions for the mould, and they are sometimes produced by growing colonies approximately at the time of sporulation (Bennett and Bentley, 1989; Calvo et al., 2002). Most of the known mycotoxins are produced by various species in the fungal genera *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria*. A single mycotoxin can be produced by multiple fungal species, and a single fungal strain can produce multiple types of mycotoxins. If conditions are favourable, moulds can grow in crops and produce mycotoxins directly in the field, as well as during the harvest, drying and storage. Because certain chemical, physical and biological conditions affect fungal growth and mycotoxin production, the distribution of mycotoxins in foodstuffs is expected to vary from year to year, depending on the production processes used (FAO, 2001; Richard, 2007).

Exposure to mycotoxins is primarily through ingestion, although it can also occur through other pathways, such as inhalation and contact. Mycotoxins can produce both acute and chronic toxicities, with symptoms ranging from deleterious effects on the gastrointestinal tract, the cardiovascular, pulmonary or central nervous systems, or other internal organs to death (FAO, 1991; FAO, 2001). Diseases and symptoms caused in humans or animals by exposure to mycotoxins are known as mycotoxicoses. Chronic toxicity is of special concern for human health since human mycotoxicoses are both less understood and less clearly defined than animal mycotoxicoses, most of which have been experimentally confirmed (Bennett and Klich, 2003; Smith et al., 1995).

By the early twenty-first century, more than 300 mycotoxins had been identified, produced by approximately 350 species of fungi (Betina, 1989; Richard, 2003). Today, the number is even higher. In 2003, Bennett and Klich estimated that the number of mycotoxins is

closer to 400, and many have since been identified, although the exact number has yet to be accurately determined. The International Agency for Research on Cancer (IARC) has evaluated the toxicity of the most important of these toxins (Table 1) (IARC, 1993), and the European Union (EU) has implemented regulations on the maximum allowable limits of the main mycotoxins in foods and feeds (Commission Regulation, 2006ab; 2010).

Scientific attention has tended to focus on those mycotoxins that have proven to be carcinogenic and/or toxic in other ways, such as aflatoxins (mainly the aflatoxins B_1, B_2, G_1, G_2 and M_1), fumonisins (mainly fumonisins B_1 and B_2), ochratoxin A, patulin, trichothecenes (mainly deoxynivalenol and the T-2 and HT-2 toxins), and zearalenone.

As this PhD thesis is focused on ochratoxin A, the following sections will provide a more detailed description of this toxin.

Table 1. Carcinogenic risk of some mycotoxins evaluated by the IARC (IARC, 2003).

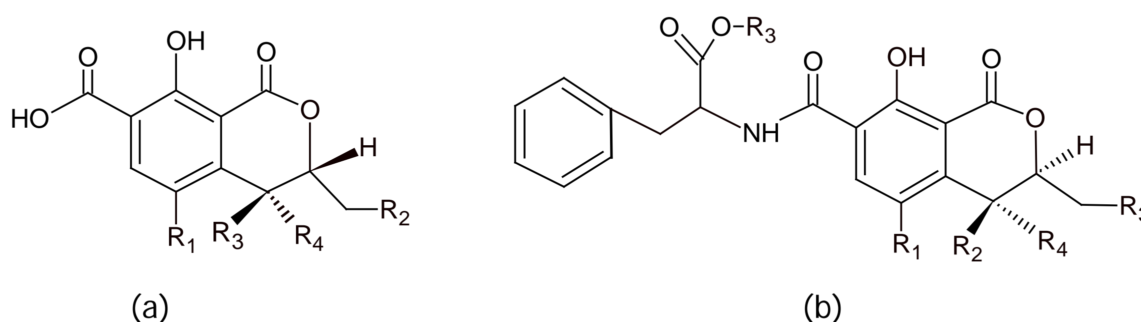
Mycotoxin	**Evidence of carcinogenicity**		**Overall evaluation of carcinogenicity to humans**
	In humans	**In animals**	
Naturally occurring mixtures of aflatoxins	S	S	Group 1
Aflatoxin B_1	S	S	Group 1
Aflatoxin B_2		L	
Aflatoxin G_1		S	
Aflatoxin G_2		I	
Aflatoxin M_1	I	S	Group 2B
Ochratoxin A	I	S	Group 2B
Toxins derived from *F. moniliforme*	I	S	Group 2B
Naturally occurring mixtures of fumonisins	I		
Fumonisin B_1		S	Group 2B
Fumonisin B_2		I	
Fusarin C		L	
T-2 toxin		L	
Toxins derived from *F. verticillioides*	I	S	Group 2B
Toxins derived from *F. graminearum*, *F. culmorum*, *F. crookwellense*	I		Group 3
Zearalenone		L	
Deoxynivalenol		I	
Nivalenol		I	
Fusarenone-X		I	
Patulin	I		Group 3

Degrees of evidence: S = sufficient, I = inadequate; L = limited; N = negative. Group 1: carcinogenic to humans; Group 2A: probably carcinogenic to humans; Group 2B: possibly carcinogenic to humans; Group 3: not classifiable as to its carcinogenicity to humans; Group 4: probably not carcinogenic to humans.

1.2 Ochratoxins

Ochratoxins are a small group of chemically related toxic fungal metabolites produced by several species of the *Aspergillus* and *Penicillium* genera. Chemically, ochratoxins are weak organic acids consisting of dihydroisocoumarin linked to β-phenylalanine (Figure 1). There are three major ochratoxins, designated as ochratoxin A, B and C. According to the

literature, ochratoxin A (OTA) and ochratoxin B (OTB) occur frequently in mouldy products (Li et al., 1997; K J van der Merwe et al., 1965). OTB is the dechlorinated analogue of OTA. The major metabolite of OTA is ochratoxin alpha (OTα), and the dechlorinated isocoumarin of OTB is ochratoxin β (OTβ); neither metabolite is linked to phenylalanine. The ethyl ester of OTA is ochratoxin C (OTC) (Moss, 1996; Xiao et al., 1995). The most important, toxic and economically significant ochratoxin is OTA (Li et al., 1997; K J van der Merwe et al., 1965).



Structure	Compound	R identity				
		R_1	R_2	R_3	R_4	R_5
a	OTα	Cl	H	H	H	-
a	OTβ	H	H	H	H	-
b	OTA	Cl	H	H	H	H
b	OTB	H	H	H	H	H
b	OTC	Cl	H	H	H	CH_3CH_3
b	4R-OH-OTA	Cl	H	H	OH	H
b	4S-OH-OTA	Cl	H	OH	H	H
b	4-OH-OTA	H	H	H	OH	H
b	10-OH-OTA	Cl	OH	H	H	H

Figure 1. Molecular structure of the major ochratoxins naturally produced by filamentous fungi. a) isocoumarin structure of ochratoxins and b) general structure of ochratoxins.

1.3 Ochratoxin A

1.3.1 Physicochemical properties and stability of ochratoxin A

OTA ($C_{20}H_{18}ClNO_6$) has a molecular mass of 403.83 g/mol. The systematic chemical IUPAC name for OTA is (2S)-2-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-2-benzopyran-7-yl]formamido}-3-phenylpropanoic acid. OTA is a colourless, crystalline compound that is soluble in polar organic solvents (chloroform, ethanol, methanol, xylene) in acid and neutral pH, slightly soluble in water, and soluble in 0.1 M sodium bicarbonate (van der Merwe et al., 1965a; van der Merwe et al., 1965b). OTA has a melting point of about 90 °C when it was crystallised from benzene as a solvate. After drying for 1 h at 60 °C, it has a melting point in the range of 168-173 °C, and the molecule is optically active ([alpha]21D: -46.8 °C (c = 2650 µmol/L)) in chloroform (van der Merwe et al., 1965b).
OTA is a weak acid, a dissociable molecule with a dissociation constant (p*K*a) approximately equal to 4 for the carboxyl group of phenylalanine and 7 for the phenolic groups (Bredenkamp et al., 1989). OTB has a higher p*K*a value (p*K*a 7.8), suggesting that the presence of the electron-withdrawing chlorine (isocoumarin ring) and carboxyl (phenylalanine) enhance the dissociation of the phenolic hydroxyl group, in contrast with the higher p*K*a of OTα (*p*K*a* 11-11.6) (Chu et al., 1972; Gillman et al., 1999).

OTA shows an intense green fluorescence under UV light in an acidic medium and a blue fluorescence in alkaline conditions (Eppley, 1968). In a basic medium (0.5 N NaOH, pH 13), the dissociated form of OTA (dissociation of the phenolic hydroxyl group) shows a high fluorescence with a red shift from 333 to 380 nm, followed by a time-dependent blue shift from 380 to 345 nm (deprotonated form). The blue shift in the UVmax of OTA is attributed to the hydrolysis of its lactone (Gillman et al., 1999). In a solution of 0.5 M Na_2CO_3 (pH 10-11), OTA shows the same red shift from 333 to 381 nm but not the time-dependent blue shift (Gillman et al., 1999). Thus, under the different conditions described above, three forms of OTA can occur (Figure 2).

OTA is a very stable mycotoxin; gamma irradiation (up to 7.5 Mrad) does not affect the OTA molecule, although it suffers photolysis during exposure to fluorescent light, and

degradation in an excess of sodium hypochlorite (Castegnaro et al., 1991; Neely and West, 1972).

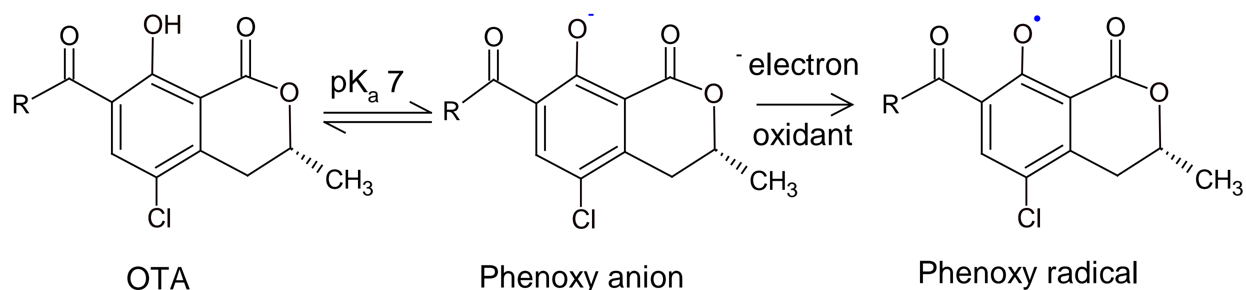


Figure 2. Oxidation of OTA at basic pH.

OTA can be stored in ethanol under refrigeration and can resist up to 3 h of high-pressure steam sterilisation (121 °C) (Neely and West, 1972; Trivedi et al., 1992). Cooking under normal conditions, and even at 170-250 °C, does not completely destroy it (Müller, 1982; Vidal et al., 2014).

1.4 OTA producers and occurrence in foods and feeds

1.4.1 OTA producers

In a large screen of fungal metabolites, van der Merwe et al. (1965a) isolated OTA from *Aspergillus ochraceus* and found it to be the most abundant and toxic ochratoxin. OTA was first reported as a contaminant of corn by Shotwell et al. (1969). Since then, several members of the *Aspergillus* genus, common in warm and temperate climates, have been described as ochratoxin producers. *Aspergillus* section *Nigri* is the largest section with species showing ability to produce OTA, including five OTA producers: *A. awamori* (Perrone et al., 2011), *A. carbonarius* (García-Cela et al., 2014; Frisvad et al., 2011; Samson et al., 2007), *A. niger* (Perrone et al., 2011; Samson et al., 2004; Varga et al., 2007), A*. sclerotioniger* (Frisvad et al., 2011; Samson et al., 2007; Serra et al., 2006), and *A. tubingensis* (Medina et al., 2005; Varga et al., 2007).

The most important OTA producer species are *A. ochraceus*, *A. westerdijkiae* and *A. steynii*, which are members of *Aspergillus* section *Circumdati*, due to their occurrence in foods. Other species belonging to the *Aspergillus* genus have also shown the ability to produce OTA. Likewise, in addition to *Aspergillus*, other species in the *Penicillium* genus have been shown to produce OTA (Table 2).

Table 2. Main OTA-producing species.

Aspergillus* section *Candidi
A. taichungensis
A. campestris
Aspergillus* section *Circumdati
A. cretensis
A. flocculosus
A. melleus
*A. ochraceus**
A. ostianus
A. persii
A. petrakii
A. pseudoelegans
A. roseoglobulosus
A. sclerotiorum
*A. steynii**
A. sulphureus
*A. westerdijkiae**
Aspergillus* section *Flavi
A. albertensis (*Petromyces albertensis*)
A. alliaceus (*Petromyces alliaceus*)
A. lanosus
Aspergillus* section *Nigri
*A. carbonarius**
*A. niger**
A. sclerotioniger
Aspergillus* section *Wentii
A. sepultus and
A. dimorphicus
Penicillium
P. nordicum
P. verrucosum[§]

*The most economically important OTA producers and [§]the most important OTA producer in northern Europe, which is associated with the cooler, damper conditions in the post-harvest. Adapted from Abrunhosa et al. (2010) and García-Cela et al. (2014).

1.4.2 Occurrence of OTA in foods and feeds

OTA has been detected worldwide in several cereals and cereal by-products, such as (Ibáñez-Vea et al., 2012), bran (Vidal et al., 2013), maize (Saleemi et al., 2012; Serrano et al., 2012), oats (Nguyen and Ryu, 2014; Vidal et al., 2013), rice (Nazari et al., 2014; Nguyen and Ryu, 2014), rye (Serrano et al., 2012), sorghum (Serrano et al., 2012) and wheat (Nguyen and Ryu, 2014; Serrano et al., 2012; Streit et al., 2013). From an economic point of view, the occurrence of OTA in other important foodstuffs, such as coffee (Drunday and Pacin, 2013; Galarce-Bustos et al., 2014; Vladimir. Ostry et al., 2013), cocoa (Brera et al., 2011; Copetti et al., 2010) and beverages like wine (Coronel et al., 2012; Ostry et al., 2013) and beer (Coronel et al., 2012), is also notable. OTA has additionally been detected in dried fruits (Coronel et al., 2012; Ostry et al., 2013) and spices (Prelle et al., 2014; Waśkiewicz et al., 2013).

Deleterious effects of mycotoxins have been documented in different animals due to the intake of contaminated feeds. In a global survey conducted with feed samples from America, Europe, and Asia, OTA was present up to 31% of the time (Rodrigues and Naehrer, 2012). In other worldwide studies (with samples from Asia, Europe, Africa, the Middle East, Oceania and America), the percentage of OTA-contaminated samples was as high as 28% (Streit et al., 2013). The highest percentage (50%) of contaminated samples was reported for wheat/wheat bran samples from North America (Rodrigues and Naehrer, 2012). The consumption by animals of feed contaminated with OTA leads to the presence of this toxin in animal-derived food products and, therefore, to its chronic ingestion by humans. In this regard, OTA has been detected in tissues from healthy pigs (20-100%), chickens (69.2%), ducks (57.8%) and geese (41.7%) (Duarte et al., 2012). OTA has also been found in the blood (Bernardini et al., 2014; Grajewski et al., 2012; Perši et al., 2012), eggs (Hassan et al., 2012; Iqbal et al., 2014; Piskorska-Pliszczyńska and Juszkiewicz, 1990) and milk (Boudra et al., 2013; Huang et al., 2014; Muñoz et al., 2014) of exposed animals.

1.5 Socio-economic impact and legislation

Millions of tonnes of agricultural and food products are produced each year. The occurrence of mycotoxins in a large percentage of them is thus a public health issue, due to their deleterious effects. International regulations on mycotoxins are most often concerned with agricultural products such as cereals. In countries where agricultural commodities, such as cereals, account for a high percentage of total national exports, high mycotoxin levels take on economic importance and can affect to international trade (APS, 2015; FAO, 1991; FAOSTAT, 2014). There are no accurate figures on the worldwide economic losses resulting from mycotoxin contamination of foods, as in addition to the direct losses of food and feed due to fungal or mycotoxin contamination, there are a variety of indirect losses, including those due to: lower productivity, losses in foreign exchange earnings, inspection, sampling and testing costs before and after shipment, the need to purchase insurance, farm subsidies to cover production losses, research costs and project support, training costs, the costs of mycotoxin decontamination, etc (APS, 2015).

It is thus essential for mycotoxin concentrations to be kept at toxicologically acceptable levels. Toxin regulations are a compromise, and regulations on mycotoxins have been put into place and are strictly enforced. Today, many countries have regulations or recommendations for mycotoxins, such as the EU member states, the USA and Canada. Table 3 shows the allowable levels of OTA in foods in the EU, and Table 4 shows the EU's recommendations on OTA levels in feedingstuffs.

Table 3. Maximum allowable levels of OTA in foodstuffs in the EU.

Foodstuff	**Maximum level (µg/kg)**	**Enactment***
Dietary foods for special medical purposes intended specifically for infants	0.5	2006
Green coffee, dried fruit other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products, spices and liquorice	0.5	2006
Wine	2	2006
Processed cereal-based foods and baby foods for infants and young children	2	2006
All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption	3	2012
Unprocessed cereals	5	2006
Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5	2006
Wheat gluten not sold directly to the consumer	8	2012
Dried vine fruit (currants, raisins and sultanas)	10	2006
Soluble coffee (instant coffee)	10	2006
Various species of spices, including *Piper* spp (pepper), *Myristica fragrans* (nutmeg), *Zingiber offinale* (ginger), *Curcuma longa* (turmeric) and *Capsicum* spp. (chili pepper), and mixtures of spices containing any one of them	15	2012

*By Commission Regulation (EC) No. 1881/2006 and Commission Regulation (EU) No. 594/2012.

Table 4. Recommended OTA levels in feedingstuffs in the EU.

Feedingstuff	Maximum level (mg/kg)[§]	Enactment*
Feed materials		2006
Cereals and cereal products	0.05	
Complementary and complete feedingstuffs		2006
Complementary and complete feedingstuffs for pigs	0.05	
Complementary and complete feedingstuffs for poultry	0.1	

[§]Concentration in a feedingstuff with a moisture content of 12%. *Commission Recommendation (EC) No.576/2006.

1.6 Bioavailability and *in vitro* bioaccessibility

The oral bioavailability (F) of a mycotoxin has been defined as the fraction of an orally ingested mycotoxin, in a given food matrix, that ultimately reaches the systemic circulation and is distributed throughout the body to exert its toxic effect (Versantvoort, 2004). Bioaccessibility (B) determination is the first step to consider when the bioavailability of a molecule in a given food matrix needs to be calculated. The bioavailability of an ingested mycotoxin depends on three different, sequential steps (Brandon et al., 2006):

a) the release of the mycotoxin from the food matrix during digestion in the gastrointestinal (GI) tract. It is in this step that the bioaccessible mycotoxin (F_B) is measured.
b) the absorption of the bioaccessible mycotoxin through the intestinal epithelial cells of the GI tract (F_A) and its subsequent transport to the blood (or lymph) stream.
c) the metabolism of the mycotoxin prior to systemic circulation (i.e. its biotransformation and excretion by the intestinal epithelium or liver). This is the so-called first-pass effect (F_M).

In light of these steps, Equation 1 defines the bioavailable fraction of an ingested mycotoxin, i.e. the fraction reaching the systemic circulation:

$$F = F_B \times F_A \times F_M \qquad \text{[Equation 1]}$$

In vitro bioaccessibility has become important because it represents the amount of the mycotoxin that could reach the blood after intestinal absorption. Bioaccessibility is given in percentages and calculated with the following equation (Equation 2):

$$B\,(\%) = [\text{mycotoxin}_{\text{chyme}}] \text{ after GI digestion} / [\text{mycotoxin}_{\text{food matrix}}] \text{ before GI digestion} \qquad \text{[Equation 2]}$$

It is worth noting that bioaccessibility has only been calculated in *in vitro* systems. The concept is only applicable to oral exposure.

1.6.1 In vitro bioaccessibility of OTA

To date, few studies have been conducted on the bioaccessibility of OTA and its relationship to different food matrices. Using the Dutch National Institute for Public Health and the Environment (Dutch name: Rijksinstituut voor Volksgezondheid en Milieu) model (Dutch acronym: RIVM model), Versantvoort et al. (2004) determined the OTA bioaccessibility in two lots of buckwheat and found that a considerable amount of OTA was released from the food matrix, from over 45% (in a buckwheat batch contaminated at 20 µg/kg) to 84% (in a buckwheat batch contaminated at 7 µg/kg). In 2005, the same authors, using the same experimental design, reported considerably higher OTA bioaccessibilities (from 86 to 116%), although the amount of mycotoxin used in the digestion model was lower in the second study (Versantvoort et al., 2005). Those data agree with the results later reported by Avantaggiato et al. (2007), who, using the TIM-1 model (TNO gastro-intestinal model-1) with artificially OTA-contaminated corn feed, found absorption levels of around 88%. In contrast, Kabak et al. (2009) once again reported much lower values for OTA bioaccessibility (22-32%) using the RIVM model with naturally OTA-contaminated buckwheat and low- and highly contaminated spiked infant foods. The bioaccessibilities found did not seem to depend on the contamination level; however,

bioaccessibility seemed to increase when spiked contamination was employed, as in such cases the toxins may not be tightly bound to the food matrix. Table 5 shows a summary of the OTA bioaccessibility studies published to date.

Table 5. Gastrointestinal *in vitro* models used for bioaccessibility or absorption determination of OTA.

Food matrix	Contami -nation	Matrix contamina-tion range (μg/kg)	Bioaccessibility/ Absorption (%)	*In vitro* model used*	Reference
Buckwheat	N	7-20	45-84	RIVM	Versantvoort et al., 2004
Food mix	N	3.3-3.5	60-6	RIVM	Versantvoort et al., 2004
Buckwheat	N	11-11.3	86-100	RIVM	Versantvoort et al., 2005
Food mix	N	1.83-2.0	114-116	RIVM	Versantvoort et al., 2005
Corn feed	S	11	88 (73 jejunum; 15 ileum)	TIM-1	Avantaggiato et al., 2007
Buckwheat	N	24.9	22	RIVM	Kabak et al., 2009
Infant food	S	3.2-13.1	29-32	RIVM	Kabak et al., 2009

N: natural; S: spiked. RIVM model: The Dutch National Institute for Public Health and the Environment. TIM-1 model: TNO (gastro-intestinal model-1)

1.7 Toxicokinetics of OTA

1.7.1 Absorption

In the gastrointestinal tract, the highest levels of OTA absorption are found in the small intestine, mainly in the proximal jejunum. The absorption mechanism for OTA is passive and increases over time, and it is affected by changes in pH. A decrease in pH results in an increase in OTA uptake (Berger et al., 2003; Kumagai, 1988); therefore, p*K*a values in the range of 4.2-4.4 (carboxylic group) and 7.0-7.3 (phenolic hydroxyl group) play an important role in OTA absorption (Galtier et al., 1979; Roth et al., 1988). In rats, the

highest OTA concentration is found in portal blood and the rate of appearance in the venous plasma increases in keeping with increases in the luminal concentration of OTA (Kumagai and Aibara, 1982); in contrast, the lymphatic route only contributes to low-dose levels of OTA (Roth et al., 1998).

1.7.2 Tissue distribution and bioavailability

Once OTA crosses the intestinal epithelium and reaches the bloodstream, it is bound to plasma proteins, mainly albumin. This fact favours the passive absorption of OTA, especially in mammalian species (more than 97% of OTA in plasma) as compared to fish (22%) (Hagelberg et al., 1989). In humans, up to 84% of OTA binds to plasma proteins within a period of 8 h (Studer-Rohr et al., 2000). In contrast, in rats the maximum OTA level is reached within 3 h; this concentration then remains unchanged before beginning to decline on day 4 and until day 14 (Mantle, 2008). The highest bioavailability values of OTA in animal species have been reported in mice (92%) and pigs (65.7%), followed by rats (61%), monkeys (57%), rabbits (55.6%), chickens (40%) and fish (1.6%) (Galtier et al., 1981; Hagelberg et al., 1989). OTA or its metabolites are mainly distributed in the blood, kidney and liver, but they are also found in the lungs, heart, spleen and bile (Aoudia et al., 2008; Fuchs and Hult, 1986; Han et al., 2013).

Interestingly, OTA levels in blood appear to be gender-dependent (Castergnaro et al., 2006; Coronel et al., 2011; Heussner et al., 2002). In rats, females have been shown to have higher blood OTA levels than males (Castegnaro et al., 2006; Mantle, 2009; Zepnik et al., 2003). In humans, blood OTA levels have also been shown to be slightly higher in females (0.81 ng/mL) than in males (0.71 ng/mL) (Coronel et al., 2011). These findings can be explained by the fact that OTA binds to α-2u-globulin protein in blood and is rapidly delivered to the renal proximal tubule epithelium. This delivery of OTA to the kidney occurs more quickly in males than females, increasing the OTA concentration in the target organ and, consequently, nephrotoxicity in males (Haighton et al., 2012; Mantle and Nagy, 2008).

With regard to age, of the different human age groups studied, the group over the age of 45 had the highest mean (0.55 ng/mL) and maximum OTA levels (10.92 ng/mL) in blood (Coronel et al., 2011).

In vivo and *in vitro* inhibition of organic anion transport with probenecid (a uricosuric drug that increases uric acid excretion in the urine) prevents OTA renal clearance, suggesting that the OTA reabsorption/excretion mechanism occurs via a transcellular organic anion transport system and, thus, that glomerular filtration of OTA is negligible and the organic anion transport system must be the specific renal mechanism associated with OTA-induced toxicity (Stein et al., 1985; Tsuda et al., 1999). The OTA clearance mechanism is related to the MRP2 transporter (Berger et al., 2003; Leier et al. 2000). MRP2 (or ABCC2; 173.681 kD) is a second member of the human multidrug resistance-associated protein (MRP) subfamily, a membrane protein implicated in the active efflux of drugs and xenobiotics. MRP2 transport is highly expressed at the apical side of the enterocytes and in other organs such as the kidney, liver, brain, etc. MRP2 plays an important role as a xenobiotic outward transporter, reducing oral bioavailability and, in the liver, contributing to bile formation and to toxin elimination via this pathway (Dietrich et al., 2003; Jedlitschky et al., 2006).

1.7.3 Half-life and excretion

OTA half-life has not been clearly defined and several factors, such as exposure time, diet, health status and the degree of serum binding, can affect the rate at which it is removed from the body. The high affinity of OTA for serum proteins partially explains its long half-life in the body and high bioavailability (Hagelberg and Hult, 1989; Kumagai, 1985). In rats, the OTA half-life is 5.93 days (Mantle, 2008). Experimental OTA half-life in humans is 35.5 days, and the elimination half-life of OTA is generally longer in blood than in tissue, due to its high affinity to bind to blood proteins (Studer-Rohr et al., 2000).

OTA, as well as its metabolites OTα and hydroxylated OTAs (4R-4-OH-OTA and 4S-4-OH-OTA), are mainly excreted in urine (Klapec et al., 2012). 4R-4-OH-OTA has been detected at higher concentrations than 4S-4-OH-OTA in the urine of rats (Stormer et al., 1980). OTA is also excreted in faeces (Kumagai and Aibara, 1982) and the OTα metabolite has been detected in the caecum and large intestine of rats, which suggests that OTα may

also be found in faeces (Storen et al., 1982; Suzuki et al., 1977). Reabsorption of OTA occurs in the proximal tubules and via enterohepatic circulation and results in the redistribution of the toxin to the body (Stein et al., 1985). OTα is not accumulated in the body and has an elimination rate of up to 90% following intraperitoneal administration in rats (Storen et al., 1982).

1.7.4 Metabolism/Biotransformation of OTA

OTA may undergo hydrolysis as a first-pass effect during the GI digestion by carboxypeptidase A, trypsin, α-chymotrypsin and cathepsin C. Digestive enzymes break the peptide bond, giving rise to a phenylalanine molecule and the non-toxic metabolite OTα, a chlorinated dihydroisocoumarin (Doster and Sinnhuber, 1972; Kumagai, 1988; Pitout, 1986). This is the main metabolic pathway by which OTA is biotransformed into a less toxic compound. OTα is also produced in *in vitro* reactions by several cell types (Faucet-Marquis et al., 2006; Han et al., 2013; Jennings-Gee et al., 2010).

In ruminant species, such as sheep (Hohler et al., 1999), goats (Nip and Chu, 1979; Ribeling et al., 1978), cows (Ribeling et al., 1978) and Holstein calves (Hult et al., 1976; Ribeling et al., 1978), intestinal microbiota enzymes are also capable of degrading the OTA. The hydrolysis reaction by microbial enzymes mainly occurs in the large intestine, although some hydrolysis can also occur in the stomach. However, the microbial enzymes involved in OTA hydrolysis have not been identified.

Peptidases are not the only enzymes involved in the detoxification of OTA, and several metabolites have been characterised *in vitro* and/or *in vivo*. OTA can be metabolised in a non-specific manner by several enzymes of the cytochrome P450 system (CYP450). The CYP450 enzymes (e.g. CYP2A6, 2B6, 2C9, 2C18, 2C19, 3A4, and 3A5) metabolise compounds to hydroxylated products (de Jonge et al., 2005; Xie et al., 2003). In the case of OTA, three different hydroxylated metabolites have been identified: 4R-4-OH-OTA, 4S-4-OH-OTA and 10-OH-OTA (Creppy et al., 1983; Stormer et al., 1983; Syvertsen and Størmer et al., 1983). The 10-OH-OTA metabolite is only produced in rabbits (Stormer et al., 1983) and the OH-OTA is not produced by sheep (Hohler et al., 1999). Figure 4 shows some metabolic reactions and the OTA metabolites produced. The biotransformation of

OTA has not been elucidated in detail and the contribution of each metabolic enzyme is still unclear; few studies have been conducted on this aspect. For example, in recombined HIH/3T3 cells (P450/LacZ) the mutagenicity of OTA increases in the presence of a stable expression of P450 enzymes 1A1, 1A2, 2C10 or 3A4 (de Groene et al., 1995; de Groene et al., 1996).

To date, several OTA metabolites have been identified *in vitro* and *in vivo* as OP-OTA, OTA methyl and ethyl ester, OTA-GSH, OTHQ, OTHQ-GSH, OTHQ-NAC, etc. (Faucet-Marquis et al., 2006; Han et al., 2013; Li et al., 1998; Malaveille et al., 1991; Tozlovanu et al., 2012). Some had been previously described in *in situ* chemical reactions (Dai et al., 2002; Frenette et al., 2008; Xiao 1995; Xiao 1996). Other OTA metabolites have yet to be defined (Faucet-Marquis et al., 2006; Jennings-Gee et al., 2010).

With regard to the metabolism of recognised OTB and OTC, the OTB is biotransformed to OTβ and OH-OTB and subsequently excreted in urine (Mally et al., 2005). The affinity of metabolising enzymes for OTB is high, since the hydrolysis and the elimination rate of this metabolite is faster than for the transformation of OTA to OTα. This fact favours the OTB elimination and its low toxicity (Mally et al., 2005; Pitout, 1986). On the other hand, OTC (the ethyl ester of OTA), has been transformed *in vivo* into OTA by an esterase and chymotrypsin. The ratio of appearance of OTA (from OTC) in blood is as fast as the absorption of OTA from the gastrointestinal tract (Fuchs et al., 1984; Hult, 2006). These findings support the fact that OTC is just as toxic as OTA (Fuchs et al., 1984; Hult, 2006; Li et al., 1997; Xiao et al., 1996).

1.7.5 Action mechanism of OTA

OTA toxicity has been attributable to its isocoumarin moiety in the structure. Several mechanisms of OTA toxicity have been proposed; the first mechanism of toxicity described in literature was the inhibition of protein synthesis by competition with phenylalanine in the phenylalanine-tRNA aminoacylation reaction (Baudrimont et al., 1997; Creppy et al., 1984). A study of microarray analysis showed that genes encode proteins of cytoskeleton were downregulated and the protein content of plasma membrane was decreased (Hundhausen et al., 2008).

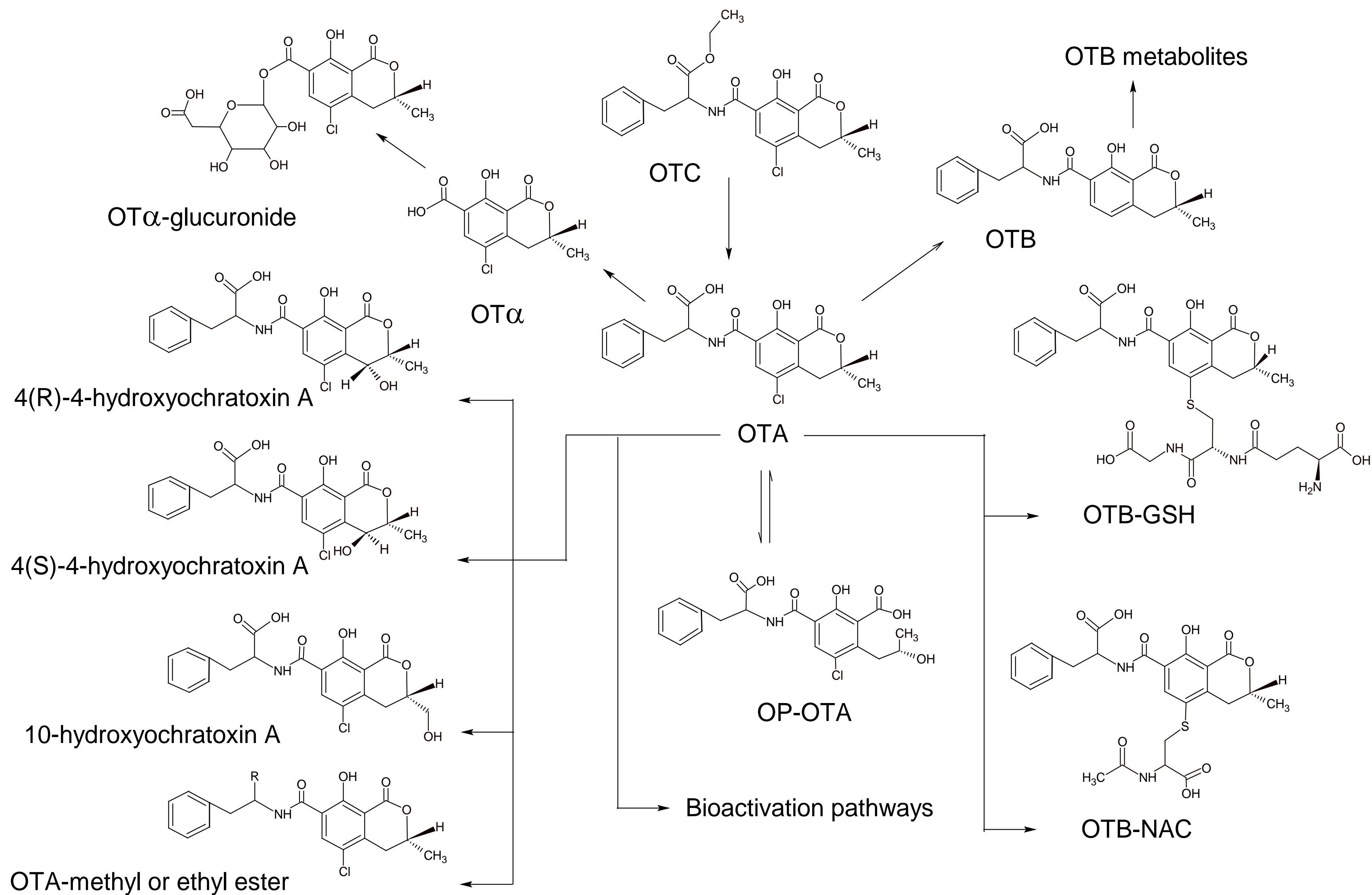


Figure 4. Proposed pathway of OTA metabolism in animals and humans.

Experimental evidence supports an important role for reactive oxygen species in the cancer development through modulation of cell signals such as the mitogen-activated protein (MAP) kinases. Protein kinases are involved in proliferation, differentiation, and apoptosis, and the intracellular calcium modulation (calcium homeostasis) (Klaunig and Kamendulis, 2004). In this regard, the results of *in vitro* studies showed that OTA has ability to inhibit the calcium (Ca^{2+}) uptake and cause a depletion of intracellular calcium stores means of an inhibitory effect on store-operated Ca^{2+} channels (SOCs) (Berndt et al., 1984; Hundhausen et al., 2008; Khan et al., 1989). The oxidative stress process was investigated on the flavoprotein NADPH-cytochrome P450 reductase, where OTA enhanced the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), results in initiation of a lipid peroxidation reaction (Omar et al., 1990). In contrast, Manderville and Pfohl-Leszkowicz (2006) reported a increase in the hydroxyl radicals level in presence of NADPH and OTA without Fe^{2+}.

OTA has also been able to alter genes involved in cell transport, immune response and inflammation, coagulation, antioxidative defence, DNA repair and mitotic regulation (Cavin et al., 2007; Gagliano et al., 2006; Luhe et al., 2003; Marin-Kuan et al., 2006). The overexpression of these mitotic regulators suggest that these may play a key role in tumour formation by OTA. Studies suggest that the metabolic activation of OTA plays an essential role in its genotoxic capacity. In case of DNA mutation assays, OTA did not cause mutation in *Salmonella typhimurium* using the Ames assay either with or without metabolic activation (Bendele et al., 1985; Kuczuk et al., 1978), but posteriorly Hennig et al. (1991) and Obrecht-Pflumio et al. (1999) identified mutations caused by OTA using the same Ames assay and bioactivated OTA metabolites by hepatic cells and kidney microsomal fraction.

The Sister Chromatid Exchange (SCE) assay (Anninou et al., 2014; Bendele et al. 1985; Föllmann et al., 1995; Lioi et al., 2004; Mosesso et al., 2008) and induction of micronucleus (MN) (Ali et al., 2011; Corcuera et al., 2015; Dönmez-Altuntaş et al., 2003; Ehrlich et al., 2002) have also been reported both positive and negative. In addition, the C8-guanine adduct of OTA (C8 dG-OTA) was detected in liver, kidney and testis of mice and pig following activation by cytochrome P450 enzymes and peroxidase enzymes (Faucet et al., 2004; Jennings-Gee et al., 2010; Mantle et al., 2010a). Results obtained

using the comet assay have been positive, which support the oxidative stress theory caused by OTA as an action mechanism (Cariddi et al., 2015; Klarić et al., 2010; Russo et al., 2005), (Ali et al., 2014; Arbillaga et al., 2007; Cariddi et al., 2015; Kamp et al., 2005; Lebrun and Follmann, 2002).

Controversial results of *in vitro* studies have been obtained and the studies have provided evidence that OTA toxicity is a plausible epigenetic mechanism, nerveless, direct genotoxicity *versus* epigenetic mechanism is a key question to classify to OTA as direct or indirect genotoxic (FAO/WHO, 2001, EFSA, 2010; IARC, 1993).

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2. Objectives and work plan

2.1 Objectives

This thesis focused on the determination of the bioaccessibility of ochratoxin A (OTA) in a food matrix and on the *in vitro* assessment of this toxin's cytotoxicity and genotoxicity on three lines of human cells.

With regard to bioaccessibility, we chose to use wine as the food in this study because previous research by our group (Belli et al., 2004; Coronel et al., 2009, 2012; Valero et al., 2008) had determined that it is one of the most OTA-contaminated matrices. To select the most suitable cytotoxic and genotoxic assays, we followed the recommendations of the OECD and the EFSA.

The objectives of this thesis fall within the objectives of the Spanish national research project "Integrated approach to simultaneous human exposure to ochratoxin A and deoxynivalenol" (project AGL2011-24862) and, more specifically, within its objective of "*in vitro* evaluation of the bioaccessibility of OTA in several food matrices, the effect of absorption and the cytotoxicity induced".

To meet this general objective, the following specific objectives were defined:

- To assess the bioaccessibility of OTA in a single food matrix (red wine) at different levels of contamination using an *in vitro* digestion model.
- To determine the *in vitro* cytotoxicity and genotoxicity of OTA in three human cell types. This objective, in turn, included the following sub-objectives:
    - To determine the bioaccessibility of OTA from red wine.
    - To investigate the *in vitro* cytotoxicity and genotoxicity of OTA in human cell cultures.
    - To determine the oxidative stress induced by OTA (and, complementarily, by DON) in human cell cultures.
    - To assess the modulation of the expression of some important genes in human cell cultures in the presence of OTA.

2.2 Work plan

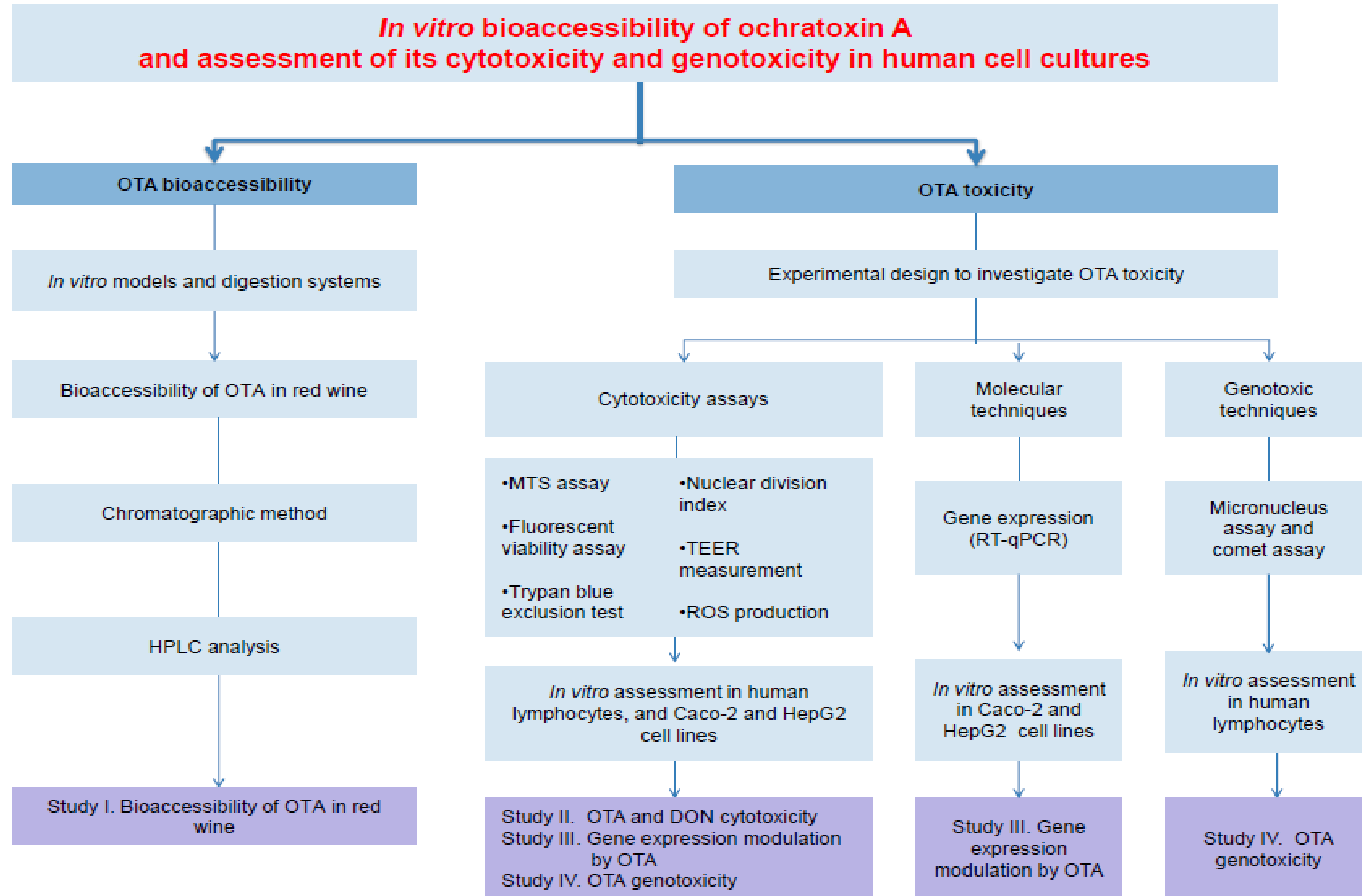

Figure 1. Work plan to perform the *in vitro* OTA bioaccessibility and toxicity studies.

3. Results

Study I.

Bioaccessibility of ochratoxin A from red wine in an *in vitro* dynamic gastrointestinal model.

González-Arias, C.A., Piquer-Garcia, I., Marin, S., Sanchis, V. and Ramos, A.J.*

Food Technology Department. Lleida University. UTPV-XaRTA, Agrotecnio Center. Av. Rovira Roure, 191. 25198 Lleida. Spain.

Abstract

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus* and *Penicillium* species with immunosuppressive, teratogenic, and carcinogenic properties. It has been determined that wine is the second largest source of OTA (10% of total OTA intake) in the European diet and that its presence, even in small doses, can be a problem in terms of long-term toxicity. In this paper, we evaluated the bioaccessibility of OTA in a spiked red wine sample under human fasting conditions using an *in vitro* dynamic digestion model that includes a continuous-flow dialysis system to simulate intestinal passage. To the best of our knowledge, this report is the first examining the bioaccessibility of OTA in wine. A liquid-liquid method was used to extract the OTA and ochratoxin alpha (OTα) from gastrointestinal juices, and the extracts were analysed by HPLC-FD. The bioaccessibility of OTA from the spiked red wine (1.0, 2.0 and 4 μg/L) was high in the gastric compartment (102.8%, 128.3% and 122.3%, respectively), whereas in the simulated intestine, it did not exceed the 26%, and the amount of OTA that crossed the dialysis membrane was very low (< 3.3%). The amount of OTα in gastric chyme ranged from 5.1 to 19.1% of the spiked OTA, whereas in the intestinal compartment it did not exceed 5%. In conclusion, in the *in vitro* system assayed, OTA exhibited a high bioaccessibility in the simulated stomach, but it decreased after the intestinal digestion and passage through the dialysis membrane.

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1.1 Introduction

Ochratoxin A (OTA) is a toxic secondary metabolite produced naturally by certain fungal species belonging to the *Aspergillus* and *Penicillium* genera. OTA is a potent nephrotoxin (IARC 2B group) that also exhibits immunosuppressive, teratogenic, and carcinogenic properties. OTA can be found in different raw materials (such as cereals, grains, and coffee beans) and foodstuffs, such as grape juice and wine (IARC, 1993). Several studies have reported that the OTA levels in wine can be reduced, but that OTA is not fully degraded or extracted during the main stages of wine making, during the fermentation process (Cecchini et al., 2006; Esti et al., 2012), wine ageing (Anli et al., 2011), or through physical treatments, e.g., wine clarification and decontamination with adsorbents (Castellari et al., 2001; Var et al., 2008).

Due to the adverse effects that OTA can have on health, the Commission Regulation 1881/2006 set a maximum level of 2 μg of OTA/L for wine and 10 mg of OTA/kg for dried vine fruit (currants, raisins, and sultanas) (EC, 2006a). Although OTA is usually detected in wine at levels below 1 μg/L, concentrations exceeding the legal limits are frequently found (EC, 2002). This is concerning because wine is the second most significant source of OTA (13-10% of the total intake) in the European diet (EC, 2002) and wines are the second most consumed alcoholic drink after beer (WHO, 2013).

It is known that OTA exposure occurs mostly by ingestion, however, a given intake of mycotoxin through food does not always reflect the amount of toxin that is able to cross the intestinal membrane. Some *in vitro* models have been developed to determine the amount of contaminant released (bioaccessible fraction) from food during its gastrointestinal passage. Release of the toxin from food in the gastrointestinal tract is a prerequisite for uptake (and bioavailability) in the body. The bioaccessible fraction is defined as the amount of free contaminant that may become available for absorption because it has been released from the matrix and solubilised in the gastrointestinal fluid (Versantvoort et al., 2004). Ochratoxin alpha (OTα) is a derivative of OTA formed in the gastrointestinal tract by the action of carboxypeptidase A and chymotrypsin (Kumagai and Aibara, 1982).

To date, no studies on the bioaccessibility of OTA have been performed in wine, a beverage consumed regularly by adults. The aim of this study was to estimate the bioaccessibility of OTA in spiked red wine through a dynamic *in vitro* gastrointestinal model simulating the digestion process, as well as the formation of OTα in the gastrointestinal juice.

1.2 Materials and Methods

1.2.1 Chemicals, reagents, and equipment

Ochratoxin A (OTA) (CAS: 303-47-9) (purity 98%) was supplied by Sigma-Aldrich (St. Louis, MO, USA), while ochratoxin alpha (OTα) (CAS: 16281-39-3) was from Romer Labs Diagnostic (Tulln, Austria). All salts used to prepare the gastrointestinal solutions and the hydrochloric acid (HCl, 37%) were supplied by Panreac, Chemical S.A. (Barcelona, Spain) or Fisher Bioreagents (Fair Lawn, NJ). Orthophosphoric acid and sodium sulphide were from Scharlau Chemical S.A. (Barcelona, Spain). Organic solvents and HPLC grade reagents were from Fisher Scientific (Leicestershire, UK). The simulated digestive system was designed with an adapted Liebig-West condenser, endfitting fluid connectors, silicone tubing (12 mm diameter) and dialysis tubing cellulose membrane from Sigma-Aldrich (D9277) with a molecular mass cut-off of 12400 Da and 32.8 mL/m of capacity (10 mm of flat width x 6 mm of diameter). A D-21FT peristaltic pump from Dinko Instruments (Barcelona, Spain) and a thermostat bath Tectron-bio-100 from J.P. Selecta, S.A. (Barcelona. Spain) were also used.

1.2.2 Wine

In this study, we used a commercial Spanish red wine made from a Tempranillo grape variety harvested in 2011 from the Castilla-La Mancha region. The initial OTA and OTα contamination in this wine was evaluated using the method described by Muñoz *et al.* (2009). In-house validation of the method was performed at three OTA and OTα contamination levels (1.0, 2.0, and 4.0 µg/L) to determine the recovery of the toxins from the matrix. Recoveries ranged from 92.4 to 100.0% (repeatability Relative Standard Deviation, RSDr: 1.30-11.54%) for OTA and 63.7-66.9% (RSDr: 15.9-20.1%) for OTα. The amount of OTA found in this wine, considering the mean recovery of the method, was

0.103 μg/L, whereas, as expected, no OTα was detected. This amount of OTA was taken into account for bioaccessibility calculations.

1.2.3 In vitro digestion procedure

The digestion model used was a slightly modified version of the technique developed by Gil- Izquierdo *et al.* (2002), including a continuous-flow dialysis system to simulate intestinal passage. The model describes a three-step procedure mimicking the digestive process in the mouth, stomach (gastric digestion), and small intestine (intestinal digestion). The constituents and concentrations of the simulated saliva, gastric juice, duodenal juice and bile juice employed were those described in Versantvoort *et al.* (2005). Digestion assays were carried out with 5 mL of red wine (spiked at 1.0, 2.0, or 4.0 μg OTA/L, with 5, 10 and 20 ng OTA). The digestion began with the addition of 6 mL of salivary juice to 5 mL of the assayed red wine, adjustment of the pH to 6.8 using 10% $NaHCO_3$ and incubation at 37 °C for 5 min. at constant orbital movement. After this treatment, gastric or gastric+intestinal digestions were developed. The gastric and intestinal mixtures were incubated for 2 h. The intestinal mixture was introduced into the dialysis tube in a continuous-flow dialysis system simulating the intestine.

The dialysis system was designed with an adapted Liebig-West condenser and end-fitting fluid connectors. The first chamber contained the dialysis tube, through which the intestinal mixture flowed during the intestinal digestion step using a peristaltic pump with a flow rate of 1 mL/min, and a phosphate buffer solution (PBS, pH 7.4) that bathed the dialysis tube. A temperate water solution was pumped from a bath through a water jacket to keep the system's temperature constant at 37 °C (Figure 1). Once the gastric and gastric+intestinal digestions were completed, the fluids (gastric, intestinal, and the dialysate) were collected and analysed. The fluids were centrifuged for 10 minutes at 100 rpm and 10 ºC, yielding a supernatant that was analysed for OTA and OTα content. Overall, for each spike level, six parallel experiments were carried out, from which three were interrupted for analysis after the gastric phase, and the remaining three underwent the intestinal phase before analysis.

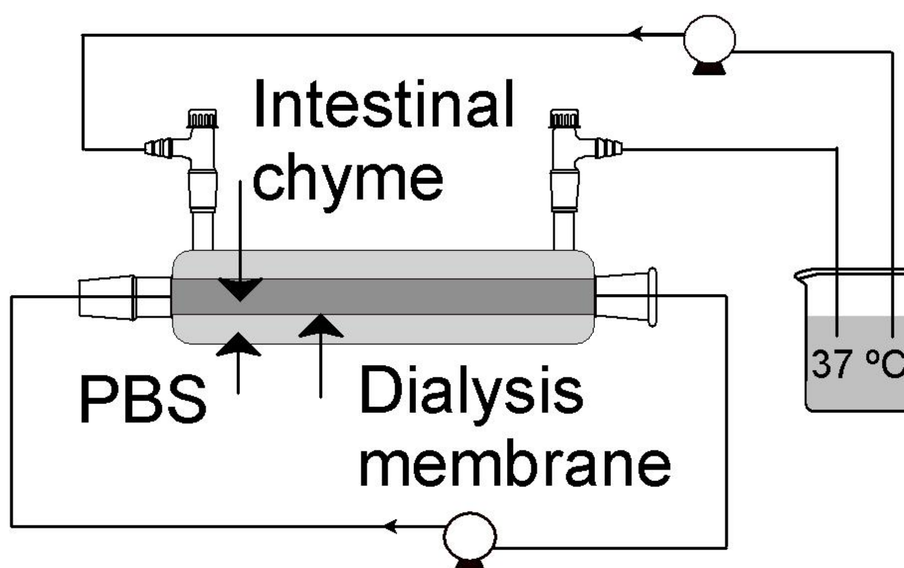

Figure 1. Schematic diagram of the simulated in vitro intestinal digestion system. PBS = phosphate buffered saline.

1.2.4 Sample analysis

1.2.4.1 Liquid-liquid extraction of OTA and OTα from the simulated physiological fluids

The extraction of OTA and OT*α*, was performed using the method described by Muñoz *et al.* (2009), with slight modifications. The gastric chyme was diluted 1:1 with $NaHCO_3$ (1%), and the pH adjusted to 3 with 6 M H_3PO_4; the intestinal chyme and dialysis samples were diluted 1:4 in H_3PO_4/NaCl (0.5 M:2 M) and the pH was adjusted to 1.6 using 10% $NaHCO_3$ (Zimmerli and Dick, 1995). Liquid-liquid OTA and OTα extractions were carried out by adding 5 mL of chloroform/isopropanol (97:3) and shaking using a magnetic stirrer for 20 min, at which time the mixture was centrifuged for 10 minutes at 1000 rpm. The organic phase was recovered and dried under a gentle stream of nitrogen at 40 °C. Samples were stored at 4 °C until HPLC analysis. To determine if the physiological fluids caused chromatographic interference, two digestions free from wine were performed following the previously described digestion procedure. After the incubation period for each digestion phase, the obtained juices were directly subjected to liquid-liquid extraction as previously described or were spiked with OTA and OTα (at 1.0, 2.0, and 3.0 μg/L) and then extracted, and the toxin was immediately analysed (Figure 2).

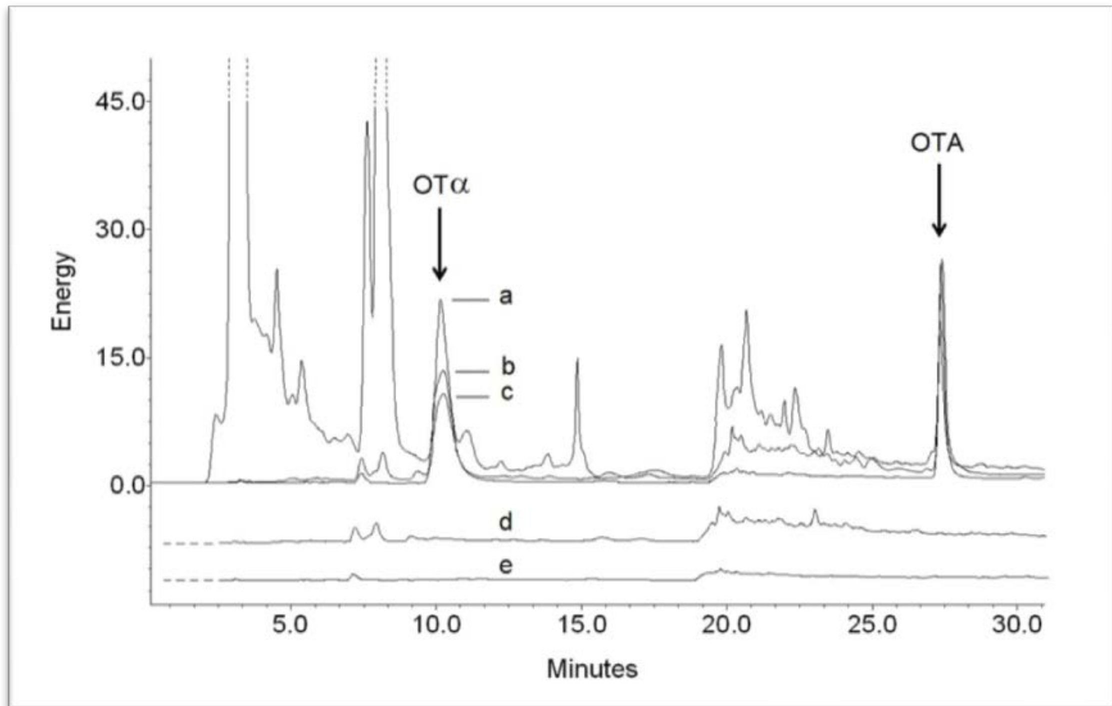

Figure 2. HPLC chromatogram of ochratoxin α (OTα) and ochratoxin A (OTA) in (a) spiked red wine, (b) spiked intestinal juice, (c) spiked gastric juice, (d) non-spiked gastric juice and (e) non-spiked intestinal juice.

1.2.4.2 High Performance Liquid Chromatography (HPLC) analysis

The HPLC analysis of OTA and OTα was performed using a Waters 2695 Separations Module coupled to a Waters 2475 Multi λ fluorescence detector. The integration software used to manage the chromatographic data was Empower 2 (2006 Waters Corporation, Database Version 6.10.00.00). Retention times were 10 min (OTα) and 27 min (OTA). Figure 2 shows the chromatograms of the extracted physiological fluids (spiked or unspiked with OTA and OTα) and a sample of the spiked wine post-digestion. The method performance was evaluated in terms of linearity, limit of detection, limit of quantification, inter-day repeatability, and recovery rates.

1.2.5 Statistical analysis

Validation to estimate the inter-day repeatability was performed with runs in triplicate. Results are shown as the means ± standard deviations. Analysis of variance (ANOVA) was used to test if there were significant differences ($p < 0.05$) in the calculated bioccessibility and for the values of mycotoxin that crossed the dialysis membrane at the various tested OTA concentrations.

1.3 Results and Discussion

1.3.1 Method validation for OTA and OTα **analysis**

The detection (LOD) and the quantification (LOQ) limits for the two toxins were experimentally determined from the calibration curve of a set of two-fold serial dilutions of the toxin standards (from 100 to 0.006 ng/mL), which was linear in the range of 0.012 to 100 ng/mL (r^2=0.995). The LOD and LOQ determined for OTA were 0.012 and 0.036 ng/mL, whereas for OTα these were 0.024 and 0.072 ng/mL, respectively. The method was repeatable for the two toxins at the three assayed spike levels, with an RSDr below 5% in most cases. OTA and OTα recoveries from the dialysis fraction were similar and usually greater than 80%. In the gastric and intestinal fractions, recoveries were lower for OTα (62-78%), although the RSDr percentages were similar to those obtained with OTA (Table 1). OTA recoveries fit the performance criteria established in the EU Regulation 401/2006 (EC, 2006b) for its official control in foodstuffs. Although there are no established performance criteria for OTα, all of the recovery percentages were homogeneous at the three spike levels. The bioaccessibility results and the amount of mycotoxin that crossed the dialysis membrane were corrected using the recovery rates.

Table 1. Recovery rates and inter-day repeatability for ochratoxin alpha (OTα) and ochratoxin A (OTA) in the digestion juices.

Digestion step	Spike level (ng/mL)	OTα		OTA	
		Recovery* (%)[1]	RSDr (%)[2]	Recovery* (%)	RSDr (%)
Gastric	1.0	62.40	2.04	89.19	2.21
	2.0	65.58	5.99	84.57	3.83
	3.0	62.76	4.17	86.12	1.56
Intestinal	1.0	78.45	3.98	78.28	11.1
	2.0	72.20	2.94	75.57	2.88
	3.0	77.11	4.74	87.58	3.32
Dialysis	1.0	83.76	2.32	85.91	3.41
	2.0	83.65	0.61	79.44	1.30
	3.0	89.55	0.88	85.07	2.56

[1]Mean of three measurements. [2]RSDr = relative standard deviation, calculated from results generated under repeatability conditions.

1.3.2 Bioaccessibility of OTA in wine

The extractable compounds from the physiological fluids did not interfere with the chromatographic analysis of OTA (Figure 2). In our study, OTA levels detected in the gastric chyme were quite high, representing a bioaccessibility of 102.81, 128.26, and 122.25% in each of the tested contamination levels (Table 2). No significant differences were determined for the three bioaccessibility values ($p > 0.05$). Our results demonstrate that, in the tested OTA concentration range, the food matrix did not affect the bioaccessibility of OTA in the gastric chyme.

Table 2. Bioaccessibility of ochratoxin A (OTA) from wine in an *in vitro* human digestion system.

OTA spiked amount (ng OTA)	Fraction	Average (ng OTA)	Bioaccessibility (% ± SD)
5	Gastric	5.14	102.81 ± 11.26
	Intestinal	1.215	24.24 ± 11.98
	Dialysis	<LOQ[1]	
10	Gastric	12.83	128.26 ± 18.01
	Intestinal	2.60	25.99 ± 9.00
	Dialysis	<LOQ	
20	Gastric	24.45	122.25 ± 15.03
	Intestinal	4.27	20.80 ± 1.38
	Dialysis	0.22	

[1]LOQ = limit of quantification: 0.036 ng/mL.

High bioaccessibility levels (86 to 116%) were determined by Versantvoort *et al.* (2005) in buckwheat, peanuts, and mixtures of these cereals (4, 11, 51 ng OTA in the digestion model). Avantaggiato *et al.* (2007) also described a high bioaccessibility of OTA (88%) from the stomach to the small intestine in a corn food at one contamination level (11 µg OTA/kg). Our results and the results of studies mentioned above are consistent with the *in vivo* data reported by Galtier (1978) and Roth *et al.* (1988). The authors attributed this behaviour to the acidic properties of OTA (Galtier, 1978; Kumagai and Aibara, 1982). However, Kabak *et al.* (2009) reported much lower bioaccessibilities; only 22% was found for buckwheat (24.9 µg OTA/kg) and 29-32% for infant food (2-13.1 µg OTA/kg), in spiked food samples. In our study, OTA levels decreased during intestinal digestion, with bioaccessibilities ranging from 21 to 26%, while less than 3.3% was detected in the intestinal dialysate (Table 2). Glahn *et al.* (1996) and Jovaní *et al.* (2001) also observed this behaviour, where the compounds that form complexes with proteins, or with mixed bile salt micelles, such as OTA, are likely to be less bioaccessible.

Results of *in vivo* experiments indicated that OTA could have highly variable bioavailability values. For example, the highest bioavailability was reported in mouse (92%), followed by pigs (65.7%), rats (61%), monkeys (57%), rabbits (55.6%), chickens (40%) and fish (1.6%) (Galtier and Alvinerie, 1981; Hagelberg et al., 1989). Unlike that of the *in vitro* systems, it has to be noted that the *in vivo* absorption in the proximal jejunum depends on gastric emptying, mechanical movements, can take place against a concentration gradient and depends on the pH at the mucosal surface of the jejunum (Kumagai and Aibara, 1982; Kumagai, 1988).

In our case, the bioaccessibility did not depend on initial mycotoxin levels ($p > 0.05$), either at the gastric or the intestinal phase. However, as with other mycotoxins, differences in bioaccessibility could also be related to the way the matrix (food or feed) is contaminated (spiked or naturally), and the bioaccessibility model employed for studying the release of mycotoxins from the matrix. Two *in vitro* models to study mycotoxin bioaccessibility have been primarily used by different authors: 1) the RIVM model (Versantvoort et al., 2004) and 2) the Gil-Izquierdo model (Gil-Izquierdo et al., 2002). A third model, the TIM-1 model, has been mainly employed in bioaccessibility assays of mycotoxins from cereals or food (Avantaggiato et al., 2003, 2004, 2007; Zeijdner et al., 2003) in porcine gastrointestinal tract conditions. An in-depth review of the conditions and digestion models is presented in our previous study on mycotoxin bioaccessibility (González-Arias et al., 2013).

Regarding formation of the OTα metabolite, in general, low amounts were detected in our *in vitro* digestion system. In the gastric chyme, OTα was detected in a range representing 5.1-19.1% of the initial OTA. In the small intestine juice, 5.1% of the metabolite was detected, but only in the digestion of the wine spiked at 4 µg OTA/mL. In the dialysis fraction, OTα was detected but not quantified because the signal was below the LOQ. Our results are in agreement with Madhyasta *et al.* (1992); the authors used an *in vitro* system based on the incubation of OTA with rat digesta, and found that most OTA hydrolysis to OTα occurs in the presence of digesta from the large intestine or caecum, but little hydrolysis was observed in the stomach contents and digesta from the small intestine, despite the fact that the pancreatic enzymes, including carboxypeptidase A, are released into the small intestine. Additionally, the biotransformation of OTA into OTα is most likely

higher for *in vivo* or *in vitro* systems involving intestinal cellular systems in comparison to our *in vitro* system, where scarcely any biotransformation was observed. In our results, as in the case of other studies, several additional factors were not taken into account, e.g., the interaction of the toxin with the intestinal microbiota in the large intestine (mainly anaerobic bacteria) (Madhyastha et al., 1992), the effect of the toxin's metabolism during the transport through the intestinal barrier and liver degradation of the oral bioavailable OTA *in vivo* (Berger et al., 2003; Schrickx et al., 2006).

1.4 Conclusions

The present study demonstrates that OTA is primarily released from the wine matrix during gastric digestion (providing high bioaccessibility at this point). However, in the small intestine the *in vivo* bioavailability of OTA may decrease due to a low bioaccessibility and, presumably, a low bioavailability. *In vitro* models are only approximations of what may occur *in vivo*, and the few studies carried out to determine the bioaccessibility of OTA in different food matrices that were naturally or artificially contaminated have yielded inconsistent results. Most *in vitro* bioaccessibility assays for mycotoxins have not been carried out for more than two hours of gastric digestion, and few matrices have been used under fasting or fed conditions. Further studies should be conducted to determine the effect that the OTA contamination level, type of contamination (natural *vs.* spiked), and food matrices exert on bioaccessibility. Additional data are required to consider these results representative of what happens under *in vivo* conditions.

Acknowledgments

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Study II.

Cytotoxicity of the mycotoxins deoxynivalenol and ochratoxin A in presence of resveratrol.

Cano-Sancho G.[1], González-Arias C.A.[1], Ramos A.J.[1], Sanchis V.[1], Fernández-Cruz M.L.[2*]

[1]Applied Mycology Unit, Food Technology Department, XaRTA-UTPV, Agrotecnio Center, University of Lleida. Av. Rovira Roure, 191. 25198 Lleida, Spain.

[2]National Institute for Agricultural and Food Research and Technology (INIA), Department of Environment, Carretera de la Coruña Km 7.5, 28040 Madrid, Spain.

Abstract

Exposure to mycotoxins through dietary food intake involves a highly complex scenario where co-occurrence of different mycotoxins has been frequently demonstrated. On the other hand, the effect of the interaction of mycotoxins with other generally considered beneficial food components, as the antioxidants, has been scarcely studied. The main goal of the present work was to assess the cytotoxic effects on Caco-2 cells of the mycotoxins deoxynivalenol (DON) and ochratoxin A (OTA), alone or combined, and to explore potential protective effects of resveratol (RES), an antioxidant frequently found in wine. In parallel, reactive oxygen species (ROS) production has also been studied as a first approach to understand the underlying mechanism of cytotoxicity. Results indicate a higher toxic effect of the mycotoxins when they are co-exposed. This increase in cytotoxicity was not accompanied by an increase in ROS production. The co-exposure of OTA or DON with RES did not result in a decrease in cytotoxicity; on the contrary, it resulted in increased cytotoxicity not associated with an increase in ROS production.

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2.1 Introduction

Ochratoxin A (OTA) (Fig. 1a) is a fungal secondary metabolite produced by some species of the genera *Aspergillus* and *Penicillium*. Sources of human exposure to OTA are mainly foodstuffs of vegetal origin, such as cereals and derivatives, grapes, musts and wines, coffee, beer, nuts and dried fruits, spices and, to a minor extent, animal by-products. OTA is a potent kidney toxin and has been classified by the IARC as a 2B cancer compound (possibly carcinogenic to humans) (IARC, 1993). It is among the strongest carcinogenic compounds in rats and mice, and its toxicological profile includes teratogenesis, nephrotoxicity and immunotoxicity (Fernández-Cruz et al., 2010). Reported *in vitro* toxic effects of OTA include inhibition of cellular proliferation, apoptosis and impairment of barrier function and, increasing membrane permeability (McLaughlin et al., 2004). OTA has been found to induce oxidative damage *in vitro* (Schaaf et al., 2002; Kamp et al., 2005; Mally et al., 2005) and *in vivo* (Petrik et al., 2003; Hsuuw et al., 2013) and to be genotoxic (Lebrun and Föllmann, 2002; González-Arias et al., 2014).

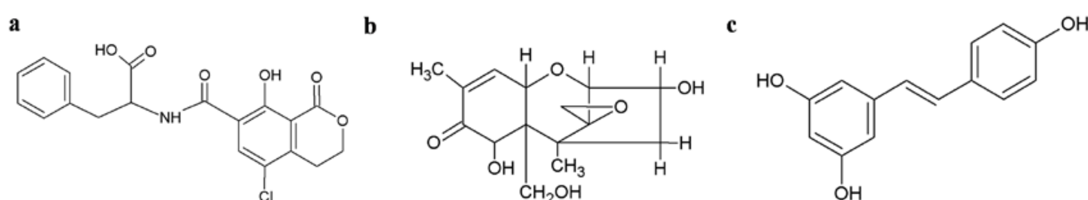

Figure 1: Molecular structure of (a) ochratoxin A, (b) deoxynivalenol and (c) resveratrol

Deoxynivalenol (DON) (Fig. 1b), is one of the most common fungal contaminants of cereals worldwide. This mycotoxin is produced by different species of *Fusarium*, thus a wide range of cereal-based foods have been reported to be contaminated by this toxin (JECFA, 2001). Acute effects of food poisoning by DON in humans are abdominal pains, dizziness, headache, throat irritation, nausea, vomiting, diarrhoea, and blood in the stool. It has also been shown that DON can act severely against the intestinal cells causing inflammation and increasing the permeability of the monolayers, resulting in a reduction of viability and immune function (Pinton et al., 2009; Van de Walle et al., 2010). DON also

increases cytokine levels, a fact that is closely related with immune function reduction and increases caspase-3 levels, a well-known apoptosis mediator (Pestka et al., 2004; Pestka, 2008). Additionally, DON induced oxidative stress has been proposed as the mechanism of DNA damage in hepatic cells HepG2 (Pestka et al., 2004).

Red wine is one of the most important beverages in the Mediterranean diet, and it is considered to have a more protective effect than white wine due to its greater content in antioxidant substances released from the grape's skin and seeds, mainly polyphenols. Red wine contains a total of 1.8 g/L of polyphenols, whereas a white wine contains only 0.2-0.3 g/L of polyphenols (Bertelli and Das, 2009). Resveratrol (RES; Fig. 1c), a polyphenolic product synthesized by a wide variety of plant fruit, including grapes, is naturally present in red wine. RES has gained considerable attention because of its potential as a chemopreventive and its anticancer properties, as well as for the evidence of decreasing heart disease and neural degeneration in animal studies (Vang et al., 2011). Among its various biological actions, RES was demonstrated to inhibit cellular survival signaling, and to interfere with apoptosis pathways, both by directly triggering apoptosis-promoting signaling cascades and by blocking antiapoptotic mechanisms (Fulda and Debatin, 2006).

Human dietary intake of food and its relationships with the digestive tract make up a very complex system in the framework of exposure to food contaminants. Most toxicity studies commonly evaluate the effect of individual mycotoxins on the target cell biological functions. However human exposure to mycotoxins is well-known to be far off this simplified paradigm, involving sophisticated interactions with a lot of different matrices and different chemical species, including other mycotoxins, but also major nutrients, fibres and natural bioactive compounds. Co-contamination of different mycotoxins in food and feed has been frequently demonstrated, being a more common event than single contamination (Streit et al., 2013). Most of the published studies are reporting the toxicological properties of mycotoxins alone, and little is known concerning the effect of a specific mycotoxin mixed with other mycotoxins and/or with bioactive compounds, which may lead to unexpected subsequent effects. DON and OTA have shown to be among the most ubiquitous mycotoxins in foods of Mediterranean countries, and thus, continuously consumed in moderate levels through dietary intake. Moreover, the mycotoxin mixtures can interact with complex food matrices containing bioactive food compounds, as RES.

Polyphenols such as RES are also widely present in the diet with the main dietary sources being fruits, wine, tea, coffee or chocolate (Burns et al., 2002; Fernández-Mar et al., 2012). These compounds are known for their beneficial effects on human health due to their antioxidants properties. Thus, the main goal of the present work was to assess the effect of DON and OTA on the viability of Caco-2 cells when these mycotoxins are combined with RES. Additionally, to try to understand the underlying mechanism of cytotoxicity, production of reactive oxygen species (ROS), as a first indicator of oxidative stress status, has been measured.

2.2 Materials and methods

2.2.1 Chemicals

DON (3α, 7α, 15-trihydroxy-12,13-epoxytrichotech-9-en-8-one, purity ≥ 98 %), OTA ((2S)-2-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-2-benzopyran-7-yl]formamido}-3-phenylpropanoic acid, purity ≥ 98%), RES (3,40,5-trihydroxystilbene, purity ≥ 99%), trypsine, dimethylsulfoxide (DMSO), L-glutamine (200 mM), penicillin-streptomycin (10.000 UI/mL-10.000 µg/mL), Minimum Essential Medium (MEM, ref 56416C, dry powder), MEM non-essential amino acids 100x (NEAA), Hepes (Bio-performance grade), chloramine-T and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (Sigma, Madrid, Spain). Dulbecco's Modified Eagle Medium (DMEM, ref. SH30022) was supplied by Thermo Scientific HyClone (Barcelona, Spain) and fetal bovine serum (FBS) by Biosera (Santa Coloma de Gramenet, Spain). CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) kit was purchased from Promega (Charbonnières, France). Solid phase extraction cartridge BondElut C18 was purchased from Varian (California, USA).

2.2.2 Cell culture and treatment

The Caco-2 cell line (ATCC HTB-37) derived from a human colorectal adenocarcinoma was purchased from LGC Standards, S.a.r.l. (Molsheim Cedex, France). Caco-2 cells were cultured at 37 °C and 5 % CO_2 in DMEM medium supplemented with penicillin (100 UI/mL), streptomycin (100 µg/mL), 15 % fetal bovine serum, 2 mM L-glutamine, and 1% NEAA. DON, OTA and RES were dissolved in DMSO and stored at -20 °C before dilution in

cell culture media. Solvent control cells received the maximal DMSO concentrations used in the treated cells (0.1%, v/v). Caco-2 cells were seeded at a concentration of 10^5 cells/well in 100 µL of culture medium in flat-bottomed 96-well plates. After culturing for 48 h, different concentrations of OTA, DON and RES or their combinations (OTA-RES, DON-RES, OTA-DON) were added to the cells. Cell lines were exposed for 6, 24 and/or 48 h, depending on the assay.

2.2.3 Stability of resveratrol in the culture medium

Yang et al. (2010) reported the oxidation of resveratrol in culture medium due to bicarbonate ions. We checked the stability of resveratrol (160 µM) in two culture media, MEM (medium without bicarbonate) and DMEM (a medium that contains 3.7 g/L of bicarbonate). Hepes (25 mM) was added to MEM to maintain the pH in a range of 7.2-7.5. Stability assays were carried out with 3 mL of culture medium (spiked at 160 µM RES). Samples were incubated at 37 °C for 12, 24 and 48 h in the same conditions described for cell culture. Three culture medium samples were incubated for each time. Samples were collected at the end of the incubation period, and RES was extracted immediately.

2.2.4 Solid-phase extraction of resveratrol and high performance liquid chromatography analysis (HPLC)

RES from culture medium samples was extracted by solid-phase extraction using a Bond Elut C18 cartridge and ethanol as eluting solvent. Samples were dried under a nitrogen stream and stored at 4 °C until analysis. RES was quantified by HPLC, using a Waters 2695 Separations Module coupled to a Waters 2475 Multi λ fluorescence detector (Waters, Milford, MA, USA). HPLC conditions were a modification of those described by Serra et al. (2009). Mobile phase was: A) acetic acid 0.2% and B) acetonitrile, according to the following gradient: 0-10 min 95% A, 10-20 min 75% A, 20-25 min 95% A, with a flow-rate of 1 mL/min. A Waters Spherisorb ODS2 C18 column (3 µm, 4.6 x 250 mm) was used and column temperature was maintained at 30 °C during analysis. Detection was performed at 280 nm, and retention time was 14.66 min. Quantification was achieved with a software integrator (Empower 2, Milford, MA, USA). RES detection (LOD) and quantification (LOQ) limits were experimentally determined from the calibration curve of a set of seven

standards (from 1 to 320 μM RES), which was linear in the range of 10-320 μM RES (r^2 = 0.989). The LOD and LOQ determined was 10 and 25 μM, respectively.

2.2.5 MTS assay

The effects on the mitochondrial activity of Caco-2 cells exposed to OTA and RES at concentrations ranging from 0.5 to 160 μM and DON (0.25-30 μM), and the combinations of OTA (20, 40 and 80 μM) or DON (0.5, 1 and 5 μM) with RES (1-100 μM), and OTA (20, 40 and 80 μM) with DON (0.5, 1 and 5 μM), after a 24 and 48 h exposure period, were studied using the MTS assay kit. The kit is composed of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) and phenazinemethosulfate (PMS), an electron coupling reagent. MTS is reduced by dehydrogenase enzymes in metabolically active cells into a formazan product that is soluble in tissue culture medium. After the 24 h or 48 h exposure period, the cells were washed with PBS, and 20 μL of a freshly prepared MTS/PMS solution in fresh medium was added to the wells. The cells were further incubated for 2-4 h. The amount of soluble formazan produced by cellular reduction of the MTS was measured by the absorbance at 490 nm on an ELISA plate reader (Genios, TECAN, Männedorf, Switzerland). Results were expressed as percentage of the control.

2.2.6 Reactive oxygen species (ROS) assay

To study the ROS production, cells were exposed for 6, 24 and 48 h to OTA and RES at concentrations ranging from 0.5 to 160 μM, and DON at 0.25-30 μM. For the combination effects study, concentrations of OTA (5 and 80 μM) or DON (1 and 30 μM) with RES (10, 40 and 160 μM), and OTA (5 and 80 μM) with DON (1, 10 and 30 μM) were tested. Intracellular ROS production was determined by using the dichlorofluorescein (DCF) assay (Wang and Joseph, 1999). DCFH-DA (100 mM) in phenol red and serum-free MEM medium was added after the incubation period and maintained during 30 min at 37 °C in the dark. Chloramine-T, an effective inducer of oxidative stress, was used as a positive control, with cells being exposed to concentrations in the range of 0.3-10 mM. Fluorescence was measured at 485 nm excitation and 535 nm emission at 37 °C, using a microplate reader. Fluorescence readings were taken every 15 min for 60 min, with the

plates being incubated at 37 °C with 5 % CO_2 between measurements. Oxidative stress was calculated as the percentage of increase in fluorescence per well over a 60-min period by the formula [(Ft60-Ft0)/Ft0 x 100]. This result was expressed as percentage of the solvent control.

2.2.7 Statistical analysis

The sodium bicarbonate effect on stability of RES in culture medium was determined by the analysis of variance ANOVA and the Bonferroni post-test. Cytotoxicity data are represented as mean ± standard deviation (SD) of three to five independent experiments (in each experiment, each concentration was applied by triplicate in the culture plates). Statistical analysis was performed using Sigma Plot version 12.0 (Jandel Scientific, San Rafael, CA, USA). Significant differences among treatments were determined by one-way repeated measures analysis of variance (RMANOVA, $p < 0.05$). Previously, normality (Shapiro-Wilk test, $p < 0.05$) and equal variance ($p < 0.05$) of the distribution was tested. Means of treatments were contrasted with respect to the control group using Dunnett's test.

2.3 Results

2.3.1 Stability of resveratrol in culture media.

Table 1 reports the decrease of RES (160 µM) over time (12-48 h) on a culture medium with or without bicarbonate. Results showed that RES concentration decreased with time in both culture media used, regardless of the presence of sodium bicarbonate in its composition. The decrease in RES level was moderate (10.5% loss) in samples incubated for 12 h in culture media with sodium bicarbonate ($p < 0.05$), and were increasing with time (19 to 41% after 24 and 48 h, respectively). Our results also show a statistical difference in RES levels due to the presence or absence of bicarbonate in the media .RES showed a moderate decrease after 24 h incubation in DMEM (19%), lower than in MEM (32.3%). However, at 48 h a minor decrease was observed in MEM (30.6%) than in DMEM (41.4%).

Table 1. Decrease of RES (160 μM) in cell culture medium with or without sodium bicarbonate.

Incubation time (h)	Resveratrol stability			
	Medium with bicarbonate (DMEM)		Medium without bicarbonate (MEM)	
	RES (μM)	(% RES loss)	RES (μM)	(% RES loss)
12	143.18 ± 7.05*	10.51	n.d.	n.d.
24	129.70 ± 5.29**, A	18.94	108.33 ± 9.22**, B	32.29
48	93.84 ± 7.53**, A	41.35	111.09 ± 3.15 **, B	30.57

Data are represented as mean ± SD of three analyses. n.d.: not determined. Stars indicate statistical differences regarding to RES control in DMEM medium (0 h) (*$p < 0.05$, **$p < 0.0005$). Different capital letters next to DMEM/MEM treatments, at the same time, mean significant differences ($p < 0.005$) due to bicarbonate.

2.3.2 MTS assay

The effect of OTA, DON and RES treatment on cell viability was tested by the MTS assay over 24 h and 48 h. Similar responses were observed between both times of exposure and only results after 48 h exposure are presented in Figure 2. A dose-response curve was obtained after OTA and DON treatment. A significant reduction in cell viability was observed with OTA at 40 μM ($p < 0.01$) (Fig. 2a) and DON 1 μM ($p < 0.001$) (Fig. 2b). The non observed effect concentration (NOEC) for OTA and DON were 20 and 0.5 μM, respectively. However, Caco-2 cells did not show any response after treatment with RES in the range 0-160 μM (Fig. 2c).

The 48 h co-exposure of DON at non-toxic concentrations (0.5 μM) with increasing concentrations (1-100 μM) of RES induced an increase in cytotoxicity at a concentration of RES of 100 μM ($p < 0.001$) (Fig. 3a). However the cytotoxic effect at concentrations of DON of 1 and 5 μM was not significantly affected by the presence of RES. On the other hand, the cytotoxicity of OTA at 80 μM increased significantly ($p < 0.05$) when co-exposed with 100 μM of RES (Fig. 3b). An increase in toxicity could also be observed when cells

were co-exposed to a non toxic concentration of OTA (20 µM) and non toxic or low concentrations (0.5 µM and 1 µM) of DON (Fig. 3c).

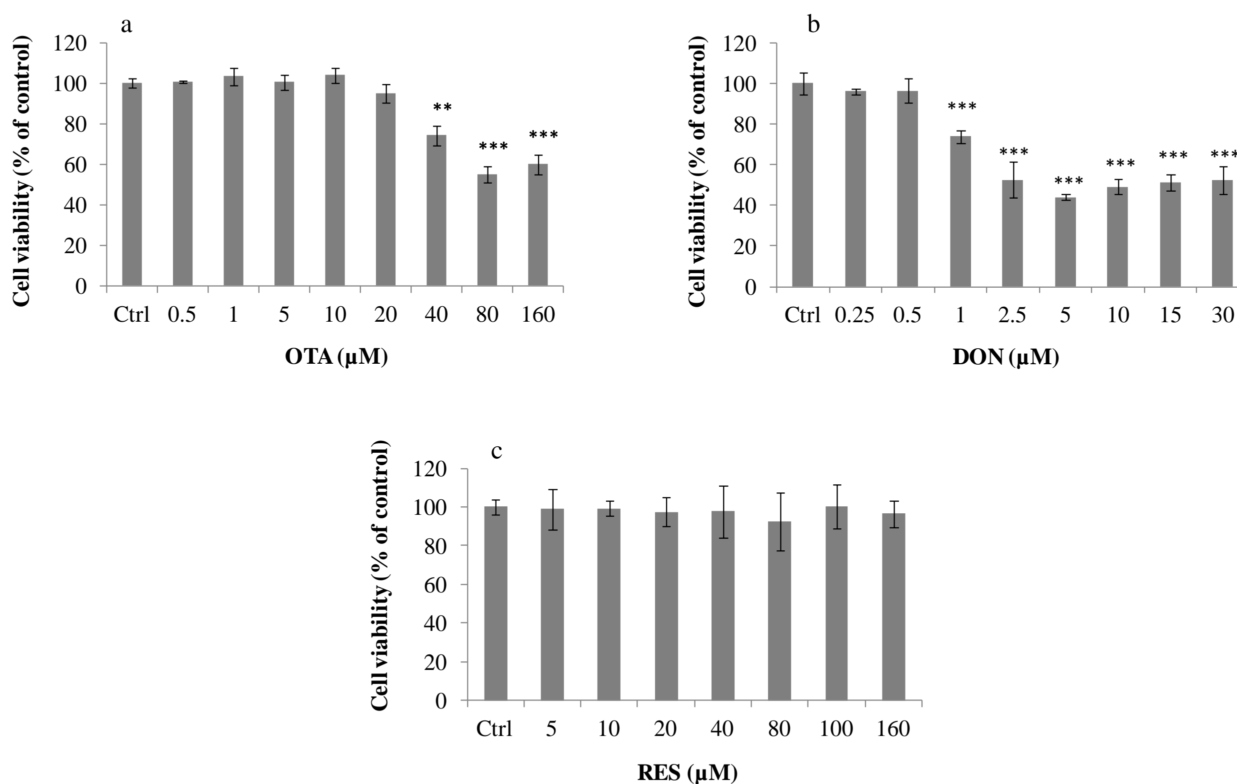

Figure 2: Effect of OTA (a), DON (b) and RES (c) on Caco-2 cell viability after 48 h exposure measured with the MTS assay. Results are expressed as percentage of viability compared with the solvent control (0.1% DMSO). Data are represented as mean ± SD of 3-5 independent experiments. Statistical differences with respect to the controls are indicated (**$p < 0.01$, ***$p < 0.001$).

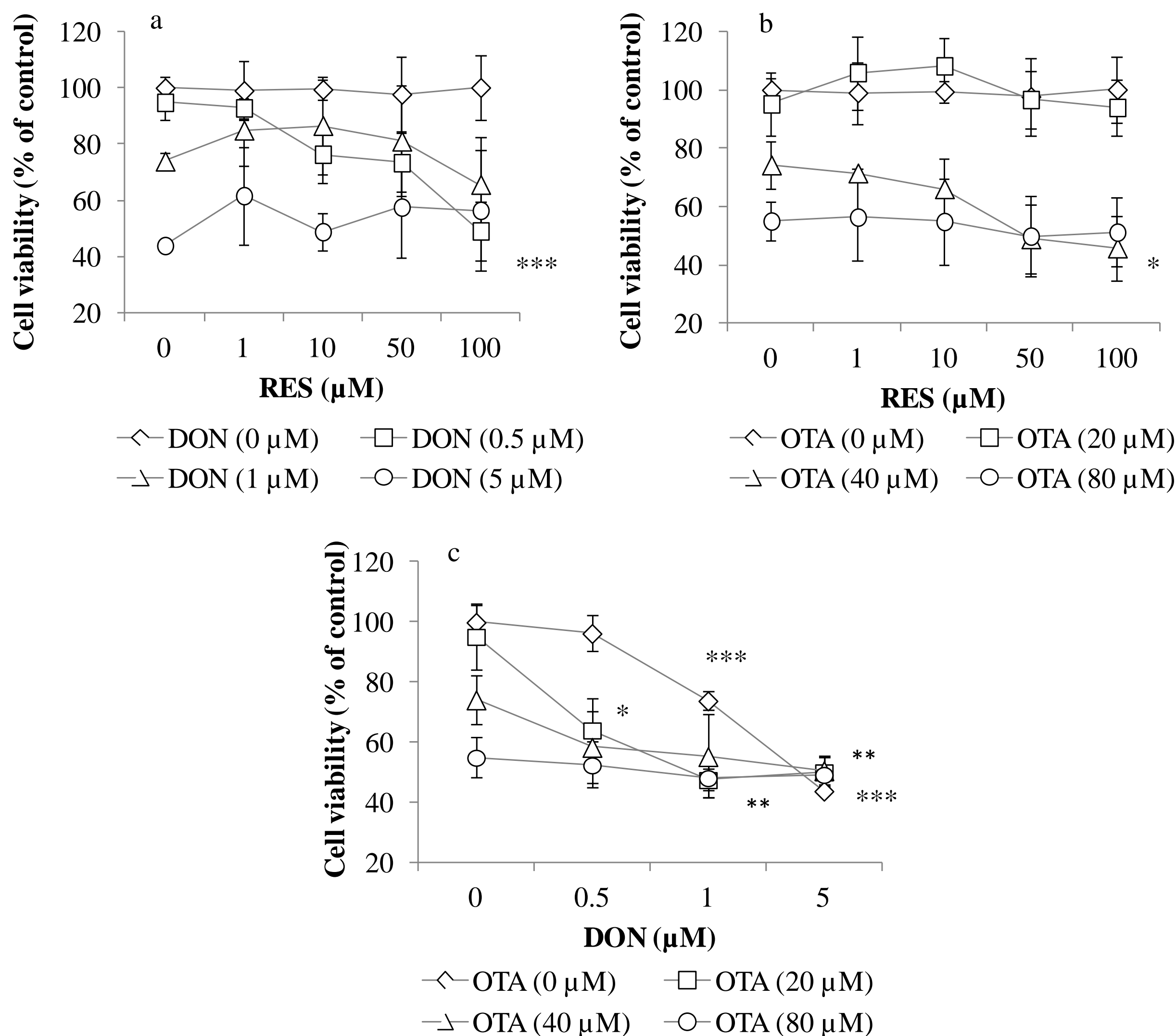

Figure 3. Combined effect of DON, OTA and RES on Caco-2 cell viability after 48 h of treatment. Results are expressed as percentage of viability compared with the solvent control (0.1% DMSO). Data are represented as mean ± SD of 3-5 independent experiments. Statistical differences with respect to the control of RES for each concentration level of DON (3a) or OTA (3b) and with respect to the control of DON for each concentration level of OTA (3c) are indicated (*p < 0.05, **p < 0.01, ***p < 0.001).

2.3.3 Reactive oxygen species (ROS) production

ROS produced by OTA, DON and RES was measured by comparison of fluorescence emitted by treated cells with the control using a dichlorofluorescein (DCF) assay (Fig. 4). Significant dose-dependent production of ROS was obtained when the cells were treated with OTA and RES for 24 h (data not shown) and 48 h (Fig. 4a and 4b), but not when treated for only 6 h (data not shown). The best response was obtained after 48 h. OTA and RES induced production of ROS at 80 and 20 μM ($p < 0.01$), respectively. However, there was not an increase in ROS production when Caco-2 cells were treated with DON during 6 and 24 h (data not shown) or 48 h (Fig. 4c).

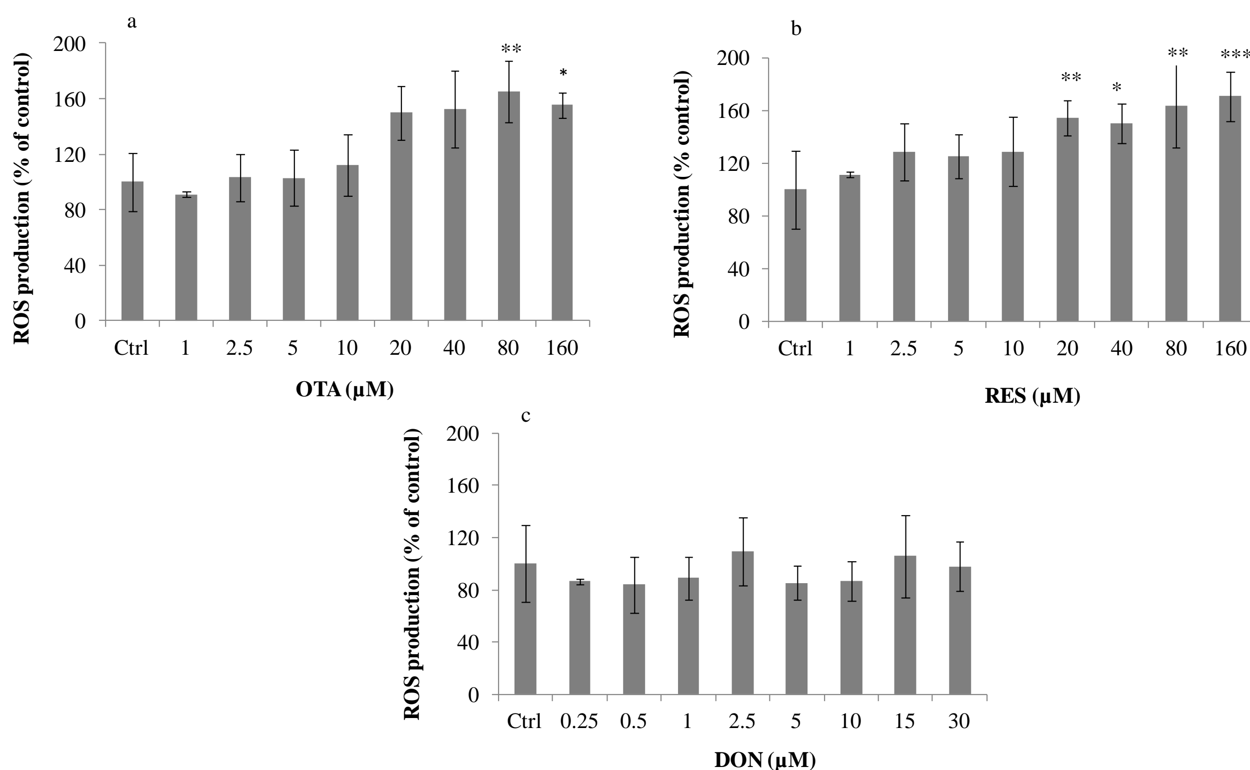

Figure 4: Individual effect of DON, OTA and RES on ROS production by Caco-2 cells after 48 h of treatment. Results are expressed as percentage of ROS production compared with the solvent control (0.1% DMSO). Data are represented as mean ± SD of 3-5 independent experiments. Statistical differences with respect to the controls are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

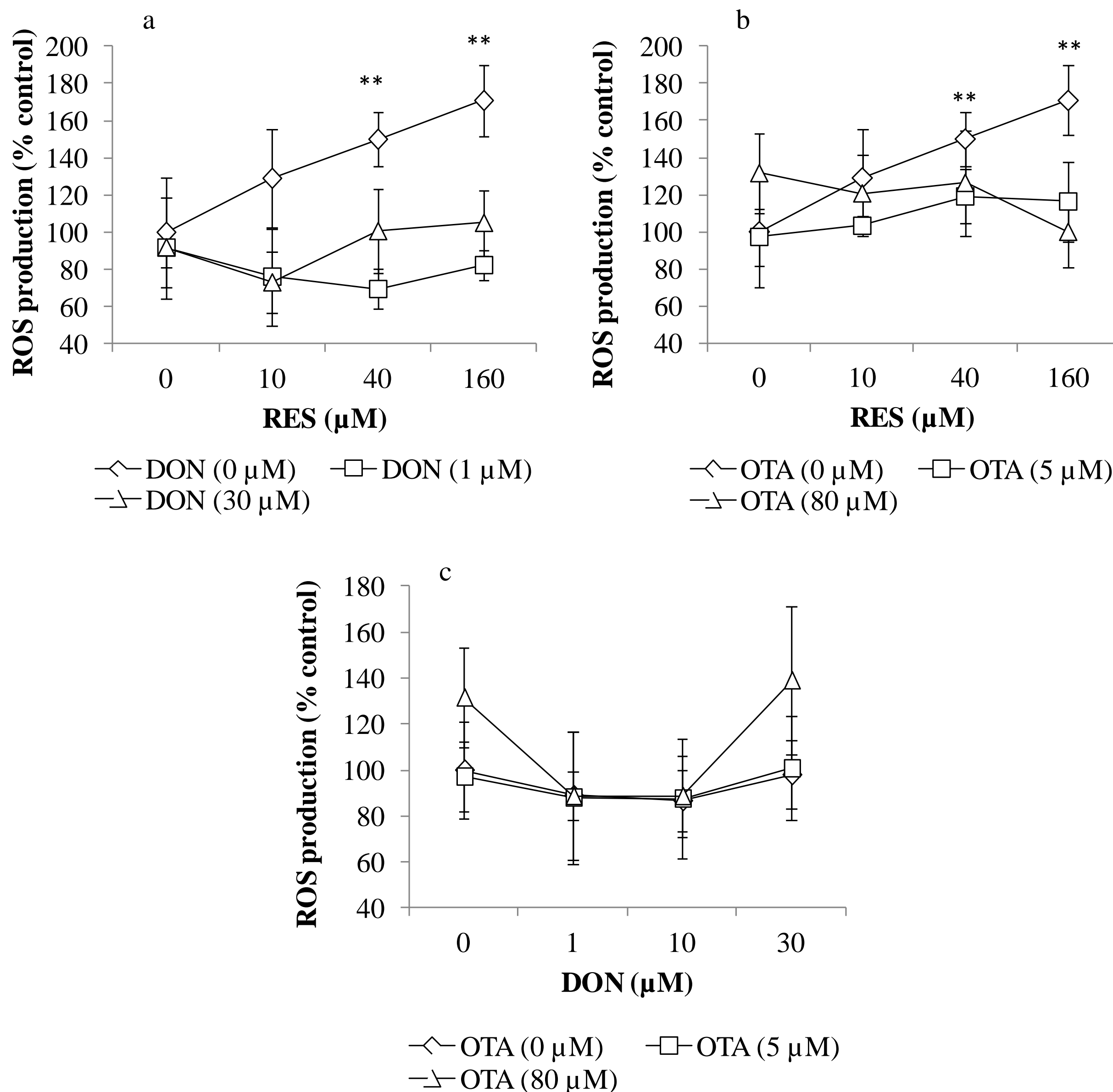

Figure 5: Combined effect of DON, OTA and RES on ROS production by Caco-2 cells after 48 h of treatment. Results are expressed as percentage of ROS production compared with the solvent control (0.1% DMSO). Data are represented as mean ± SD of 3-5 independent experiments. Statistical differences with respect to the control of RES for each concentration level of DON (5a) or OTA (5b) and with respect to the control of DON for each concentration level of OTA (5c) are indicated (**$p < 0.01$).

Binary mixtures of DON and OTA with different concentrations of RES (10, 40 and 160 µM) were tested (Fig. 5). To study the effects of DON on the ROS production, a low and a high cytotoxic concentration of DON (1 µM and 30 µM) were selected for these co-exposure studies. RES produced a notable increase of ROS levels when it was treated individually, however this ROS production decreased completely when co-exposed with DON at both concentration levels (Fig. 5a). To study the effects of the co-exposure of OTA with RES a non ROS-producing and non-cytotoxic concentration (5 µM), and a high cytotoxic and high ROS-producing concentration (80 µM) of OTA were selected. Combination of OTA at both doses with RES blocked the production of ROS by RES (Fig. 5b). Mixtures of OTA-DON didn't increase the ROS production observed for the highest concentration of OTA (80 µM) (Fig. 5c).

2.4 Discussion

In this study we have used the human cell line Caco-2 to study the possible toxic effects of the presence in foods of two different mycotoxins when they are present alone or together as well as when the antioxidant RES is also present in food. Caco-2 cell model is an accepted intestinal model widely implemented to assess the effect of chemical compounds on the intestinal function. Hence, undifferentiated and proliferating Caco-2 cells share some characteristics with crypt enterocytes. Studies focusing to assess the trans-cellular transport, barrier function and permeability properties are performed with differentiated cells, which exhibit structural and molecular characteristics similar to villous enterocytes. Cytotoxicity, viability and proliferation assays may be carried out also with un-differentiated (proliferating) Caco-2 phenotypes, which share some characteristics with crypt enterocytes (Manda et al. 2015; Sambruy et al 2001). Bony et al (2006) reported that proliferating cells were found to be more sensitive to DON than differentiated cells, therefore this model can be considered as the most conservative approach for hazard characterization.

In the present work we have shown that the degradation of RES in DMEM, a medium with sodium bicarbonate, is lower than 20% after 24 h of incubation and around 40% after 48 h. These results do not agree with those found by Yang et al. (2010) that reported a 96% of RES degradation after 24 h of incubation at 37 °C when it was added at 200 µM to the

Base Modified Eagle (BME) medium, a medium with bicarbonate (2.2 g/L) in its composition. Our results also show that, independently of the presence or absence of bicarbonate, the decrease in RES levels after 24 h (18.94% and 32.29%) and 48 h (41.35% and 30.57%) of incubation is relatively similar.

Regarding ROS production, Yang et al. (2010) reported a production of high levels (90 µM) of H_2O_2 after incubating RES for 24 h in a medium with bicarbonate. Nakamura et al. (2003) demonstrated a significant toxicity in HeLa cells after a 15 min exposure to H_2O_2 at concentrations as low as 0.06 mM (2 µg/mL). However, our results show that after 24 h and 48 h of exposure, RES is capable to increase ROS levels but not after 6 h, and no induction of cytotoxicity was observed with RES. This suggests that the enhanced production of ROS by RES in our study is mainly due to RES itself.

Both assayed mycotoxins are cytotoxic for the Caco-2 cell line. The NOEC and the lowest observed effect concentration (LOEC) were 0.5 and 1 µM for DON and 20 and 40 µM for OTA, respectively, indicating a highly toxic effect, especially for DON. Contrary to our results, Calvert et al. (2005) didn't find cytotoxicity according to the MTT assay for the Caco-2 cell line after 48 h exposure to 0.34-1.7 µM of DON, but HeLa and Hep-2 cells were very sensitive to DON ≥ 0.34 µM. Also in the mammalian kidney epithelial (Vero) cells the cytotoxicity of DON, evaluated by the NR and MTT assays over 24, 48 and 72 h, showed IC_{50} values ranging from 3.30 to 10 µM (Ruiz et al., 2011). Berger et al. (2003) reported the toxic effect of OTA on Caco-2 cells with the MTT assay. They determined a lower IC_{50} (0.4 µM) after a 48 h exposure period. However, different results are reported in the HepG2 cell line. The IC_{50} of OTA, as measured by the NR assay and the MTT assay, was 35 µM after 24 and 48 h exposure (Hundhausen et al., 2005; Zheng et al., 2013). As expected, RES didn't show cytotoxic effects up to concentrations of 160 µM. However, Sergent et al. (2005) found a slight cytotoxic effect of RES on Caco-2 cells after 48 h of treatment at 100 µM.

To investigate the capacity of these compounds and their combinations to induce oxidative stress, production of ROS was also measured. Studies carried out with individual compounds showed that only OTA and RES increased ROS levels with NOECs and LOECs of 40 and 80 µM for OTA and 10 and 20 µM for RES, respectively. The implication of

oxidative stress in the cytotoxicity induced by OTA has been previously suggested (Schaaf et al., 2002; Petrik et al., 2003; Kamp et al., 2005; Mally et al., 2005; Hsuuw et al., 2013). In our study, the production of ROS is associated with a parallel reduction in viability, suggesting the same conclusion of other authors. Interestingly, although RES is known for its anti-oxidative properties, it was able to produce high levels of ROS at concentrations as low as 20 μM in the Caco-2 cell line. Juan et al. (2008) have also reported the generation of mitochondrial ROS in the human colon cancer cell line (HT-29) exposed to RES (150 μM) for 4 h. Despite this ROS production, the basal redox status of the cells was not disturbed and no cytotoxicity was recorded with this compound in the cell line studied. From our results, the cytotoxicity induced by DON might not be explained by an oxidative stress mechanism. Previous results points to an apoptotic mechanism (Petska et al., 2004; Pestka, 2008). Recently, Ma et al. (2012) demonstrated the capacity of DON to induce apoptosis in the HT-29 cell line at very low concentrations ranging from 0.8 to 3.4 μM. They showed that the mechanism of apoptosis was caused by mitochondrial dysfunction and subsequent release of cytochrome c into the cytoplasm and successive activation of caspase-9 and caspase-3.

There have been few works published to date attempting to contribute on the knowledge of mycotoxins interactions with bioactive compounds such as dietary polyphenols. The most common approach followed involves pre-treatments with the bioactive compound and a subsequent treatment with the mycotoxin (Hundhausen et al., 2005; Bosch-Saadatmandi et al., 2006; Costa et al., 2007). In real exposure scenarios, interactions between the bioactive compounds and mycotoxins will occur simultaneously, which suggest that pre-treatment may not be the best approach.

In this study using a co-exposure approach, we didn't found a protective effect when OTA was co-exposed with RES. In fact, co-exposure of OTA at concentrations of 40 μM (moderately toxic dose) with the highest concentration of RES (100 μM) resulted in an increase in cytotoxicity. Sergent et al. (2005) showed that the Caco-2 absorption of OTA, at concentrations that should be easily encountered in the gut, is increased in the presence of RES. This would imply a greater bioavailability of the mycotoxin which could increase the toxicity of this compound. It could be that this also happens in the Caco-2 cell line, although this possibility has not been checked in this study. Co-exposure of DON with

RES did neither result in a protective effect of the polyphenols to the toxic effects of this mycotoxin. Moreover, in the case of DON, the presence of RES at high doses (100 μM) increases the toxicity of a non-cytotoxic dose of DON (0.5 μM). In contrast, Kolesarova et al. (2012) showed in porcine ovarian granulosa cells that RES protected cells from the reproductive toxicity induced by DON. Co-exposure of OTA and DON with RES resulted in the complete abrogation of ROS levels induced by RES. This finding is difficult to explain especially in the case of the co-exposure of RES with the non-toxic and non-ROS producing dose of OTA (5 μM). The decrease in ROS production of RES observed when this compound is co-exposed with toxic doses of DON (1 μM and 30 μM) or OTA (80 μM) could be the result of loss in cell viability. The mechanism triggering the cytotoxic effect of DON and OTA enhanced by RES at a non- toxic and a sub-toxic concentration respectively cannot be completely elucidated with the present data. Several studies have reported that RES modulates multiple vias that elicite to apoptosis and cell damage. Fouad et al. (2013) and Pasciu et al. (2010) have related a response mediated by the caspase-3 and the cells damage observed. Zhai et al. (2015) reported that RES inhibits proliferation and induces apoptosis of fibroblasts through the mechanism involving transforming growth factor and Smad proteins (TGF-b1/Smads) signaling pathway. On the one hand, TGF-b1 is a multifunctional cytokine that controls a diverse set of cellular processes (cell proliferation, differentiation, and apoptosis). On the other hand, Smad proteins are downstream messengers of TGF-b signaling pathways, conveying the TGF-b-mediated extracellular signals to the nucleus (Zhai et al., 2015).

When both mycotoxins were co-exposed at non-toxic concentrations of DON and OTA (0.5 and 20 μM, respectively) a significant increase in cytotoxicity was observed. Also the toxic response induced by DON (1 μM) increased significantly with increasing doses of OTA (40 and 80 μM), indicating that cocktails of these mycotoxins can suppose a risk for consumers. This increase in cytotoxicity could not be explained by an increase in ROS production. To our knowledge this is the first study where the *in vitro* toxicity of the co-exposure of DON and OTA has been studied. Other authors have reported different interactions between binary and tertiary combinations of DON or OTA with other mycotoxins (T-2 toxin, zearalenone, beauvericin, fumonisin B1, nivalenol, fusarenon-X) resulting in antagonism, synergism or additive effects depending on the combinations, the

cell lines, the concentrations and the assays used (Ruiz et al., 2011; Alassane-Kpembi et al., 2013).

Our results elucidated the complex behavior of mixtures of chemicals and bioactive compounds and the need of further research to better understand the biological responses and potential effects on the human health. Although the extrapolation of *in vitro* models to humans is very complex and requires an accurate approach, the concentration doses used in the present study are expected in the range of those levels expected from a common European diet. For example, the mean and highest (percentile 95) exposure levels of DON in Spain were estimated to be 0.37 and 1.9 mg/kg bw/day (Cano-Sancho et al., 2011). Thus, a mean intestinal dose of 0.5 μM can be expected in a relevant group of population. RES can be detected in Spanish wine at mean concentrations of 4.6 mg/L (Martelo-Vidal and Vázquez, 2014), leading to estimate an intestinal dose of 20 μM. While the risk assessment frameworks are developed on the basis of animal or human dose-response studies with isolated mycotoxins, potential interactions with other mycotoxins and bioactive compounds are missed. We focused the study to assess the simultaneous exposure of DON and OTA, the most concerning mycotoxins in Spain (Cano-Sancho et al., 2011: Coronel et al. 2012) with RES, a polyphenol widely consumed on the Spanish households. Despite the risk characterization did not reveal a concern for the human health, in the present study we elucidated toxicological issues commonly misplaced on risk assessment, such as the potential interaction of bioactive compounds at sub-toxic doses of mycotoxins. Future studies should confirm these results on animal models and assess the interactions with other bioactive compounds, as well as to translate the effect of these interactions on the risk characterization frameworks.

2.5 Conclusion

The results from our study showed that the co-exposure of both mycotoxins increased significantly the cytotoxicity of these compounds in Caco-2 cells without increasing the ROS production. The co-exposure of OTA or DON with RES did not result in a decrease in cytotoxicity; on the contrary, it resulted in increased cytotoxicity not associated with an increase in ROS production. Further studies should be conducted to elucidate the mechanisms underlying the toxic effects observed in the co-exposures and confirm these

findings in animals. Further studies should be conducted to elucidate the mechanisms underlying the toxic effects observed in the co-exposures and confirm these findings in animals. These results also elucidated a need to better understand the interactions with other bioactive compounds present in the diet, and evaluate the impact on risk assessment.

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Study III.

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

González-Arias, CA., Crespo-Sempere, A., Marín, S., Sanchis, V., Ramos, A.J.*

Food Technology Department. Lleida University. UTPV-XaRTA, Agrotecnio Center. Av. Rovira Roure 191. 25198 Lleida. Spain.

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 of treatment 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 *CYP450*, *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.

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3.1 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 CYP450 enzymes expressed in the liver have a major role in the *in vitro* and *in vivo* biotransformation of OTA and, therefore, in its 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).

Human N-acetyltransferase 2 (encoded by the *NAT2* gene) is involved in the metabolism of drugs and xenobiotics during phase II metabolism. Lack of NAT2 function is also associated with higher incidences of cancer and drug toxicity. NAT2 shows a restricted tissue distribution, and high levels are found in the human liver and intestine (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.

Some *in vitro* and *in vivo* assays indicate that the level of expression of cyclooxygenase-2 (*COX-2*) and lipoxygenase-5 (*5-LOX*) are associated with the presence of OTA (Ferrante et al., 2006; Kumar et al., 2013; Ramyaa et al., 2014). *COX-2* and *5-LOX* gene expression 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).

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 secondary hepatic metabolism, using Caco-2 and HepG2 cells. 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.

3.2 Methodology

3.2.1 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^{TM} 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).

3.2.2 Cell culture

The human colon cell line Caco-2 (HTB-37) and the hepatic 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% RH 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.

3.2.3 Viability assays

3.2.3.1 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 0.5% 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.

3.2.3.2 Trans epithelial electrical resistance (TEER) measurement

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. The maintenance of cell monolayers was performed as described in the co-culture section. 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 $\Omega \cdot cm^2$, according to the equation 1:

$$TEER_{monolayer} = (Resistance_{monolayer} - Resistance_{blank}) \cdot (\text{filter growth area in } cm^2) \quad \text{Equation 1}$$

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

3.2.4 Caco-2/HepG2 co-culture system

3.2.4.1 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.

3.2.4.2 Co-culture system and OTA treatments

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 0.5% of the final volume in the transwell system for all of the treatments.

3.2.5 Gene expression

3.2.5.1 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.

3.2.5.2 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 °C for 2 minutes followed by 40 cycles of 15 s at 95 °C and 30 s at 58 °C 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.

3.2.6 Statistics

Three experiments were run in triplicate, and two technical replicates were run for each of the treatments that constitute the experiment. 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.

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).

3.3. Results

3.3.1 MTS assay

Relative viability measured by the metabolic reduction of MTS is shown in Table 2. In general terms, the viability was slightly lower in Caco-2 cells (53.81-80.57 %) than in HepG2 cells (66.28-87.75 %) at 24 h. The mean values show that treatment with 1 μM OTA for 24 h resulted in viabilities of approximately 81% (Caco-2 cells) and 88% (HepG2 cells), similar to the results observed after treatment with 180 μM OTA for 12 h in both type of cells (85 and 84%, respectively). Additionally, the higher OTA concentrations did not substantially affect Caco-2 cell viability at 3 and 12 h.

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).

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

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 ± 5.00[a]
	5	112.20 ± 3.32[a]	97.96 ± 3.01[a]	78.37 ± 3.34[ab]
	15	102.60 ± 2.98[a]	96.80 ± 2.46[a]	72.17 ± 4.32[ab]
	45	107.08 ± 2.37[a]	86.18 ± 1.21[a]	76.14 ± 8.23[ab]
	60	99.18 ± 3.01[a]	90.99 ± 2.70[a]	74.00 ± 6.34[ab]
	75	104.36 ± 2.61[a]	95.50 ± 6.78[a]	66.26 ± 3.57[ab]
	90	104.81 ± 3.54[a]	91.15 ± 2.47[a]	66.34 ± 4.59[ab]
	180	95.85 ± 5.14[a]	84.98 ± 4.72[a]	53.81 ± 6.03[b]
HepG2	1	98.53 ± 0.48[ab]	97.48 ± 2.65[a]	87.75 ± 1.66[a]
	5	97.34 ± 2.83[ab]	101.38 ± 2.71[b]	91.06 ± 1.50[ab]
	15	91.81 ± 2.02[ab]	105.67 ± 3.37[ab]	85.58 ± 1.30[c]
	45	100.85 ± 3.33[ab]	106.32 ± 2.68[ab]	91.53 ± 2.04[ab]
	60	95.97 ± 2.54[ab]	105.20 ± 2.93[ab]	90.66 ± 2.04[c]
	75	95.55 ± 2.81[ab]	103.97 ± 3.84[ab]	88.43 ± 2.06[c]
	90	88.98 ± 2.06[bc]	98.34 ± 2.75[bc]	88.64 ± 1.51[c]
	180	81.58 ± 2.37[c]	83.74 ± 2.20[c]	66.28 ± 2.21[d]

Relative viability was calculated respect to solvent control (0.5% 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.

3.3.2 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 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 3 or 12 h treatments showed a slight but significant decrease in TEER values. Additionally, 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).

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

	TEER Ω•cm^2 (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.

3.3.3 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.

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.

Regarding the results of the hepatic cells, OTA induced both up- and down-regulation (Figure 2). Unlike the results obtained in Caco-2 cells, the *CYP3A4* expression increased significantly after 12 h of 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 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.

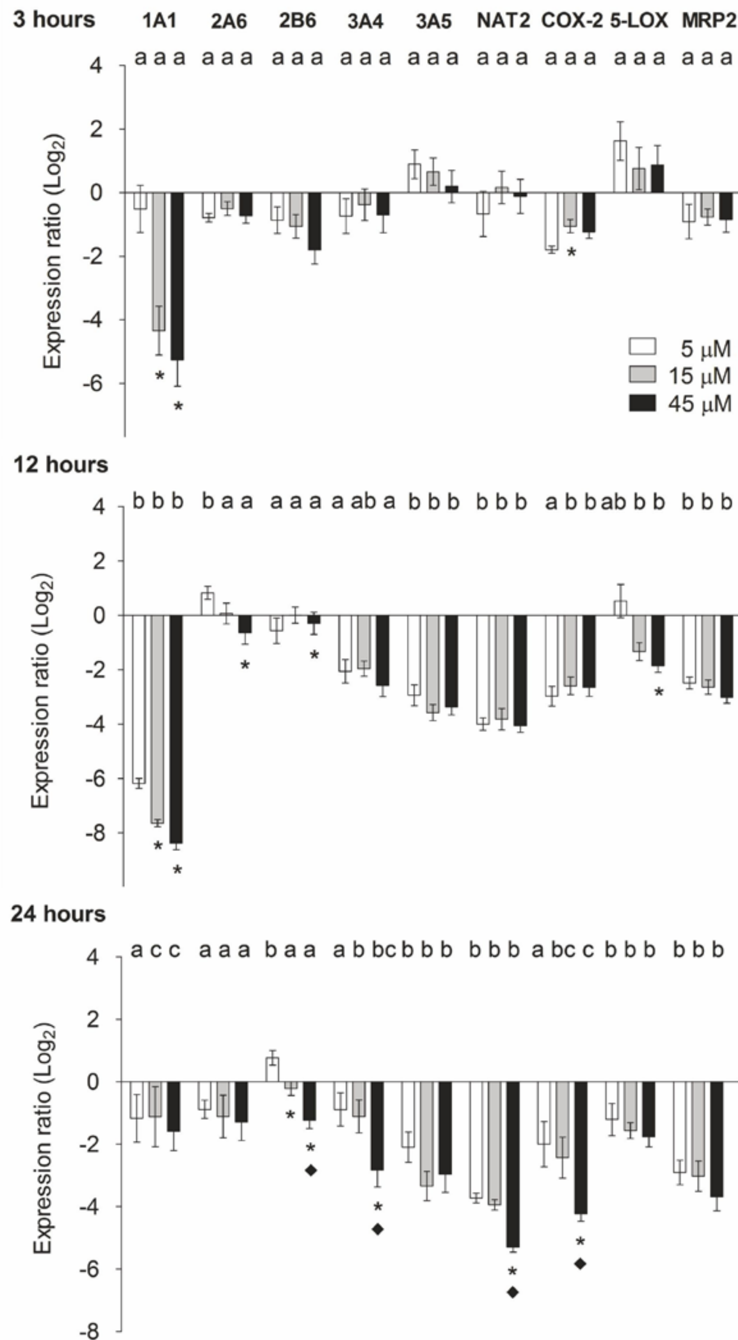

Figure 1. Relative gene expression ratio (in logarithmic scale) in differentiated Caco-2 cells exposed to 5, 15 and 45 OTA µM. The corresponding ratios of relative expression respect to GAPDH gene were calculated through a mathematical model (equation 2). Different letters (a, b, c) mean differences within time of treatment (vertical), whereas different symbols (asterisks or rhombus) mean a statistical significance (p < 0.05) for the same gene and between different OTA treatments during the same period of exposure. *Statistically significant respect to 5 µM or ♦respect to 15 µM.

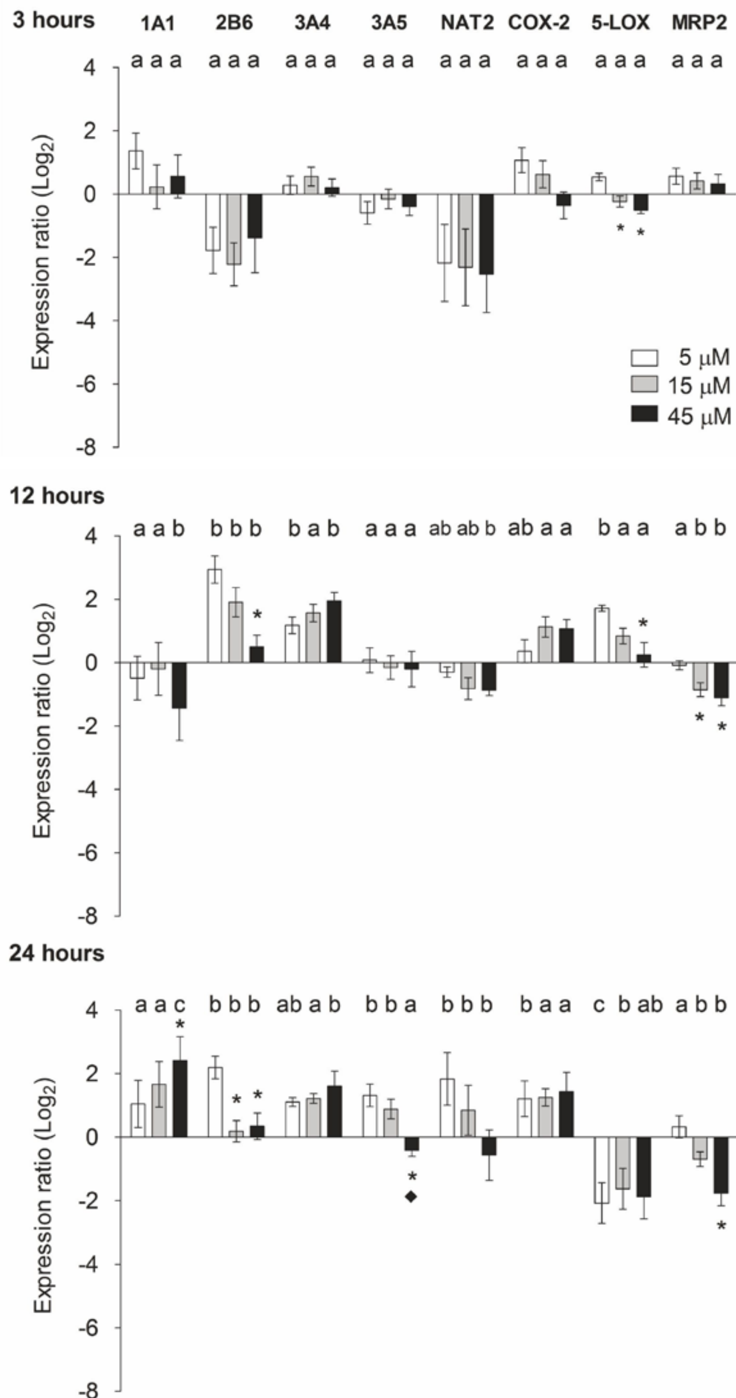

Figure 2. Relative expression ratio (in logarithmic scale) in HepG2 cells exposed to 5, 15 and 45 OTA μM. The corresponding ratios of target genes of relative expression respect to GAPDH gene were calculated through a mathematical model (equation 2). Different letters (a, b, c) mean differences within time of treatment (vertical), whereas the symbols (asterisks or rhombus) indicate a statistical significance ($p < 0.05$) for the same gene and between different OTA treatments during the same period of exposure. *Statistically significant respect to 5 μM or ♦respect to 15 μM.

3.4 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 Clària, 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

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Study IV.

Low doses of ochratoxin A induce micronucleus formation and delay DNA repair in human lymphocytes.

Cyndia A. González-Arias[a], Alma B. Benítez-Trinidad[b], Monserrat Sordo[c], Mª Lourdes Robledo-Marenco[b], Irma M. Medina-Díaz[b], Briscia S. Barrón-Vivanco[b], Sonia Marín[a], Vicente Sanchis[a], Antonio J. Ramos[a], Aurora E. Rojas-García[b*]

[a]Food Technology Department. Lleida University. UTPV-XaRTA, Agrotecnio Center. Av. Rovira Roure 191. 25198 Lleida. Spain.

[b]Laboratorio de Contaminación y Toxicología Ambiental. Secretaría de Investigación y Posgrado, Universidad Autónoma de Nayarit. Los fresnos, C.P. 63155. Tepic, Nayarit, México.

[c]Departamento de Genética y Toxicología Ambiental. Instituto de Investigaciones Biomédicas, U.N.A.M. Ciudad Universitaria, Coyoacán, C.P. 04510. México D.F., México.

Abstract

Contamination of food and feed commodities by fungal mycotoxins has attracted great interest because many of these mycotoxins are responsible for different diseases, including cancer and other chronic illnesses. Ochratoxin A (OTA) is a mycotoxin naturally present in food, and long-term exposure to food contaminated with low levels of OTA has been associated with renal cancer. In the present study, the cytotoxicity, cytostaticity, and genotoxicity of OTA (0.075, 0.15, 1.5, 5.0, and 15 μM) in human lymphocytes were evaluated. The comet assay and the modified comet assay (DNA repair assay), which use N-hydroxyurea (NHU) to detect non-repaired lesions produced by OTA, and the cytokinesis-blocked micronucleus (CBMN) assay were used. Treatments with OTA were not cytotoxic but caused a cytostatic effect in human lymphocytes at 15 μM. OTA (0.075-5

μM) produced a slight increase in the percentage of DNA in the comets and a delay in the DNA repair capacity of the lymphocytes. Micronucleus (MN) induction was observed at OTA concentrations of 1.5 and 5 μM. In conclusion, our results indicate that OTA induces DNA stable damage at low doses that are neither cytotoxic nor cytostatic, and OTA delays DNA repair kinetics. These two findings indicate that OTA affects two pivotal events in the carcinogenesis pathway.

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4.1 Introduction

Ochratoxin A (OTA) is a fungal toxin produced by species of the *Aspergillus* and *Penicillium* genera. It was first described by Van der Merwe et al. (1965). Foodstuffs for human and animal consumption are frequently contaminated with OTA worldwide, and the main contaminated foods are cereals, dried fruits, coffee, grapes, wine, and beer. The consumption of OTA-contaminated food is known to have a close relationship with the development of diseases, such as Balkan Endemic Nephropathy (BEN), a chronic tubule interstitial renal disease observed in settlements along the Danube River in the Balkan Peninsula (Stoev, 1998; Vrabcheva et al., 2004).

OTA consists of a dihydroisocoumarin with a para-chlorophthalic group that is linked to an L-β-phenylalanine by an amino bond (Moss, 1996). OTA is mainly nephrotoxic, but other toxic effects have been described, including teratogenicity, neurotoxicity, immunotoxicity, and genotoxicity. The nephrotoxic mechanisms of OTA have been studied *in vitro* and *in vivo*, but the mechanisms through which OTA exerts carcinogenic effects are not entirely understood (IARC, 1993; Benford et al., 2001; Abouzied et al., 2002). Two hypotheses are still discussed when dealing with the carcinogenicity of OTA. The first hypothesis suggests that the carcinogenicity of OTA is linked to its epigenetic nature because OTA produces effects in target cells that either indirectly lead to neoplasic transformation or facilitate the development of neoplasms from cytogenetically transformed cells (O'Brien and Dietrich 2005; Schilter et al., 2005; Turesky, 2005). The second hypothesis suggests that its carcinogenicity is due to genotoxic mechanisms (Manderville, 2005; Mantle et al., 2010a; Pfohl-Leszkowicz and Castegnaro, 2005). In any case, OTA has been classified as a possible group 2B human carcinogen by the International Agency for Research on Cancer (IARC, 1993). It has also been classified in the germ cell mutagen group 3B (MAK Value Documentation, 2006) and recommended to be regulated as a non-threshold carcinogen (Kuiper-Goodman et al., 2010).

Several *in vitro* studies using microbial and mammalian models have suggested that OTA does not act as a direct genotoxic compound. Some studies have reported that OTA shows no mutagenic capacity in the Ames assay, either with or without metabolic activation (Bendele et al., 1985). However, other studies have shown that OTA has mutagenic

capacity using the same Ames assay when OTA metabolites were formed by hepatocytes in culture (Hennig et al., 1991) or were generated by a kidney microsomal fraction (Obrecht-Pflumio et al., 1999).

Additionally, controversial results have been obtained with the Sister Chromatid Exchange (SCE) assay. Although an OTA treatment did not increase the SCE frequency in human peripheral blood lymphocytes (Cooray, 1984; Mosesso et al., 2008) or in the bone marrow of Chinese hamsters (Bendele et al., 1985), (Föllmann et al., 1995; Lioi et al., 2004) and Anninou et al. (2014) detected an increase in the SCE frequency with and without metabolic activation in response to treatment with OTA.

DNA adduct formation has been reported in many *in vitro* and *in vivo* studies and results from the direct covalent binding of OTA to DNA, which is related to the metabolic activation of OTA (El Adlouni et al., 2000; Mantle et al., 2010; Pfohl-Leszkowicz and Castegnaro, 2005; Hadjeba-Medjdoub et al., 2012). However, three different studies failed to identify DNA adducts following OTA exposure (Gautier et al. 2001; Gross-Steinmeyer et al., 2002; Mally et al., 2005).

Chromosomal instability caused by OTA has also been measured using the micronucleus (MN) assay. This assay is a tool to evaluate the clastogenic capacity of compounds that can induce chromosomal aberrations and aneugenicity through mitotic spindle dysfunction (Fenech, 2007). Some MN assays performed with OTA did not show a dose-response relationship. However, in most studies, the concentrations tested were higher than 12 µM (4.8 µg/mL) (Degen et al., 1997; Ehrlich, et al., 2002; Knasmüller et al., 2004; Fuchs et al., 2008).

The aim of this study was to evaluate the genotoxic potential of OTA in human lymphocytes through the comet and repair assays and through the cytokinesis-blocked micronucleus (CBMN) technique. This study also assessed the effects of low concentrations of OTA, which have not been frequently assayed in past research.

4.2 Materials and methods

4.2.1 Chemicals

The following products were obtained from Sigma-Aldrich (Steinheim, Germany): low-melting-point agarose, regular agarose type IIA, cytochalasin-B (Cyt-B) from *Drechslera dematioidea* (ref. C6762), ethylenediamine tetraacetic acid (EDTA) (ref. E9884), fluorescein diacetate (25 mg/mL) (ref. F7378), hydrogen peroxide (H_2O_2) at 30%, mitomycin C (ref. M4287), phytohemagglutinin (PHA) from *Phaseolus vulgaris* (red kidney bean) (ref. L4144), RPMI-1640 medium (ref. R4130), trypan blue solution (ref. T8154) and trypsin solution (1X) (ref. T3924).

Standard OTA (98% purity) was obtained from Chem Service® (ref. O1877) (West Chester, PA, USA), and ethanol 96° was obtained from Jalmek® (ref. A3380) (Nuevo León, México). The L-glutamine solution (100X) (ref. 25030) and 100X nonessential amino acid solution (ref. 11140-050) were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA).

Microscopy Hemacolor (ref. 111661) was obtained from Merck KGaA (Darmstadt, Germany). Ethidium bromide (ref. 161-0433), Tris (ref. 161-0716) and triton X-100 (ref. 1610407) were obtained from BioRad (Hercules, California. USA). Dimethyl sulfoxide (DMSO) for molecular biology (ref. D8418), glacial acetic acid, methanol, sodium hydroxide (NaOH), and sodium chloride (NaCl) were supplied by JT-Baker (Center Valley, PA, USA), and Petri dishes (1.9 cm^2) were obtained from Corning (ref. 3526) (New York, USA).

4.2.2 Cell culture and OTA treatments

Heparinized venous blood samples from healthy non-smoking male donors of 21-23 years of age were used for the experiments.

For the comet assay, a volume of 20 µL of whole blood in 1 mL of RPMI-1640 medium (without supplement) was treated with OTA for 3 h at 37 °C.

For the CBMN assay, a volume of 0.5 mL of whole blood was cultured in 7 mL of RPMI-1640 medium supplemented with 1% L-glutamine and non-essential amino acids (100x). The lymphocytes were stimulated with PHA (7.1 µg/mL) and cultured for 72 h at 37 °C. Cytochalasin B (6 µg/ml) was added in the last 24 h of culture to accumulate cells that had divided only once. OTA was added 24 h after the beginning of the PHA stimulation, and the OTA incubation lasted for the remaining 48 h of the assay. The OTA doses assayed were 0.075, 0.15, 1.5, 5.0 and 15 µM (ethanol: DMSO, 3:2) for both techniques. The percentage of solvent was less than 0.05% of the final volume.

4.2.3 Cell viability tests

4.2.3.1 Double staining with fluorescein diacetate and ethidium bromide

The cytotoxicity was evaluated using the fluorescent dyes fluorescein diacetate (FDA) and ethidium bromide (EtBr). The OTA-treated cells were resuspended, and 100-µL aliquots of each treatment group were transferred to new tubes and centrifuged at 3000 rpm for 2 min. The supernatant was removed, and the cell pellet was maintained on ice until use. For the cell viability analysis, a fresh staining solution was prepared with 30 µL of FDA in acetone (5 mg/mL), 200 µL of EtBr in phosphate buffer saline (200 µg/mL), and 4.8 mL of PBS. The cell pellet was resuspended in 20 µL of the FDA/EtBr solution, placed on a slide, and covered with a coverslip. Cell counting was performed with an Axio Scope A1 fluorescence microscope (Carl Zeiss, Gottingen, Germany) using the 20X objective. The living cells could be visualized in green, whereas the dead cells could be visualized in red; a total of 200 cells were counted for each treatment.

4.2.4 Comet assay

The alkaline comet assay was performed as described by Tice et al. (2000). After OTA treatment, button cells were mixed with 150 µL of low-melting-point agarose (0.5%) at 37 °C, placed on slides that had been precoated with a layer of regular agarose (0.5%) and allowed to polymerize at 4 °C. Another layer of low-melting-point agarose was added and allowed to solidify. The slides were placed for 24 h in Coplin jars with 50 mL of lysis solution (2.5 M NaCl, 100 mM EDTA-Na_2 and 10 mM Tris, pH>10) at 4 °C, 5 mL of DMSO and 0.5 mL of Triton X-100. Prior to electrophoresis, the slides were incubated for 20 min

in an alkaline buffer (10 M NaOH, 200 mM EDTA-Na_2, pH>13), which is capable of detecting DNA damage, including single-strand breaks (SSB), alkali-labile sites (ALS), and DNA-DNA/DNA-protein cross-linking. After alkali unwinding, the slides were run by electrophoresis at 25 V and 300 mA for 20 min, neutralized, and fixed. The slides were stained with EtBr for analysis and prepared in duplicate per treatment. Slides were randomized and coded to blind the scorer. A total of 100 individual cells were screened per treatment (50 cells from each slide). The scoring was performed in a fluorescence microscope Carl Zeiss Axio Scope A1 with 20X objective. The percentage of DNA was determined using the *Image Comet software version 2.2.*

The results are presented as the means from three independent experiments. To determine the kinetics of DNA repair in the lymphocytes exposed to OTA, a modified comet assay was conducted. Four Eppendorf tubes (T0 (DNA damage produced by a second known exposition); T30 and T60 (two repair times in minutes) and TNHU (10 µL of N-hydroxyurea was used as positive control for DNA repair) were used for each of the following conditions: whole blood lymphocytes treated with OTA (1.5 and 5 µM), a positive control (10 µL, 30% H_2O_2) and a negative control (ethanol:DMSO <0.05%). All of the tubes were incubated for 3 h at 37 °C. After incubation, the samples were treated with 10 µL of H_2O_2 (30%) for 10 min at room temperature, centrifuged, washed with 1 mL of culture medium and centrifuged again. Slides were prepared for the T0 sample as described above. The T30 and T60 samples were incubated for their respective repair times, and the tubes were then centrifuged prior to slide preparation. TNHU was also incubated for 60 min.

4.2.5 Cytokinesis-block micronucleus assay (CBMN)

4.2.5.1 Nuclear index (NI)

To evaluate the effect of OTA on the mitogenic response of lymphocytes, the NI was evaluated according to a method described by Eastmond and Tucker (1989). The viable cells (n=200) were scored to determine the frequency of cells with one, two, three, or four nuclei, and the NI was calculated according to $NI = (M1+2(M2)+3(M3)+4(M4))/N$, where M1 to M4 represent the number of viable cells with one to four or more nuclei and N represents the total number of viable cells scored.

4.2.5.2 CBMN technique

The evaluation of MN was performed using the CBMN technique with criteria established through the work conducted by Fenech *et al.* (2003). Mitomycin C was used as a positive control for MN induction. The slides were codified, and the examination of the slides was performed blindly. One thousand binucleated cells per slide were evaluated, and the resulting MN frequency was expressed as the number of MN in 1000 binucleated cells.

The mitogenic response of lymphocytes and the frequency of MN were evaluated using a light microscope (Carl Zeiss Axiostar Plus, Gottingen, Germany) at 1000X magnification.

4.2.6 Statistical analysis

Statistical analysis were conducted using the GraphPad Prism 5.01 and Stata 8.0 programs (Stata statistical software, Stata Corporation, College Station, TX, USA). The effects of OTA on cell viability, percentage of binucleated cells and NI were analysed using the analysis of variance (ANOVA) and the Bonferroni post-test. The results of the MN and comet assays were analysed with Mann Whitney and Dunn's tests. P values of $p<0.05$ were considered to be statistically significant.

4.3 Results

4.3.1 Cytotoxicity and cytostaticity

At the doses assayed (0.075-15 µM OTA), the viability of the lymphocytes was approximately 80% or slightly higher, and no significant differences were noted between the treated and the solvent control cells. In addition, OTA did not statistically decrease NI or the percentage of binucleated cells at concentrations lower or equal to 5 µM compared with the solvent control. However, a cytostatic effect was observed at a concentration of 15 µM (Table 1).

Table 1. Citotoxicity and citostaticity in human lymphocytes treated with ochratoxin A

	Treatment	Viability (%)	P-value	Nuclear index	P- value	Binucleated cells (%)	P-value
OTA (μM)	0.075	88.3 ± 9.3	0.647	1.9 ± 0.2	0.89	55.2 ± 11.8	0.92
	0.15	89.0 ± 9.1	0.525	1.8 ± 0.2	0.85	50.7 ± 19.1	0.53
	1.5	89.0 ± 6.8	0.419	1.8 ± 0.1	0.47	54.1 ± 13.2	0.80
	5	90.6 ± 9.3	0.290	1.7 ± 0.1	0.10	54.7 ± 7.5	0.85
	15	88.0 ± 7.6	0.645	ND		ND	
Mitomycin C (1 μM)	(+)	90.0 ± 4.7	0.016	1.4 ± 0.24	<0.0001	15.8 ± 10.4	<0.0001
Control	(-)	87.3 ± 6.8	0.831	1.8 ± 0.1	0.56	60.3 ± 20.3	0.21
Solvent (DMSO %)	0.05	86.6 ± 4.8		1.9 ± 0.2		61.1 ± 10.2	

Data represent the means ± SD of three independent experiments run in triplicate. P-values are significantly different than the dissolvent control ($p<0.05$). ND: No data, the amount of cells was not sufficient to complete one thousand binucleated cells per slide.

4.3.2 Comet Assay

The results obtained from the comet assay showed that although no differences were found in the tail length in the concentration range assayed (data not shown), a slight increase was observed in the percentage of DNA at the 0.075, 1.5 and 5 μM treatments (Fig. 1) without a dose-response pattern. In addition, according to the repair assay results, although the lymphocytes repaired their DNA within 30 min following oxidative treatment (H_2O_2), co-exposure to H_2O_2 and OTA delayed their repair capacity for a period of 60 min, until then, the DNA percentage was similar to control ($p>0.05$), suggesting that OTA can alter the capacity of lymphocytes to repair their DNA (Fig. 2).

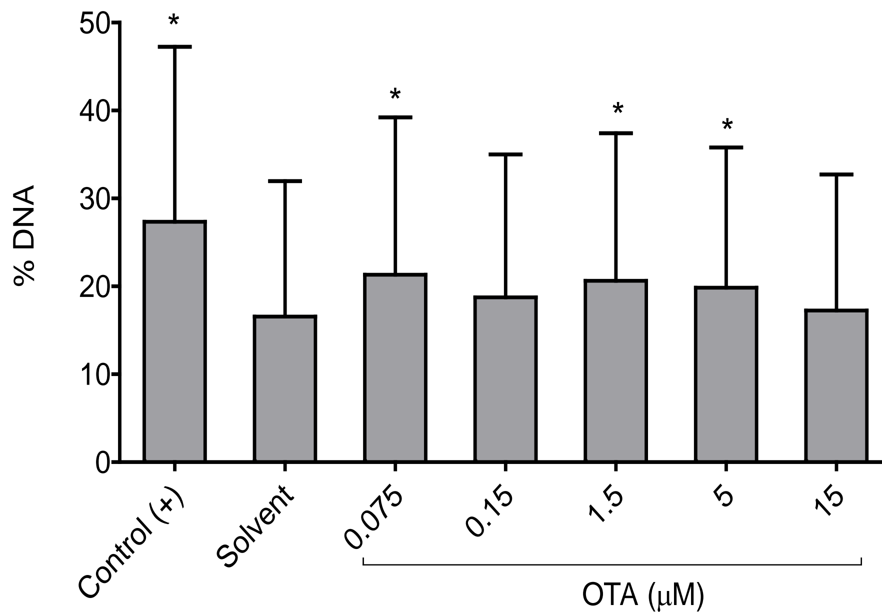

Figure 1. DNA damage (measured as DNA percentage, %DNA) in human lymphocytes after 3 h exposure to OTA. Positive control: 10 μL H_2O_2 (30%), solvent control: 0.05% ethanol:DMSO (3:2). Each value represents the mean ± SD of three independent experiments run in duplicate. *Significantly different than the solvent control ($p<0.05$).

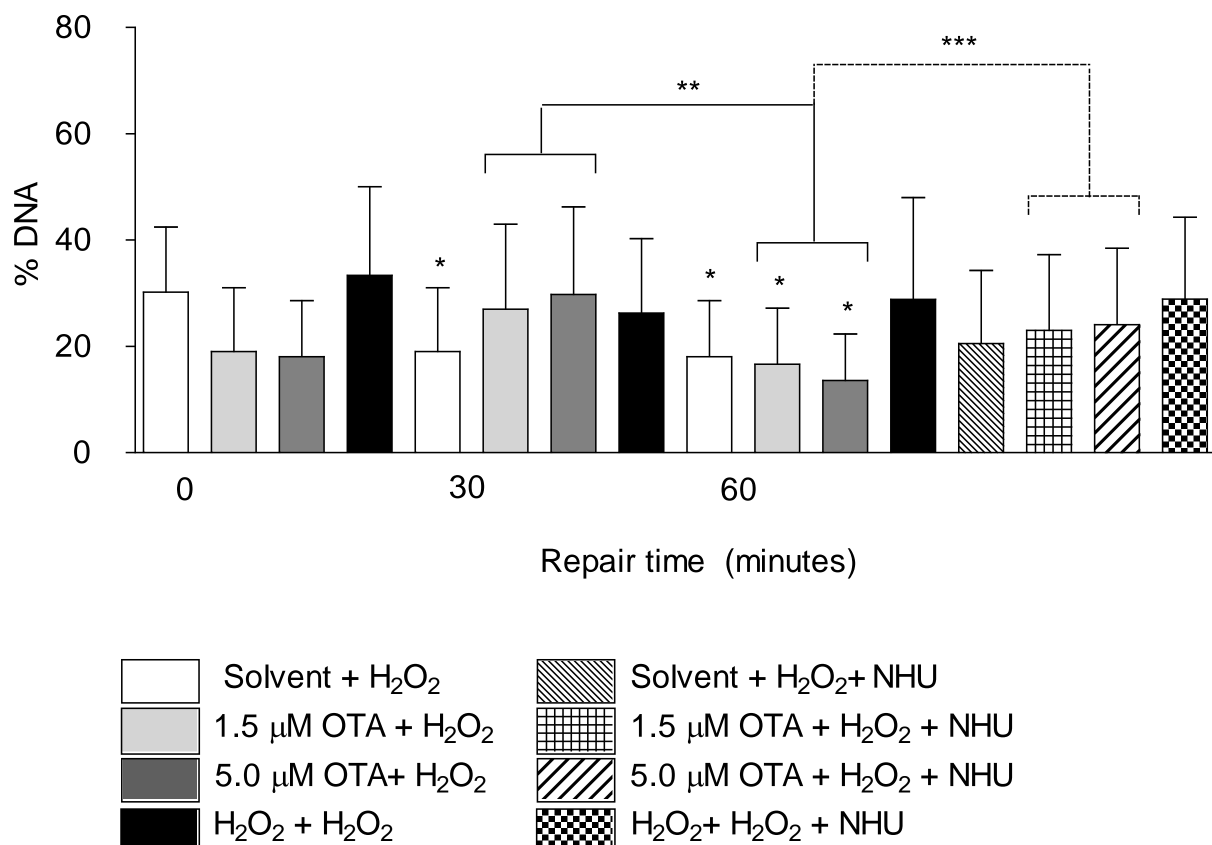

Figure 2. Kinetics of DNA repair in human lymphocytes treated with OTA (1.5 and 5 μM) measured as DNA percentage at 0, 30, and 60 min. The response of lymphocytes to 10 mM NHU (N-hydroxyurea) was determined at 60 min. Each value represents the mean ± SD of three independent experiments run in duplicate. *Significantly different from the solvent control at time 0, and ** Repair effect at time 60 (min) is significantly different from the OTA + H_2O_2 treatment at time 30 (min) ($p<0.05$). ***OTA treatments with NHU are different from the OTA + H_2O_2 treatments at time 60 (min) without NHU ($p<0.05$).

4.3.3 CBMN assay

Fig. 3 shows the formation of MN in binucleated human lymphocytes after OTA exposure. OTA induced a slight but significant increase in the MN frequency in cells treated with OTA concentrations of 1.5 µM and 5 µM compared with the solvent control. MN induction was not scored accurately in the cells treated with OTA at a concentration of 15 µM due to the cytostatic effect observed.

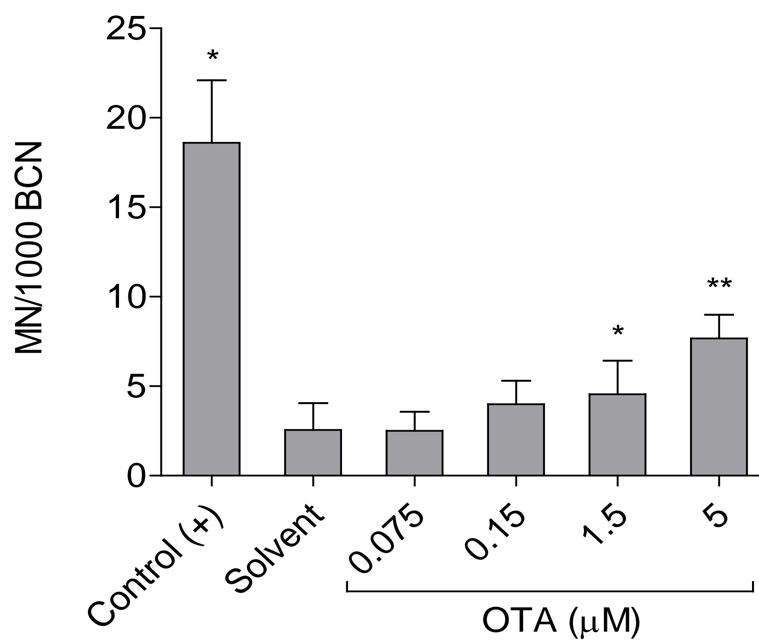

Figure 3. Effect of OTA on the formation of MN in binucleate human lymphocyte cells. Values are means ± SD of three independent experiments run in triplicate (1000 binucleated cells were analysed per treatment). Positive control: 1 µM mitomycin C; solvent control: 0.05% ethanol:DMSO (3:2). *Significantly different than the solvent control ($p<0.05$). **Significantly different than the 1.5 µM treatment ($p<0.05$).

4.4 Discussion

Several studies have shown that the levels of OTA found in food are often in the range of μg/kg (ppb). In foods intended for direct human consumption, such as grape juice, wine, or beer, the mean contamination levels in the EU were 0.56 μg/kg, 0.36 μg/kg and 0.03 μg/kg, respectively (Jørgensen, 2005), making these items important sources of OTA in the diet. OTA is associated with chronic toxicity following long periods of exposure to relatively low levels of this mycotoxin, and therefore toxicity tests must necessarily be carried out at low OTA levels. For this reason, lower levels of OTA than those commonly referenced in the literature were included in this study.

OTA has been reported as either a carcinogen or non-carcinogen compound (IARC, 1993; JECFA, 2006; 2010), and diverse tests and cell types have been used to investigate its genotoxic potential. Among them, the CBMN assay is a well-known comprehensive system that provides data from the DNA damage measures of cytostaticity and cytotoxicity (Fenech, 2007). In contrast, the comet assay measures DNA damage by assessing strand breaks in single cells. In this study, we performed the alkali version to detect single- and double-strand breaks and the comet assay to assess DNA repair. These studies provide data on the mechanism of damage and on the capacity of cells to block the base excision repair system, which can allow the accumulation of breaks for longer periods of time.

The results of the cytotoxicity assay performed in this study are consistent with other reports, in which the viability was above 70-80% when the OTA concentration was lower than 20 μM (Golli-Bennour et al., 2010; Ali et al., 2011), indicating that the viability values were higher than the recommended level (cytotoxic limit) for performing the *in vitro* CBMN assay (OECD, 2010) and for the *in vitro* comet assay (Tice et al., 2000). Regarding the cytostatic effect of OTA, our data showed that this mycotoxin, at a concentration of 15 μM, decreased the NI in human lymphocytes. Dönmez-Altuntaş et al. (2003) reported that OTA did not decrease the percentage of binucleated cells at concentrations ranging from 100 pM to 10 μM; however, the authors also reported the opposite results at higher concentrations (25 μM), and these latter results were consistent with our findings.

In our study, the OTA treatments did not appear to increase the tail length (data not shown) but did increase the percentage of DNA at concentrations of 0.075, 1.5 and 5 μM, although not in a dose-dependent manner. In addition, when an oxidative stress (co-incubation with H_2O_2) was applied in conjunction with the OTA treatments to evaluate the repair capacity of the lymphocytes, the DNA repair of the lymphocytes was found to be delayed to a time point of 60 min. Our results thus agree with those previously reported by Lebrun and Föllmann (2002), who found that OTA induced single-strand breaks in Madin-Darby canine kidney (MDCK) cells at high concentrations (up to 100 μM) in a dose-dependent manner. The authors also observed an increase in the genotoxic effect of OTA when an external metabolizing enzyme system (S9-mix from rat liver) was added, although the damage was completely repaired within 2 h. Additionally, in two studies performed by Ali et al. (2011; 2014), a non-significant increase in the DNA damage (% tail intensity, 4 h of treatment, without S9 treatment) was found with 5 μM OTA in three different cell lines (TK6, CHO and L5178Y tK+/-). However, L5178Y tK+/- cells were more sensitive than the other two cell lines at 10 μM OTA. A dose-response pattern was observed in the range of 20 to 50 μM OTA in the TK6 and CHO cells and in the range of 10 to 100 μM OTA in the L5178Y tK+/- cells.

Russo et al. (2005) found that human fibroblasts treated with OTA for 72 h (6-50 μM) exhibit an exposure time-dependent increase in the percentage of damaged DNA. This study also suggested the involvement of oxidative stress (due to an increase in ROS) in the OTA genotoxicity, which is also consistent with the work of Schilter et al. (2005) and Zheng et al. (2013). This finding is further supported by a study conducted by Kamp et al. (2005) in the V79 and CV-1 cell lines and in primary rat kidney cells, which revealed a slight increase in the basic DNA damage (percent of DNA) without treatment with DNA repair enzymes (formamidopyrimidine-DNA glycolyase and endonuclease III) in both cell lines. However, this study was performed using only a short exposure time (1 h) and a high OTA concentration (≥500 μM).

Based on their work with human leukocytes, Klarić et al. (2010) reported that OTA does not cause genotoxicity at concentrations of 1 and 5 μM (1 h of exposure), as measured by the tail length extension. However, the tail intensity and tail moment parameters were significantly higher, which is consistent with our results.

Although the mechanism that leads to OTA genotoxicity, as measured through the comet assay, is not fully understood, evidence from *in vitro* (with different cell types) and *in vivo* studies suggests the role of oxidative stress (Lebrun and Föllmann, 2002; Arbillaga et al., 2007; Hadjeba-Medjdoub et al., 2012; Aydin et al., 2013; Hibi et al., 2013a; Hibi et al., 2013b; Ali et al., 2014; Yang et al., 2014). It has also been reported that OTA biotransformation produces metabolites that are more genotoxic than the original toxin. However, it is important to note that this type of DNA damage can be effectively repaired (Lebrun and Föllmann, 2002; Simarro et al., 2006).

The results obtained in the present study show that OTA produces a slight but significant MN induction at concentrations as low as 1.5 μM (1.8 times more MN than in the solvent control) and 5 μM (3 times more MN than in the solvent control). Similar results were observed in seminal vesicle cell cultures treated with 12 μM OTA for 6 h (Degen et al., 1997).

Several studies have reported that the OTA-based MN induction is dependent on the concentration of OTA at doses higher than 5 μM (Degen et al., 1997; Dopp et al., 1999; Knasmüller et al., 2004; Fuchs et al., 2008; Klarić et al., 2008). In contrast, studies conducted at low OTA concentrations have reported MN induction but have not shown a dose-response pattern (Ehrlich et al., 2002; Dönmez-Altuntaş et al., 2003; Ali et al., 2011). Unlike the studies previously mentioned, our results with the CBMN assay showed that OTA is genotoxic at concentrations as low as 1.5 μM. The mechanism underlying OTA-induced genotoxicity has been the subject of controversy. Some studies have suggested that genotoxicity results from the direct covalent binding of OTA to DNA (El Adlouni et al., 2000; Pfohl-Leszkowicz and Mandeville, 2012). In contrast, Dopp et al. (1999) determined that 3 h of exposure to 10 μM OTA causes an induction of MN production in Syrian hamster embryo (SHE) fibroblasts. Ehrlich et al. (2002) reported that only 1 h of OTA treatment (61.9 μM) is sufficient to increase the MN frequency in HepG2 cells, with an effect that was approximately 30% lower than when the cells were exposed for 24 h at the same concentration.

In the case of human lymphocytes, Mosesso et al. (2008) reported that OTA did not cause an increase in the MN frequencies at concentrations ranging from 5.3 to 53.2 μM in both

the absence and presence of S9 metabolism. However, it is important to consider the high cytotoxicity (32-67%) and the low values of the cytokinesis block proliferation index (CBPI) (1.17-1.08) reported in this study. Additionally, Dönmez-Altuntaş et al. (2003) treated a lymphocyte culture (48 h) with OTA at concentrations of 100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM and 25 µM. At the highest concentration, the authors reported that OTA induced MN but also led to a clear decrease in the percentage of binucleated cells observed.

The available evidence suggests that OTA increases MN production by inducing clastogenic events (60-70%) more commonly than aneugenic events due to chromosomal breakage (Degen et al., 1997; Dopp et al., 1999; Knasmüller et al., 2004). To this end, Dopp et al. (1999) reported that OTA is an effective inducer of DNA damage and serves to disrupt intracellular calcium homeostasis and actin stress fibres. The study concluded that OTA has a predominantly clastogenic mode of action.

In contrast, the mechanisms through which OTA can cause a clastogenic effect in both comet and MN assays have not been well established. Some studies have reported that OTA can induce cell cycle arrest in the G1 phase by down-regulating the expression of CDK4 and the cyclin D1 protein and by limiting apoptosis in human peripheral blood mononuclear cells (hPBMC) *in vitro* (Liu et al., 2012). Kuroda et al. (2014) demonstrated that OTA also produces an increase in γ-H2AX expression and was capable of inducing double-strand breaks (DSBs) in DNA in a rat model. OTA also increases the mRNA and protein expression levels of homologous recombination (HR) repair-related genes (Rad51, Rad18 and Brip1) and genes involved in G2/M arrest (Chek1 and Wee1) and the S/G2 phase (Ccna2 and Cdk1) in a dose-dependent manner.

It is important to note that inconsistencies in the OTA genotoxicity results could be due to differences in the concentrations of OTA, the characteristics of the particular cell lines, the exposure time, the specific genotoxicity endpoint analysed, and the criteria used to assess a valid study and/or a positive response (Ali et al., 2011). Furthermore, it is important to remember that OTA is known for its rather high affinity to serum proteins. It is therefore possible that the serum content of the cell cultures under different culture conditions could affect the bioavailability and toxicity of OTA in *in vitro* systems (Degen et al., 1997).

Human lymphocytes are frequently exposed to low amounts of OTA under *in vivo* conditions. The amount of OTA in the plasma/serum of healthy volunteers from 23 European countries and several additional countries from around the world ranged from 0.15 to 9.15 ng/mL, with a mean of 0.45 ng/mL (74% of positive samples) (Coronel et al., 2010). Although these levels appear low, OTA has been associated with the induction of adverse effects in humans, especially in some specific cell types, such as urothelial cells. However, questions regarding the OTA genotoxicity at low concentrations remain unsolved (JECFA, 2006; 2010).

In conclusion, our results indicate that OTA induces DNA stable damage at low doses, which are neither cytotoxic nor cytostatic, and delays the DNA repair kinetics, thus inducing pivotal events in the carcinogenesis pathway of xenobiotics.

Conflict of interest

The authors declare that they have no conflicts of interest.

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4. General discussion

The research conducted in this PhD thesis was carried out to fulfil one of the objectives of the Spanish national research project "Integrated approach to simultaneous human exposure to ochratoxin A and deoxynivalenol" (project AGL2011-24862) for which the Applied Mycology Unit of the University of Lleida received a grant. The overall aims of the project were the "*in vitro* evaluation of the bioaccessibility of deoxynivalenol (DON) and ochratoxin A (OTA) in several food matrices, the effect of absorption and the cytotoxicity induced" and were thus clearly linked to the proposed objectives of this thesis. To accomplish these objectives, the studies carried out for this thesis were divided into two research lines – i) OTA bioaccessibility and ii) OTA toxicity – as presented in the "Objectives and work plan" section.

Due to the ubiquity of mycotoxins, the food matrix to be used in the bioaccessibility study was selected based on previous research by our research group on OTA exposure (Belli et al., 2004; Coronel et al., 2009, 2012; Valero et al., 2008) showing that red wine is one of the most frequently OTA-contaminated foods.

The OTA toxicity studies were planned taking into account previous results reported in the literature (Ali et al., 2011; Degen et al., 1997; Ehrlich et al., 2002; Fuchs et al., 2008; Knasmüller et al., 2004) and following the recommendations of the European Food Safety Authority (EFSA) on conducting toxicity and genotoxicity studies (EFSA, 2011), as well as the guidelines for *in vitro* assays published by the Organisation for Economic Co-operation and Development (OECD, 2010).

The results of our work are discussed in detail in the "Results and discussion" sections of each of the studies (Studies I to IV) comprising the Results chapter of this thesis. This "General discussion" section offers an overview of the results obtained, highlighting the most important findings and comparing them with those reported to date in the literature. It likewise addresses certain aspects of the experimental designs and methodological points that are not fully considered in the discussion sections of the corresponding studies.

4.1 Bioaccessibility of OTA

As mentioned in the Introduction section, bioaccessibility has only been evaluated in *in vitro* systems. Methodologically, *in vitro* bioaccessibility is a rapid and relatively inexpensive alternative for predicting relative bioavailability (Brandon et al., 2006). One of the aims of this PhD thesis was to assess OTA bioaccessibility in red wine at different levels of contamination. To this end, it was conducted a highly detailed review of the digestion models that have been used to study mycotoxin bioaccessibility (see Gonzalez-Arias et al., 2013; section 3.2 *In vitro* models used in bioaccessibility or absorption of studies on mycotoxins, page 192). In this regard, we concluded that *in vitro* digestion models (based on human or animal physiology and developed for compounds other than mycotoxins) are useful tools that can be used in our area of interest. These models usually simulate oral, gastric and small intestine digestion and, occasionally, include simulation of the large intestine. Moreover, most digestion models are developed to be easily operated and thus require minimal features for implementation, unlike the highly sophisticated models as TIM-1 and TIM-2 dynamic models. TIM systems mimic the gastric emptying and intestinal transit times, and a computer-controlled system introduces the simulated salivary, gastric, biliary and pancreatic juices at the appropriate moment. After comparing the numerous methods described in the literature, we selected two digestion models (based on a wide use in mycotoxins bioaccessibity studies and on the best composition of the digestions juices): 1) the RIVM model (Versantvoort et al., 2005) and 2) the Gil-Izquierdo model (Gil-Izquierdo et al., 2002), both *in vitro* digestion models developed and validated to evaluate digestion in simulated human conditions (Gil-Izquierdo et al., 2002; RIVM, 2004).

To assess the bioaccessibility of OTA in red wine, we decided to use the composition of digestive juices described in the RIVM model (Annex 2) and in the *in vitro* dynamic digestion model described by Gil-Izquierdo (Study I, Figure 1). As for the analytical method used to quantify the bioaccessibility of OTA in red wine (Study I), we chose the HPLC method described by Muñoz et al. (2010); the use of immunoaffinity columns was rejected because OTα is not recovered, since the antibodies are specific for OTA. The liquid-liquid extraction methods described by Muñoz et al. (2010) (for the OTA extraction from the gastric chyme) and Zimmerli and Dick (1995) (for the OTA extraction from the

intestinal chyme) were selected, and the in-house validation of the method yielded good recoveries for both toxins (62.4 to 89.55% for OTα and 75.57 to 89.19% for OTA). The EU has no established performance criteria for OTα analysis in foodstuffs, but all OTA recovery rates met the performance criteria established in EU Regulation 401/2006 (EC, 2006) for official control in foodstuffs, which may be used as a reference.

During the liquid-liquid extraction, we found that the gastrointestinal digestion samples were so large that handling them could be troublesome. The sample volumes ranged from 22 to 45 mL, depending on the chyme studied (gastric or intestinal), plus the extraction solvent added for OTA purification. Despite the technical difficulties, the results from Study I had little variability (a small standard deviation). Variability of results has been described by the US Environmental Protection Agency (EPA) and the Bioaccessibility Research Group of Europe (BARGE) in bioaccessibility studies of other contaminants. For example, in one study using a single soil sample, the bioaccessibility of arsenic varied from 10% to 59% and lead bioaccessibility ranged from 3% to 90% (EPA, 2005). Nevertheless, *in vitro* assessment of bioaccessibility is considered a good indicator of the maximal oral bioavailability, which can be used for risk assessment of a realistic worst-case scenario for a given compound in a food product (Brandon et al., 2006; EPA, 2005).

Our study of OTA bioaccessibility in red wine (Study I) found that the bioaccessibility depended on the section of the simulated gastrointestinal tract considered. Thus, the bioaccessibility of OTA in the simulated stomach was 102.8%, 128.3%, and 122.3% for the contamination levels assayed in the wine (5, 10 and 20 ng OTA, respectively). The percentage of bioaccessible OTA drastically decreased in the simulated intestinal section (ranging from 21% to 26%), in the presence of lipase and bile salts. With regard to the inherent properties of OTA, compounds that form complexes with proteins or mixtures of bile salt micelles (such as OTA) have been found to be less likely to be bioaccessible (Jovaní et al., 2001), unlike what happens in gastric conditions, where the lower number of proteins and low pH favour OTA stability, due to its acidic properties (Galtier, 1977; Kumagai and Aibara, 1982).

We also investigated the hydrolysis of OTA to OTα by gastrointestinal peptidases based on the initial OTA level in the wine (Study I). While this process was evidenced in our *in vitro*

digestion system, the formation of OTα was relatively low in both gastric (5.11-19.12% detected, from wine spiked at 20 ng OTA) and intestinal (only 5.1% detected) chyme. In the dialysed fraction, OTα was detected but it was not quantified because the signal was below the LOQ. Hydrolysis of OTA to OTα is probably higher in *in vivo* or *in vitro* systems involving intestinal cellular systems (enterocytes) than in our *in vitro* dynamic gastrointestinal system, where OTα formation was scarce. As in other studies of *in vitro* bioaccessibility, it is important to note that several biological factors were not taken into account for the formation of OTα, e.g. the interaction of the toxin with the intestinal microbiota in the large intestine (mainly anaerobic bacteria) (Madhyastha et al., 1992) or the metabolism of the toxins during their transport through the intestinal barrier (Berger et al., 2003; Schrickx et al., 2006).

Regarding the bioaccessibility results from other works, the bioaccessibility of OTA in the gastric chyme reported in this PhD thesis (102.8%, 128.3%, and 122.3%, as mentioned above) was as high as in other studies using the RIVM method (reported bioaccessibility percentages of 25 to 115%), in contrast with the OTA bioaccessibility in the intestinal chyme (21-26%). Interestingly, our results on bioaccessibility in the intestinal chyme were similar to those from studies including a dialysis membrane, as in the Gil-Izquierdo method (25-71%) (See the Table 3 from Annex I, Gonzalez-Arias et al., 2013). To this end, it should be noted that the *in vitro* digestion model used to assess bioaccessibility (static or dynamic) can influence the results obtained. Additionally, the use of different compositions of digestive juices can affect the bioaccessibility data obtained, as contradictory results have been reported when different rates of enzymes and food have been used (BARGE, 2015; Gonzalez-Arias et al., 2013).

Hence, the determination of oral bioaccessibility will have a strong significance as long as the results are similar to those from *in vivo* assays. In this regard, the EPA (2005; 2007) has recommended both the inter-laboratory validation of methods and the validation of results with *in vivo* data. We have not carried out an *in vivo* study but the bioaccessibility found in our bioaccessibility study does agree with results from *in vivo* studies (Galtier et al., 1981; Hagelberg et al., 1989). We obtained high values of *in vitro* bioaccessibility for OTA (102.8-128.3% in gastric chyme), in keeping with the *in vivo* bioavailability data in humans and in the most important animal species. For example, in humans, up to 84% of

OTA has been detected in plasma (Studer-Rohr et al., 1995); in animal species, the highest bioavailability has been reported in mice (92%), followed by pigs (65.7%), rats (61%), monkeys (57%), rabbits (55.6%), chickens (40%) and fish (1.6%).

Regardless of the contamination level assayed, OTA in red wine proved to be highly bioaccessible in gastric chyme, but not in intestinal chyme. Unlike the *in vitro* bioaccessibility, the *in vivo* bioavailability could increase, as OTA absorption depends on gastric emptying, mechanical movements, and the pH at the mucosal surface of the intestine (Gonzalez-Arias et al., 2013).

Other way to assess the bioaccessibility are the *in vitro* cultures using Caco-2 cells, a cell line from human epithelial colorectal adenocarcinoma. The Caco-2 cells differentiated monolayers achieve morphological/physiological function, like intestinal epithelium, during its growth in cell cultures. Therefore, mycotoxins passing through cell membrane can be measured in cell culture media. Normally, data are presented as relative percentage of toxin or as concentration in the basolateral compartment (in a Transwell® plates). In humans, an intervention study was conducted in human volunteers, using a chlorophyllin adsorbent (Egner et al. 2001). In *in vivo* studies, results are reported as bioavailability or presence of compounds in urine. Additionally to toxicokinetics of toxins, toxicity data can be obtained from animal/cellular models with/without adsorbents (toxin-DNA levels and albumin adducts, and biotransformation) and the results may be extrapolated and applied to human conditions for risk assessment (Breinholt et al., 1999; Kensler; et al., 1998). To our knowledge, only a study in humans has been published regarding ochratoxin bioavailabilty (Studer-Rohr et al., 1995). The results showed that OTA has a bioavailabity of 84% in plasma, similar to the observed in animal species, as mentioned above.

4.2 In vitro assessment of OTA toxicity

Three studies (Studies II, III and IV) were planned in the context of this PhD thesis for the *in vitro* assessment of OTA toxicity. The primary human lymphocytes and two human cell lines (Caco-2 and HepG2 cells) were used to perform the assays. To assess OTA toxicity, we selected several cytotoxicity assays widely used in the *in vitro* assessment of the toxicity of compounds, as well as an array of three genotoxic tests from amongst the

most common and well-accepted by the EFSA. These tests/assays helped to: a) predict potential genotoxic carcinogens, and b) contribute to understanding the mechanism of action recommended by the EFSA Scientific Committee (EFSA, 2011). There is likewise a need to avoid unnecessary animal tests (EFSA, 2011). A third endpoint not investigated in this thesis was the heritable damage in humans caused by OTA; this has been addressed in other studies with either positive or negative (Mosesso et al., 2008; NIH, 1989; Zepnik et al., 2001) results.

To establish the range of OTA doses for the treatments in this research, a review of the cytotoxic data on OTA was carried out. The most important references to this end were the studies by Akman et al. (2012), Ali et al. (2011), Degen et al. (1997), Ehrlich et al. (2002), IARC (1993), JECFA (2001), Kamp et al. (2005), Kuiper-Goodman et al. (2010), Lioi et al. (2004), and Simarro Doorten et al. (2006). We performed the cytotoxicity, genotoxicity and gene expression assays using doses lower than 45 µM for the treatments, since the chronic toxicity caused by OTA has been found to be associated with long periods of exposure to low levels of toxin (IARC, 1993; JECFA, 2001; Kuiper-Goodman et al., 2010).

4.2.1 Cytotoxicity of OTA in cultured human cells

The first stage of the cytotoxic assay was carried out in independent assays with primary human lymphocytes, and the Caco-2 cell and HepG2 cell line (Studies II, III and IV). The viability values (expressed as the percentage of viability compared to the solvent control) obtained in the different assays were over 60%, as recommended for the performance of both *in vitro* cytokinesis-block micronucleus (CBMN) assays (OECD, 2010) and *in vitro* comet assays (Tice et al., 2000).

Figure 1 summarises the viability values obtained in the three studies. These results were obtained using the MTT assay (Studies II and III), the trypan blue exclusion test and fluorescein diacetate assay (Study IV). In general, no large differences were found in cell viability in the three studies based on the different cell types used. We were thus able to use a similar range of OTA doses in all three studies conducted as part of this PhD thesis.

A higher loss of viability was observed in Caco-2 cells treated with doses higher than 75 µM OTA for 24 h (Studies III) and 48 h exposure (Studies II).

The viability values from the three studies (Studies II, III and IV) were similar to those reported by Ali et al. (2011) (using CHO cells and TK6 cells) and Ramyaa et al. (2014) (with HepG2 cells). Viability was approximately 70% when the OTA concentration was lower than 45 µM. The results from Study II show that OTA (80 µM) caused viability to fall to 60% in Caco-2 cells after a period of 48 h. Hundhausen et al. (2005) and Zheng et al. (2013) independently found that HepG2 cells were more sensitive than Caco-2 cells. A 50% loss in viability was detected in HepG2 cells treated with 35 µM OTA after an exposure of between 24 and 48 h (Hundhausen et al., 2005; Zheng et al., 2013). As with OTA nephrotoxicity, the highest decrease in viability was observed in OK cells, a cell line from the kidney of an adult female opossum. The exposure of OK cells to OTA at concentrations as low as 10 µM caused cell viability to fall to 60% or less after 24 and 48 h (Faucet-Marquis et al., 2006; Hadjeba-Medjdoub et al., 2012).

In order to compare the cytotoxic effect of OTA with that of other mycotoxins, a viability assay was performed using Caco-2 cells exposed to DON and Caco-2 cells co-exposed to OTA and DON (Study II). DON was chosen because it is a mycotoxin commonly found in co-occurrence with OTA in foods (Streit et al., 2013). Since the toxicity of DON was higher than the toxicity of OTA, co-exposure to OTA (20, 40 and 80 µM) and DON (0.5, 1 and 5 µM) increased the toxicity for the cells. Co-exposure to the mycotoxin mixture resulted in a loss of cell viability (64.1%) at lower concentrations of OTA (20 µM) and DON (0.5 µM) as compared to that observed in the single treatments, in which OTA and DON at the referred doses led to 95.18% and 96.27% cell viability, respectively.

There are many ways to assess viability/cytotoxicity and each one provides information about different cellular processes (Stoddart, 2011). As noted in the work plan section, this PhD thesis used different methods to assess OTA cytotoxicity. The most interesting findings are described below. For example, in the study of MN induction (Study IV), the cytostaticity caused by OTA was also determined. In this case, the cytostatic effect was determined at 15 µM OTA using human lymphocyte cells.

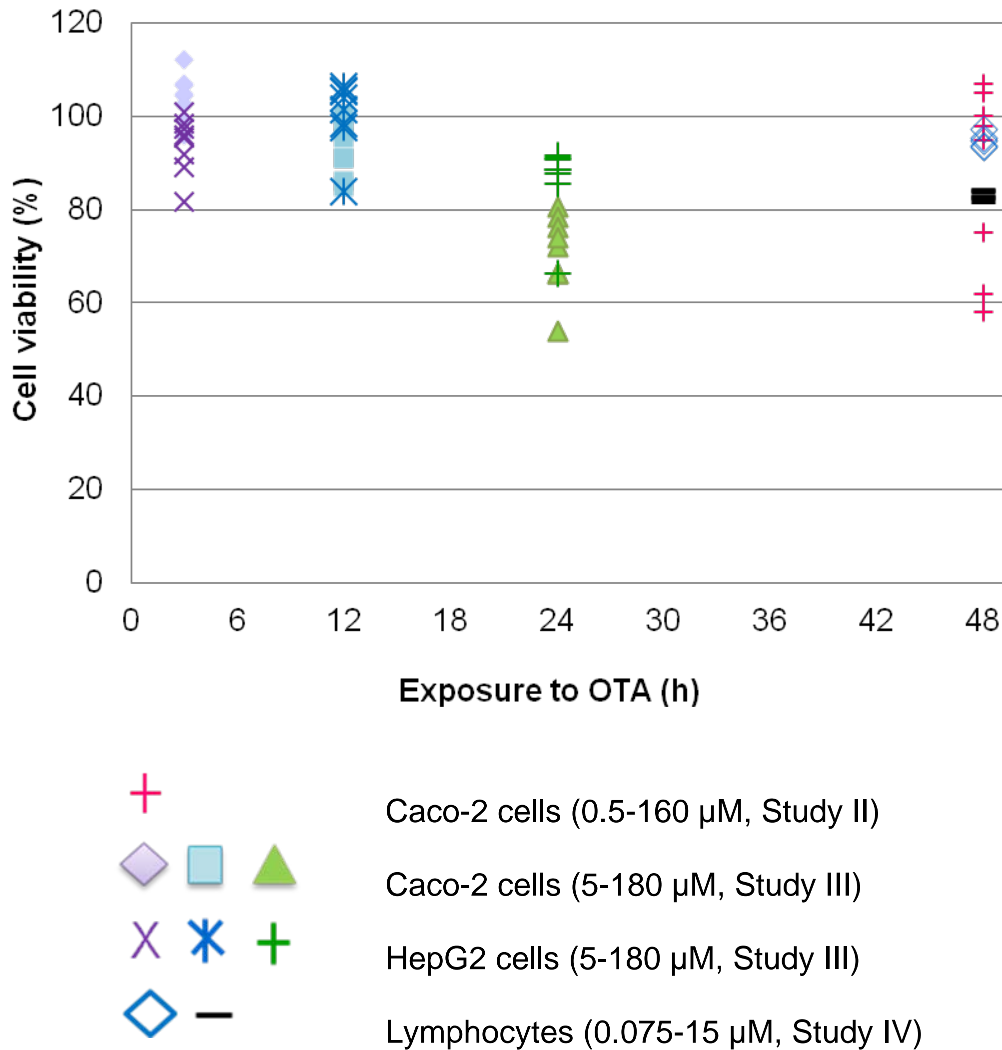

Figure 1. Mean of cell viability (percentage relative to controls) in Caco-2, HepG2 and lymphocites exposed to OTA. The viability assays used to determine the effect of OTA on cell viability were: an MTT assay (Study II and III) and the trypan blue exclusion test (—) and fluorescein diacetate assay (◇) (Study IV).

Interestingly, this OTA concentration did not cause a high loss of viability (88% cellular viability) compared to the solvent control. The cytostatic effect refers to the suppression of cell growth (cellular biomass) and proliferation (cell cycle arrest) (Fenech, 2007; MacLaine et al., 2008). Neither biomass nor cell cycle arrest were determined during the different assays conducted in this thesis, but a decrease in cellular density could be observed under

a microscope compared to the cell cultures treated in control conditions (DMSO as vehicle control), or with the lower concentrations of OTA (Studies II, III and IV).

The study of membrane integrity and ROS production are other methods for assessing cytotoxicity. Both assays were conducted on Caco-2 cells (differentiated and undifferentiated, respectively). To evaluate membrane integrity, the change in transepithelial electrical resistance (TEER) before and after treatment with OTA was recorded using a TEER system (Figure 2a and 2b). The results of the TEER measurements showed that the Caco-2 monolayers (cells grown in a transwell plate, Figure 2c) were depolarised (decrease in TEER) after 24 h of exposure to OTA, regardless of the OTA concentration.

Interestingly, in our study (Study III), TEER decrease and loss of cell viability were detected in cell cultures exposed to OTA for 24 h. The mean TEER values of the monolayers exposed for 24 h were 63.6%, 61.5% and 64.6% for 5, 15 and 45 μM OTA, respectively, in sharp contrast to the results of the TEER measures at 3 h and 12 h, in which OTA did not cause any significant decrease. Our findings revealed cellular damage to the intestinal epithelial cell tight junction proteins and increased cell permeability.

Maresca et al. (2001) recorded a 50% decrease in TEER after treatment with 100 μM OTA (48 h of exposure), which was related to a decrease in whole cell protein content, the inhibition of cell growth and morphological changes. Like Maresca et al. (2001), we did not observe a time-dependent decrease in TEER over a 24 h period. Possibly, such a time-dependent response was not observed due to the low range of OTA doses assayed (0.001-1 μM OTA for 48 h) (Maresca et al., 2001).

With regard to ROS production due to OTA exposure, both *in vitro* (Kamp et al., 2005; Mally et al., 2005; Schaaf et al., 2002) and *in vivo* (Hsuuw et al., 2013; Petrik et al., 2003) studies have been described in the literature. Our findings suggest that low doses of OTA do not cause significant ROS production in undifferentiated Caco-2 cells (Study II). Only cells treated with > 80 μM OTA for 48 h showed an increase in ROS production with a parallel reduction in cell viability. This finding showed that the ROS produced after a long period of exposure to OTA (48 h) can be neutralised by the antioxidant mechanisms in the cell (Study II), but also that the pathway of oxidative damage to DNA acts early (as

observed in human lymphocytes) (Study IV) (this latter finding will be discussed in greater detail in the section on OTA genotoxicity). It is therefore possible that OTA concentrations higher than 20 μM play an important role in cytotoxicity due to a free radical-mediated mechanism in short periods of exposure.

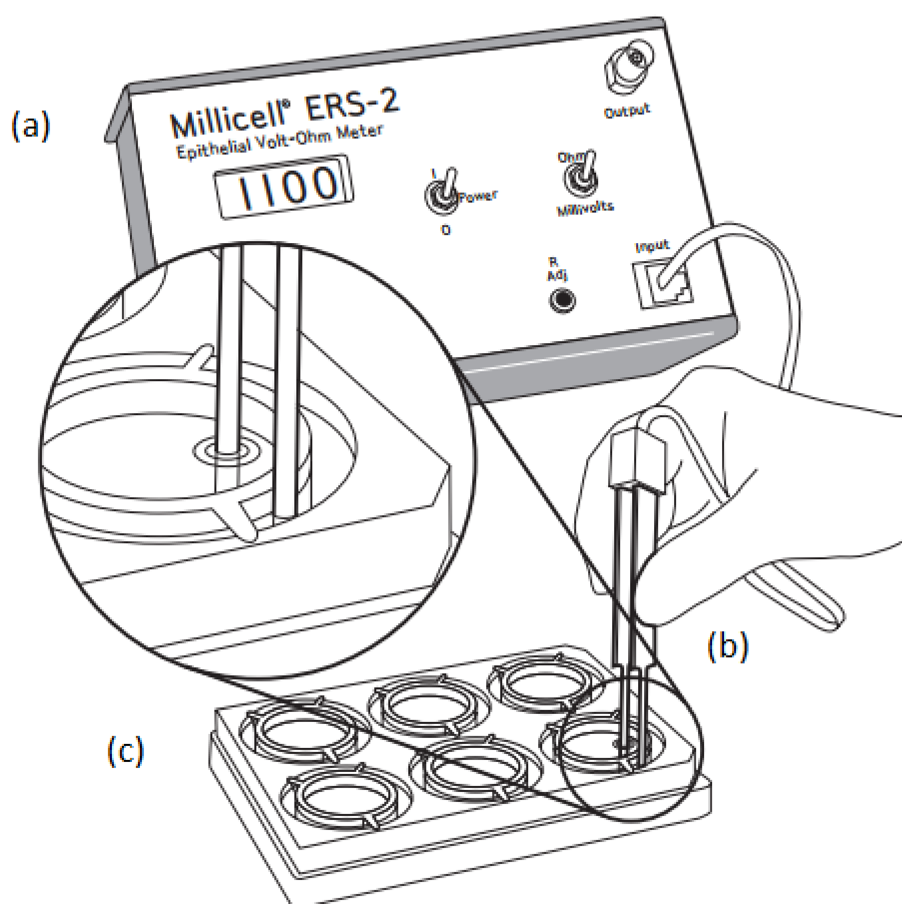

Figure 2. Transepithelial electrical resistance (TEER) measurement: a) Millicell-ERS-2 model by Millipore® (Bedford, MA); b) STX01 electrode; and c) transwell plate with microporous membrane inserts.

This study also looked at the capacity of OTA to induce oxidative stress in combination with DON, as well as the protective effects of resveratrol (RES) (an antioxidant present in grapes and wine) in Caco-2 cells (Study II). The oxidative stress induced by these three

compounds was measured in terms of the fluorescence intensity of the DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) hydrolysed by metabolically active cells. The OTA-DON mixtures did not increase the ROS production observed at the highest concentration of OTA (80 μM), but a reduction in the cellular antioxidant defence of Caco-2 cells was observed after treatment with a mixture of OTA (80 μM) and RES (40 μM and 160 μM RES). Other authors have also described the non-protective effect of antioxidant compounds, including increased ROS production. For example, Juan et al. (2008) reported increased mitochondrial ROS in a human colon cancer cell line (HT-29) exposed to RES (150 μM) for 4 h, and Badding et al. (2014) reported a positive association between loss of viability and the non-protective effect of the antioxidant *N*-acetyl-L-cysteine in RAW 264.7 mouse macrophages. In case of resveratrol, a polyphenolic compound described as a strong antioxidant, its antioxidant activity is associated with its ability to scavenge free radicals, donate hydrogen, break radical chain reactions, chelate metals, and quench singlet oxygen *in vitro* and *in vivo* (Duthie et al., 2000; Perron et al., 2009).

As mentioned in study II, the mechanism triggering the cytotoxic effect of DON and OTA enhanced by RES or the mechanism triggering the enhanced production of ROS by RES itself, respectively, cannot be completely elucidated with the present data. The research conducted in the Study II of this thesis is the first study in which the *in vitro* toxicity of the co-exposure of DON and OTA has been studied in the presence of RES.

Despite of an increase in ROS levels from *in vitro* assays (Study II), epidemiological studies have revealed a positive association between the consumption of phenolic-rich foods and the prevention of oxidative stress-related diseases by dietary antioxidants (Vang et al., 2011; Willcox et al. 2008), being RES one of the most abundant antioxidant compound in the diets (Burns et al., 2002; Fernández-Mar et al., 2012). Results in literature support our findings, and it was described that RES can modulate multiple vias that elicite to apoptosis and cell damage. For example: 1) cells damage related with a response mediated by the caspase-3 (Fouad et al., 2013; and Pasciu et al., 2010); 2) the proliferation inhibition and apoptosis induction through the mechanism involving transforming growth factor and Smad proteins (TGF-b1/Smads) (Zhai et al., 2015). Further investigations will be required to elucidate an action mechanism with the Study II conditions.

4.3 Gene expression

In order to assess the effect of OTA on the expression of certain genes, the mRNA levels were quantified using real-time-qPCR. To carry out this study (Study III), we used an *in vitro* cell co-culture system (Figure 3) to mimic initial passage through the intestine and hepatic metabolism, using Caco-2 and HepG2 cells. To assess the effect of OTA over time, we assayed periods of 3, 12 and 24 h of exposure. The *CYP1A1*, *CYP2A6*, *CYP2B6*, *CYP3A4* and *CYP3A5* genes from the *CYP450* family; the *NAT2* xenobiotic metaboliser involved in Phase II; the *COX-2* and *5-LOX* inflammatory markers; and the *MRP2* transporter were studied.

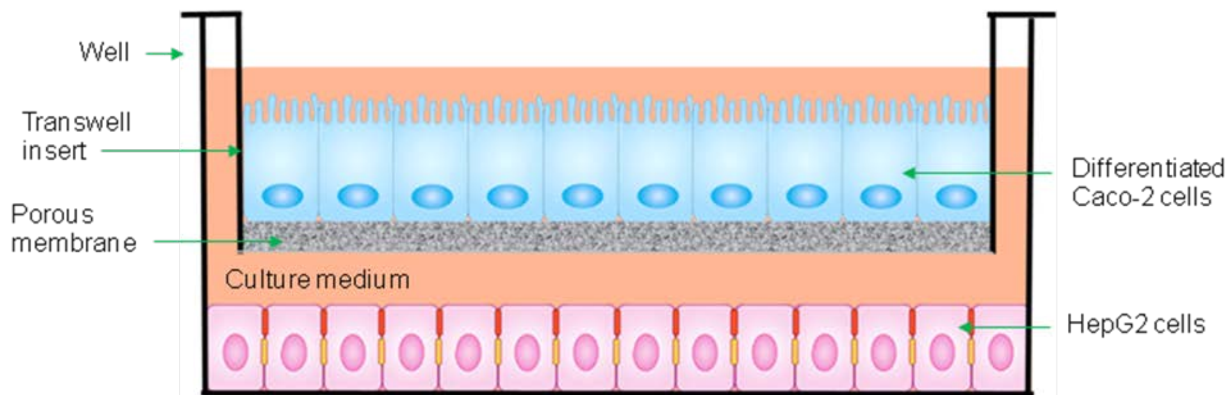

Figure 3. Co-culture of differentiated Caco-2 cells and HepG2 cells in a transwell plate with microporous membrane inserts.

The results (Study III) in differentiated Caco-2 cells showed that expression was down-regulated in most genes. Amongst the genes from the *CYP450* family, the *CYP1A1* gene was the most down-regulated by OTA in all treatments. Monolayer exposure to OTA for 12 h was found to have the greatest effect on *CYP1A1* expression (52-fold decrease), whereas *CYP1A1* was less modulated by OTA in cells exposed for 24 h.

The *CYP2A6* and *CYP2B6* genes from the *CYP2* family were slightly down-regulated by OTA within the first 3 hours. No additional significant changes were detected in cells exposed for 12 and 24 h. Interestingly, OTA did not modulate the expression of the

CYP3A4 gene in cells exposed for 3 h. Nonetheless, treatment with 45 µM (24 h) caused a significant difference in the expression level of *CYP3A4*. To date, few studies have been conducted on the toxicodynamics of OTA in relation to the genes that encoded for metabolising enzymes in the intestinal epithelium, as research has tended to focus on absorption mechanisms. In this sense, with regard to *CYP1A1*, our results for gene expression differ from those of the study performed at the protein level by Ribonnet et al. (2011). They measured the activity of the CYP1A1 and CYP3A4 enzymes from Caco-2 cells and found that OTA increased CYP1A1 activity and inhibited CYP3A4 activity.

With regard to the results obtained in hepatic cells, OTA caused both up- and down-regulation. Unlike the results obtained in Caco-2 cells, the expression level of *CYP1A1* showed a significant up-regulation, mainly in cells exposed for 24 h. In the case of hepatocytes, *CYP3A4* expression increased significantly after 12 h, and the expression level was stable for up to 24 h of exposure to OTA. In contrast, *CYP3A5* did not show a significant response to exposure to OTA, as exposure to 45 µM OTA for 24 h had only a slight down-regulation effect. As for the *CYP2* family in hepatic cells, *CYP2A6* mRNA was not detected, and the *CYP2B6* gene was down-regulated within the first 3 h, whereas an up-regulation effect was observed in cells exposed to OTA for 12 and 24 h.

The above results for gene expression over time (3, 12 and 24 h) suggest that the changes in the expression level are the result of (Chung et al., 2008; Morgan, 2001; Ramyaa and Padma, 2013):

a) stress signals caused by toxicity,
b) an adaptive response that promotes the controlled generation of ROS, nitric oxide or arachidonic acid metabolism.

This was observed in Study II, where OTA induced different level of ROS production depending on the OTA dose assayed, as well as in Study III, where changes were detected in the mRNA expression levels of genes involved in the metabolic response and inflammation (to be discussed in greater detail below).

In parallel, the expression of the *NAT2* gene was assessed. The *NAT2* gene has a very strong epidemiological association with a variable risk of urinary bladder cancer, possibly due to impaired detoxification of carcinogenic metabolites in the liver (Hein et al., 2000; Husain et al., 2007). To our knowledge, the expression of the *NAT2* gene has not been studied previously in cell cultures after exposure to OTA. Only Lebrun and Follmann (2002) reported a high correlation between *NAT2* polymorphisms and DNA damage in an *in vitro* culture of OTA-exposed human urothelial cells. It is important to highlight the results obtained with the *NAT2* gene in this thesis (Study III): amongst the genes tested, the *NAT2* gene was the second most OTA-modulated gene in Caco-2 cells (after a period of 12 and 24 h of OTA exposure); this stands in contrast to HepG2 cells, in which the *NAT2* gene was both up- and down-regulated.

The inflammatory response was evaluated by determining the mRNA levels of the *COX-2* and *5-LOX* genes. In Caco-2 cells, OTA presence caused a higher decrease in *COX-2* mRNA expression than in *5-LOX* mRNA expression in a time-dependent manner. This is in keeping with what has been observed for most genes, but in contrast to what has been observed in the HepG2 cells, in which OTA caused changes in the expression level over time. For example, the inflammatory markers were slightly modulated by OTA at 3 h of treatment, but after 12 h of exposure, both *COX-2* and *5-LOX* genes appeared up-regulated. As in other studies, the *5-LOX* gene did not maintain the same level of expression over the course of the 24 hours of exposure to OTA. In this regard, Kumar et al. (2013) and Ramyaa et al. (2014) described an increase in the mRNA level over a period of less than 24 h.

Regarding the mechanism of OTA secretion in the cells, our results suggest a possible impairment in OTA secretion at the intestinal and hepatic levels due to *MRP2* repression, especially in the enterocytes. The repression of the *MRP2* gene suggests an increase in *in vivo* bioavailability due to a decreased first-pass effect in the small intestine, as the function of the MRP2 transporter is the active transport of anionic conjugates and drug extrusion from the intestine, although it is also expressed in the liver, kidney-proximal tubules and other cells (Jedlitschky et al., 2006). At the hepatic level, the *MRP2* repression supports the finding that OTA is not highly excreted in bile, resulting in an increase in its plasmatic half-life.

The induction of genes in response to xenobiotics has been previously studied, and the mechanism is largely understood. For instance, it is known that *CYP450* genes show similar responses 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). In the case of OTA, some genes have been linked to OTA metabolism/biotransformation (Marin-Kuan et al., 2006). Nevertheless, the mechanism of action through which the *CYP450* and other genes can be suppressed remains unclear (Marin-Kuan et al., 2006; Ramyaa and Padma, 2013).

In summary, our transwell cell system allowed us to obtain data from both types of cells during the same OTA exposure, mimicking intestinal absorption and hepatic metabolism. The analysis of gene expression showed that the modulation of gene expression by OTA was higher in the Caco-2 cells than in the HepG2 cells. The *CYP1A1* gene was the most modulated by OTA in Caco-2 cells, followed by the *CYP3A4* and *CYP3A5* genes. In contrast, in HepG2 cells, *CYP2B6* was highly regulated at 3 and 12 h as compared with the other cytochrome genes. Moreover, the *CYP1A1* gene was slightly modulated during the first 12 hours, but over-expression was observed at 24 hours. Our data support the findings on the effects of OTA on the *COX-2* and *5-LOX* genes during liver metabolism. Thus, the *COX-2* and *5-LOX* genes may be proposed as early inflammatory markers of OTA exposure. All this suggests that OTA acts on the same genes in a different way depending on whether the toxin is in the enterocytes or the hepatocytes. Additionally, we were able to provide evidence of the effect of OTA on the *NAT2* gene expression, which had not been previously reported.

4.4 Genotoxicity of OTA

Genotoxicity is a broad term and refers to processes that alter genetic materials (DNA, histones and chromosomes). Genotoxic processes have been described in both germinal and somatic cells. We used the alkaline comet assay and the MN test to assess OTA genotoxicity *in vitro*. Both the comet assay and the MN test provide data on damage to DNA through different action mechanisms (both direct and indirect) (Collins, 2014; Fenech et al., 2003). A DNA repair assay with N-hydroxyurea (NHU) was carried out to obtain

data on the effect of OTA on cells' DNA repair systems (Collins, 2004). This repair assay is a modified comet assay and was conducted in conjunction with a second comet assay in order to compare the repair capacities of cells exposed to OTA and NHU. In the latter case, only the OTA concentrations that caused significant damage in the DNA (as revealed in the first comet assay) were used in the DNA repair assay.

The alkaline comet assay was conducted for 3 h, and the results after the exposure to OTA showed that the mycotoxin had caused DNA single- and double-strand breaks. An increase in the percentage of DNA in the tails of the comets was observed in cells treated with 0.075, 1.5 and 5 μM OTA, although no dose-response pattern was detected. The results of the comet assay provide direct evidence regarding DNA damage at low doses. Possibly, these OTA doses do not generate detectable free radicals, as was observed in Study II, in which low OTA doses did not result in increased ROS production.
As mentioned above, the repair of specific DNA lesions by cellular repair processes was monitored by means of a DNA repair assay (Study IV). Thus, to enable comparison of the effects caused by OTA, we exposed cells to 1.5 or 5 μM OTA (concentrations that increased the percentage of DNA in the comet tails) jointly with NHU and hydrogen peroxide (H_2O_2). The repair assay was conducted in parallel with a second comet assay to compare the delay in the repair pathway within a period of 0-60 minutes. The use of short periods of time has been suggested in the literature (Collins, 2004), as strand breaks are quickly repaired during the activation of the excision repair pathway. This mechanism can be inhibited by co-exposure to DNA synthesis inhibitors, such as NHU.

In this study, 1.5 and 5 μM OTA inhibited DNA repair in the presence of 10 mM NHU by blocking DNA synthesis, leading to an accumulation of breaks. The accumulation of breaks resulted in an increased percentage of DNA compared to non-NHU-exposed cells. Interesting results were observed using the repair assay in lymphocytes. For example, OTA treatment with NHU led to different results from OTA+H_2O_2 treatment at 60 min without NHU. Similarly, in the OTA+H_2O_2 treatment, the repair effect at 60 min was different from that observed at 30 min. Güerci et al. (2009) used NHU to investigate the repair activity of human lymphocytes. As in this study, those authors assayed ethyl and methyl methanesulfonate (compounds with alkylating activity) during a repair assay with 10 μM NHU, as in the present thesis. Methanesulfonates did not cause delays in the repair

pathway in human lymphocytes co-exposed to 10 µM NHU. Like OTA (Group 2B), these methanesulfonate compounds are also classified as Group 2 by the IARC (Group 2A and Group 2B, respectively). It can thus be stated that OTA is able to inhibit a repair pathway that other compounds from the same group (Group 2) cannot, indicating that OTA causes damaged DNA to accumulate in cells.

Additionally, an MN test was performed (Study IV) using the same range of micromolar OTA concentrations. The identification of MN showed a significant increase in lymphocytes treated with 1.5 and 5 µM OTA, for which the mean MN was 4.6 ± 1.8 and 7.7 ± 1.3 MN/1000 binuclear cells, respectively. The MN induction was not quantified in cells treated with 15 µM OTA due to the cytostatic effect observed, and discussed in the section on cytotoxicity above. In this case, OTA cytostaticity was evidenced by high cell viability (88%) in the absence of binuclear cells in treatments done with 15 µM OTA.

OTA-based MN induction has been reported in the literature at doses higher than 5 µM (Degen et al., 1997; Dopp et al., 1999; Fuchs et al., 2008; Klarić and Pepeljnjak, 2008; Knasmüller et al., 2004). Other studies conducted with a wide range of OTA concentrations have reported MN induction at doses higher than 25 µM, and have not shown a dose-response pattern (Ali et al., 2011; Dönmez-Altuntaş et al., 2003; Ehrlich et al., 2002). Unlike the aforementioned studies, our results with the MN assay showed that OTA is a micronuclei inducer at concentrations as low as 1.5 µM. With regard to the statistics of the results of the comet assay, Figure 1 and 2 of Study IV (page 122 and 123) showed a high standard deviation. The protocol of the comet assay was published by Tice et al. (2000), but with regard to the statistical treatment of data, the authors does not provide any useful guidance. At the International Workshop on Genotoxicity Test Procedures, the expert panel did not infom of the most appropriate statistical method(s) to be used, and there was no consensus in this regard (Tice el al., 2000). Nowadays, the prevailing idea is that the kind of sample used (animals per group or cultures per treatment, number of gels per animal/culture and number of measured cells per gel) has a big influence on the result, as with the comet assay the risk in neglecting the hierarchical nature of data must be taken into account (Tice et al., 2000). Our results of Study IV are shown as the mean ± SD of three independent experiments run in duplicate, and the size sample by slide was higher than 50 cells/slide. An experimental desing like this is well

accepted, and it was pointed out that experimental data from comet assays do not show a normal distribution (Tice et al., 2000). Until now, literature describes multiples ways, as suggestions, to carry out a statical analysis in the comet assays (Bright et al., 2011; Wiklund et al., 2003).

In summary, Study IV allowed us to conclude that OTA is an MN inducer that also causes stable damage to DNA through an oxidative via without a dose-response pattern at doses that do not cause losses in viability or cytostaticity. Our results moreover showed an indirect mechanism of action of OTA – the inhibition of the DNA repair pathway – in addition to MN induction and the induction of DNA single-strand and double-strand breaks.

Recent decades have seen considerable debate about the genotoxicity of OTA and its role in the carcinogenic process (Akman et al., 2012; González-Arias et al., 2014; Mosesso et al., 2008; Pfohl-Leszkowicz and Castegnaro, 2005; Turesky, 2005; Yang et al., 2014). This debate over the carcinogenicity of OTA has not yet been resolved, and data such as ours suggest that exposure to low doses of this toxin can cause serious damage. The many effects of OTA have attracted significant public health attention; consequently, OTA surveys in foods and feeds have been conducted worldwide.

International and national food safety organisations (EFSA, JECFA, ILSI, Health Canada) have reviewed these data and defined a risk assessment of OTA in foods to provide insight into its health significance (JECFA, 2008; Kuiper-Goodman et al., 2010). The approaches used by these expert groups to establish the safe levels of OTA exposure have been based on uncertainty factors (key events compatible with a threshold effect). As a result, amongst the possible mechanisms of action, oxidative stress has been presented as one of the most probable (JECFA, 2007; Kuiper-Goodman et al., 2010).

The results of the study conducted as part of this thesis (Study IV) indicate that OTA is a genotoxic mycotoxin. Moreover, other cellular processes (ROS induction at high doses and gene expression modulation) and the effect of OTA dose on cells have been implicated in the toxicity induced by this mycotoxin, as observed in Studies II and III. These findings indicate that OTA affects pivotal events in the carcinogenesis pathway, and all these

mechanisms of action have been related to OTA toxicity at non-cytotoxic doses during long-term exposure (González-Arias et al., 2014; Horvath et al., 2002; Qi et al., 2014).

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5. Conclusions

- The *in vitro* dynamic digestion system used is a good tool to assess the bioaccessibility of OTA and may help researchers understand and monitor the complex processes of transformation that take place in *in vivo* gastrointestinal digestion. In red wine, and at the assayed concentrations (1.0-4.0 µg/L), ochratoxin A (OTA) bioaccessibility was not affected by the initial contamination level in an *in vitro* simulated gastrointestinal system. OTA showed a high bioaccessibility after gastric digestion (103-128%) at pH 2-3, whereas at the intestinal level, at pH 7.2-8.0 and in the presence of bile salts, OTA bioaccessibility decreased to levels below 26%. This fact suggests that *in vivo* absorption of OTA could depend on pH and other environmental conditions.

- Low doses of OTA did not cause less than 65% cell viability in the three human cell types assayed; however, they did cause cytostaticity by inhibiting cell proliferation in human lymphocyte cells and decreasing transepithelial electrical resistance in polarised monolayers of Caco-2 cells.

- ROS production occurred in parallel to a significant decrease in the viability of cells treated with the highest OTA concentrations. A joint treatment with OTA and DON increased cytotoxicity in Caco-2 cells, whereas the antioxidant resveratrol did not protect against the deleterious effect of these mycotoxins.

- In an *in vitro* co-culture system with Caco-2 and HepG2 cells, OTA caused a down-regulation in the gene expression of most of the *CYP450* genes in differentiated Caco-2 cells; in contrast, both up- and down-regulation were detected at different exposure times in gene expression in HepG2 cells. The *CYP1A1* gene was highly modulated by OTA in all treatments in both Caco-2 and HepG2 cells, while the *CYP3A5* gene was the second most OTA-modulated gene in Caco-2 cells. The *CYP2B6* and *CYP3A4* genes were moderately modulated in HepG2 cells, while the *CYP1A1* gene was weakly modulated by OTA in HepG2 cells.

- *NAT2* gene expression was strongly down-regulated by OTA in Caco-2 cells, but was usually moderately down-regulated in HepG2 cells, except when exposed to OTA for 24 h.

- The expression of the *MRP2* transporter gene decreased in the presence of OTA, suggesting an increase in *in vivo* bioavailability through a lowering of the first-pass effect in the small intestine and poor excretion of OTA in the bile.

- In general, longer exposure times to OTA had stronger effects on gene expression modulation and membrane integrity than increasing the OTA dose used in the treatments.

- OTA proved to be a genotoxic mycotoxin at low doses (0.075-5 μM) in human cell cultures. OTA induced micronuclei, and, in the comet assay, led to an increase in the DNA percentage and DNA accumulation in the comet tails, indicating DNA damage and a delay in the DNA repair system, respectively.

6. Further research

Since both the BARGE and the EPA recommend the validation of methods to assess the bioaccessibility of compounds, complementary *in vivo* assays should be conducted to validate the bioaccessibility data for OTA obtained using the *in vitro* digestion model described in this research.

OTA bioaccessibility should be studied in other liquid or solid food matrices, as well as in the presence of other mycotoxins. In this regard, the efficacy of the *in vitro* system employed could be compromised, as the recoveries and chromatographic determination of toxins could be profoundly affected due to the matrix effect. Thus, validation of the system for more complex foods will be necessary in future.

The expression levels of the *CYP1A1*, *CYP2B6*, *CYP3A4* and *CYP3A5* genes should also be investigated at the protein level in the presence of OTA, in order to understand the real OTA effect on gene expression modulation and to establish a positive/negative correlation. In addition, there is a need to investigate the involvement of the *NAT2* gene during OTA metabolism more deeply.

This research found that OTA is able to modulate the expression of the *COX-2* and *5-LOX* genes. Nevertheless, more studies are needed before these genes can be proposed as early inflammatory markers of OTA exposure.

Particular attention should be given to the OTA doses that cause cytostaticity and DNA damage, which have not been related to the production of ROS at detectable levels in the cells, in order to determine an alternative mechanism of action linked to the doses assayed in the treatments.

Using the transwell system employed in this thesis, an *in vitro* study of the biotransformation of OTA into other metabolites should be carried out to complete the toxicity studies conducted in this research. To this end, it should be noted that such a study is currently being conducted.

Annex I

Review

Mycotoxin bioaccessibility/absorption assessment using *in vitro* digestion models: a review.

González-Arias, C.A., Marín, S., Sanchis, V. and Ramos, A.J.*
Food Technology Department. Lleida University. UTPV-XaRTA, Agrotecnio Center.

Av. Rovira Roure 191. 25198 Lleida. Spain. ajramos@tecal.udl.es

Abstract

In the evaluation of the oral bioavailability of a mycotoxin, the first step is the determination of its bioaccessibility, i.e., the percentage of mycotoxin released from the food matrix during digestion in the gastrointestinal (GI) tract that could be absorbed through the intestinal epithelium. As a first approximation to the problem, different *in vitro* digestion models have been recently used for bioaccessibility calculation, thereby avoiding the use of more complex cell culture techniques or the use of animals in expensive *in vivo* experiments.

In vitro methods offer an appealing alternative to human and animal studies. They usually are rapid, simple and reasonably low in cost, and can be used to perform simplified experiments under uniform and well-controlled conditions, providing insights not achievable in whole animal studies. The available *in vitro* methods for GI simulation differ in the design of the system, the composition of the physiological juices assayed, as well as in the use or not of intestinal microbiota. There are models that only simulate the upper part of the GI tract (mouth-stomach-small intestine), whereas other methods include the large intestine, so that the model chosen could have some influence on the bioaccessibility data obtained.

Bioaccessibility depends on the food matrix, as well as on the contamination level and the way the food/feed is contaminated (spiked or naturally).

This review focuses on the currently available data regarding the *in vitro* digestion models for the study of the bioaccessibility or absorption of mycotoxins, detailing the characteristics of each digestion step and the importance of the physiological juices employed during digestion. The effect that different factors play on the mycotoxin release from food matrix in the GI tract is also considered, and existing data on bioaccessibility of the main mycotoxins are given.

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1. Introduction

Mycotoxins are a wide group of fungal secondary metabolites that exert multiple toxic effects on humans and animals. Some mycotoxins can cause autoimmune illnesses, have allergenic properties, and some of them are teratogenic, carcinogenic, mutagenic, nephrotoxic or estrogenic (CAST, 2003). Although hundreds of mycotoxins exist, the most important for public health are aflatoxins (AFs, aflatoxins AFB_1, AFB_2, AFG_1 and AFG_2 being the main ones), ochratoxin A (OTA), patulin (PAT), fumonisins (FBs) zearalenone (ZEA), and the trichothecene group, among them deoxynivalenol (DON), nivalenol (NIV), T-2 and HT-2 toxins being the most important. Recently, other mycotoxins - the so-called emerging mycotoxins, such as fusaproliferin, beauvericin (BEA), enniatins (ENs), moniliformin and the *Alternaria* toxins - are attracting the attention of researchers.

The major mycotoxin-producing fungal genera are *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* (Moss, 1992; Sweeney and Dobson, 1998). The growth of mycotoxigenic strains of these fungi on crops, either in the field or during storage, could lead to the accumulation of mycotoxins in a great variety of foods. Besides, the metabolism of ingested mycotoxins could result in modified mycotoxins, as happens when aflatoxin B_1 (AFB_1) is converted by hydroxylation to aflatoxin M_1 (AFM_1), mycotoxin mainly present in milk as a result of AFB_1 metabolism in cow and other mammals (Prandini et al., 2009).
Natural occurrence of mycotoxins in food has been broadly documented. Thus, mycotoxins have been widely detected in food of vegetal origin, mainly in cereals (barley, wheat, corn, oat, etc.) and their by-products (Marín et al., 2012; Rodrigues and Naehrer, 2012), as well as in nuts, dried fruits, spices, cocoa, coffee, beer, wine, and fruits, particularly apples (Bellí et al., 2002; Fernández-Cruz et al., 2010; Molyneux et al., 2007; Placinta et al., 1999; Santos et al., 2010; Turcotte et al., 2011). Mycotoxins also enter the human food chain via meat or other animal products such as eggs, milk and cheese as a result of contaminated livestock feed (Chen et al., 2012; Meyer et al., 2003).
This huge variety of food matrices in which mycotoxins occur can have a very significant effect on the bioavailability of mycotoxins, as complex and diverse reactions can occur between mycotoxin and the food matrix, which could interfere in the way these toxins are absorbed through the intestinal tract.

On the other hand, in the last years many structurally related compounds generated by plant metabolism or by food processing have been described in mycotoxin-contaminated commodities, which can co-exist together with the native toxins. These mycotoxin derivatives (named conjugated or "masked" mycotoxins) may have a very different chemical behaviour, thus they can easily escape routine analyses. Nevertheless, these forms could be hydrolysed to their precursors in the digestive tracts of animals or could exert toxic effects comparable to those imputable to free mycotoxins (De Saeger and van Egmond, 2012; Galaverna et al., 2009). This can make in some occasions very difficult to establish a clear relationship between the amount of ingested toxin and the toxic effects observed, and expected, for a given amount of mycotoxin. All these facts could help to explain the so-called "fumonisin paradox" (i.e. the fact that apparently low contaminated commodities induce severe toxic effects in animals), whereby the oral bioaccessibility of this mycotoxin could be affected by different factors, one of them the uptake of fumonisin B_1 (FB1) strongly conjugated to the food matrix or FB_1 derivatives with higher bioavailabilities (Shier, 2000). This could also happen with other mycotoxins.

So, knowing the amount of mycotoxin ingested may not be enough for exposure assessment. Knowing the amount of toxin that becomes available for absorption through the intestinal epithelium (which will be the measure of its bioaccessibility) is also required. Different factors, as pH changes, enzymatic activities, etc., play an important role during the gastrointestinal transit of mycotoxins and thus affect bioaccessibility.
To determine the bioaccessibility of mycotoxins (or in same cases, the absorption), and as a first approximation to the problem, different *in vitro* digestion models have been used, avoiding the use of more complex cell cultures or the ethically questionable use of animals in *in vivo* experiments.

This review focuses on the currently available data regarding the *in vitro* digestion models for the study of the bioaccessibility of mycotoxins, detailing the characteristics of each digestion step and the importance of the physiological juices employed during digestion. The effect that different factors play on the mycotoxin release from food matrix in the GI tract is also considered, and existing data on bioaccessibility of the main mycotoxins are given.

2. Bioaccessibility and bioavailability

The amount of mycotoxin consumed via food does not always reflect the amount of this compound that is available to exert its toxic action in a target organ of the body, as only a part of the ingested compound will be bioavailable. Thus the oral bioavailability (F) of a mycotoxin has been defined as the fraction of an orally ingested mycotoxin, in a certain food matrix, that finally reaches the systemic circulation and is distributed throughout the entire body to exert its toxic effect (Versantvoort, 2004). This definition assumes that toxicity is exerted by the parent compound and not by formed metabolites.

In fact, the oral bioavailability comprises three different and sequential processes (Brandon et al., 2006):

a) the release of the mycotoxin from the food matrix during digestion in the GI tract. In this step we are measuring the bioaccessible mycotoxin (F_B).
b) the absorption of the bioaccessible mycotoxin through the intestinal epithelial cells of the GI tract (F_A), being transported to the blood (or lymph) stream.
c) the metabolism of the mycotoxin previous to systemic circulation (i.e., the biotransformation and excretion by the intestinal epithelium or the liver), the so-called first pass effect (F_M).
So, the equation that defines the bioavailable fraction of an ingested mycotoxin, that is, the fraction that reaches the systemic circulation, is defined by:

$$F = F_B \times F_A \times F_M \quad (1)$$

Bioaccessibility (B) has become important because it represents the amount of the mycotoxin that can reach the blood after intestinal absorption. It is worth to mention that bioaccessibility has been calculated only in *in vitro* systems. This concept is only applicable to oral exposure. Other routes of exposure do not depend on the process described above. Bioaccessibility is given in percentages and calculated with the following formula:

$$B (\%) = [mycotoxin_{chyme}] \text{ after GI digestion} / [mycotoxin_{food\ matrix}] \text{ before GI digestion} \quad (2)$$

Physiologically speaking, bioaccessibility refers to the amount of toxin that is liberated from the food matrix in the stomach and is available for absorption in the small intestine (Peijnenburg and Jager, 2003; Ruby et al., 1996). Bioaccessibility (some times called *digestibility*) involves all the events that occur before intestinal and hepatic presystemic metabolism, and that take place during the digestion of food until the macronutrients and micronutrients can be assimilated into the cells of the intestinal epithelium (Fernandez-García et al., 2009), and can be considered as an indicator for the maximal oral bioavailability of the toxin, which can be used for realistic worst case risk assessment of the toxin in a consumer product (Brandon et al., 2006).

For a mycotoxin administered in solution, as it is with drinking water, the bioaccessibility of the toxin is assumed to be 100%. The toxin does not need to be mobilized from the matrix as it is already in solution and, thereby, available for absorption in the intestine. After ingestion of other matrices such as feed or food, the toxin may be partially or totally released from their matrix during digestion in the GI tract. Only the bioaccessible fraction is available for transport across the intestinal epithelium and can contribute to the internal exposure (Versantvoort et al., 2004).

The food matrix mainly affects the bioaccessibility, whereas absorption and metabolism depend more on the toxin specific properties and on the animal physiology and, therefore, the food matrix is expected to have less influence on theses processes (Brandon et al., 2006). Thus the bioaccessibility of a given mycotoxin can differ according to the considered food, as has recently been demonstrated in the case of the bioaccessibilities of DON in different types of Italian pasta (Raiola et al., 2012b), of BEA in wheat crispy breads with different fiber concentrations (Meca et al., 2012b) and of PAT in different apple products (Raiola et al., 2012a).

3. *In vitro* digestion models

As described before, the bioaccessibility depends on the mycotoxin and the food matrix considered. This implies that for health risk assessment of the more important mycotoxin-contaminated foods, it would be convenient to obtain food-specific results of oral bioavailability in order to better adjust the legal limits of different food groups. Thus, *in*

vitro digestion models based on human or animal physiology have been developed, not only for mycotoxins, but also for other areas of application. Most researches have been devoted to investigation of bioaccessibility of food components and contaminants, but also of toxics on soils (Avantaggiato et al., 2003; Boisen and Eggum, 1991; Brandon et al., 2006; Döll et al., 2004; Dominy et al., 2004; Garret et al., 1999; Gil-Izquierdo et al., 2002; Larson et al., 1997; Miller et al., 1981; Minekus, 2005; Minekus et al., 1999, Oomen et al., 2003; Ortega et al., 2009; Ruby et al., 1999; Savoie, 1994; Versantvoort et al., 2004, 2005). Most of the *in vitro* digestion models simulate, in a simplified manner, the digestion processes in mouth, stomach, and small intestine, often obviating some physiological processes that occur during digestion, such as peristalsis or the existence of intestinal microbiota.

Digestion is a well known process in which the breaking down of food into smaller components that can be absorbed by the bloodstream and distributed throughout the body takes place. Briefly, in humans, and in general in the monogastric animals, digestion is a sequential process that begins with a mechanical and chemical digestion in the mouth, where food is chewed and mixed with saliva (rich in amylases) and where many polysaccharides are breaking down. Then, the stomach continues smashing the food and breaking food constituents mechanically and chemically with the aid of pepsine and some gastric lipases; mainly protein and peptide degradation takes place, although some lipolysis also occurs (Forte, 1996). In the small intestine, where absorption of nutrients is mainly conducted, the presence of lipids in the duodenum stimulates the secretion of bile salts, phosphatidylcholine, and cholesterol from the gall bladder and pancreatic fluids (containing pancreatic lipase/colipase, etc.) from the pancreas. Water and minerals are reabsorbed back into the blood in the colon (large intestine), together with some vitamins, such as biotin and vitamin K produced by bacteria (Conigrave and Young, 1996).

The *in vitro* digestion models try to mimic this layout, especially in the first three compartments of the GI tract (because mycotoxin absorption takes place mainly in the small intestine). Main parameters to control are temperature (although if simulates human GI tract all reactions are carried out at 37 °C), time in each compartment, pH changes, ionic strength, gastric/intestinal juice composition and enzymatic activities.

The main features that are requested to an optimum *in vitro* dynamic digestion model are (Minekus, 2005; Versantvoort et al., 2004;):

1) The model must be similar and representative of the physiological processes in the human body (or in the considered animal).
2) Digestion must be a dynamic process which helps to food disintegration and absorption. Biochemical reactions, flow (hydrodynamics) and mechanical forces must be in accordance with the kinetics of digestion. The rate of release (emptying) should be controlled for a quick or prolonged release.
3) The system should allow simulating fasted or fed conditions.
4) The model should include anaerobic conditions and presence of typical GI microbiota.
5) The methodology should be easy and applicable, robust and reproducible.

Most of the designed models attempt to fulfill with the first four requirements, the latter being the more difficult to achieve due to methodological complications related to the anaerobic assay conditions.
With regard to the type of models used, most models are static GI models which simulate the transit through the digestive tract by sequential (compartmentalized) exposure of the food to simulated mouth, gastric and small intestinal conditions. These models are a good first approximation to the problem, as they are of easy performance, and generally allow rapid processing of a large number of samples, but represent in a lesser extent the GI physiological reality.

On the other hand, dynamic GI models mimic the gradual transit of ingested compounds through the simulated compartments of the GI tract, giving a more realistic simulation. In these models, successive physiological conditions in the stomach and segments of the intestines of humans and animals are closely simulated. Digestion products and other small molecules are absorbed from the different intestinal compartments by dialysis. These models usually take into account factors as gastric emptying patterns, GI transit times in combination with changing pH values, variable concentration of electrolytes, enzymes and bile salts, absorption of water and, in some cases, microbial activity during passage of the food through the entire GI tract (Zeijdner et al., 2004).

There are many biological conditions which differ from *in vitro* and *in vivo* systems. The best approximation to the composition of digestive juices ensures better mimicked digestion in the GI tract and the results may be approximate to those observed *in vivo*. However, in some cases the results do not agree with those observed in *in vivo*, as will be seen below for FB_1 *in vitro* simulation.

3.1 Main physiological components of the in vitro models

3.1.1 Saliva

Digestion is a physiological process that starts in the mouth with a mechanic action during which salivary fluids initiate the hydrolytic processes, the central nervous system is stimulated and the cephalic phase of digestion is initiated. Salivary fluid is an exocrine secretion consisting of approximately 99% water, containing a variety of electrolytes (sodium, potassium, calcium, chloride, magnesium, bicarbonate, phosphate) and proteins, including enzymes (mainly amylases and, in a minor extent, lipases), immunoglobulins and other antimicrobial factors (lysozyme, peroxidase systems, lactoferrin, histatins and agglutinins), hormones, mucosal glycoproteins, traces of albumin and some polypeptides and oligopeptides of importance to oral health. There are also glucose and nitrogenous products, such as urea and ammonia (Rantonen, 2003).

Saliva production is stimulated by visual and olfactory stimuli, sour taste, chewing and the presence of food particles in the mouth. Composition of saliva depends on the flow rate: at higher flow rates, sodium, calcium, chloride, bicarbonate and amylases increase, whilst phosphate concentrations and mucin decrease, and the potassium concentration show little change (Versantvoort et al., 2004).

The salivary α-amylase, which acts as an endoglycosidase, hydrolyzes starch and related R-1,4-linked polysaccharides starting the starch digestion and its transformation to oligosaccharides and monosaccharides. The lingual lipase is a triacylglycerol lipase that hydrolyzes dietary lipids on the carboxylic ester to produce diglycerols. This hydrolysis continues in the stomach by the gastric lipase, but the activity of lingual lipase has been described between pH 2 and 6.4, indicating that the lingual lipase is active from the mouth to the small intestine where the activity decreases at pH 6.9, while gastric lipase is still active in the intestine (Liao et al., 1984).

Mucin lubricates oral surfaces, provides a protective barrier between underlying hard and soft tissues and the external environment, and aid in mastication, speech and swallowing. Saliva behaves as a buffer system to protect the mouth. Urea acts as a buffer present in total salivary fluid; it is a product of aminoacid and protein catabolism that causes a rapid increase in biofilm pH by releasing ammonia and carbon dioxide when hydrolyzed by bacterial ureases.

In the main *in vitro* models, simulated saliva consists of a simplified version of this complex biological fluid, containing electrolytes (KCl, KSCN, NaH_2PO_4, $NaSO_4$, NaCl and $NaHCO_3$), urea and α-amylase (Gil-Izquierdo et al., 2002), whereas other models also uses uric acid and mucin (Versantvoort et al., 2005). Generally, the pH value used in this fluid is around 6.8. However, salivary digestion is omitted in some *in vitro* models, as the TIM-1 dynamic gastrointestinal model (Minekus et al., 1995), because it is considered that it does not represent great changes in matrices or components of interest.

3.1.2 Gastric juices

The gastric phase is activated when the acid secretion begins and finishes when the stomach contents reach the duodenum to start the intestinal phase. Gastric juice is secreted by the gastric glands of the stomach, and its production is regulated through specific neural and hormonal pathways, by the eating act and by the presence of food in the stomach. In the adult human, the stomach typically secretes about 2-3 liters of gastric juice per day. The three major constituents of gastric juice are the mucus, the enzymes and the aqueous component, the production of hydrochloric acid being a key factor as produces a significant drop in pH values (Forte, 1996).
Gastric mucin is a large glycoprotein which is thought to play, together with $NaHCO_3$ secretion, a major role in the protection of the gastrointestinal tract from acid, proteases, pathogenic microorganisms, and mechanical trauma.

The main enzyme in gastric juice is pepsin, although other enzymes like the gastric lipase are also present. Pepsin is actually a heterogeneous group of endoproteases responsible for the proteolytic activity of gastric juice (Forte, 1996). Gastric juice lowers the pH of the gastric content, due to secretion of hydrochloric acid. Gastric pH values have been

reported between 1 and 3 (Allen and Flemström, 2005; Jolliffe et al., 2009; Kong and Singh, 2009), while during digestion the pH can increase up to 7.5 (Kong and Singh, 2009). This pH change is important for the activation of pepsinogens precursors (Vertzoni et al., 2005) and to stabilize pepsin that has optimal proteolytic activity in the same pH range (i.e., pH 1-3).

When gastric juice is neutralized as it passes into the duodenum, pepsin is denatured and thus eliminated from further digestive function. The lipids are emulsified and micellizated in the stomach and the small intestine, respectively (Carey et al., 1983). The acidic pH optimum for lipolysis is from 3.5 to 6.0 and lipase activity achieves a wide range of pH which allows the enzyme to act in the stomach where the postprandial pH is from 4.5 to 5.5. In the small intestine the pH range is between 5.0 and 6.5 and lipase activity is proportional to the bile concentration after ingestion (Liao et al., 1984).

In the *in vitro* models, gastric juice is often simulated only with a strong decrease of the pH, but more complete systems include gastric juices containing pepsin (Gil-Izquierdo et al., 2002), and also several electrolytes (NaCl, KCl, $CaCl_2$, $NaHCO_3$), a lipase and bovine trypsin (Versantvoort et al., 2005). The gastric juice used in the TIM-1 dynamic gastrointestinal model (Minekus et al., 1995), one of the more complete, includes pepsin, mucin, glucose, glucuronic acid, urea, glucoseamine hydrochloride, BSA and several salts (NaCl, NaH_2PO_4, KCl, $CaCl_2 \cdot 2H_2O$, NH_4Cl and HCl).

The pH value of the gastric juice during the *in vitro* GI simulation varies between models. In some cases the pH is a constant value, usually low (1.3-2) (Gil-Izquierdo et al., 2002; Versantvoort et al., 2004), but in other models pH values decrease during simulation from higher values (7-5) to lower values at the end (3-2), in a gradual or discontinuous way (Döll et al., 2004; Minekus et al., 1995). The gastric pH value is a crucial factor in the bioaccessibility determination as it is essential for the activity of pepsin, enzyme that can contribute to the release of hidden mycotoxins from proteins of the food matrix (Dall'Asta et al., 2009). However, some conjugated mycotoxins, as the DON-3-glucoside are resistant to acidic conditions, thus it is extremely unlikely that this compound can be hydrolyzed into DON in the stomach (Berthiller et al., 2011).

3.1.3 Intestinal juices

Following gastric digestion, the stomach releases food into the duodenum through the pyloric sphincter. Duodenum receives pancreatic enzymes from the pancreas and bile from the liver and gallbladder. These fluids are important in aiding digestion and absorption. Peristalsis also aids digestion and absorption by churning up food and mixing it with intestinal secretions. The rest of the small intestine, located below the duodenum, consists of the jejunum and the ileum. These parts of the small intestine are largely responsible for the absorption of fats and other nutrients. The intestinal wall releases mucus, which lubricates the intestinal contents, and water, which helps dissolve the digested fragments. The main components of all the biological fluids implied in the intestinal digestion of food are:

- The exocrine pancreatic secretions, which mainly contain pancreatic enzymes as proteases (in zymogenic form, including trypsinogen, chymotrypsinogen, procarboxypeptidase), lipases (that degrade triglycerides into fatty acids and glycerol), cholesterol esterase, phospholipase, nucleases and amylase.
- The liver secretes bile and bicarbonate into the small intestine. The bile secretion contains bile salts, lecithin, cholesterol and bilirubin.
- The small intestine secretes watery mucus (that protects the intestinal mucosa from auto-digestion by proteases and acid) and hormones (like secretin, somastotin, motilin, cholecystokinin and the gastric inhibitory peptide). In the small intestine there are numerous "brush border" enzymes whose function is to further cleave the already-broken-down products of digestion into absorbable particles. Some of these enzymes include: sucrose, lactase, maltase and other disaccharides.
- The large intestine secretes mucus (for lubrication and mechanical protection), and bicarbonate and potassium ions (for protection from bacterial acid).

Given this high number of chemical compounds forming part of the intestinal secretions, it is understandable that *in vitro* models must simplify the components of the intestinal tract to the main components, especially those related with enzymatic properties. Thus *in vitro* small intestine simulation basically includes the main duodenal electrolytes (among which the calcium salts are very important), pancreatin (a mixture of several digestive enzymes

produced by the exocrine cells of the pancreas: amylase, lipase and protease) and bile salts (Gil-Izquierdo et al., 2002; Minekus et al., 1995). Complementarily, some models add other components as mucin, lipases, BSA and urea (Versantvoort et al., 2005).

Calcium ion (Ca^{2+}) acts as a co-factor required for enzymatic activity at a low concentration, as 40 mM is enough to make, for example, lipolysis to increase (Brownlee et al., 2010; Kimura et al., 1982). This ion is usually added in simulated intestine digestion systems as $CaCl_2$, at concentrations ranging from 0.3 to 22.2 g/L (see Table 1).

Gastrointestinal enzymes generally have a greater resistance to irreversible denaturation, but in the different parts of the intestine of healthy subjects there can be a broad pH range, which can vary from pH 5.9 to pH 9 (Brownlee et al., 2010). pH values employed for the *in vitro* intestinal fluids used with mycotoxins have ranged from 6 to 8.2.

Bile salts have a severe impact on bioaccessibility, as they have the capacity of surfacting and decreasing the surface tension, consequently, creating an apolar environment in the interior of bile salt micelles for hydrophobic contaminants and thereby increase their solubility (Oomen et al., 2004).

3.1.4 Microbial interactions

The interaction of digestive microbiota with mycotoxins has been predominantly studied in models simulating the rumen of ruminants. Thus, *in vitro* metabolism of different mycotoxins (AFB_1, AFG_2, DON, diacetoxyscirpenol and T-2 toxin) by bacterial, protozoal and ovine ruminal fluid preparations has been determined by Westlake et al. (1989). Mobashar et al. (2012) have recently studied the contribution of the different microbial populations of rumen on OTA degradation using the *in vitro* Hohenheim gas incubator system. Authors have found that in contrast to the opinions in many publications, the bacterial (and not the protozoal) community played the dominant role in ruminal OTA degradation. Similarly, *in vitro* ruminal degradation of AFB1 has been described (Jiang et al., 2012). However, the most commonly *in vitro* models used for determination of bioaccessibility of mycotoxins in human or monogastric systems ignore what happen in the large intestine, as it is in the small intestine where absorption of mycotoxins occurs.

Besides, simulation of the large intestine, for its implications in fermentation processes that take place on this location, necessarily involves the use of microbiota of human or animal origin, under anaerobic conditions, making more difficult the operating procedure.

Laboratory systems to simulate the large intestine have been used successfully (Minekus, 2005), but they generally do not combine physiological concentrations of metabolites with physiological numbers of fecal microorganisms. The TIM-2 large intestinal system developed at the TNO Nutrition and Food Research Center (The Netherlands) simulates human and monogastric large intestine, and use a complex, metabolic active microbiota of human origin. The fecal inoculum used contains a mixture of total anaerobes, facultative anaerobes and methanogenic bacteria, as well as Enterobacteriaceae, *Bacteroides*, *Bifidobacterium* and *Lactobacillus*. This model is kept always under anaerobic conditions by flushing with nitrogen (Minekus et al., 1999). At the moment, and to our knowledge, this system has not been used with mycotoxins, unlike what happened with its brother system, the TIM-1, which simulates the small intestine.

The effect of different probiotic bacteria on the bioaccessibility of AFB_1 and OTA (Kabak et al., 2009) and the main four AFs (Kabak and Ozbey, 2012b) has been studied using the *in vitro* model developed by Versantvoort et al. (2005), but in this case the bacteria used (different *Lactobacillus* and *Bifidobacterium* strains) were employed as mycotoxin adsorbents in a system that only simulates the stomach and small intestine. Thus no physiological interactions were expected and anaerobic conditions were not employed.

3.1.5 Other factors: temperature, peristalsis and transit (incubation) time

Temperature used for *in vitro* bioaccessibility assays of mycotoxins always simulates the physiological temperature of the human being, so 37 °C has been the selected temperature for all the studies. Regarding gastrointestinal movements, the mechanical action and hydrodynamic flow created by the contraction waves of the stomach muscles also play a critical role in gastric digestion. Movements in the stomach are agitation mechanics that help to mix the food with the components of gastric juice to obtain a homogeneous bolo. Stomach contractions generate a fluid flow of the gastric contents that cause a shearing effect on the food surface. Similarly, in the small intestine peristaltic

waves not only move food along the intestine, but also mix the food chyme to help in the digestive process. In the *in vitro* models usually used with mycotoxins these physiological movements are simulated in different ways, as the use of orbital shakers (Gil-Izquierdo et al., 2002), head-over-heels rotation (Versantvoort et al., 2004), movement of tubes in a water bath (Döll et al., 2004) or the employment of peristaltic valve pumps (Minekus et al., 1995). The influence of the movement system selected has not been properly evaluated in the bioaccessibility assays conducted with mycotoxins.

The gastric emptying is regulated by different factors as the volume of the meal, its osmotic pressure and the caloric content of the food. Gastric emptying is also the final expression of complex hormonal interactions and digestive electrical signal driven by the neuronal net and juice secretions. The half-emptying time in healthy subjects occurred around 90 minutes (in a range from 60 to 277 minutes), although there are great differences between solid and fluid meals (Versantvoort et al., 2004). However, after a heavy meal completely emptying of the stomach can take up to 16 hours (Davenport, 1984). Incubation time in the stomach conditions usually used in *in vitro* models ranged from 2 to 4 hours. With regard to residence time in the small intestine, the mean transit time is of the order of 3 hours (range 1 to 6 hours), 2-3 hours being the time usually employed in the *in vitro* models.

3.2 In vitro models used in bioaccessibility or absorption studies on mycotoxins

Related to mycotoxins, one of the sequential discontinuous models most frequently used has been that developed by Gil-Izquierdo et al. (2002), a modification of a previous method described by Miller et al. (1981) to study iron bioavailability. This method, with slight modifications (Meca et al., 2012ab; Raiola et al., 2012ab), consists of two sequential steps: an initial saliva/pepsin/HCl digestion for 2h at 37 °C to simulate the mouth and the gastric conditions, and a second digestion with bile salts/pancreatin for 2.5h at 37°C to simulate duodenal digestion.

Similarly, the method proposed by Versantvoort et al. (2004), and known as RIVM model, has been widely used. This system comprises a three-step procedure simulating digestive processes in mouth, stomach and small intestine. In each compartment digestion takes

place after addition of simulated physiological juices and incubation at 37 °C for a time relevant for the considered compartment. The system has been employed mainly to simulate human GI tract (Brandon et al., 2006; De Nijs et al., 2012; Kabak et al., 2009, 2012ab; Motta and Scott, 2009; Versantvoort et al., 2004, 2005). A very similar compartmentalized approach, but simpler in terms of composition of simulated physiological juices, has been described by Gawlik-Dziki et al. (2009), and has been used for AFB1 bioaccessibility calculations (Simla et al., 2009).
Döll et al. (2004) developed an *in vitro* model to simulate the effect of the GI tract on DON and ZEA and some potential detoxifying agents. The *in vivo* conditions (pH, temperature and transit time) mimic the porcine GI tract as reviewed by Dänicke et al. (1999) and Clemens et al. (1999). The system consists in a sequential incubation of the mycotoxins in (0.1 M, , pH 5) phosphate-citrate buffer during 2 h followed by a pH decrease to 3.0 using 300 µL of ortho-phosphoric acid (85%), pH value that is maintained during 2 h (4 h in all, simulates gastric digestion). Thereafter, the pH is increased to 6.0 by adding 600 µL NaOH (12 M) and incubation for 3 h after adjusting the pH to 7.0 with 400 µL NaOh (12 M). All the incubations are carried out in a water bath regulated at 37 °C.

The dynamic *in vitro* gastrointestinal model developed at the TNO, known as TIM model has been widely used in the determination of absorption of mycotoxins and other contaminants (Minekus, 1998; Minekus *et al*, 1995, 1999). TIM model is a multi compartmental, continuous, dynamic, computer-controlled system which closely simulates the *in vivo* conditions of the stomach and small intestine (TIM-1) and large intestine (TIM-2) of humans and monogastric animals. The system simulates the peristaltic movements in the GI tract, mixing and moving the contents gradually through the stomach and the intestine. This system allows the simulation of the gastric emptying and intestinal transit times, and a computer-controlled system introduces the simulated salivary, gastric, biliary and pancreatic secretions at the appropriate moment. Absorption of water and digested food compounds from the small intestinal compartment is achieved by the use of hollow fiber membrane systems that are hooked up at the middle and end part of the small intestine simulating the jejunum and ileum (Zeijdner et al., 2004). In the large intestinal model (TIM-2) a complex metabolic active microbiota of human origin performs the fermentation of undigested food components (Minekus, 2005). The system has been used simulating the human situation for babies, young adults and elderly people (Dominy et al.,

2004; Minekus, 1998; Oomen et al., 2002) and, on the other hand, the dog, pig and calves situation (Avantaggiato et al., 2003, 2004, 2005, 2007; Minekus, 1998; Smeets-Peeter et al., 1998, 1999). This system seems to be one of the more complete and realistic of those available, but it has also some limitations as there are no mucosal cells inside the model, there is no immune system and there are no real feed back mechanisms, except for pH and intestinal water absorption (Zeijdner et al., 2004). Table 1 shows the main methods used to evaluate the bioaccessibility or absorption of mycotoxins, as well as their main characteristics.

4. *In vitro* bioaccessibility/absorption data of mycotoxins

Although simulated gastric and intestinal fluids have been used extensively in the evaluation of the stability of the adsorbent-mycotoxin union (Ramos, 1996a; Scheideler, 1989), it was not until the development of the *in vitro* GI models described previously that effective evaluation of the bioaccessibility/absorption of mycotoxins in different food matrices has begun. In the next sections, available data on bioaccessibility or absorption of mycotoxins, calculated using the *in vitro* models previously explained, will be described (Tables 2 and 3).

4.1 Aflatoxins

In 2003, Zeijdner *et al.* using the TIM-1 GI *in vitro* model tested the absorption of AFB_1 in an experiment designed for the evaluation of the efficacy of a natural magnesium smectite in pig GI tract. Considering only the data from the control, without adsorbent, it could be observed that in a contaminated feed with 20 µg/kg AFB_1 (65% natural contamination),

Table 1.- *In vitro* digestion models used for mycotoxin bioaccessibility determination.

		TIM-1	Döll	RIVM	Gil-Izquierdo
General	**Type model**	Dynamic	Static	Static	Static
	First time assayed with mycotoxins	2003	2004	2004	2012
	Matrix/Food assayed	Wheat/Corn	Phosphate-citrate buffer 0.1M, pH5	Peanut slurry/Buckwheat	Crispy bread/Apple derivatives/Italian pasta
	Mycotoxin assayed over time	ZEA, AFB1, DON, NIV, FBs, OTA	DON, ZEA	AFB1, OTA	ENs/BEA/PAT/DON
	Simulated physiology	Pig	Pig	Human	Human
	Temperature of assay	37 °C	37 °C	37 °C	37 °C
	Juice movement	Peristaltic valve pumps	Tubes in a water bath	Head-over-heels rotation at 55 rpm	Stomacher/Orbital shaker
	Computer	Yes	No	No	No

	assisted				
Mouth simulation		No	Yes	Yes	Yes
	Saliva composition	--	Phosphate-citrate buffer	10 mL KCl 89.6 g/L, 10 mL KSCN 20 g/L, 10 mL NaH_2PO_4 88.8 g/L, 10 mL $NaSO_4$ 57 g/L, 1.7 mL NaCl 175.3 g/L, 20 mL $NaHCO_3$ 84.7 g/L, 8 mL urea 25 g/L, 290 mg α-amylase, 15 mg uric acid, 25 mg mucin	KCl 89.6 g/L, KSCN 20 g/L, NaH_2PO_4 88.8 g/L, $NaSO_4$ 57 g/L, NaCl 175.3 g/L, $NaHCO_3$ 84.7 g/L, urea 25 g/L, 290 mg α-amylase
	pH	--	5.0	6.8	6.8
	Incubation time	--	2 hours	5 minutes	30 seconds

Table 1. Continued.

		TIM-1	Döll	RIVM	Gil-Izquierdo
Stomach simulation		Yes	Yes	Yes	Yes
	Gastric juice composition	NaCl 3.1 g/L, KCl 1.1 g/L, $CaCl_2$ 0.15 g/L, $NaHCO_3$ 0.6 g/L, porcine pepsin, fungal lipase, bovine trypsin	Only pH adjustment to pH 3 with ortho-phosphoric acid 85%	15.7 mL NaCl 175.3 g/L, 3.0 mL NaH_2PO_4 88 g/L, 9.2 KCl 89.6 g/L, 18 mL $CaCl_2 \cdot 2H_2O$ 22.2 g/L, 10 mL NH_4Cl 30.6 g/L, 6.5 mL HCl 37% g/g, 10 mL glucose 65 g/L, 10 mL glucuronic acid 2g/L, 3.4 mL urea 25 g/L, 10 mL glucosamine hydrochloride 33 g/L, 1 g BSA, 2.5 g pepsin, 3 g mucin	Pepsin (14,800U in HCl 0.1 N)
	pH	Gradually decreasing from 7 to 2	5 (2h) + 3 (2h)	1.30	2.0
	Incubation time		4 hours	2 hours	2 hours

Small intestine simulation		Yes	Yes	Yes	Yes
	Small intestine juice composition	Duodenal electrolyte solution: NaCl 5.0 g/L, KCl 0.6 g/L, $CaCl_2$ 0.3 g/L. Pancreatin solution at 7% Porcine bile exttract at 4%	Only pH adjustment to pH 6 with a 12 M NaOH	Duodenal juice: 40 mL NaCl 175.3 g/L, 40 mL $NaHCO_3$ 84.7 g/L, 10 mL KH_2PO_4 8 g/L, 6.3 mL KCl 89.6 g/L, 10 mL $MgCl_2$ 5 g/L, 180 µL HCl 37% g/g, 4 mL urea 25 g/L, 9 mL $CaCl_2 \cdot 2H_2O$ 22.2 g/L, 1g BSA, 9 g pancreatin, 1.5 g lipase	Pancreatin (8mg/mL), bile salts (50 mg/mL)

Table 1. Continued

		TIM-1	Döll	RIVM	Gil-Izquierdo
Small intestine simulation				Bile juice: 30 mL NaCl 175.3 g/L, 68.3 mL NaHCO3 84.7 g/L, 4.2 mL KCl 89.6 g/L, 150 µL HCl 37% g/g, 10 mL urea 25 g/L, 10 mL $CaCl_2 \cdot 2H_2O$ 22.2 g/L, 1.8 g BSA, 30 g bile	
	pH	6.5 (duodenum), 6.7 (jejunum), 7.2 (ileum)	6	8.1 (duodenal juice), 8.2 (bile juice)	6.5
	Incubation time		3 hours	2 hours	2 hours
Large intestine simulation		No	Yes	No	No
	Large intestine juices composition	Optionally, a TIM-2 large intestinal system for microbiological	Only pH adjustment to pH 7 with a 12 M NaOH		

		studies is available. Not evaluated with mycotoxins			
	pH		7		
	Incubation time		3 hours		
	Incubation time		3 hours		
Output	**Specific treatment**	Dialysis through hollow fiber membranes with a cut-off of approx. 5000D	No	No	No
	Sample centrifugation	Yes	2100 *g* - 10 min	2750 *g* - 5 min	4000 rpm - 10 min to 1 h
	Sample filtration	Yes	No	No	No

Table 1. Continued.

		TIM-1	**Döll**	**RIVM**	**Gil-Izquierdo**
Output	**Observations**	Total digestion time 6 h More information in Avantaggiato et al. (2003)	More information in Döll et al. (2004)	More information in Versantvoort et al. (2004, 2005)	More information in Meca et al. (2012a)

the amount of mycotoxin absorbed was of 47% (Zeijdner et al., 2003, 2004). This result was confirmed by Avantaggiato et al. (2007), using the same *in vitro* model trying to assess the efficacy of a carbon/aluminosilicate-based product on a multicontaminated feed containing AFB_1, FB_1, FB_2, DON, ZEA and OTA, as a 46% absorption for AFB_1 was observed, although the initial level of contamination was almost 10-fold higher (193 µg/kg). The AFB_1 absorption occurred mainly (approx. 75%) at the upper part of the system (simulating jejunum) and less at the ileum.

Shortly after, Versantvoort et al. (2004), in the first document where the term "bioaccessibility" is applied in the field of mycotoxins, described the bioaccessibility of AFB_1 from peanut slurries using the RIVM model for GI human tract simulation. Data obtained using a low amount of AFB_1 (3 ng AFB_1, from 0.5 g of a peanut slurry contaminated at 6 µg/kg) or a higher amount (27 ng AFB_1, from 4.5 g of a peanut slurry containing 6 µg/kg) showed similar results of bioaccessibility, ranging between 80 and 81%, considerably higher than the previous works. The bioaccessibility of AFB_1 from 9 peanut slurries ranging from 0.6 to 14 µg/kg in the chyme (1.5 to 36 µg/kg contamination level of peanuts) was determined and it was observed that a more or less constant bioaccessibility percentage of 90% at each contamination level was found. The bioaccessibility rose to 104-111% when AFB_1 was in a food mix containing a standard meal plus the peanut slurry (1 and 3 ng AFB_1 in the digestion model). To test the robustness of the digestion model, some changes were applied, as the decrease of the pH in the small intestinal compartment, the prolongation of the incubation time in the small intestine section (4h instead 2h), or doubling the bile concentration, but none of these variations affected the bioaccessibility of AFB_1 from the food mix. This data were confirmed with very slight modifications (83-84% bioaccessibility in peanut slurries, 108-115% in food mix) shortly thereafter by the same authors (Versantvoort et al., 2005), and by Simla et al. (2009) in ground corn (92.5-98.1% bioaccessibility) and peanut (91.2-97.0% bioaccessibility).

Table 2. GI *in vitro* models used for bioaccessibility or absorption determination of aflatoxins.

Mycotoxin	Matrix	Contami-nation	Matrix contamination range	Bioaccessibility /Absorption	*In vitro* model used	Reference
AFB1	Feed	S	20 µg/kg	47%	TIM-1*	Zeijdner et al., 2003
	Peanut slurries	N	0.6-14 µg/kg	around 90%	RIVM	Versantvoort et al., 2004
	Peanut slurries	N	6 µg/kg	80-81%	RIVM	Versantvoort et al., 2004
	Food mix	N	0.5 µg/kg	104-111%	RIVM	Versantvoort et al., 2004
	Peanut slurries	N	6 µg/kg	83-84%	RIVM	Versantvoort et al., 2005
	Food mix	N	0.2-0.4 µg/kg	108-115%	RIVM	Versantvoort et al., 2005
	Corn feed	S	193 µg/kg	46% (35% jejunum; 11% ileum)	TIM-1*	Avantaggiato et al., 2007
	Ground corn	S	1-20 mg/kg	93-98%	Gawlik-Dziki et al., 2009	Simla et al., 2009
	Peanut slurries	S	1-20 mg/kg	91-97%	Gawlik-Dziki et al., 2009	Simla et al., 2009

	Pistachio nuts	N	109.1 μg/kg	86%	RIVM	Kabak et al., 2009
	Infant food	S	1.76-3.75 μg/kg	88-94%	RIVM	Kabak et al., 2009
	Peanut	S	1.86-9.62 μg/kg	90-95%	RIVM	Kabak et al., 2012b
	Pistachio	S	1.89-9.66 μg/kg	90-96%	RIVM	Kabak et al., 2012b
	Hazelnut	S	1.84-9.54 μg/kg	89-95%	RIVM	Kabak et al., 2012b
	Dried figs	S	1.87-9.71 μg/kg	91-99%	RIVM	Kabak et al., 2012b
	Paprika	S	1.83-9.44 μg/kg	86-94%	RIVM	Kabak et al., 2012b
	Wheat	S	1.87-9.49 μg/kg	85-90%	RIVM	Kabak et al., 2012b
	Corn	S	1.88-9.43 μg/kg	88-93%	RIVM	Kabak et al., 2012b
AFB2	Peanut	S	0.53-2.78 μg/kg	86-92%	RIVM	Kabak et al., 2012b
	Pistachio	S	0.54-2.81 μg/kg	88-92%	RIVM	Kabak et al., 2012b
	Hazelnut	S	0.53-2.75 μg/kg	86-90%	RIVM	Kabak et al., 2012b
	Dried figs	S	0.54-2.78 μg/kg	88-90%	RIVM	Kabak et al., 2012b
	Paprika	S	0.53-2.72 μg/kg	84-85%	RIVM	Kabak et al., 2012b

Table 2. Continued.

Mycotoxin	Matrix	Contami-nation	Matrix contamination range	Bioaccessibility /Absorption	*In vitro* model used	Reference
AFB2	Wheat	S	0.52-2.63 µg/kg	83-85%	RIVM	Kabak et al., 2012b
	Corn	S	0.52-2.65 µg/kg	84-88%	RIVM	Kabak et al., 2012b
AFG1	Peanut	S	1.81-9.57 µg/kg	89-93%	RIVM	Kabak et al., 2012b
	Pistachio	S	1.84-9.61 µg/kg	91-95%	RIVM	Kabak et al., 2012b
	Hazelnut	S	1.83-9.51 µg/kg	91-94%	RIVM	Kabak et al., 2012b
	Dried figs	S	1.85-9.50 µg/kg	91-94%	RIVM	Kabak et al., 2012b
	Paprika	S	1.81-9.37 µg/kg	85-91%	RIVM	Kabak et al., 2012b
	Wheat	S	1.83-9.43 µg/kg	88-89%	RIVM	Kabak et al., 2012b
	Corn	S	1.87-9.40 µg/kg	87-92%	RIVM	Kabak et al., 2012b
AFG2	Peanut	S	0.52-2.70 µg/kg	84-87%	RIVM	Kabak et al., 2012b
	Pistachio	S	0.53-2.75 µg/kg	87-88%	RIVM	Kabak et al., 2012b
	Hazelnut	S	0.52-2.73 µg/kg	84-86%	RIVM	Kabak et al., 2012b
	Dried figs	S	0.53-2.77 µg/kg	87-91%	RIVM	Kabak et al., 2012b
	Paprika	S	0.51-2.70 µg/kg	81-86%	RIVM	Kabak et al., 2012b

AFG2	Wheat	S	0.50-2.61 μg/kg	81-84%	RIVM	Kabak et al., 2012b
	Corn	S	0.51-2.63 μg/kg	82-85%	RIVM	Kabak et al., 2012b
AFM1	UHT milk	N/S	0.011-0.939 μg/L	81-86%	RIVM	Kabak et al., 2012a

N: natural, S: spiked. *TIM-1 model determines in vitro intestinal absorption of the bioaccessible mycotoxin.

Kabak et al. (2009), who also used the RIVM in vitro model found similar data, with an AFB_1 bioaccessibility that ranged from the 86% in naturally contaminated pistachio nuts, to the 88-94% in artificially contaminated infant food. These authors also found that the bioaccessibility of AFB_1 could be reduced in a 37% by the addition of a probiotic bacteria (*Lactobacillus acidophilus* NCC12). Although other probiotics were used (from *Lactobacillus* and *Bifidobacterium* genera), results were not conclusive when the standard deviations were taken into account.

These authors extended their studies on AFs bioaccessibility to the four main Aflatoxins (Kabak and Ozbey, 2012) and to AFM_1 (Kabak and Ozbey, 2012a). In the first case, a wide range of artificially contaminated food matrices were assayed (peanut, pistachio, hazelnut, dried figs, paprika, wheat and corn) finding bioaccessibilities that ranged from 85.1-98.1% for AFB_1, 83.3-91.8% for AFB_2, 85.3-95.1 for AFG_1 and 80.7-91.2% for AFG_2. The bioaccessibilities of all four toxins were independent of the 3 spiking levels (2, 5 and 10 µg/kg for AFB_1 and AFG_1, and 0.6, 1.5 and 3 µg/kg for AFB_2 and AFG_2) and the 7 different food matrices assayed. The highest AFB_1 bioaccessibility value was reported in dried figs (average 94.4%), whereas the lowest was detected in wheat samples (average 87.2%). Again, these authors studied the effect of the addition of probiotic bacteria (from *Lactobacillus* and *Bifidobacterium* genera), finding a bioaccessibility reduction that could reach in the best situation a 35.6% reduction.

With regard to AFM_1, results found in spiked and naturally contaminated UHT milk, with contamination levels ranging from 0.011 to 0.939 µg/L, showed bioaccessibilities around 80.5-86.3% (Kabak et al., 2012a). There were no significant differences among the spiked and naturally contaminated milk samples. Assuming that the bioaccessibility of AFs, including AFM_1, would be around 80-90%, depending on the matrices and contamination levels considered; it can be concluded that AFs are almost completely released from the food matrices during digestion, implying a high toxicological risk.

4.2 Ochratoxin A

At present, there are few studies on the bioaccessibility of OTA and its relation to different food matrices. Versantvoort et al. (2004), using the RIVM model, determined the OTA

bioaccessibility in two lots of buckwheat, finding that a considerable amount of OTA, above 45%, was released from the food matrix. Bioaccessibilities seemed to be not dependent of the amount of food in the digestion model, the presence of other components in the food matrix or some variations in parameters of the *in vitro* model (as lowering the intestinal pH, prolongation of the transit time or doubling the bile concentration). Values obtained ranged from 45% (in a buckwheat batch contaminated at 20 μg/kg) to 84% (in a buckwheat batch contaminated at 7 μg/kg). However, in 2005, the same authors using the same experimental design reported bioaccessibilities of OTA considerably higher. Thus, values ranging from 86 to 116% were described, although in this second report the amount of mycotoxin used in the digestion model was lower (Versantvoort et al., 2005). Those data agree with the results later reported by Avantaggiato et al. (2007) who found, using the TIM-1 model with an artificially OTA contaminated corn feed, absorptions near 88%. Most of the OTA was released from the food matrix in the jejunal dialysate, showing a high absorption in the upper part of the small intestine, whereas in the ileal dialysate absorption of the ingested OTA was limited to approximately 15%.

However, Kabak *et al.* described again in 2009 much lower values for OTA bioaccessibility (22-32%) using the RIVM model with naturally OTA contaminated buckwheat or with low- and high-contaminated spiked infant foods. Bioaccessibilities found seemed not to be contamination level dependent, but it seemed that bioaccessibility increased if spiked contamination was employed, as in this case the toxin may not be tightly bound to the food matrix. Besides, the authors suggested that the difference found between the bioaccessibility of AFB_1 and OTA (AFB_1:OTA ratio at 3:1, according to their data) should be taken into account in risk assessment, as AFB_1 presents a higher toxicological risk.

It seems obvious that more data are required to more accurately assess OTA bioaccessibility. The effect that OTA contamination level, type of contamination (natural *vs.* spiked) and food matrix exerts in bioaccessibility must be more profoundly studied in this mycotoxin.

4.3 Patulin

Raiola et al. (2012a) determined the bioaccessibility of PAT in apple juices and purees by using an *in vitro* model based on the sequential steps of GI digestion defined by Gil-Izquierdo et al. (2002) (gastric and duodenal digestion), pepsin, pancreatin and bile extract used were demineralised using a Chelex-100 resin, as described by Jovaní et al. (2004). Unlike the original Gil-Izquierdo method, a brief salivary digestion was added and no dialysis of the pepsin digest was carried out. Commercial PAT-free apple products were contaminated with 50 µg PAT/L (juices) or 25 µg PAT/kg (purees) and digested. A higher bioaccessibility was observed in apple juices with pulp (67.3-70.9%), followed by puree samples (55.7-58.2%). Apple and pear nectar (38.9%) and clarified apple juices (25.3-28.6%) showed much lower bioaccessibilities. The authors suggested that these data should be taken into account in the risk assessment of this toxin, as high bioaccessibilities could be found in apple juices, frequently ingested by children over 3 years.

Similar high PAT bioaccessibilities were found by Brandon et al. (2012) in apple products using the RIVM digestion model. The bioaccessibility of PAT was assayed using two different amounts of food per digestion tube (2.0 and 4.5 g, representing half a meal and a normal amount of dinner). Home-made apple sauce made from apples contaminated with *Penicillium expansum*, and spiked apple sauce and baby fruit were assayed alone or in combination with other foods as yoghurt, biscuits or a standard Dutch dinner. Level of PAT contamination ranged from 99.8 (spiked foods) to 110-485 µg/kg (naturally contaminated). Results showed that bioaccessibility was similar for the two amounts of food and that bioaccessibility values were high, ranging between 55 and 100%.

4.4 *Fusarium* mycotoxins

4.4.1 Zearalenone

Avantaggiato et al. (2003) using the TIM-1 model that simulates the porcine gastrointestinal tract determined the GI absorption of ZEA. Contaminated feed (820 µg ZEA/kg), containing in its composition artificially contaminated wheat, was pumped into the GI-model. Intestinal absorption of the food-released ZEA was measured analysing the dialysates at the jejunal and ileal locations of the system. A 32% absorption was observed at the jejunal+ileal compartment, although absorption occurred mainly in the jejunal part

of the model (22%). Absorption at the stomach+duodenal compartment was considerably lower (4%). Authors considered that almost all the ZEA released from the food matrix during digestion was rapidly absorbed, a result consistent with the *in vivo* data obtained by Ramos et al. (1996b) in rats that showed a high rate of ZEA absorption through the intestinal lumen by a passive process. These results were confirmed by Zeijdner et al. (2004) using a naturally contaminated feed (100 µg ZEA/kg). Feed was digested in a TIM-1 model during six hours, obtaining again a 22% of absorption in the jejunal compartment and a 10% in the ileal compartment. Avantaggiato et al. (2007) reported an absorption of 25% (16% jejunum; 9% ileum) using a naturally contaminated feed containing 1.3 mg ZEA/kg. The authors concluded that neither the level of ZEA in the feed nor the origin of contamination (natural or spiked) has an effect on the intestinal absorption of ZEA, setting an average value of 28% of the matrix-released mycotoxin. On the other hand, absorption of ZEA in the TIM-1 model was lower and slower than the observed for other mycotoxins as FBs, DON, OTA or AFB_1. Consequently, ZEA absorption took place in the distal part of the small intestine, with a higher absorption in the ileum compartment compared with the rest of mycotoxins.

4.4.2 Deoxynivalenol and nivalenol

Simultaneous absorption of DON and NIV was evaluated using the porcine TIM-1 model by Avantaggiato et al. (2004). A meal containing DON and NIV spiked ground wheat was employed for absorption determination. Contamination level of the meal was 560 µg DON/kg and 760 µg NIV/kg. Results showed that both toxins were simultaneously absorbed in the small intestine, DON absorption being 2.4 times higher that NIV uptake. A total absorption of 51% was observed for DON, 44% of which takes place in the jejunum and the rest in the ileum compartment. On the other hand, NIV showed a 21% absorption, 18% of which corresponding to jejunum. The authors suggested that the higher absorption of DON with regard to NIV (ratio 5:2) may be due to its higher hydrophobicity. However, using the same *in vitro* model, higher values for DON absorption (68% at jejunum; 6% at ileum) were found later using 10-fold naturally contaminated grains (Avantaggiato et al., 2007).

Table 3.- GI *in vitro* models used for bioaccessibility or absorption determination of OTA, PAT and *Fusarium* mycotoxins.

Mycotoxin	Matrix	Contami-nation	Matrix contamination range	Bioaccessibility/ Absorption	In vitro model used	Reference
OTA	Buckwheat	N	7-20 µg/kg	45-84%	RIVM	Versantvoort et al., 2004
	Food mix	N	3.3-3.5 µg/kg	60-62%	RIVM	Versantvoort et al., 2004
	Buckwheat	N	11-11.3 µg/kg	86-100%	RIVM	Versantvoort et al., 2005
	Food mix	N	1.83-2.0 µg/kg	114-116%	RIVM	Versantvoort et al., 2005
	Corn feed	S	11 µg/kg	88% (73% jejunum; 15% ileum)	TIM-1*	Avantaggiato et al., 2007
	Buckwheat	N	24.9 µg/kg	22%	RIVM	Kabak et al., 2009
	Infant food	S	3.2-13.1 µg/kg	29-32%	RIVM	Kabak et al., 2009
PAT	Cloudy apple juice	S	50 µg/L	67-71%	Gil-Izquierdo et al., 2002 and Jovaní et al., 2004	Raiola et al., 2012a
	Apple	S	25 µg/kg	56-58%	Gil-Izquierdo et al.,	Raiola et al., 2012a

	puree				2002 and Jovaní et al., 2004	
	Apple and pear nectar	S	50 µg/L	39%	Gil-Izquierdo et al., 2002 and Jovaní et al., 2004	Raiola et al., 2012a
	Clarified apple juice	S	50 µg/L	25-29%	Gil-Izquierdo et al., 2002 and Jovaní et al., 2004	Raiola et al., 2012a
	Apple derived products	N-S	99.8-485 µg/kg	55-100%	RIVM	Brandon et al., 2012

Table 3. Continued.

Mycotoxin	Matrix	Contami-nation	Matrix contamination range	Bioaccessibility/ Absorption	In vitro model used	Reference
ZEA	Feed meal	S	820 µg/kg	32% total (22% jejunum; 10% ileum)	TIM-1*	Avantaggiato et al., 2003
	Feed	N	100 µg/kg	32% total (22% jejunum; 10% ileum)	TIM-1*	Zeijdner et al., 2004
	Grain	N	1300 µg/kg	25% (16% jejunum; 9% ileum)	TIM-1*	Avantaggiato et al., 2007
DON	Feed meal	S	560 µg/kg	51% total (44% jejunum; 7% ileum)	TIM-1*	Avantaggiato et al., 2004
	Grain	N	5600 µg/kg	74% total (68% jejunum; 6% ileum)	TIM-1*	Avantaggiato et al., 2007
	Pasta	N	73.8-387	Child: 20% gastric;	Gil-Izquierdo et al.,	Raiola et al., 2012b

			µg/kg	10% duodenal Adult: 42%% gastric; 24% duodenal	2002	
	Infant formula	S	2222 µg/kg	65%	RIVM	De Nijs et al., 2012
DON-3-G	Infant formula	S	2778 µg/kg	55%	RIVM	De Nijs et al., 2012
NIV	Feed meal	S	760 µg/kg	21% total (18% jejunum; 3% ileum)	TIM-1*	Avantaggiato et al., 2004
FBs	Grain	N	19900 µg FB1/kg 5900 µg FB2/kg	104% total FB1 (87% jejunum; 17% ileum) 89% total FB2 (75% jejunum; 14% ileum)	TIM-1*	Avantaggiato et al., 2007
TB FB1	Corn flakes	N	92 µg/kg	51%	RIVM	Motta and Scott, 2009

Table 3. Continued.

Mycotoxin	Matrix	Contami-nation	Matrix contamination range	Bioaccessibility/ Absorption	In vitro model used	Reference
ENNs	Crispy bread	S	1.5 and 3.0 µmol/g	85-91% ENA 69-73% ENA1 69-82% ENB 74-84%ENB1	Gil-Izquierdo et al., 2002	Meca et al., 2012a
BEA	Wheat crispy bread	S	5 and 25 mg/kg	around 40% duodenal digestion around 30% duodenal+colonic digestion	Gil-Izquierdo et al., 2002 modified	Meca et al., 2012b

N: natural, S: spiked *TIM-1 model determines in vitro intestinal absorption of bioaccessible mycotoxin.

Recently, Raiola et al. (2012b) determined the bioaccessibility of DON in pasta samples using the human *in vitro* model described by Gil-Izquierdo et al. (2002), slightly modified. In most of the samples, the *in vitro* model was adjusted to simulate child digestion (higher gastric pH, reduced amount of pepsin, pancreatin and bile salts) and in this case the mean value for DON bioaccessibility was 19.5% (ranging from 2.12% to 38.41%) for the gastric compartment and 9.7% for the duodenal compartment (range 1.11-17.91%). In one sample where adult digestion was simulated, the bioaccessibilities found were considerably higher (32.81% average; 41.49% for gastric and 24.13% for duodenal compartments). Although bioaccessibilities found in the adult model were higher, authors postulate that, considering the small dimension of the child intestinal tract, the released DON present in this place could probably produce more damage to the intestinal enterocytes respect to that in an adult.

Regarding masked mycotoxins, a conjugated form of DON, the DON-3-ß-D-glucoside (DON-3-G) could be present in DON-contaminated plants, as glucosylation represents a major route to detoxify xenobiotics for plants. In fact, it has been proven that in some cases mean DON-3-G contamination exceeded the DON contamination (Sasanya et al., 2008). A major concern is the hydrolysis of the DON-3-G conjugate back to its toxic precursor DON during gastrointestinal passage. This fact could clearly influence the real bioaccessibility value of this toxin in a food from which only DON contamination is known. For this reason DON reversion from DON-3-G was determined using an *in vitro* experiment that mimics the digestion conditions at the GI tract (acid hydrolysis of DON-3-G using 0.02-0.2 M HCl, pH 1.7-0.7; artificial stomach juice containing pepsin; artificial non-microbial gut juice containing pancreatin) (Berthiller et al., 2011). Results showed that neither the acidic hydrolysis nor the GI enzymatic simulations resulted in DON regeneration. However, metabolic activity of some usual GI bacteria could produce the hydrolysis of DON-3-G to DON. Several *Enterococcus* species (*E. durans*, *E. faecium* and *E. mundtii*), but also *Enterobacter cloacae*, *Lactobacillus plantarum* and *Bifidobacterium adolescentis* were able to cleave DON-3-G in the semi-anaerobic conditions assayed, releasing up to values from 6 to 62% of DON after 8h, depending of the considered species. Similarly the hydrolytic enzyme cellobiase produces a 73% DON-3-G hydrolysis in 18h, suggesting that DON- 3-G could be cleaved and DON released in the GI tract of plant-based cellulose-foraging ruminants (Berthiller et al., 2011).

Once the possibility of a GI reversion of DON-3-G was stated, De Nijs et al. (2012) calculated the possible transformation of this masked mycotoxin to DON using the RIVM *in vitro* digestion model. Thus, an infant formula with 2778 µg DON-3-G/kg was introduced into the system, but DON was not detected in the chyme of the digested samples (this means that, in theory, less than 5% of DON-3-G was hydrolysed to DON). The level of DON-3-G detected after digestion was 55% (that is, the bioaccessible fraction). When samples were only spiked with DON (2222 µg/kg) the amount of DON detected after digestion was 65%, and DON-3-G was not detected. These results confirm the previous data, but as this model does not consider the existence of intestinal microbiota, the question of whether the DON-3-G hydrolysis at this location would lead to an increase in the DON uptake remains unanswered.

4.4.3 Fumonisins

Absorption of FB_1 and FB_2 were determined by Avantaggiato et al. (2007) in a naturally contaminated blend of grains by using the TIM-1 *in vitro* model. Absorption of both mycotoxins takes place mainly in the simulated jejunum (87% FB_1, 75% FB_2) and in a lesser extent in the ileum (17.3% FB_1, 13.9% FB_2). Thus, average intestinal absorption was 104% for FB_1 and 89% for FB_2, indicating that release of FBs from the food matrix and, consequently, absorption in the simulated intestinal tract is an almost complete fact and that absorption of bioaccessible fumonisins would take place mainly in the upper part of the small intestine. On the other hand, maximum absorption occurred in the first 2 hours of digestion. These results do not agree with those from *in vivo* experiments, as it is known that FB_1 has very species-specific toxicity and a very low bioavailability when administered orally, resulting from low uptake coupled with efficient biliary excretion (Shier, 2000). Low oral FB_1 bioavailability has been demonstrated in swine (3-6%) (Prelusky et al., 1994) and laying hens (0.7%) (Vudathala et al., 1994), whereas in cows no FB_1 or known metabolites were found in the plasma of orally administered cows, indicating no or very limited bioavailability in ruminants (Prelusky et al., 1995). These discrepancies between the results obtained *in vitro* and highlight the limitations of *in vitro* models.

It has been hypothesized that the totally hydrolyzed FB_1 metabolite HFB_1 is the real molecule involved in fumonisin toxicity, is more polar than FB_1 and *in vivo* studies demonstrated its higher absorption in rats (Hopmans et al., 1997). However, recent *in vivo* studies wih piglets have shown that FB1 hydrolysis strongly reduces its toxicity both in the GI tract and the liver (Grenier et al., 2012) these findings suggest that HFB1, rather than being the molecule implicated in FB1 toxicity, represents a mechanism for detoxification. Effects of HFB1 on the intestine have been poorly studied, and most of the data have been obtained from in vitro studies with intestinal cell lines.

It has been demonstrated that although HFB_1 could be absorbed in a dose-dependent manner by the human colon adenocarcinoma Caco-2 cells, while FB_1 was not absorbed by human colon adenocarcinoma Caco-2 monolayer of cells. In spite of this, HFB1 was not accumalated inside the cells, and no viability alterations or barrier damage was observed (De Angelis et al., 2005). Similarly, in vivo HFB1 ingestion assays showed that this compound did not induce toxic effect in the piglet intestine, as intestinal integrity was not altered and intestinal immunity was not compromised (Grenier et al., 2012).
Dall'Asta et al. (2009) addressed the problem created by the presence of hidden (masked) FBs in the food matrix, as we explained before regarding DON-3-G.

With regard to extractable FBs (expressed as the sum of FB_1, FB_2 and FB_3) an increase of 30-50% in FB concentration was observed after *in vitro* digestion of naturally contaminated corn flours using the RIVM model, which greatly affects the bioaccessibility determination. The authors suggested that the binding mechanism of FBs in raw corn is mainly due to an association with macromolecules such as starch and proteins. Additionally, no hydrolyzed or partially hydrolyzed FBs were found in the chyme. Besides, the total FBs found after digestion are slightly higher than those obtained after an alkaline hydrolysis, suggesting that masked FBs are native forms, which can be released under conditions found in the GI tract. As a clear example of how the presence of masked mycotoxins could affect bioaccessibility measurements, in a reference material with a declared FBs contamination of 3036 µg/kg, an amount of 8010 µg/kg of free FBs was detected after the *in vitro* digestion (Dall'Asta et al., 2009).

Similarly, Motta and Scott (2009) studied the bioaccessibility of total bound FB_1 (TB FB_1) in naturally contaminated corn flakes using the RIVM model. Corn flake samples assayed had a low level (23 µg/kg TB FB_1) or high level (92 µg/kg TB FB_1) of contamination. Results showed that in the case of the high level contaminated corn flakes a 51% (range 37-64%) bioaccessibility of TB FB_1 was found, showing that masked mycotoxins could be bioaccessible for absorption at the small intestine. In addition, authors suggested that TB FB_1 could be a substrate for the intestinal bacteria and could be also hydrolyzed to regenerate the FB_1 or to create other hydrolyzed metabolites, increasing the possibility of exposure to this group of toxins after ingestion.

4.4.4 Emerging Fusarium mycotoxins

Fusarium species are also responsible for the production of another group of bioactive compounds considered as "minor" mycotoxins. This group includes enniatins (ENA, ENA1, ENB and ENB1), fusaproliferin and beauvericin (Meca et al., 2010). All of these toxins belong to the group of "emerging" mycotoxins, which are neither routinely determined, nor legislatively regulated; however, the evidence of their incidence is rapidly increasing. Enniatins represent an emerging food safety issue because of their extensive incidence, documented in recent decades, in various small grain cereals (Santini et al., 2012). Bioaccessibility of ENNs has been evaluated by the *in vitro* method of Gil-Izquierdo et al. (2002) on spiked wheat crispy breads (Meca et al., 2012a). Thus, breads were contaminated with ENA, ENA1, ENB and ENB1 at two concentrations each (1.5 and 3.0 µmol/g) and gastric and duodenal bioaccessibilities were calculated. At the gastric compartment, bioaccessibility ranged between 69.0% (ENA1 at 1.5 µmol/g) and 91.0% (ENA at 3 µmol/g), whereas at duodenal compartment the range was between 68.6% (ENB at 1.5 µmol/g) and 87.3% (ENA at 3 µmol/g). The enniatin that showed the highest bioaccessibilities was the ENA, and the mean bioaccessibility considering the four compounds analyzed resulted in 80%. Given the fact that cereals are frequently contaminated, that it has been demonstrated that ENs can exert toxic activity at low micromolar concentrations in mammalian cells (Meca et al., 2011), and that high bioaccessibilities were found these data must be taken into account in future legislative actions.

A similar study was conducted by the same group with beauvericin (Meca et al., 2012b). BEA has also shown to be toxic in *in vitro* studies with human cell lines, including the intestinal Caco-2 cells (Prosperini et al., 2012). Gil-Izquierdo et al. (2002) model was used, with slight modifications; a simulation of the large intestine (colonic digestion) was employed introducing a high inoculum (10^{14} cfu/mL) of several characteristic bacteria of the GI tract after the duodenal digestion. Wheat crispy breads were spiked with 5 or 25 mg BEA/kg; bioaccessibility was around 40% until duodenal digestion and around 30% until duodenal+colonic digestion, with higher bioaccessibilities at the lower BEA dose employed. When crispy breads were produced with different concentrations of soluble alimentary dietary fibers, bioaccessibilities were drastically reduced at the duodenal digestion, but results were quite variable when considered the joint duodenal+colonic digestion. Thus, some fibers as chitosan medium MW and galattomannan considerably reduced BEA bioaccessibility when used at 5%. On the contrary, the use of fructooligosaccharides, inulin or pectin increases the BEA bioaccessibility after duodenal+colonic digestion, mainly at low BEA levels (5 mg/kg).

5. Conclusions

According to the reviewed studies, it can be concluded that bioaccessibility of mycotoxins greatly depends on the considered toxin, as well as on the food matrix in which experiments are carried out. As a general rule it can be established that bioaccessibility of AFs, FBs and ENNs shows high values, generally ranging between 70 and 100%, while PAT, DON and BEA show intermediate values, around 30-70%, and ZEA presents lower values. The bioaccessibility of OTA has proven to be very variable, with values near 100% in some cases but below 30% in others.

The great variability of results between different mycotoxins and, for the same mycotoxin among the different studied matrices, highlights the need for further studies on bioaccessibility of these fungal metabolites, increasing the number and types of studied foods. The true knowledge of the percentage of mycotoxin that can be absorbed in the small intestine, from the initially present in food, would enable a more accurate risk assessment.

It is also necessary to increase the number of studies with naturally contaminated foods, as the mycotoxin-matrix binding has shown to be stronger, which could affect the bioaccessibility values. On the other hand, when working with spiked samples it is necessary that the mycotoxin contamination level fits the natural contamination found in foods. In the same sense, a special attention has to be paid to masked mycotoxins, as an overestimation of bioaccessibility could occur due to an incorrect estimation of the bioaccessible toxin after the digestive process, in relation to the initially known amount of toxin.

Determination of the internal exposure is a good approach to improve the risk assessment of a mycotoxin. *In vitro* methods offer an appealing alternative to human and animal studies. They can be rapid, simple and reasonably low in cost, can be used to perform simplified experiments under uniform and well-controlled conditions, and may provide insights not achievable in whole animal studies, but they are not free of some weaknesses. In one hand, in many cases they do not take into account important physiological factors as the lack of intestinal mucosa, enterohepatic cycling, and immune system and, in most cases they do not take into account the existence of an intestinal microbiota which possesses enzymatic capabilities that can decisively influence the release of the mycotoxin from the food matrix or the bioconversion of these toxins in the GI tract. On the other hand, these models usually do not take the large intestine into account, as absorption of toxins mainly takes place in the small intestine.

Combined use of these *in vitro* digestion models with other techniques that employ intestinal cell lines, as *in vitro* intestinal absorption models that use Caco-2 cells (De Nijs et al., 2012), conducting for instance transepithelial transport studies (Meca et al., 2012a, Prosperini et al., 2012) may offer a more complete picture of what happens in the intestinal tract during intestinal digestion.

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Annex II

Composition of digestive juices

Table 1. Composition of digestive juices for the *in vitro* digestion model.

Solutions§	Saliva	Gastric jucie	Duodenal juice	Bile juice
Inorganic solution	10 mL KCl 89.6 g/L 10 mL KSCN 20 g/L 10 mL NaH_2PO4 88.8 g/L 10 mL $NaSO_4$ 57 g/L 1.7 mL NaCl 175.3 g/L 20 mL $NaHCO_3$ 84.7 g/L	15.7 mL NaCl 175.3 g/L 3.0 mL NaH_2PO_4 88.8 g/L 9.2 mL KCl 89.6 g/L 18 mL $CaCl_2$ 19.1 g/L 10 mL NH_4Cl 30.6 g/L 6.5 mL HCl 37% g/g	40 mL NaCl 175.3 g/L 40 mL $NaHCO_3$ 84.7 g/L 10 mL KH_2PO_4 8 g/L 6.3 mL KCl 89.6 g/L 10 mL $MgCl_2$ 5 g/L 180 μL HCl 37% g/g	30 mL NaCl 175.3 g/L 68.3 mL $NaHCO_3$ 84.7 g/L 4.2 mL KCl 89.6 g/L 150 μL HCl 37% g/g
Organic solution	8 mL urea 25 g/L	10 mL glucose 65 g/L 10 mL glucuronic acid 2 g/L 3.4 mL urea 25g/L 10 mL glucosamine hydrochloride 33 g/L	4 mL urea 25 g/L	10 mL urea 25 g/L

Table 1. continued.

Solutions[§]	**Saliva**	**Gastric jucie**	**Duodenal juice**	**Bile juice**
Add to mixture organic + inorganic solution	290 mg α-amylase 15 mg uric acid 25 mg mucin	1 gr BSA 2.5 gr pepsin 3 g mucin	9 mL CaC_{l2} 19.1 g/L 1gr BSA 9 g pancreatin 1.5 g lipase	10 mL $CaCl_2$ 19.1 g/L 1,8 g BSA 30 g bile
pH	6.8 ± 0.2	1.30 ± 0.02	8.1 ± 0.2	8.2 ± 0.2

[§]The inorganic and organic solutions are filled up to 500 mL with distilled water. After mixing the inorganic and organic solutions, some further constituents are added and dissolved. If necessary, the pH of the juices is adjusted to the appropriate value. Adapted from Versantvoort et al. (2014).