VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia

Departament de Medicina Preventiva i Salut Pública, Ciències de l'Alimentació, Toxicologia i Medicina Legal

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Tesi Doctoral Internacional

ESTUDIOS *IN VITRO* DE LOS MECANISMOS DE TOXICIDAD DE LAS MICOTOXINAS

IN VITRO STUDIES OF MYCOTOXIN TOXICITY MECHANISMS

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La Dra. Guillermina Font Pérez, Catedrática del área de Toxicología y la Dra. María José Ruiz Leal, Profesora Titular de Toxicología,

CERTIFICAN QUE:

Dª Celia Fernández-Blanco Gómez, licenciada en Farmacia, ha realizado bajo nuestra dirección el trabajo que lleva por título: "*In vitro* studies of mycotoxin toxicity mechanisms". Y autorizamos la presentación para optar al título de Doctor Internacional.

Y para que así conste, expiden y firman el presente certificado

Burjassot (Valencia), 2017

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7. Interaction effects of eninatin B, deoxinivalenol and alternariol in Caco-2 cells. *Toxicology Letters, 241 (2016) 38-48. Impact factor 3.263*
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9. *An in vitro investigation on the cytotoxic and nuclear receptor transcriptional activity of the mycotoxins fumonisin B1 and beauvericin. Toxicology Letters 257 (2016) 1–10. Impact factor 3.263*
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La presente Tesis Doctoral Internacional se engloba dentro de los siguientes proyectos y red:

- Evaluación del riesgo de las micotoxinas emergentes de *Fusarium* (AGL2010-17024/ALI).

- Estudio de las micotoxinas y metabolitos en alimentos y muestras biológicas, de la toxicidad y de los procesos de descontaminación (AGL2013-43194-P).

- Red nacional sobre las micotoxinas y hongos toxigénicos y de sus procesos de descontaminación (MICOFOOD).

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LISTA DE ABREVIATURAS

AB: Azul alamar

Ac-DEVD: N-acetil-L-α-aspartil-L-α-glutamil-N-(2-carboxil-1-formiletil)-L-valinamida

AECOSAN: Agencia Española de Consumo, Seguridad Alimentaria y Nutrición

AFB1: Aflatoxina B1

AFB2: Aflatoxina B2

AFG1: Aflatoxina G1

AFG2: Aflatoxina G2

AITC: Isotiocianato de alilo

ALL: Toxinas de *Alternaria*

Alt a1: Alérgeno de *Alternaria*

ALT: Altenueno

AME: Alternariol monometil-eter

AO: Acridina metacromática naranja

AOH: Alternariol

AOVE: Aceite de oliva virgen extra

AP: Compartimento apical

ATX: Altertoxina

ATX-I: Altertoxinas I

ATX-II: Altertoxinas II

ATX-III: Altertoxinas III

A431: Células de carcinoma epidérmico

BEA: Beauvericina

BL: Compartimento basolateral

BrdU: Bromodeoxiuridina

BRL3A: Células de hígado de rata

BSA: Albúmina sérica bovina

Caco-2 TC7: Células clonadas de adenocarcinoma de colon humano

Caco-2: Células de adenocarcenoma de colon humano

CAT: Catalasa

CDNB: 1-cloro-2,4-dinitrobenceno

CFU-GM: Células madres formadoras de colonias eritromegacariocíticas

CHL: Células de pulmón de hámster chino

CHO-K1: Células de ovario de hámster chino

CIT: Citrinina

CMXRos: 1H,5H,11H,15H-xanteno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-io, 9-[4-(clorometil)fenil]-2,3,6,7,12,13,16,17-octahidro

Cyp 450: Citocromo P 450

DAPI: 4 ',6-diamino-2-fenilindol

DAS: Diacetoxiscirpenol

DCF: Diclorofluoresceína

DEVD: N-acetil-L-α-aspartil-L-α-glutamil-N-(2-carboxil-1-formiletil)-L-valinamida

DHE: Dihidroetidio

DiOC6: Ioduro de 3,3'-dihexiloxacarbocianina

DMSO: Dimetilsulfóxido

DON: Deoxinivalenol

DXR: Doxorubicina

EAs: Alcaloides ergóticos

EC_{50}: Concentración media efectiva

ED: Disruptores endocrinos

EFSA: Agencia Europea de Seguridad Alimentaria

EGC: Galato de epicatequina

EGCG: 3- galato de epigalocatequina

EMA: Fluorocromo etidio monoazida

ENNs: Eniatinas

ENN A: Eniatina A

ENN A_1: Eniatina A_1

ER-TA: Activación transcripcional del receptor de estrógeno

E2: 17-β-Estradiol

ENN B: Eniatina B

ENN B_1: Eniatina B_1

FAO: Organización para la Agricultura y la Alimentación

FBs: Fumonisinas

FB1: Fumonisina B1

FB2: Fumonisina B2

FC: Fluorocromo

FDA: Diacetato de fluoresceína

FITC: Fluoresceína isotiocianato

FUS: Fusaproliferina

GCS: Γ-glutamil cisteína sintetasa

GL: Glucosinolatos

GPX: Glutatión peroxidasa

GR: Glutatión reductasa

GSH: Glutatión reducido

GSSG: Glutatión oxidado

GST: Glutatión-*S*-transferasa

H295R: Células humanas adrenocorticales

H2-DCFDA: Diclorodihidrofluoresceína diacetato

H_2O_2: Peróxido de hidrógeno

H4TG: Células de hígado de rata

HCA: High content analysis

HCT116: Células de carcinoma de colon humano

HDAC: Histona deacetilasa

Hek-293: Células embrionarias humanas del riñón

HeLa: Células de cáncer cérvico-uterino

Hepa-1c1c4 y Hepa-1c1c12: Células hepáticas de ratón

HepG2: Células de hígado humano

HO342: Hoechst 33342

HPLC: Cromatografía líquida de alta resolución

HT-2: Toxina HT-2

HT29: Células de carcinoma de colon humano

INT: Cloruro de 2-(4-yodofenil)-3-(4-nitrofenol)-5-fenil tetrazolio

i.p: Intraperitoneal

IARC: Agencia Internacional de Investigación sobre el Cáncer

IC_{50}: Concentración inhibitoria media

ICH: Intercambio de **c**romátidas hermanas

IEC: Células del epitelio intestinal

Ig E: Inmunoglobulina E

IPEC-1: Células intestinales porcinas

ITC: Iisotiocianatos

JC-1: 5,5',6, 6'-tetracloro-1,1', yodurode 3, 3' tetraetilbenzimidazolilcarboxiamina

JECFA: Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios

L929: Fibroblastos de ratón

LC: Células porcinas neonatales de Leyding

LDH: Ensayo de la lactato deshidrogenasa

LLC-PK1: Células epiteliales de riñones porcinos

L-O2: Células humanas de hepatocitos

L-OOH: Peróxidos lipídicos

LPO: Peroxidación lipídica

MCF-7: Células de mama humana

MDA: Malonildialdehído

MDCK: Células de hígado de perro

MitoSOX: Indicador de superóxido mitocondrial

MLC: Células de linfoma de ratón

MMP: Potencial de membrana mitocondrial

MMS: Metil metanosulfonato

MN: Micronúcleos

MON: Moniliformina

MTT: Ensayo de la sal de tetrazolio

MTX: Micotoxina

NADPH: Nicotinamida adenina dinucleótido fosfato

NEM: N-etilmaleimida

NIH3T3: Células de fibroblastos de rata

NIV: Nivalenol

NR: Rojo neutro

$O^{1/2}$: Oxígeno singlete

$O_2\bullet^-$: Anión Superóxido

OCDE: Organización para la cooperación y el desarrollo económico

OH·: Radical Hidroxilo

OSV: Células de vesículas seminales ovinas

OTA: Ocratoxina A

OTB: Ocratoxina B

P4: Células de ovario (específicas para la hormona de la progesterona)

PAT: Patulina

PC: Proteínas totales

p.c: Peso corporal

PI: Ioduro de propidio

PK-15: Células de riñón de cerdo

pNA: p-nitroanalina

PTP: Transición de la permeabilidad mitocondrial

Quer: Quercetina

RAW 264.7: Macrófagos de roedor

RGA: Gen reportero de luciferasa

RGAs: Células con gen reportero (estrógenos, andrógenos, progestágeno y glucocorticoides)

Rh123: Rodamina 123

RNasa A: Ribonucleasa A

ROO·: Radical peróxido

ROS: Especies reactivas del oxígeno

RSV: Resveratrol

S.c*:* Subcutánea

SCIRI: Sistema Coordinado de Intercambio Rápido de Información

SMS: Células del músculo liso

SOD: Superóxido dismutasa

SRB: Sulforhodamina B

SS-I Soyasaponina I

STE: Esterigmatocistina

SYRB: Cianinas asimétricas (fluoróforos 4S)

T-2: Toxina T-2

T-47D: Células de mama humanas

TBA: Ácido tiobarbitúrico

TBARS: Sustancias reactivas del ácido tiobarbitúrico

TeA: Ácido tenuazoico

TEN: Tentoxina

TK6: Células linfoblasticas

TMRE: Tetrametilrodamina etil éster

TMRM: Tetrametilrodamina de metilo

UDPGA: Ácido uridín difosfato glucurónico

UGTs: Uridin glucuronil tranferasas

V79: Células de pulmón de hámster chino

Vero: Células epiteliales de riñón de mono

WHO: Organización mundial de la salud

XOD: Xantina oxidasa

ZEA: Zearalenona

α-ZOL: α -zearalenol

β-ZOL: β -zearalenol

ΔΨ: Potencial de Membrana

3T3: Células de fIbroblastos de rata

4-HDA: 4-hidroxi-alquenal

RESUMEN

Se ha llevado a cabo la evaluación *in vitro* de los efectos producidos por micotoxinas de *Alternaria* y *Fusarium* en células de mamífero. Se ha evaluado la citotoxicidad individual del alternariol (AOH), alternariol monometil éter (AME), beauvericina (BEA), deoxinivalenol (DON), eniatina B (ENN B), fumonisina B1 (FB1), zearalenona (ZEA) y α-zearalenol (α-ZOL) en células de adenocarcinoma de colon humano (Caco-2) donde únicamente se obtuvieron valores de IC_{50} para la ENN B, DON, BEA y α-ZOL. La evaluación de la citotoxicidad combinada entre mezclas de micotoxinas en células Caco-2 mostró efecto sinérgico en las combinaciones de AOH+AME, DON+AOH y ENN B+AOH; efecto aditivo en la combinación DON+ENN B y efecto antagonista en la combinación terciaria DON+AOH+ENN B.

Se estudió la bioaccesibilidad y biodisponibilidad del AOH, ZEA y α-ZOL. Se evaluó la bioaccesibilidad mediante el método de digestión estático *in vitro,* siendo el α-ZOL más bioaccesible a nivel gástrico y duodenal que la ZEA. Se observó una baja biodisponibilidad en las tres micotoxinas ensayadas con las células Caco-2/TC7, siendo el AOH el más biodisponible.

Teniendo en cuenta la biodisponibilidad de las micotoxinas y los escasos estudios de mecanismos de toxicidad conocidos, se estudió la interacción de las micotoxinas con los componentes y la alteración de actividades celulares. Los resultados obtenidos demostraron que el AOH bloquea el ciclo celular en la fase G2/M, causa pérdida del potencial de la membrana mitocondrial y produce apoptosis a través de la vía mitocondrial. Además, se evaluó el daño causado por el AOH a nivel del ADN mediante el ensayo del cometa y se observó un incremento del daño dependiente de la concentración.

Debido a la biodisponibilidad de las micotoxinas, se determinó la actividad estrogénica de algunas muy prevalentes como la FB1 y la BEA, observándose que la BEA produce mayor actividad estrogénica sobre células que la FB1.

Dado que un mecanismo de citotoxicidad es el estrés oxidativo, se determina la capacidad del AOH para generar especies reactivas de oxígeno (ROS), evidenciándose que el AOH en las células Caco-2 produce ROS inmediatamente tras la exposición en todas las

concentraciones ensayadas. Una de las consecuencias de las ROS es la oxidación de los lípidos de las membranas celulares, es decir, la generación de peroxidación lipídica (LPO). Los resultados obtenidos demostraron que el AOH aumenta significativamente la producción de LPO.

Tras los resultados obtenidos se procedió a determinar la eficacia del sistema de defensa intracelular (enzimático y no enzimático) frente al estrés oxidativo. Estos indicaron un incremento de la actividad de la superóxido dismutasa (SOD) a todas las concentraciones de AOH expuestas en las células Caco-2 y que la actividad enzimática de la catalasa (CAT) fue más eficaz que la glutatión peroxidasa (GPx), eliminando peróxido de hidrógeno. Además, se demostró que el glutatión (GSH) y las enzimas implicadas en el ciclo del glutatión participan de manera activa en la defensa celular frente al AOH.

Por otra parte, se estudió el efecto protector de vitaminas y antioxidantes de la dieta mediterránea frente a la exposición al AOH. Los resultados demostraron que los antioxidantes del aceite de oliva virgen extra previenen el daño celular producido por el AOH cuando se exponen simultáneamente. Mientras que la quercetina, considerada el polifenol en mayor cantidad ingerido diariamente en la dieta, no presentó efecto citoprotector frente al AOH, la soyasaponina I, saponina en mayor proporción en las legumbres y con efecto antioxidante, sí mostró efecto citoprotector tras la exposición de AOH.

Para concluir, se estudió el isotiocianato de alilo como estrategia de mitigación para prevenir el crecimiento de los hongos en los alimentos y evitar la presencia de micotoxinas en la dieta. El isotiocianato de alilo reaccionó mejor con la ZEA que con el α-ZOL, reduciendo más de la mitad de la concentración inicial de ZEA. De esta forma, el isotiocinato de alilo podría considerarse una buena estrategia de reducción de las micotoxinas de los alimentos.

SUMMARY

In vitro evaluation of the effects produced by Alternaria and Fusarium mycotoxins in mammalian cells has been carried out. Individual cytotoxicity of alternariol (AOH), alternariol monomethyl ether (AME), beauvericin (BEA), deoxynivalenol (DON), eniatin B (ENN B) fumonisin B1 (FB1), zearalenone (ZEA) and α-zearalenol (α-ZOL) in human colon adenocarcinoma (Caco-2) cells have been evaluated, where only IC50 values for ENN B, DON, BEA and α-ZOL were obtained. The evaluation of the combined cytotoxicity between mixtures of mycotoxins in Caco-2 cells showed synergistic effects in AOH + AME, DON + AOH and ENN B + AOH combinations; Additive effect on DON + ENN B combination and antagonist effect on the tertiary combination DON + AOH + ENN B.

Bioaccessibility and bioavailability of AOH, ZEA and α-ZOL were studied. In order to evaluate the bioaccessibility, the static digestion method was applied in vitro, being α-ZOL more bioaccessible at the gastric and duodenal levels than ZEA. Low bioavailability was observed in the three mycotoxins tested with Caco-2 / TC7 cells, being AOH the most bioavailable.

Taking into account the bioavailability of mycotoxins and the few studies on toxicity mechanisms, the interaction of mycotoxins with the components and the alteration of cellular activities were examined. The results obtained demonstrated that AOH blocks the cell cycle in the G2 / M phase and produces apoptosis and necrosis through the mitochondrial pathway. The loss of potential of mitochondrial membrane after AOH exposure suggested that mitochondria plays an important role in the induction of apoptosis / necrosis. In addition, the damage caused by AOH at the DNA level was assessed through the Comet assay and an increase in concentration-dependent damage was observed.

Generally, mycotoxins are bioavailable, although some are absorbed faster than others. For this reason, the estrogenic activity of some high prevalent mycotoxins, such as FBI and BEA, was analised. It was noticed that BEA had greater estrogenic activity in cells than FB1.

Because a mechanism of cytotoxicity is the oxidative stress, the ability of AOH to generate reactive oxygen species (ROS) is determined. The results obtained demonstrated that AOH produces ROS immediately after the exposure to every concentration tested. One of the most studied consequences produced by ROS is the lipid oxidation of cell membranes, namely, the generation of lipid peroxidation (LPO). The results obtained demonstrated that AOH significantly increases LPO production.

Following the results obtained, we proceeded to determine the effectiveness of the intracellular defense system (enzymatic and non-enzymatic) against oxidative stress. The results indicated an increase in superoxide dismutase (SOD) activity at all concentrations of AOH exposed in Caco-2 cells and that catalase (CAT) enzyme activity was more effective than glutathione peroxidase (GPx) on the elimination of hydrogen peroxide. In addition, it was demonstrated that glutathione (GSH) and the enzymes involved in the glutathione cycle are actively involved in cell defense against AOH.

On the other hand, the protective effect of vitamins and antioxidants of the Mediterranean diet in front of exposure to AOH was studied. The results showed that the antioxidants of the extra virgin olive oil prevent cell damage produced by the AOH when exposed simultaneously. Quercetin, considered the greatest amount of polyphenol ingested daily, did not present a cytoprotective effect against AOH; while the soyasaponin I, the saponin that is present in legumes in a higher proportion and that has antioxidant effect, showed a cytoprotective effect after exposure of AOH.

In conclusion, allyl isothiocyanate was studied as a mitigation strategy to prevent the growth of fungi in food and to avoid the presence of mycotoxins in the diet. The allyl isothiocyanate reacted better with the ZEA than with the α-ZOL, reducing more than half the initial concentration of ZEA. Therefore, allyl isothiocyanate could be considered a good strategy to mitigate mycotoxins in food.

1.INTRODUCCIÓN

INTRODUCTION

1. INTRODUCCIÓN

1.1 Micotoxinas: Aspectos generales

Las micotoxinas son compuestos químicos naturales de bajo peso molecular, producidos por determinados hongos pertenecientes principalmente a los géneros *Aspergillus, Fusarium, Alternaria y Penicillium* (Tabla 1). Los cultivos representan una buena fuente de nutrientes para los hongos. Por ello, las micotoxinas suelen encontrarse en una gran variedad de productos agrícolas, siendo los contaminantes naturales de los alimentos más extendidos a nivel mundial (Mareca et al., 2010).

Tabla 1. Tipos de micotoxinas y principales especies de hongos micotoxigénicos productores (Marin et al., 2013).

Micotoxina	**Acrónimo**	**Principales especies productoras**
Aflatoxinas B1, B2, G1, G2	AFB1 AFB2 AFG1 AFG2	*Aspergillus* sección *flavi*
Alcaloides ergóticos	EAs	*Claviceps purpurea, C. fusiformis, C. africana, Neotyphodium spp.*
Alternariol	AOH	*Alternaria* alternata
Alternariol monometil éter	AME	*A. alternata, A. solani*
Ácido tenuazónico	TeA	*A. alternata*
Altertoxinas	ALT	*A. tenuissima*
Altenueno	ATX	*A. alternata*
Tentoxina	TEN	*A. alternata*

Micotoxina	**Acrónimo**	**Principales especies productoras**
Beauvericina	BEA	*Fusarium sporotrichioides, F. poae, F. langsethiae, F.* sección *liseola, F. avenaceum*
Eniatinas A_1,A_2,B_1,B_2	ENN A1 ENN A2 ENN B1 ENN B2	*F. avenaceum, F. tricinctum*
Fumonisinas B_1, B_2	FB1, FB2	*Fusarium* sección *liseola*
Fusaproliferina	FUS	*F. poae, F. langsethiae, F. sporotrichioides* *F. proliferatum, F. subglutinans*
Moniliformina	MON	*F. avenaceum, F. tricinctum, F.* sección *liseola*
Nivalenol y Deoxinivalenol (tricotecenos tipo B)	NIV DON	*F. graminearum, F. culmorum, F. cerealis*
Ocratoxina A	OTA	*Aspergillus* sección *circumdati* *A.* sección *nigri* *Penicillium verrucosum*
Patulina	PAT	*P. expansum, Bysochlamis nívea, Aspergillus clavatus*
Toxina HT-2 y toxina T-2 (tricotecenos tipo A)	HT-2 T-2	*F. acuminatum, F. poae* *F. sporotrichioides, F. langsethiae*
Zearalenona	ZEA	*F. graminearum (F. roseum), F. culmorum, F. equiseti, F. cerealis, F. verticillioides, F. incarnatum*

Las micotoxinas son consideradas metabolitos secundarios porque no son necesarios para el crecimiento y reproducción del organismo que las sintetiza. Son, simplemente, un producto de los procesos metabólicos primarios. Las funciones de las micotoxinas no se han establecido claramente, pero se cree que desempeñan un papel en la eliminación de otros microorganismos compitiendo en el mismo entorno y, a su vez, ayudan a los hongos parásitos a invadir los tejidos del huésped.

Las micotoxinas son contaminantes naturales producidos por hongos ubicuos y el hombre siempre ha estado expuesto a estos compuestos a través de la alimentación. Los alimentos más habitualmente contaminados por micotoxinas son los productos del sector primario: cereales, frutas y verduras, oleaginosas, café, cacao, especias y frutos secos (Richard, 2007). La invasión por estos hongos puede producirse durante la precosecha (en campo) o en las etapas de la postcosecha (almacenamiento, transporte y procesamiento), generando importantes pérdidas económicas en el comercio nacional e internacional debido a la disminución de la productividad de animales y/o cosechas, y efectos sobre la salud de las personas y animales (FAO, 2001). Las condiciones ambientales son muy importantes en la contaminación por micotoxinas antes de la cosecha del grano y de cultivos oleaginosos, ya que el crecimiento y el buen estado de las plantas y la competitividad de hongos micotoxigénicos se ven muy afectados por la temperatura y la humedad, debido a que los organismos heterotróficos necesitan agua y algunos nutrientes esenciales para vivir (Wilson, Abramson, 1992). Así, mientras las especies que pertenecen a los géneros *Fusarium y Alternaria* precisan un alto contenido en humedad para crecer y, normalmente, producen micotoxinas en el campo, las especies de los géneros *Penicillium y Aspergillus* proliferan durante el trasporte y almacenamiento de la materia prima,

pues requieren bajos contenidos en humedad (CAST, 2003; Sanchis et al., 2007). Es destacable que una misma cepa toxigénica puede producir diferentes micotoxinas y que una micotoxina puede ser sintetizada por diferentes hongos.

La cantidad de micotoxinas necesarias para producir efectos adversos en la salud varía dependiendo de las características del estado del sistema inmunológico de cada individuo (Bräse et al., 2009).

La exposición a micotoxinas puede producir efectos agudos y crónicos con resultados dañinos en el sistema nervioso central, cardiovascular, respiratorio y en el aparato digestivo hasta daños cancerígenos, mutagénicos, teratógenos e inmunodepresores (Golli-Bennour y Bacha., 2011). Los efectos tóxicos producidos por micotoxinas se conocen desde inicios de la Edad Media, cuando se describió el fuego de San Antonio; hoy se conoce que fue consecuencia de productos de alcaloides ergóticos producidos por el cornezuelo del centeno (*Claviceps purpurea*) (Beardall y Miller, 1994). La importancia de las micotoxinas como un problema de salud se reconoció durante la postguerra en Japón y Rusia, dónde los tricotecenos contaminaron cosechas de arroz y produjeron la muerte de un gran número de personas (Marasas et al., 1984). A finales del siglo XX, una gran mortandad de pavos en el Reino Unido condujo al descubrimiento de las aflatoxinas y, posteriormente, a su gran potencial cancerígeno (Soriano, 2007). En la actualidad, la presencia de micotoxinas en los alimentos, constituye un grave problema de salud pública a nivel mundial. La Organización para la Agricultura y la Alimentación (FAO) considera que, al menos el 25% de las cosechas mundiales, están contaminadas por micotoxinas (FAO/WHO, 2001). En España, el Sistema Coordinado de Intercambio Rápido de Información (SCIRI), bajo la dirección de la Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN)

recibió 585 notificaciones en el año 2014 referentes al peligro químico, de las cuales el 19 % correspondían a micotoxinas fúngicas (SCIRI, 2015).

Respecto al marco legislativo vigente, la evaluación del peligro ocasionado por las micotoxinas ha conducido al Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios (JECFA) y a la Agencia Europea de Seguridad Alimentaria (EFSA, European Food Safety Authority) a establecer niveles máximos permitidos de micotoxinas en alimentos y piensos (Reglamento (CE) no 1881/2006; Egmond V, 2007). Y, aunque en la actualidad se han identificado más de 400 micotoxinas, únicamente se han adoptado medidas legislativas para 13 de ellas.

1.1.1 *Fusarium sp:* Generalidades y toxicidad

Fusarium sp. es un extenso género de hongos filamentosos ampliamente distribuido a nivel mundial. Este género es considerado el mayor productor de micotoxinas que infectan cereales (SCF, 2002). Las principales micotoxinas producidas por las especies de *Fusarium* son las fumonisinas (FBs), tricotecenos, zearalenona (ZEA) y las micotoxinas emergentes, que incluyen, entre otras, las eniatinas (ENNs) y beauvericina (BEA).

1.1.1.1 Deoxinivalenol

Los tricotecenos se encuentran divididos principalmente en cuatro grupos: A, B, C y D. Los grupos A y B son los comúnmente encontrados en alimentos, siendo el grupo B el más fitotóxico (Bennett y Klich, 2003). El Deoxinivalenol (DON) (Fig. 1) es una micotoxina perteneciente a los tricotecenos del grupo B. La sintomatología asociada a una toxicidad aguda incluye diarrea, pérdida de peso, anorexia, desnutrición, disfunción endocrina, hemorragias intestinales severas y

alteraciones inmunitarias (Creppy, 2002; Ndossi et al., 2012, Yang et al., 2014). El DON se clasifica por la Agencia Internacional de Investigación sobre el Cáncer (IARC) en el grupo 3 (IARC, 1993). A nivel celular, el DON inhibe la proliferación celular, la síntesis proteica de ARN y ADN, e induce apoptosis (Rotter et al., 1996; Marin et al., 2013; Ruiz et al., 2011 b).

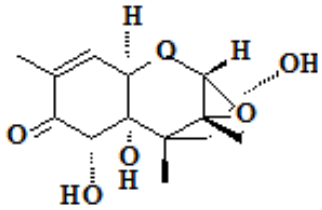

Figura 1. Estructura química del DON

1.1.1.2 Fumonisina B1

Las fumonisinas (FBs) se pueden clasificar en cuatro series: A, B, C y P. No obstante, las FBs pertenecientes a la serie B (B1, B2, B3 y B4) son las que mayoritariamente se aíslan en alimentos, siendo la FB1 (Fig. 2) la más prevalente, ya que puede constituir hasta un 70 % de todas las FBs presentes en los alimentos (Rheeder et al., 2002). El mecanismo molecular de toxicidad de la FB1 está relacionado con la desregulación del metabolismo de los esfingolípidos, debido a la elevada similitud estructural con ellos (Merrill et al., 2001; Soriano et al., 2005).

La sintomatología de una intoxicación aguda por FB1 incluye dolor abdominal, borborigmo y diarrea. A nivel crónico, los efectos fisiopatológicos son la leucoencefalomalacia, edema pulmonar porcino, toxicidad renal y hepática, cáncer de hígado y carcinoma esofágico humano (Kellerman et al., 1990;

Gelderblom et al., 1997; Hussein et a.l, 2001). Debido a la asociación entre la FB1 y el cáncer, ha sido clasificada por la IARC dentro del grupo 2B (IARC, 1993).

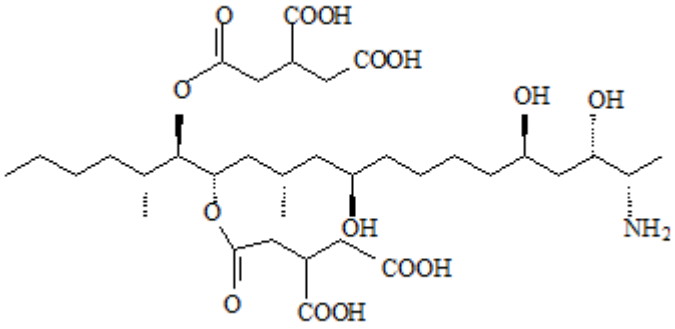

Figura 2. Estructura química de la FB1

1.1.1.3 Zearalenona y metabolitos

La zearalenona (ZEA) (Fig. 3) es una micotoxina estrogénica no esteroidea que se encuentra principalmente en cereales. La ZEA se absorbe de forma rápida y se metaboliza en el organismo dando lugar al α-zearalenol (α-ZOL) y al β-zearalenol (β-ZOL).

La toxicidad de la ZEA y algunos de sus metabolitos, especialmente el α-ZOL, está asociada a problemas de trastornos de la reproducción, fertilidad y genotoxicidad en algunos animales, y posiblemente en humanos, ya que tienen la capacidad de unirse de forma competitiva a los receptores estrogénicos (Frizzell et al., 2011; Sun et al., 2015). Recientes estudios han demostrado que la ZEA puede alterar la integridad de las células intestinales porcinas (Marin et al., 2015; Taranu et al., 2015). Además, produce citotoxicidad y estrés oxidativo, alterando el sistema de defensa antioxidante (Ferrer et al., 2009; Tatay et al., 2014, 2016; Wu et al., 2014). A pesar de los estudios disponibles hasta la fecha, la IARC no

dispone de suficientes evidencias científicas para clasificar esta micotoxina como un posible cancerígeno, por lo que la ZEA y sus metabolitos han sido clasificados dentro del grupo 3 (IARC, 1993).

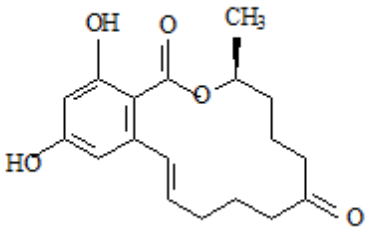

Figura 3. Estructura química de la ZEA

1.1.1.4 Eniatina B

Las eniatinas (ENNs) presentan una estructura de depsipéptido cíclico. Las principales variantes son las ENNs A, A1, B y B1 junto con cantidades menores de ENN_S C, D, E y F. La ENN B (Fig. 4) es la más frecuente en los alimentos y la más estudiada. La ENN B presenta un amplio rango de actividades biológicas, habiéndose descrito una serie de propiedades antimicrobianas, insecticidas y herbicidas, así como actividad antibiótica sobre organismos patógenos (Klaric et al., 2010). Las ENNs suponen un problema para la salud humana y animal debido a sus propiedades ionofóras, ya que tienen la capacidad de formar complejos estables con iones metálicos alcalinos (Logrieco et al., 2003; Kamyar et al., 2004). La formación de estos complejos da lugar a un aumento de la permeabilidad de las membranas biológicas y a la formación de canales catión-selectivos en las membranas celulares, los cuales pueden afectar a la homeostasis celular y al desacoplamiento de la fosforilación oxidativa. Asimismo, se ha demostrado que produce citotoxicidad y daño en el ADN, debido a la generación de estrés

oxidativo e inducción de la apoptosis y necrosis a través de la vía mitocondrial (Meca et al., 2011; Lu et al., 2013;Prosperini et al., 2013b; 2014; Juan-García et al., 2015;).

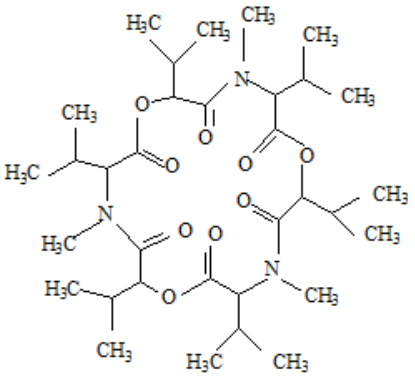

Figura 4. Estructura química de la ENN B

1.1.1.5 Beauvericina

La beauvericina (BEA) (Fig.5) es un hexadepsipéptido cíclico que posee una amplia gama de propiedades biológicas, siendo un compuesto insecticida, antibacteriano, antihelmíntico y antifúngico (Ganassi et al., 2002; Meca et al., 2010a; Xu et al., 2010). La BEA presenta el mismo mecanismo de toxicidad que las ENNs, ya que también tiene actividad ionófora (Chen et al., 2006). La formación de poros en la membrana mitocondrial incrementa la permeabilidad mitocondrial y permite la liberación de factores apoptogénos, citocromo c, acumulación de Ca, ROS…, que conducen a la muerte celular(Jow et al., 2004). Asimismo, la BEA presenta citotoxicidad, alteración de las enzimas antioxidantes y efecto genotóxico (Ferrer et al., 2009; Klaric et al., 2010; Mallebrera et al., 2014; 2015; Prosperini et al., 2013a).

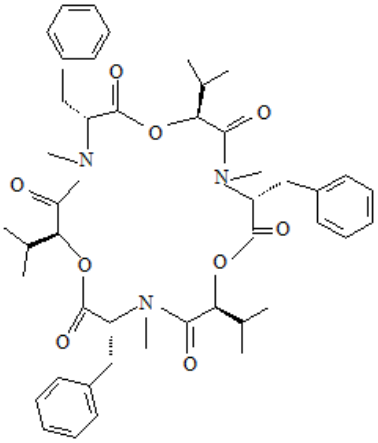

Figura 5. Estructura química de la BEA

1.1.2 *Alternaria sp:* Generalidades y toxicidad

Las micotoxinas del género *Alternaria* se desarrollan en los cultivos agrícolas pero también crecen en el suelo, textiles, madera en descomposición y abono (Gravesen et al., 1994; Patriarca et al., 2007). Este género fue definido originalmente en 1816 y, desde entonces, se han descrito numerosas especies de *Alternaria sp.*

Los metabolitos de *Alternaria* exhiben diferentes actividades biológicas tales como propiedades antimicrobianas, fitotóxicas y citotóxicas. Por ejemplo, la porritoxina de la especie endofítica de *Alternaria* se ha estudiado como agente quimioprotector del cáncer (Horiuchi et al., 2006). La depudecina es un metabolito de la especie *A. brassicicola,* inhibidor de la histonadeacetilasa (HDAC), que presenta un potencial antitumoral (Kwon et al., 1998). Algunos metabolitos de *Alternaria,* tales como el ácido tenuazónico (TeA) y la tentoxina (TEN) se han estudiado como posibles herbicidas (Lou et al., 2013).

Las micotoxinas de *Alternaria* desempeñan un papel importante en la patogénesis de plantas, siendo la causa de la mancha marrón del tabaco, manzana, pera y de otras enfermedades en la postcosecha (Rotem, 1994). El género *Alternaria* causa importantes problemas en tomates, ya que es uno de los alimentos más frecuentemente contaminados, produciendo cancro en el tallo del tomate. Dicha podredumbre negra, generada particularmente en la época de maduración, causa severas pérdidas económicas, especialmente a la industria conservera (Pavón et al., 2012).

Estudios *in vitro* evidencian que algunas micotoxinas de *Alternaria* provocan genotoxicidad en bacterias y células de mamíferos (Tiessen et al., 2013). También, se han observado eventos clastogénicos y de inducción de rotura del ADN en diferentes células de mamíferos (Lehmann et al., 2006). La inhibición de las topoisomerasas (enzimas responsables de la regulación del superenrollamiento del ADN genómico y participación en los procesos esenciales de las células como replicación, transcripción, recombinación, reparación, etc.), así como la producción de daño oxidativo, podrían explicar la rotura del ADN (Lehmann et al., 2006; Wollenhaupt et al., 2008; Fehr et al., 2009).

Las micotoxinas más importantes de *Alternaria* son: alternariol (AOH), alternariol monometil-eter (AME), altenueno (ALT), TeA, TEN, toxinas de Alternaria (ALL) y las altertoxinas I, II, III (ATX-I, -II, -III). Estas, a su vez, pertenecen a cinco clases estructurales (Bottalico y Logrieco, 1998): derivados de perileno (ATX-I, -II, -III); derivados del ácido tetrámico (TeA); derivados dibenzo-α-pironas (AOH, AME, ALT); toxinas de AAL y tetrapéptido cíclico (TEN). Las estructuras químicas de estas micotoxinas se describen en la Figura 6.

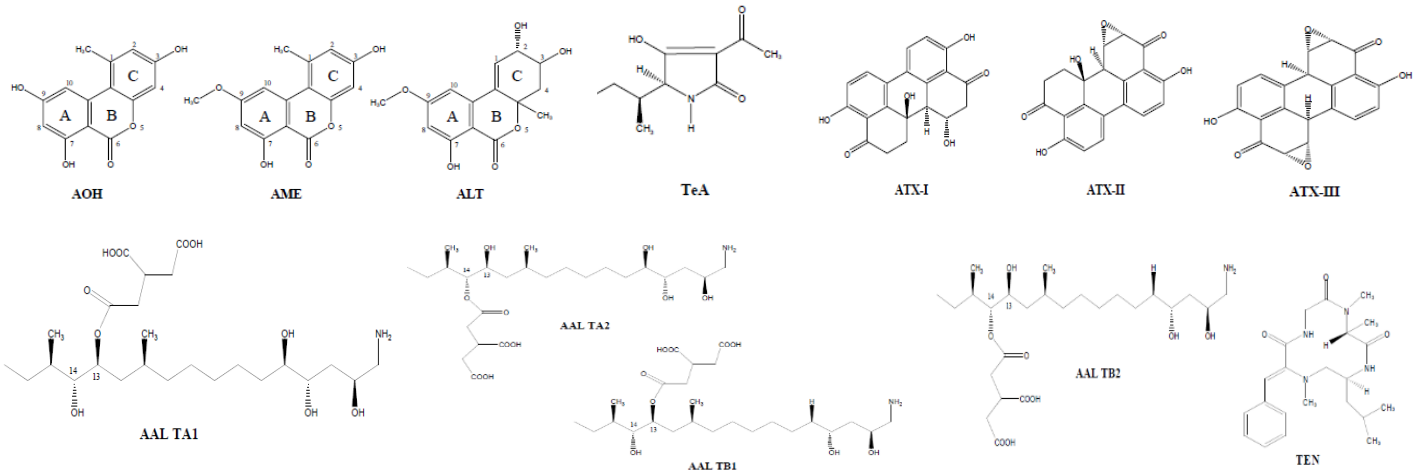

Figura 6. Estructura química de las micotoxinas de *Alternaria*

AAL-TA1 ((2R)- ácido 1,2,3-Propanetricarboxilico, 1-[(1S,3S,9R,10S,12S)-13-9,10,12-trihidroxi-1-[(1R,2R)-1-hidroxi-2-metilbutil]-3- metiltridecil] éster); **AAL-TA2** (2R)- ácido 1,2,3-Propanetricarboxilico,1-[(1R,2S,4S,10R,11S,13S)-14-amino-2,10,11,13-tetrahidroxi-4-metil-1-[(1R)-1-methilpropil]tetradecil] éster; **AAL-TB1** 2R)- ácido 1,2,3-Propanetricarboxilic, 1-[(1S,3S,10R,12S)-13- amino-10,12-dihidroxi-1-[(1R,2R)-1-hidroxi-2-methilbutil]-3- metiltridecil] éster; **AAL-TB2** 2R)- ácido 1,2,3-Propanetricarboxílico, 1-[(1R,2S,4S,11R,13S)-14- amino-2,11,13-trihidroxi-4-metil-1-[(1R)-1- metilpropil]tetradecil] éster; **ALT** (2α, 3α, 4aβ-tetrahidro-2,3,7-trihidroxi-9-metoxi-4a-metil-6H dibenzo [b,d] piran-6-ona); **AME** (3,7-dihidroxi-9-metoxi-1-metil-6H-dibenzo [b,d] piran-6-ona); **AOH** (3,7,9-trihidroxi-1-metil-6H-dibenzo [b,d] piran- 6-ona); **ATX-I** (1, 2, 7, 8, 12b-pentahidro-1, 4, 6b, 10-tetrahidroxiperileno- 3,9-diona); **ATX-II** ([perilo(1,2-b)oxireno-7,11-dion,7a,8a,8b,8c,9,10- hexahidro-1,6,8c-trihidroxi-, (7aR,8aR,8bS,8cR); **ATX-III** ([perilo (1,2-b: 7,8-b') bisoxireno-5,10-diona, 1a, 1b, 5a, 6a, 6b, 10a-hexahidro-4,9-dihidroxi-]); **TeA** (3-acetil-5-sec-butil-4-hidroxi-3-pirrolin-2-ona); **TEN** (Ciclo[N-metil-L-alanil-L-leucil-(αZ)-α,β-didehidro-metilfenillalanilglicilo]).

Las micotoxinas de *Alternaria* se encuentran con frecuencia en una gran variedad de productos vegetales frescos y procesados (Tabla 2). La presencia de micotoxinas de *Alternaria* en cereales es muy común debido al almacenamiento de los granos en condiciones favorables para el crecimiento del hongo (Logrieco et al., 2003). También, se ha detectado la presencia de AME y TeA en preparados infantiles que contenían cereales en su composición (Scoot et al., 2012). En semillas oleaginosas como la colza, el girasol, el sésamo y la linaza se ha determinado la presencia de AOH y AME (Visconti et al., 1986; Ostry et al., 2004; 2008), al igual que en legumbres como las lentejas y la soja (Barros et al., 2011). (Kralova et al., 2006; Barkai-Golan et al., 2008).

En numerosas frutas y verduras se ha detectado la presencia de micotoxinas de A*lternaria*. El consumo humano directo de frutas y hortalizas putrefactas con infección fúngica visible es improbable. Sin embargo, es frecuente encontrarlas en alimentos procesados como salsas de tomate, conservas, mermeladas, vino o zumos de fruta (Fernández-Cruz et al., 2010). No se descarta que las micotoxinas, aunque se eliminen de las zonas visiblemente alteradas, se puedan transferir a los tejidos sanos circundantes. Por otra parte, las industrias transformadoras no siempre disponen de métodos eficientes para detectar y eliminar completamente las materias primas con alteración fúngica (Pavón et al., 2012).

En la Tabla 2 se esquematizan las especies fúngicas responsables de cada una de las micotoxinas, así como los alimentos que pueden verse implicados en su contaminación.

Tabla 2. Presencia de micotoxinas de *Alternaria* en alimentos.

Micotoxina	Especie productora	Alimentos implicados
Ácido tenuazónico (TeA)	*A.alternata* *A.brassiciola* *A.brassicae* *A.citri* *A.jaoinica* *A.kikuchiana* *A.mali* *A.pori* *A.racina* *A.tenuissina*	Aceitunas Alimentos infantiles a base de cereales Bebidas alcohólicas Especias Frutas cítricas Hortalizas y derivados Manzanas y zumo Naranja Limón Pimienta Remolacha roja Semillas de girasol Sorgo Tomate Trigo
ALL-toxina	*A.alternata*	Granos de cereales y derivados
Altenueno (ALT)	*A.alternata* *A.arborescens* *A.citri* *A.gaisen* *A.porri* *A.tenuissina*	Aceites de semillas Granos de cereales y derivados Hortalizas y derivados Semillas de oleaginosas
Alternariol (AOH)	*A.alternata* *A.arborescens* *A.brassicicola* *A.citri* *A.cucumerina* *A.dauci* *A.gaisen* *A.tenuissima*	Avena Bebidas alcohólicas Especias Hortalizas y derivados Legumbres Limón Manzana y zumo Naranja Pimiento Semillas de girasol Tomate Trigo

Micotoxina	Especie productora	Alimentos implicados
Alternariol monometil éter (AME)	*A.alternata* *A.arborescens* *A.brassicae* *A.brassicicola* *A.citri* *A.cucumerina* *A.dauci* *A.gaisen* *A.kikuchiana* *A.longipes* *A.mali* *A.porri* *A.solani* *A.tenuissima*	Aceitunas Alimentos infantiles a base de cereales Bebidas alcohólicas Cebada Centeno Especias Frutas cítricas Hortalizas y derivados Legumbres Limón Manzana y zumo Melón Naranja Pimienta Pimiento Semillas de girasol Sorgo Tomate Trigo
Altertoxina I-II-II (ATX)	*A.alternata* *A.arborescens* *A.brassicae* *A.gaisen* *A.longipes* *A.mali* *A.radicina* *A.tenuissima*	Manzana y zumo Sorgo
Tentoxina (TEN)	*A.alternata* *A.mali* *A.porri* *A.tenuissima*	Granos de cereales y derivados Aceites de semillas Hortalizas y derivados

Datos obtenidos de Kralova et al., 2006; Soriano, 2007; Barkai-Golan et al., 2008; Ostry et al., 2008; Barros et al., 2011; Pavón et al., 2012.

La EFSA ha determinado los niveles diarios de ingesta de las micotoxinas de *Alternaria* para los grupos de población comprendidos entre los 18 y 65 años (EFSA, 2011). Dichos niveles son de 1.9- 39 ng/kg de peso corporal (pc) para el AOH, 0.8-4.7 ng/kg de pc para el AME, 36-141 ng/kg de pc para el TeA y 0.01-7 ng/kg de pc para la TEN. Para los niños pequeños, la estimación es de 3.8 - 71.6 ng/kg de pc para el AOH, 3.4 - 38.8 ng/kg de pc para el AME, 1.614 -100 ng/kg de pc para el TeA, 1.6 - 33.4 ng/kg de pc para la TEN (EFSA, 2016). Para realizar estas estimaciones se han tenido en cuenta únicamente alimentos de origen vegetal, puesto que no se ha demostrado la presencia de micotoxinas de *Alternaria* en alimentos de origen animal (EFSA, 2011, 2016).

La exposición a las toxinas de *Alternaria* ha sido relacionada con la aparición de una gran variedad de efectos adversos en la salud de personas y animales en función del tipo de micotoxina implicada (Schracher et al., 2001). La incidencia de *Alternaria* en infecciones humanas no es muy elevada. *A. alternata y A. tenuissima* producen alternariosis cutánea, que se manifiesta en forma de placas pardorojizas papulonodulares, pustulosas o ulcerocostrosas, localizadas en superficies corporales expuestas, siendo rara la diseminación sistémica (Vieira et al., 2006). La alternariosis cutánea se considera una infección oportunista que aparece en personas inmunodeficientes. En la mitad de los casos, la alternariosis aparece en pacientes con enfermedades subyacentes, especialmente con trasplante de órganos sólidos y el síndrome de Cushing (Guerin et al., 1991).

Asimismo, *Alternaria* es uno de los principales géneros fúngicos causantes de alergias (Päivi et al., 2006). *A. alternata* es la principal especie productora de alérgenos del género, causando reacciones cutáneas positivas en el 70% de los pacientes alérgicos a antígenos fúngicos. El principal alérgeno es el Alt a 1, al que reconocen los anticuerpos IgE de más del 90% de los pacientes alérgicos a *A.*

alternata. La respuesta alérgica se presenta clínicamente como reacciones asmáticas de tipo inmediato mediadas por IgE (de Vouge et al., 1998). Los síntomas más significativos incluyen: irritaciones de ojos, nariz y garganta; sequedad de la piel; síntomas generales: dolor de cabeza, fatiga mental y pérdida de capacidad de memoria a corto plazo. Igualmente, *A. alternata* y A. infectoria se han relacionado con la aparición de rinosinusitis, oculomicosis, y onicomicosis (Pastor,Guarro, 2008).

Las micotoxinas producidas por el hongo *Alternaria* son tóxicas en ratas, embriones de pollo y cultivos celulares humanos (Griffin y Chu, 1983). Son mutagénicas en varios sistemas *in vitro* y cancerígenos en ratas alimentadas con piensos contaminados (Yekeler et al., 2001). Por otra parte, el consumo de alimentos contaminados con *A. alternata* se ha relacionado con una elevada incidencia de cáncer de esófago humano (Liu et al., 1992).

1.1.2.1 Principales toxinas de *Alternaria*: AOH y AME

- **Toxicocinética y Metabolismo**

Estudios *in vivo* e *in vitro* han demostrado que el AME posee una mala absorción en el tracto gastrointestinal; no obstante, la proporción que es absorbida es ampliamente metabolizada y persistente en los tejidos (Pollock et al., 1982; Pfeiffer et al., 2007). Tras la incubación de AME con microsomas NADPH-fortificados de hígado de rata, se observaron metabolitos oxidativos del AME (Pfeiffer et al., 2007). Igualmente, cuando AOH y AME se incubaron con fragmentos de hígado de rata, se observaron productos de biotransformación oxidativa por el Cyp P450 que, junto con la presencia de enzimas de la fase II, produjeron catecoles y productos de biometilación, es decir, compuestos O-metilados (Burkhardt et al., 2011). La importancia de los catecoles radica en que son capaces de formar intermediarios reactivos tales como quinonas y

semiquinonas. Estas son capaces de unirse al ADN, dando lugar a aductos de ADN, e incluso, de producir especies reactivas del oxígeno (ROS) (Solhaug et al., 2012). Sin embargo, estos mecanismos aún no han sido confirmados para AOH y AME.

El AME y el AOH (Fig. 6) tienen 2 y 3 grupos hidroxilo fenólicos respectivamente, por lo que se espera la formación de metabolitos conjugados. Cuando el AME y el AOH son incubados con microsomas del ácido uridíndifosfato glucurónico (UDPGA) y microsomas intestinales y hepáticos de ratas, cerdos y/o seres humanos, se conjugan formando glucurónidos tales como AME-3-O-glucurónido, AOH-3-O-glucurónido y AOH-9-O-glucurónido (Pfeiffer et al., 2009). Ambas micotoxinas de *Alternaria* se glucuronidan fácilmente en los tejidos hepáticos y extra hepáticos. Este efecto se confirmó para ambas micotoxinas en células derivadas de adenocarcinoma de colon humano (Caco-2), ya que, aunque estas células tienen baja actividad del Cyp P450, presentan enzimas uridin glucuronil transferasas (UGTs) y sulfotransferasas activas (Burkhardt et al., 2009).

Por tanto, aunque el AOH no se absorbe fácilmente en el tracto gastrointestinal, estudios *in vitro* y estudios preliminares en ratas sugieren que el AOH, una vez biotransformado en el hígado y excretado vía biliar al duodeno, se absorbe rápidamente desde el lumen intestinal y alcanza la sangre portal en forma de aglicona, glucurónido y sulfato(Burkhardt et al., 2011).

- **Efectos tóxicos**

En adelante, se indican los estudios relacionados con la citotoxicidad, estrés oxidativo y daño al ADN producido por micotoxinas de *Alternaria*, principalmente AOH y AME.

Los estudios *in vivo* del AOH y AME son escasos. En la Tabla 3 se muestran los ensayos correspondientes a toxicidad aguda, crónica, estudios de reproductividad, desarrollo y genotoxicidad.

Tabla 3. Efectos tóxicos del AOH y AME mediante ensayos *in vivo*

ESPECIE	MICOTOXINA	VIA Y DOSIS	TIPO ENSAYO	EFECTOS	REFERENCIAS
Ratón	AOH AME	i.p. 100,200, 400 mg/kg p.c.	Aguda	Espasmos gástricos	Pero et al., 1973
	AOH	*s.c*. 100 mg/kg p.c. día - días 9-12 de la gestación - días 13-16 de la gestación	Reproducción y desarrollo	Aumento de muertes. Reabsorción parcial o total del feto	Pero et al., 1973
	AOH	200 y 1000 mg/kg p.c. AOH radiomarcado	Toxicocinética	Baja absorción sistémica, 90% de la dosis total se excreta por las heces y hasta 9% a través de la orina. Niveles significantes en sangre (0.5 µM)	Schuchardt et al., 2014
		3 x 2000 mg/kg de AOH (0, 24, y 45 h), tras 48 h los ratones se sacrifican	Genotoxicidad Ensayos de micronúcleos y del cometa	Nose observó efecto genotóxico en la médula ósea ni en el tejido hepático	
	AME	Alimento: 50mg/kg p.c. 10 meses	Caricinogenicidad	Displasia de la mucosa esofágica	Yekeler et al., 2001

ESPECIE	MICOTOXINA	VIA Y DOSIS	TIPO ENSAYO	EFECTOS	REFERENCIAS
Rata	AME AOH	Alimentada 21 días con 39 mg/kg/día (AOH) y 24 mg/kg/día (AME)	Crónica	No evidencia de toxicidad	Sauer et al., 1978
Hámster	AME	*i.p*. 200 mg/kg p.c. el día 8 de la gestación	Reproducción y desarrollo	Dosis tóxicas para la madre. Aumento de reabsorciones y disminución del peso fetal	Pollock et al., 1982b
Embrión de pollo	AOH AME	i.p. 1000 (AOH), 500 (AME) µg por huevo	Aguda	No mortalidad	Griffin y Chu, 1983.

Abreviaturas:AOH*:* alternariol; AME: alternariol monometil éter*; i.p.*: intraperitoneal; *sc.:* subcutánea; p.c.: peso corporal

1.2 Toxicidad i*n vitro*

Numerosos ensayos *in vitro* han sido desarrollados durante la última década para evaluar la toxicidad aguda de diversos productos químicos. Estos ensayos son indicadores sencillos y rápidos de toxicidad, desarrollados con la intención de predecir los efectos tóxicos de dichos productos en animales y humanos, mediante reducción del uso de animales de experimentación, refinamiento de los métodos aplicados si se usan animales de experimentación o reemplazo de los animales por métodos alternativos, como es el caso de los ensayos *in vitro*.

Los ensayos *in vitro* son una alternativa a los ensayos tradicionales con animales, no sólo porque limitan el uso de animales, sino porque además proporcionan mayor rentabilidad. Son ética y moralmente más aceptables que los ensayos *in vivo*, ya que los animales vivos no se exponen a los tóxicos de ensayo. Los sistemas *in vitro* son utilizados para estudiar los mecanismos de acción y presentan numerosas ventajas como la versatilidad del diseño experimental, resultados más rápidos y fiables, menos coste económico, automatización y monitorización. Permiten estudiar las acciones del compuesto sobre una determinada población celular o fracción subcelular aislada, en la que se presuponga la diana principal o secundaria. Con ello, se evitan las interferencias producidas por otros órganos, células u orgánulos sobre la diana de estudio. Los ensayos se realizan en condiciones controladas, sin interacciones, requieren menores cantidades de los productos ensayados y los resultados presentan mayor reproducibilidad. Sin embargo, también presentan, entre otras limitaciones, la simplicidad de los resultados, complejidad de la interpretación y la dificultad de la extrapolación al hombre. A continuación, se describen diversos parámetros que se pueden determinar mediante la toxicidad *in vitro*.

1.2.1 Ensayos de viabilidad celular

La citotoxicidad basal implica la alteración de la función o la estructura celular, siendo similar la sensibilidad en tipos celulares de distinto origen (Ekwall, 1983). A lo largo de los años, se ha conjugado una gran variedad de pruebas y criterios para evaluar los efectos que presentan las células después de su exposición a un determinado compuesto químico.

Se han descrito múltiples ensayos para la determinación de la citotoxicidad de las micotoxinas. Entre los ensayos más frecuentes se encuentran: el ensayo de la sal de tetrazolio (MTT), el ensayo del rojo neutro (NR), el ensayo de contenido en proteínas totales (PC), el ensayo de la fluoresceína (FDA), el ensayo de ioduro de propidio (PI), el ensayo del azul alamar (AB), el ensayo de tripán azul, el ensayo de la lactato deshidrogenasa (LDH) y high content analysis (HCA).

El ensayo del MTT es simple y se usa para determinar la proliferación celular dada por el número de células presentes en el cultivo. El MTT (bromuro de 3(4,5 dimetil-2-tiazoil)-2,5- difeniltetrazólico), es captado por las células y reducido por la enzima succinato deshidrogenasa mitocondrial a su forma insoluble formazán. El producto púrpura de la reacción, el formazán, queda retenido en las células vivas y puede ser liberado mediante la solubilización con dimetil sulfóxido (DMSO) (Ruiz et al., 2006). La proliferación celular es proporcional a la cantidad de formazán púrpura producido por las células viables. El colorante NR (3-amino-7-dimetilamino-2-metilfenazina) sólo puede ser captado por las células vivas que lo acumulan en sus lisosomas. Este colorante débilmente catiónico penetra a través de las membranas celulares por difusión pasiva y se une intracelularmente a grupos carboxílicos y/o fosfatos de la matriz lisosomal (Ferrer et al., 2009).

Existen diferentes métodos para la cuantificación de PC. Entre los más comunes, se encuentran el método del azul de Coomassie, el método de Bradfort y el método de la sulforodamina (SRB). El azul de Comassie es un colorante derivado del trifenilmetano y tiñe proteínas al unirlas con el colorante Coomassie Blue G-250 (Pichardo et al., 2007). El mecanismo se basa en la unión del colorante azul brillante con residuos aromáticos y arginina en las proteínas, generando un azul intenso tras dicha unión. Sin embargo, la SRB es un colorante de aminoxantano, rosado brillante, capaz de unirse electrostáticamente a cationes. En condiciones ácidas, la SRB aumenta su afinidad por los aminoácidos básicos de las proteínas, y se fija selectivamente a estos, proporcionando un índice del contenido de proteína celular (Monks et al., 1991). Mediante citometría de flujo se puede caracterizar las células vivas o muertas de una población celular usando colorantes vitales como el PI, que tiñe los ácidos nucleícos cuando penetra en las células muertas y emite fluorescencia en el rojo, o el diacetato de fluoresceína (FDA), que es captado por las células vivas cuyas enterasas lo hidrolizan a fluoresceína que emite fluorescencia en el verde (Sandström et al., 2000). En la técnica del AB, el ingrediente activo es la resazurina, de color azul, permeable a las células. Al entrar en las células, la resazurina se reduce a resorufina, que produce fluorescencia roja o rosa. Las células viables realizan esta reacción, generando así una medida cuantitativa de la viabilidad (Ahmed et al., 1994). El azul de tripán es un método de tinción por exclusión que permite diferenciar células vivas de células muertas. Las células vivas con la membrana celular intacta no se colorean debido a que la membrana celular es selectiva. Por el contrario, sí atraviesa la membrana de las células muertas, lo que genera que se muestren de color azul bajo el microscopio.

Otro método muy utilizado en la determinación de la viabilidad celular es la evaluación de la actividad de la enzima LDH, una enzima estable normalmente presente en el citosol de todas las células, la cual es rápidamente liberada al exterior cuando hay daño celular. La actividad de la LDH se determina mediante una reacción enzimática en la que la LDH oxida el lactato dando piruvato, el cual reacciona con una sal de tetrazolio convirtiéndola en formazán. El incremento en la cantidad de formazán producido en el sobrenadante de cultivo se correlaciona directamente con el número de células muertas.

El HCA se considera una poderosa herramienta de detección, que se compone de un microscopio automatizado y una aplicación de software biológico, que puede adquirir, procesar y analizar datos de imagen obtenidos a partir de ensayos celulares específicos basados en fluorescencia. Esto permite analizar diversos parámetros de forma simultánea y cuantificar cambios visuales celulares o subcelulares.

En la Tabla 4 se observan los ensayos de viabilidad celular realizados con micotoxinas de *Alternaria.*

Tabla 4. Ensayos de viabilidad celular con micotoxinas de Alternaria.

CÉLULAS	MICOTOXINA	ENSAYO	CONDICIONES	RESULTADOS	REFERENCIA
Células primarias de endometrio porcino	AOH	MTT	0-12.5 μM;24 h	Disminuye la viabilidad desde 3.12 μM	Wollenhaupt et al., 2008
HCT116	AOH	FDA	10-200 μM; 24 h	IC_{50}=65 μM	Bensassi et al.,2012; 2015
	AME		0- 200 μM; 24 h	Disminución de la viabilidad dosis-dependiente desde 10 μM. IC_{50} = 120 μM	Bensassi et al., 2011
			10-200 μM; 24 h	$IC_{50=}$= 120 μM	Bensassi et al., 2015
HepG2	AOH	MTT	0-100 μM; 24, 48 y 72 h	IC_{50}= 65 -96 μM	Juan-García et al., 2015
HT29	AOH AME	SRB	0.1-50 μM; 24 h	50 μM disminuye la viabilidad entre un 10-15%	Tiessen et al., 2013
	AOH TeA	Azul de tripán	0.01-1 μM; 12 y 24 h	No disminuye la viabilidad	Schwarz et al., 2012
		SRB	0.01-1 μM; 24 y 72 h		

CÉLULAS	MICOTOXINA	ENSAYO	CONDICIONES	RESULTADOS	REFERENCIA
HT29 A431	AOH AME	Azul de tripán LDH	0.1-50 μM; 1 h 0.1-50 μM; 1h	-Azul de tripán: disminuye la viabilidad en un 85% -LDH: AOH y AME no disminuye la viabilidad	Fehr et al., 2009
H295R RGAs	AOH	AB MTT	H295R: 0.1-10000 ng/mL; 24 y 48 h RGAs : 50-10000 ng/mL; 48 h	AB: No disminuye la viabilidad MTT: disminuye la viabilidad a 5000 y 10000 ng/ml	Frizzell et al., 2014
MDCK, H4TG, NIH3T3	AAL		0, 1, 10 y 200 μM; 48 h	IC_{50}= 25 μM (MDCK) IC_{50}= 25 μM (H4TG) IC_{50}=200 μM (NIH3T3)	Abbas y Shierir, 1995
MLC, V79	AOH	No específico	0- 30 μM; 24 h	30 μM: disminuye la viabilidad un 90%	Brugger et al., 2006
P4	AOH AME	MTT	0.8-25.6 μM AOH; 48 h 6.4-100 μM AME; 48 h	12.8 μM (AOH y AME): disminuye la viabilidad	Tiemann et al., 2009

CÉLULAS	MICOTOXINA	ENSAYO	CONDICIONES	RESULTADOS	REFERENCIA
RAW 264.7	AOH	citometría de flujo	15 y 30 μM; 24 y 48 h. Fluorocomo: PI	-24 h: disminuyen la viabilidad entre el 66% (15 μM) y el 76% (30 μM) -48 h: disminuye la viabilidad entre el 66% (15 μM) y el 89% (30 μM)	Solhaug et al., 2013
		AB y NR	0- 60 μM; 24 y 48 h	-NR: IC_{50} =49.65 μM (24 h) -AB: IC_{50} =78.01 μM (24 h) IC_{50} =30 μM (48 h)	Solhaug et al., 2012
V79	AOH	MTT	0- 50 μM; 6 h	Inhibición de la proliferación celular a 25 y 50 μM	Lehmann et al., 2006
3T3, CHL, L-O2	TeA	MTT	12.5-400 μg/mL; 24, 48 y 72 h	-3T3:IC_{50}=34,7 μg/mL (24 h); IC_{50} = 26,37 μg/mL (48 h); IC_{50}= 26,13 μg/mL (72 h). -CHL:IC_{50}= 63,45μg /mL (24h); IC_{50}= 50,16 μg/mL (48 h); IC_{50}= 33,7 μg/mL (72h). -L-02: IC_{50}= 117,77 μg/mL (24 h); IC_{50}= 65,19 μg/mL (48 h); IC_{50} = 38,65 μg/mL (72 h).	Zhou y Qiang, 2008
		PC	12.5-400 μg/mL, 72h	IC_{50} = 37,37 μg/mL (3T3) IC_{50} =56,28 μg/mL (CHL) IC_{50} =68,79 μg/mL(L-O2)	

Abreviaturas: AB: azul alamar; ALL: toxinas de alternaría; AME: alternariol monometil- eter; AOH: alternariol; A431: células humanas de carcinoma de vulva; Caco-2: células de adenocarcinoma de colon humano; CHL: células de pulmón de hámster chino**;** EC_{50}: concentración media efectiva; FDA: ensayo de la fluoresceína diacetato; HCT116: células de cacinoma de colon humano; HepG2: células de hígado humano; HT29: células de carcinoma de colon humano; H295R : células humanas adrenocorticales; H4TG: células de hígado de rata; IC_{50}: concentración inhibitoria media; L-O2: células humanas de hepatocitos; LDH: ensayo de la lactato deshidrogenasa; MDCK: células de hígado de perro; MLC: células de linfoma de raton ; NIH3T3: células de fibroblastos de rata; MTT: ensayo de la sal de tetrazolio; NR: ensayo de rojo neutro; PC: ensayo de proteínas totales; P4: células de ovario (específicas para la hormona de la progesterona); RAW 264.7: macrófagos de roedor; RGAs: células con gen reportero (estrógenos, andrógenos, progestágeno y glucocorticoides); SRB: ensayo de la sulforhodamina B; TeA: ácido tenuazónico; V79: células de pulmón de hámster chino; 3T3: fibroblastos de rata.

1.2.2 Citotoxicidad de combinación de micotoxinas

Un hongo micotoxigénico es capaz de producir más de una micotoxina y, generalmente, diferentes hongos pueden infectar un mismo alimento. Teniendo en cuenta que la dieta humana es una combinación de alimentos, es muy importante la presencia de múltiples micotoxinas y sus posibles efectos sinérgicos. Igualmente, se debe considerar que la estructura química de las micotoxinas es muy diversa y no se dispone de información acerca del mecanismo de acción de un gran número de éstas (Speijers, 2004).

Los efectos sinérgicos se producen cuando los efectos combinados de dos o más micotoxinas son mucho mayores que los efectos individuales de cada micotoxina. La exposición a más de una micotoxina puede ser igual a la respuesta originada por cada toxina de manera individual, siendo éste un efecto aditivo. Cuando al combinar varias de ellas disminuyen los efectos tóxicos respecto a los producidos por las micotoxinas de forma individual, se le llama efecto antagonista.

Hasta la fecha, se han realizado un gran número de trabajos de investigación sobre las interacciones entre las micotoxinas en cultivos celulares, especialmente con micotoxinas de *Penicillium y Fusarium* (DON, toxina T-2, ZEA, ENNs, FB1, BEA,...) (Bernhoft et al., 2004, Heussner et al., 2006; Kouadio et al., 2007; Klarić et al., 2008, 2010; Ruiz et al., 2011a, 2011b; Ficheux et al., 2012; Lu et al., 2013; Prosperini et al., 2013; Tatay et al., 2013, 2014; Anninou et al., 2014; Zouaoui et al., 2016). Estos trabajos estudian la acción conjunta de dos o más micotoxinas combinadas en una mezcla y expuestas a diferentes tipos de células. La mayoría de los estudios de interacción *in vitro* se llevan a cabo con dosis únicas de cada micotoxina de manera individual y dosis en combinación. Sin embargo, la

evaluación de la toxicidad combinada es todavía un campo complejo donde se tiene en cuenta el tipo y la magnitud de la interacción, ya que ésta puede variar dependiendo de la concentración. En la Tabla 5 se exponen los resultados obtenidos en estudios de combinación de micotoxinas de *Alternaria* y otros géneros. Cabe destacar que se han aplicado varios métodos matemáticos para evaluar la interacción. Entre los diferentes utilizados para estudiar la citotoxicidad combinada encontramos el análisis de isobologramas. Este método se introdujo hace más de 30 años por Chou y Talalay (1984) para estudiar los efectos al combinar fármacos. En la última década, se ha aplicado con éxito este modelo matemático para determinar el tipo de interacción *in vitro* entre dos o más micotoxinas del genero *Penicillium* o *Fusarium* (Bernhoft et al., 2004; Luongo et al., 2006, 2008; Ruiz et al., 2011a, 2011b; Lu et al., 2013; Prosperini et al., 2013; Tatay et al., 2013, 2014; Alassane-Kpembi et al., 2015; Zouaoni et al.,2016).

El análisis de isobologramas implica el trazado de las curvas de dosis-efecto para cada compuesto y sus combinaciones en varias concentraciones diluidas mediante el uso de la siguiente ecuación (Figura 7):

$$fa/fu = (D/D_m)^m$$

Figura 7. La ecuación del efecto medio.

Donde *D* es la concentración del producto, *Dm* es la dosis que produce el efecto medio (por ejemplo, IC_{50}, EC_{50}, o LD_{50}), *f*a es la fracción afectada por la concentración de *D* (por ejemplo, porcentaje de inhibición / 100), *fu* es la fracción no afectada (por lo tanto, *f*a = 1 - *fU*), y m es el coeficiente que establece la forma de la relación dosis-efecto, siendo *m*= 1, *m*>1 o *m*<1 (Chou y Talalay, 1984). Por lo

tanto, el método tiene en cuenta tanto los parámetros de potencia (*Dm*) como de forma (*m*).

Chou y Talalay (1984) introdujeron el índice de combinación (CI) para la cuantificación del sinergismo o antagonismo en *n* números de compuestos, por lo que la ecuación general para la combinación de n-compuestos se convierte en (Fig. 8):

$$^{n}(\mathrm{CI})_x = \sum_{j=1}^{n} (D_j/D_x)_j = \frac{(D_x)_{1-n}\{[D]_j \sum_{j=1}^{n} [D]\}^{1/mj}}{(D_m)_j\{(\mathrm{fax})_j/[1-(\mathrm{fax})_j]\}}$$

Figura 8. Ecuación del índice de combinación.

Donde $^{n}(CI)_x$ es el índice de combinación para *n* compuestos (por ejemplo, micotoxinas) en la inhibición de x% (por ejemplo, inhibición de la proliferación); $(Dx)_{1\text{-}n}$ es la suma de la concentración de los *n* compuestos que ejercen inhibición de x% en la combinación, $\{[D]_j/\sum_{j=1}^{n}(D)\}^{1/mj}$ es la proporción de la concentración de cada uno de los *n* compuestos que ejerce inhibición x% en la combinación; y $(Dm)_j$ $\{(fax)_j$ / [1- $(fax)_j]\}$ es la concentración individual de cada compuesto que ejerce la inhibición x%. A partir de esta ecuación obtenemos CI <1, = 1, y> 1 que indica, respectivamente, sinergia, efecto aditivo y antagonismo.

Tabla 5. Ensayos de citotoxicidad combinada con micotoxinas de *Fusarium*, *Penicillium* y *Alternaria*.

CÉLULA	COMBINACIÓN	CONDICIONES	RESULTADO	REFERENCIAS
Caco-2	ENN B+ENN A1 ENN B1+ENN A1 ENNB+ENN B1 ENN A+ENN A1 ENN A+ENN B1 ENN A +ENN B, ENN A+ ENN A1+ ENN B ENN A+ ENN B+ ENN B1 ENN A+ ENN A1+ ENN B1 ENN A2+ ENN B+ ENN B1 ENN A+ ENN A1+ ENN B+ ENN B1	Combinaciones binarias: 0.625 - 5.0 µM (radio=1:1) Combinaciones terciarias y cuaternarias: 1.25 -5.0 µM (radio= 1:1:1 y 1:1:1:1); 24 h	ENN B+ENN A1,ENN B1+ ENN A1, ENN A+ ENN A1+ENN B= efecto sinérgico ENN B+ ENN B1=efecto antagonista ENN A+ ENN A1, ENN A+ ENN B1, ENN A + ENN B, ENN A+B+B1, ENN A+A1+B+B1, ENN A+A1+B1, ENN A2+B+B1= efecto aditivo	Prosperini et al., 2014
CFU-GM	DON + BEA DON + FB1 DON + T-2 DON + ZEA T-2 + ZEA BEA +ENB	DON= 40, 60, 100 Nm; BEA: 0.064, 0.64, 3.2 µM; FB1: 0.5, 1, 2 µM T-2: 0.5, 1, 1.6; ZEA: 0.2,1, 10 µM; 14 días.	DON+BEA=efecto sinérgico DON+ZEA , DON+T-2, ZEA+T-2 y BEA+ENNB=efecto aditivo DON+FB1=efecto antagonista	Ficheux et al., 2012

CÉLULA	COMBINACIÓN	CONDICIONES	RESULTADO	REFERENCIAS
CHO-K1	ENN A+ ENN A1 ENN A+ ENN B ENN A1+ ENN B1 ENN A+ ENN A1+ ENN B ENN A+ ENN A1+ ENN B1 ENN A+ ENN B+ ENN B1 ENN A1+ ENN B+ ENN B1	Combinaciones binarias (1:1): 0.625 - 5 µM Combinaciones terciarias (1:1:1): 0.3125 - 2.5 µM, 24 h	Las combinaciones binarias tuvieron un efecto aditivo. A las concentraciones más elevadas en combinaciones con ENN A se observó efecto sinérgico. En concentraciones menores de las combinaciones de ENNs A+A1+B1 y ENNsA1+B+B1 se observó efecto antagonista.	Lu et al., 2013
	BEA+DON+T-2 BEA+DON BEA+T-2 DON+T-2	DON: 0.25-4 µM BEA: 0.78-12.5 µM T-2: 0.006-0.1 µM BEA + DON, ratio = 3:1; BEA + T-2,ratio = 125:1; DON + T-2, ratio = 40:1)	DON + BEA y DON + T-2 presentaron efecto antagonista a 24, 48 y 72 h. BEA + T-2 presentaron sinergismo a 24, 48 y 72 h. BEA + DON + T-2 presentaron 24 y 48h sinergismo y antagonismo a 72 h.	Ruiz et al., 2011 b

CÉLULA	COMBINACIÓN	CONDICIONES	RESULTADO	REFERENCIAS
	ZEA + αZOL ZEA+ βZOL αZOL + βZOL ZEA+ α ZOL+ βZOL	ZEA: 12.5 - 50 µM αZOL y βZOL: 6.25 - 25 µM; 24, 48 y 72 h Radio: ZEA + α-ZOL y ZEA + β-ZOL =2:1 α-ZOL + β-ZOL= 1:1 y ZEA + α-ZOL + β-ZOL =2:1:1.	Combinaciones binarias: Efecto sinérgico a bajas concentraciones y efecto aditivo a altas concentraciones. Combinaciones terciarias: efecto antagonista a concentraciones bajas y efecto sinérgico a altas concentraciones.	Tatay et al., 2013, 2014
	BEA+PAT BEA+STE STE+PAT BEA+PAT+STE	STE: 0.78 - 6.25 µM. BEA: 0.156 - 1.25 µM PAT: 0.049 - 0.39 µM; 24, 48 y 72 h Radios: STE + PAT y STE+BEA= 5:1 BEA+ PAT= 3.2:1 y STE+ BEA + PAT= 5:3.2:1	Combinaciones binarias y terciaria, a bajas concentraciones tienen efecto sinérgico y a altas efecto aditivo.	Zouaoui et al., 2016
HepG2	ZEA + αZOL ZEA+ βZOL αZOL + βZOL ZEA+ α ZOL+ βZOL	ZEA: 12.5 - 100 µM αZOL y βZOL: 6.25 - 100 µM; 24, 48 y 72 h Radio: ZEA + α-ZOL y ZEA + β-ZOL =2:1 α-ZOL + β-ZOL= 1:1 y ZEA + α-ZOL + β-ZOL =2:1:1.	Combinaciones binarias: Efecto sinérgico y aditivo dependiendo de las concentraciones. Combinaciones terciarias: efecto antagonista a concentraciones bajas y efecto aditivo a altas concentraciones.	Tatay et al., 2014

CÉLULA	COMBINACIÓN	CONDICIONES	RESULTADO	REFERENCIAS
L929	DON+T-2 ZEA+FB1	ZEA + FB1: 1.9-3.9 ng/ml T-2 + NIV: 0.5-2.9 ng/ml, 24 h	En las concentraciones menores de combinaciones de ZEA + FB1 y T-2 + NIV hubo efecto aditivo.	Tajima et al., 2002
Linfocitos de sangre periférica humana	DON + DAS T-2 + DAS T-2 + NIV DAS + NIV NIV + DON T-2 + DON	Concentraciones de T-2 y DAS: $2\ 10^{-8}$, $4\ 10^{-9}$, $2\ 10^{-9}$ y $4\ 10^{-10}$ M Concentraciones de NIV y DON: $4\ 10^{-6}$, $2\ 10^{-6}$, $4\ 10^{-7}$ y $2\ 10^{-7}$ M; 24 h	Las combinaciones con NIV presentan efecto aditivo Las combinaciones con DON efecto antagosnista	Thuvander et al., 1999.
LLC-PK1	CIT + OTA OTB + OTA PAT + OTA CIT + OTB PAT + OTB OTA + OTB	OTA: 10 - 14 µM OTB: 25 - 50 µM PAT: 0.1 - 1.25 µM CIT: 70 - 170 µM 24, 48, 72 y 96h	Todas las combinaciones presentan efecto aditivo, siendo mayor en el siguiente orden: CIT + OTA > OTB + OTA > PAT + OTA y CIT + OTB > PAT + OTB > OTA + OTB	Heussner et al., 2006.
PK-15	FB1 + BEA FB1 + OTA BEA + OTA FB1 + BEA + OTA	Combinación binaria y terciaria (1:1), (1:1:1) 0.05, 0.5 y 5 µg/ml; 24 y 48 h	La combinación binaria y terciaria presentó efecto aditivo a 5 µg/ml a 24 y 48 h.	Klarić et al., 2008
	BEA+OTA	0.1 + 1; 0.1 + 5; 0.5 + 1; 0.5 + 5 µM; 24 h	Efecto aditivo (0.1 + 5 y 0.5 + 5 µM) Efecto sinérgico (0.1 + 1 y 0.5 + 1 µM).	Klarić et a., 2010

CÉLULA	COMBINACIÓN	CONDICIONES	RESULTADO	REFERENCIAS
Vero	BEA + DON+T-2 BEA + DON BEA + T-2 DON + T-2	T-2= 0.001 - 0.05 µM DON = 0.25 8 µM BEA = 0.78 - 25 µM Radio: BEA + DON= 3:1 BEA + T-2= 250:1 DON + T-2 = 60:1 BEA+ DON + T-2=1250:400:1	Combinaciones binarias y terciarias presentaron efecto antagonista.	Ruiz et al., 2011a

Abreviaturas: BEA: Beauvericina; Caco-2: células de adenocarcinoma de colon humano;CFU-GM: células madres formadoras de colonias eritromegacariocíticas; CHO-K1: células de ovario de hámster chino; CIT:Citrina; DAS: Diacetoxiscirpenol; DON: Deoxinivalenol;ENN A, A1, B, B1, B2: Eniatina A, A1, B, B1, B2; FB1: Fumonisina B1; HCT116: células de carcinoma de colon humano; NIV: Nivalenol; LLC-PK1: células epiteliales de riñones porcinos; L929: fibroblastos de ratón; OTA: Ocratoxina A; OTB: Ocratoxina B; PAT: Patulina; PK-15: células de riñón de cerdo; STE: Esterigmatocistina; T-2: toxina T-2; Vero: células epiteliales de riñón de mono; ZEA: Zearalenona; αZOL: αZearalenol; βZOL; βZearalenol.

1.2.3 Proliferación celular

El ciclo celular es un conjunto ordenado de sucesos que culmina con el crecimiento de la célula y la división en dos células hijas (Fig.9). El ciclo celular se divide en dos fases (Alberts et al., 2008):

Fase 1. Interfase: Es el periodo de tiempo entre dos mitosis sucesivas y ocupa la mayor parte del tiempo del ciclo celular. En esta fase, la actividad metabólica es muy alta, ya que la célula aumenta de tamaño y duplica el material genético. Los periodos de la interfase son los siguientes:

-Fase G1: Es la primera fase de crecimiento. Dura hasta la entrada en la fase S. Hay una intensa actividad biosintética de ARN y proteínas. Las células que no entran en mitosis permanecen en la fase G0 o Sub G1 (estado de reposo o quiescencia). Este es un estado propio de células diferenciadas que entran en quiescencia o que van a morir (apoptosis).

-Fase S: Una vez doblado su tamaño se inicia la duplicación del ADN, la síntesis de histonas y la duplicación de los centrosomas (en células animales). Es importante tener en cuenta que no todo el ADN se está replicando a la vez. Si se detectan roturas del ADN, mediante los sistemas de control, la copia del resto del ADN se detiene.

-Fase G2: Es la segunda fase de crecimiento donde hay un ligero aumento de tamaño celular. Acaba con el inicio de la condensación de los cromosomas y la entrada en mitosis. Durante esta etapa, sin embargo, se comprueba si ha habido errores durante la replicación del ADN y si se ha producido su duplicación completa. Si estos defectos son detectados, la célula no entrará en fase M y el ciclo celular se detendrá hasta que los daños sean reparados o el ADN sea completamente copiado.

Fase 2. Mitosis y citocinesis: Fase M. Es la división celular en la que una célula progenitora se divide en dos células hijas idénticas.

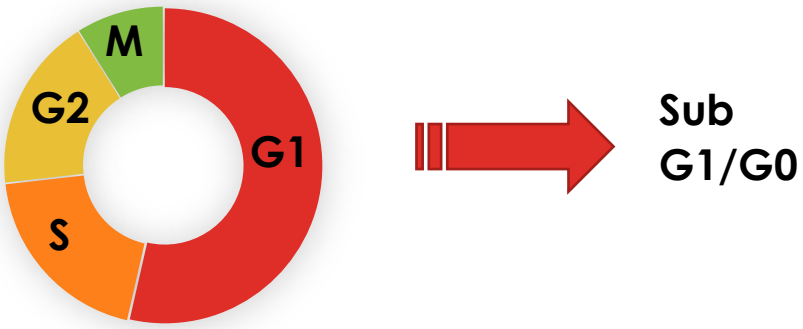

Figura 9. Representación de las fases del ciclo celular. Sub G1/G0= estado de reposo o quiescencia; G1: fase de crecimiento; S: fase de síntesis; G2: fase de crecimiento 2; M: mitosis.

Son varios los efectos que las micotoxinas pueden ejercer sobre el ciclo celular, los cuales pueden inducir parada en distintas líneas celulares en las diferentes fases del mismo (G1, S y G2/M) (Brugger et al., 2006; Fleck et al., 2012; Juan-García et al., 2013; Solhaugh et al., 2012, 2013; Prosperini et al., 2013). Esto es debido a la activación de diferentes puntos de control del ciclo celular que aseguran la progresión ordenada y previenen la mitosis aberrante en respuesta a una serie de eventos, incluyendo daños en el ADN (Visanji et al., 2004).

La citometría de flujo es una técnica de análisis celular multiparamétrica que cuantifica la cantidad de ADN en cada fase del ciclo celular. Esta técnica proporciona información útil sobre la acción citotóxica de las micotoxinas. Para estudiar el contenido de ADN y la proliferación celular se necesitan fluorocromos que tras la unión con el ácido nucleico emita una señal fluorescente. Hay cuatro

grupos de fluorocromos básicos que tienen diferencias en el espectro de absorción: (Ormerod, 1990).

1. Fenantridinas: del tipo del bromuro de etidio y PI. Especificidad por el ADN y ARN intercalándose en la doble cadena. Excitación en el azul/verde-rojo. Precisan de células muertas y fijadas, disminuye la intensidad de la señal y la especificidad de la unión secundaria a los grupos fosfato, proteasas, ADN estructura Z y bromodeoxiuridina.

2. Bismenimidazoles y afines: tipo Hoechst y DAPI (4',6-diamino-2-fenilindol). Tienen elevada especificidad por el ADN y se unen preferentemente a las bases de adenina-timina (AT) del ADN. Causan excitación en el ultravioleta-azul y tiñen células vivas.

3. Cromomicina y similares: como la cromomicina A3, mitramicina y olivomicina. Se unen preferiblemente a las bases de citosina-guanina (CG) del ADN. Producen excitación en el espectro ultravioleta-azul/verde. Requieren del ión magnesio (Mg) para la unión y pueden teñir células vivas.

4. Otros: quinacrina (intercalante), naranja de acridina (unión al ADN y ARN bicatenario, emisión diferente). Excitación en el espectro verde-rojo.

En la Tabla 6 se observan los ensayos de proliferación celular con micotoxinas de *Alternaria.*

Tabla 6. Ensayos de proliferación celular con micotoxinas de *Alternaria*.

CÉLULAS	MTX	ENSAYO	FC	CONDICIONES	RESULTADOS	REFERENCIAS
Células primarias porcinas endometriales	AOH	Ciclo celular	PI	0.078 y 12.5 μM; 24 y 48 h	24 h: no hay cambio 48 h: aumento de las células en fase G0 /G1 a 12.5 μM (40.7%) y 0.78 μM (17.5%). Las células en fase S disminuyen a 12.5 μM (39.5%) y 0.78 μM (67.5%).	Wollenhaupt et al., 2008
HCT116	AOH AME	Fragmentación Nuclear del ADN	PI y RNasa	50 μM; 48 h	La población hipodiploide aumentó en un 21%.	Bensassi et al., 2012
Hepa-1c1c4 Hepa-1c1c12	AME	Ciclo celular	PI/DAPI	5- 40 μM; 24h	Bloqueo en la fase G2/M en células Hepa-1c1c7 expuestas a AME y en células Hepa-1c1c12 al AOH.	Burkhardt et al., 2012
HT29	ATX II	Ciclo celular	PI	0.1 y 1 μM; 12 y 24 h	Aumentan las células en fase G0/G1 tras 24 h de incubación con 1 μM ATX II.	Schwarz et al., 2012

CÉLULAS	MTX	ENSAYO	FC	CONDICIONES	RESULTADOS	REFERENCIAS
Ishiwaka y V79	AOH	Proliferación celular	PI/DAPI	0.5-10 μM; 48 y 72 h: Ishiwaka 6 h: V79	Las células disminuyen en la fase G1 y aumentan en la fase S y G2/M. El índice mitótico se reduce dependiendo del tiempo y la concentración.	Lehmann et al., 2006
MLC y V79	AOH	Ciclo celular	PI/DAPI	0- 30 μM; 24 h	Aumentan las células V79 en fase G2/M (desde 15.3% hasta62.6 %) de manera concentración y tiempo dependientes.Aumentan las células MLC en fase G2/M (desde 21.4% a 37.1%) tras ≥20 μM.	Brugger et al., 2006
RAW 264.7	AOH	Ciclo celular	PI/RNasa	30 μM; 24 h.	El 77.4% de células se bloquean en la fase G2.	Solhaug et al., 2012
			PI	15 y 30 μM; 6, 24 y 48 h	Acumulación de células en la fase S (15 μM) y en la fase G2/M (30 μM) a las 48 h de exposición.	Solhaug et al., 2013

CÉLULAS	MTX	ENSAYO	FC	CONDICIONES	RESULTADOS	REFERENCIAS
RAW 264.7	AOH	Cambio nuclear	Hoechst 33342	15 y 30 μM; 24-72 h	Aumento del tamaño nuclear provocando una morfología anormal.	Solhaug et al., 2013
V79	ATX II AOH AME	Ciclo celular	PI/DAPI	ATX II: 0-0.75 μM AOH: 0-20 μM AME: 0-40 μM 24h	La ATX II no afecta al ciclo celular. El AOH y el AME producen acumulación de células en la fase G2/M a 10 μM	Fleck et al., 2012

Abreviaturas: AME: alternariol monometil- éter; AOH: alternariol; ATX II: altertoxina II; DAPI: 4'-6-diamino-2-fenilindol; FC: fluorocromo; HC T116: células de carcinoma de colon humano; Hepa-1c1c4 y Hepa-1c1c12 : células hepáticas de ratón; HT29: células de carcinoma de colon humano; MLC: células de linfoma de ratón; MTX: micotoxina; PI: yoduro de propidio; RAW 264.7: macrófagos de roedor; RNasa A : ribonucleasa A; V79: células de pulmón de hámster chino.

1.2.4 Estrés oxidativo

El estrés oxidativo consiste en un desequilibrio entre especies oxidantes y antioxidantes, favoreciéndose los procesos pro-oxidantes capaces de oxidar lípidos, proteínas y ácidos nucleicos, que resultan en un cambio estructural y funcional celular que puede causar la muerte celular.

1.2.4.1 Especies reactivas de oxígeno

La oxidación es un proceso bioquímico donde existe una pérdida de electrones siempre asociado a otro de captación de electrones. Estas reacciones se las conoce como reacciones redox. A través de estas reacciones se genera energía, la cual es necesaria para que la vida exista. Sin embargo, en la respiración no sólo se obtiene energía, también se producen especies reactivas de oxígeno (ROS), entre las que se encuentran los radicales peróxidos (ROO·), radical hidroxilo (OH·), anión superóxido ($O2\bullet^{-}$), el peróxido de hidrógeno (H_2O_2) y el singlete de oxígeno ($\frac{1}{2}O_2$).

Las ROS no sólo se generan por la respiración celular, sino también por oxidantes exógenos como las micotoxinas. En determinadas circunstancias, donde se produce el desequilibrio entre las velocidades de producción y de destrucción de las moléculas tóxicas, da lugar a un aumento en la concentración celular de las ROS causando el estrés oxidativo (Ferrer et al., 2009; Meca et al., 2010 b; Prosperini et al., 2013a, 2013b; Tatay et al., 2016).

En los últimos años, se han utilizado sondas fluorescentes para determinar la generación intracelular de las ROS en tiempo real. En este sentido, la sondas que más se utilizan son la dihidroetidio (DHE), el MitoSox, la dihidrorodamina 123 (DHR 123) y la diclorodihidrofluoresceína (H_2-DCF) (Wojtala et al., 2014).

La DHE es la forma reducida del bromuro de etidio. Debido a su alta reactividad y a su eficacia en la difusión pasiva en las células, el DHE se utiliza para detectar superóxidos citosólicos. Tras su reacción con el anión superóxido, el DHE forma un color fluorescente rojo cuya máxima longitud de excitación y emisión tiene lugar a 500 y 580 nm respectivamente (Martinez-Pastor et al., 2010).

MitoSOX es una sonda utilizada para medir la producción de superóxido en la matriz mitocondrial. Debido a que el MitoSOX es un derivado catiónico del DHE, su reacción con los $O2^{\bullet -}$, es muy similar a la del DHE. Sin embargo, reacciona con el superóxido de forma más rápida que ésta en la mitocondria, donde se oxida por el superóxido para fomar 2-hidrometiletidio, cuya longitud de excitación y emisión es de 510 y 580 nm, respectivamente (Robinson et al., 2006).

La DHR123 (no fluorescente) se difunde a través de las membranas celulares y es oxidada por las ROS al fluorocromo rodamina 123 (RH123) que emite fluorescencia.

La H_2-DCFDA detecta el peróxido de hidrógeno en las células, permeabilizándose libremente a través de la membrana plasmática e hidrolizándose en el citosol formando el anión carboxilato (DCFH). La oxidación de H_2DCF a diclorofluoresceína (DCF) es un proceso de dos pasos: primero, formación del radical DCF y, a continuación, se oxida a DCF en una reacción con el oxígeno molecular. Como resultado, la molécula se detecta a una longitud de excitación y emisión de 495 nm y 520 nm respectivamente. (Liu et al., 2007; Bouaziz et al., 2008; Ferrer et al., 2009; Meca et al., 2010 b; Prosperini et al., 2013 a, b).

En la Tabla 7 se observan los resultados obtenidos con ensayos que determinan las ROS realizados mediante ensayos *in vitro* con micotoxinas de *Alternaria.*

Tabla 7: Determinación de ROS en células expuestas a micotoxinas de *Alternaria*

CÉLULAS	MICOTOXINA	ENSAYO	CONDICIONES	RESULTADOS	REFERENCIA
HCT116	AME	H2- DCFDA MitoSOX	120 μM; 24 h	No se observó generación de ROS. Los niveles del anión superóxido mitocondrial aumentaron en un 28% respecto al control.	Bensassi et al., 2011
	AOH	H2-DCFDA MitoSOX	50 μM; 24 h	No se observó generación de ROS. Los niveles del anión superóxido mitocondrial aumentaron aprox. 30 veces respecto al control.	Bensassi et al.,2012
	AOH AME	H2- DCFDA MitoSOX	10-200 μM; 24 h	No se observó generación de ROS. Los niveles del anión superóxido en la mitocondria aumentaron aprox. un 22.5%, 15% y 35 % en presencia de 25 μM de AOH; 25μM de AME y la combinación de 25 μM AOH+AME, respectivamente.	Bensassi et al., 2015
HT29	AOH AME TeA	H2- DCFDA	AOH y AME: 0.1-50 μM; 1 h TeA: 0.2 - 200 μM; 1 h	Tras 50 μM de AOH y AME, la generación de ROS aumentó 2.5 veces respecto al control. No se observaron cambios con el TeA.	Schwarz et al., 2012

CÉLULAS	MICOTOXINA	ENSAYO	CONDICIONES	RESULTADOS	REFERENCIA
	AOH AME	H2-DCFDA	0.1-50 μM; 1 h	Tras 50 μM de AOH y AME, la generación de ROS aumenta 2.5 y 1.8 veces respectivamente respecto al control.	Tiessen et al., 2013
RAW 264.7	AOH	H2-DCFDA	30 μM; 0-7 h	Aumentó la generación de ROS tras 30 μM de AOH a partir de los 30 min.	Solhaug et al., 2012

Abreviaturas: AOH: alternariol; AME: alternariol monometil-éter; Caco-2: células de adenocarcinoma de colon humano; HCT116: células de cacinoma de colon humano; HT29: células de carcinoma de colon humano; H2-DCFDA: 2',7' diclorodihidrofluoresceína diacetato; MitoSOX: indicador de superóxido mitocondrial; RAW 264.7: macrófagos de roedor; ROS: especies reactivas de oxígeno; TeA: ácido tenuazoíco.

1.2.4.2 Peroxidación lipídica

Los lípidos son constituyentes estructurales y funcionales de las membranas biológicas. Las modificaciones de la estructura o alteración de la función que desempeñan pueden conducir a la muerte celular. La peroxidación lipídica (LPO) es un proceso patológico relacionado con la toxicidad del H_20_2 y del estrés oxidativo. Es una reacción en cadena de degradación de ácidos grasos poliinsaturados de las membranas que se descomponen en aldehídos como el malondialdehído (MDA), los 4-hidroxi-alquenales (4-HDA) y algunos isoprostanos. La determinación de la LPO se basa en la detección de los subproductos generados, el MDA, 4-HDA y el 8-isoprostano. Para cuantificar los aductos del 4-HDA se utilizan anticuerpos y se determinan mediante western blot o ELISA y, para el 8-isoprostano, también se utiliza el método ELISA.

El método más comúnmente utilizado para determinar la degradación de ácidos grasos como el MDA por micotoxinas es el método TBARS. Las sustancias reactivas al ácido tiobarbitúrico (TBAR) provocan modificaciones del transporte a través de las proteínas de membrana y de la actividad enzimática celular y, como consecuencia, pueden causar la muerte celular (Mary et al., 2012). Este se basa en la reacción del MDA con el ácido 2-tiobarbitúrico (TBA) para formar aductos cromógenos y fluorescentes de MDA-TBA muy estables y que se pueden cuantificar por espectrofotometría de absorción visible o por fluorimetría (Liu et al., 2006; Ferrer et al., 2009; Meca et al., 2010c; Prosperini et al., 2313 a, b; Awad et al., 2014) (Fig. 10).

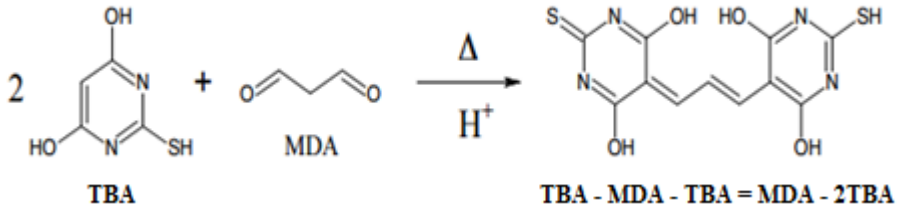

Figura 10. Mecanismo de la reacción entre el ácido tiobarbitúrico y el malondialdehído para la cuantificación de aductos de MDA - TBA.

Se ha observado un aumento de LPO tras la exposición a diferentes micotoxinas como las ENNs (Prosperini et al., 2012), BEA (Ferrer et al., 2009; Prosperini et al., 2013; Mallebrera et al., 2015), PAT (Liu et al., 2007; Ferrer et al., 2009), ZEA (Koaudio et al., 2007; Ferrer et al., 2009), FB1 (Koaudio et al., 2007; Meca et al., 2010 b; Mary et al., 2012) y DON (Koaudio et al., 2007; Yang et al., 2014). Actualmente, no se disponen de datos de LPO en líneas celulares expuestas a micotoxinas de *Alternaria*.

1.2.5 Potencial de membrana

La membrana plasmática contiene canales proteicos que permiten a los principales iones celulares (Na^+, K^+, Ca^{2+} y Cl^-) atravesarla a distintas velocidades a favor del gradiente de concentración. Los gradientes de concentración de iones y el movimiento de iones a través de la membrana crean una diferencia de potencial eléctrico entre el interior y el exterior de la membrana celular. A esta diferencia de potencial eléctrico se la denomina Potencial de Membrana (ΔΨ). El ΔΨ representa el elemento fundamental de toda la actividad eléctrica de las células. En la mayor parte de las células, el valor de dicho potencial varía entre -60 ó -70 mV (el signo negativo indica que el interior de la célula presenta carga negativa respecto al exterior). Esta diferencia de gradientes y el potencial

eléctrico a través de la membrana dirigen muchos procesos biológicos. Así, por ejemplo, la apertura y cierre de canales de Na^+, K^+, Ca^{2+} y Cl^-, es esencial para la conducción de impulsos nerviosos a través de los axones de células nerviosas y el aumento en la concentración de Ca^{2+}, importante señal reguladora (contracción muscular, secreción de hormonas). Muchas micotoxinas presentes en la naturaleza actúan a menudo sobre canales iónicos, originando diferencias en el potencial eléctrico. El ΔΨ queda reducido cuando la energía del metabolismo está alterada, generalmente durante apoptosis (Shapiro, 2000). Una gran variedad de eventos claves en la apoptosis están focalizados en las mitocondrias. Estos incluyen: liberación de activadores de caspasas (por ej., Citocromo c), cambios en el transporte de electrones, pérdida del ΔΨ, alteraciones en el proceso de óxido - reducción celular, participación de la familia de las proteínas pro- (Bax, Bad, Back y p53) y antiapoptóticas (Bcl-2 y Bcl-x) (Dornetshuber et al., 2007; Ivanova et al., 2012).

La medida del ΔΨ se lleva a cabo por medio de colorantes lipófilos que se acumulan tras atravesar la membrana celular. Las células con membranas dañadas no pueden mantener o generar un gradiente electroquímico, por lo que se altera el ΔΨ considerando estas células como muertas. Las estructuras de estas células se liberan al medio y finalmente se descomponen y lisan. La integridad de la membrana puede detectarse por métodos de exclusión de colorante. Las células con membranas intactas son impermeables a colorantes cargados. De esta manera, si las células pierden la integridad de membrana, los colorantes pueden entrar en las células, emitiendo la fluorescencia tras la unión a los ácidos nucleicos.

Varios colorantes catiónicos lipofílicos fluorescentes, como la tetrametilrodamina de metilo (TMRM); tetrametilrodamina etil éster (TMRE), Rh

123, yoduro de 3,3'-dihexiloxacarbocianine (DiOC6), y 5, 5', 6, 6'-tetracloro-1, 1', yoduro de 3, 3' tetraetilbenzimidazolilcarbocianina (JC-1) miden directamente el ΔΨ (Prosperini et al., 2013b; Mallebrera et al., 2016).

Estos colorantes cargados se acumulan dentro de las mitocondrias en proporción inversa al Δψ de acuerdo con la ecuación de Nernst (Fig. 11). En mitocondrias hiperpolarizadas, donde el interior es más negativo, se acumulará mayor cantidad de colorante y, en las mitocondrias despolarizadas (con un interior menos negativo) se acumula menos colorante (Perry et al., 2011).

$$E = E° - \frac{RT}{nF}\log(Q)$$

Figura 11. Ecuación de Nernst.

Siendo: E, el potencial corregido del electrodo; E^o, el potencial en condiciones estándar (los potenciales se encuentran tabulados para diferentes reacciones de reducción); R, la constante de los gases; T, la temperatura absoluta (escala Kelvin); n, la cantidad de moles de electrones que participan en la reacción; F, la constante de Faraday (aproximadamente 96500 °C/mol); Log (Q), el logaritmo natural de Q que es el cociente de la reacción.

Hasta la fecha, únicamente Bensassi et al., (2012 y 2015) han estudiado el potencial de membrana con micotoxinas de *Alternaria.* La investigación realizada por Bensassi et al., (2012) se centró en el potencial de membrana tras la exposición de las células HCT 116 a 50 µM de AOH durante 6, 16, 24 y 48 h. En el estudio realizado por Bensassi et al., (2015), l*as* células HCT116 fueron expuestas al AOH y al AME de manera individual (25 µM de AOH y AME) y combinada (25:25 µM de AOH y AME). Mediante el fluorocomo CMXRos (1H,5H,11H,15H-Xanteno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-io,9-[4-(clorometil)fenil]-

2,3,6,7,12,13,16,17-octahidro se midió el Δψ en ambos estudios y se observó una pérdida de potencial tras la exposición individual de ambas micotoxinas, siendo dicha pérdida mayor tras la exposición combinada.

1.2.6 Apoptosis/Necrosis

El número de células en los diferentes tejidos está determinado por un balance homeostático entre la proliferación de células nuevas y la muerte de células agotadas o seniles, existiendo una tasa o ritmo de relación proliferación/muerte que varía de un tejido a otro (Hess et al., 1998).

Existen dos mecanismos de muerte celular. Uno se produce como consecuencia de una lesión celular masiva con inflamación conocida como necrosis, mientras que el otro causa muerte celular a través de un mecanismo controlado genéticamente, no inflamatorio, dependiente de la energía celular, llamado apoptosis. Entre los cambios producidos en la apoptosis, se incluyen los cambios morfológicos, dónde se observan cambios en la organización de la membrana citoplasmática, aparición de condensación de la cromatina y aparición de fragmentación nuclear consecuencia de la degradación de ADN. La desregulación de la apoptosis está asociada a enfermedades como cáncer, neurodegeneración, autoinmunidad, miocardiopatías y otras alteraciones como las observadas en el proceso inflamatorio inmune (Hetts, 1998).

Se han descrito múltiples métodos para la determinación de células apoptoticas y necróticas. Para determinar los cambios en la célula apoptótica, se pueden utilizar tanto la microscopia óptica, como la microscopia electrónica. Para detectar la degradación del ADN, se utiliza el marcaje del ADN en células individuales adicionando nucleótidos marcados con fluorescencia, o bien, se pueden detectar por métodos de inmunohistoquímica. Este método se conoce

como método de TUNEL (transferase-mediated dUTP nick endlabeling) y se puede utilizar tanto para la detección por medio de citometría de flujo, como por microscopia fluorescente (Gavrieli et al., 1992). La determinación de la activación de las moléculas que disparan el proceso de apoptosis también ha adquirido gran importancia en su detección.

Las caspasas son un grupo de proteínas pertenecientes a las cisteín-proteasas, caracterizadas por presentar un residuo de cisteína que media la ruptura de otras proteínas. Las caspasas están presentes en forma de pro-caspasa hasta que una señal las activa. Una vez que las primeras caspasas han sido procesadas y activadas, éstas desencadenan una señal en cascada activando a otras caspasas. La activación de esta cascada de amplificación trae como consecuencia la inactivación de un gran número de proteínas estructurales, de señalización, reguladoras y transcripción y del metabolito de ácidos nucleicos, que desemboca en la muerte celular (Logue y Martin, 2008).

Las caspasas iniciadoras como las caspasas-8 y 9 procesan las formas inactivas de las caspasas efectoras como las caspasas-3 y 7, activándolas. Las caspasas efectoras, una vez activadas, procesan a su vez otros sustratos proteicos que mediarán en las distintas vías de apoptosis. La iniciación de estas reacciones en cascada está regulada por inhibidores de caspasas. Por ello, la activación de la caspasa-3 y 7 se utiliza como marcador temprano de la apoptosis tanto por citometría como por fluorescencia. Uno de los marcadores más utilizados es el DEVD-pNA. Las caspasas reconocen la secuencia de aminoácidos DEVD (secuencia de escisión de las caspasas-3). El pNA es un fluorocromo que se encuentra unido a DEVD. La actividad de la caspasa se puede cuantificar por espectrometría dependiendo del pNA libre.

Otro método muy utilizado es la evaluación de la simetría de la membrana celular. Durante la apoptosis, la célula mantiene la integridad de la membrana, permaneciendo la característica de ser semipermeable, lo cual conlleva cambios en su simetría. La fostatidil serina es una molécula orientada hacia el interior de la célula y, cuando la célula entra en el proceso de muerte por apoptosis, la fosftatidil serina sale hacia el exterior de la membrana celular. Se han desarrollado métodos que permiten detectar la presencia de fosfatidilserina en el exterior de la membrana celular. La anexina V es una molécula que no es capaz de difundir a través de la membrana y que tiene una alta afinidad por la fosfatidil serina. El isotiocianato de fluoresceína (FITC) se une a la Anexina V, de manera que mediante citometría de flujo, se detectan las células marcadas con FITC-anexina V, las cuales se encuentran en apoptosis (Moreno et al., 2000). También se utiliza el método de la Calceina/cobalto para monitorizar la activación de la membrana plasmática, de forma que una vez el poro se abre, la calceina se libera de la matriz mitocondrial emitiendo fluorescencia.

La fluorescencia del fluorocromo DIOC-6 con el medio es mucho mayor cuando se incorporan en las membranas o se unen a biomoléculas lipófilas tales como proteínas. Son débilmente fluorescentes en agua, pero altamente fluorescentes y muy fotoestables cuando se incorporan en las membranas.

La citometría de flujo parece ser la metodología de elección para estudiar diversos aspectos de la necrosis y apoptosis. Gran variedad de ensayos se han desarrollado sobre los cambios en las propiedades de la membrana plasmática. Debido a que la membrana intacta de células vivas excluye los colorantes catiónicos, los ensayos basados en la exclusión de estos fluorocromos son comúnmente utilizados para investigar la viabilidad celular. Tanto el fluorocromo etidio monoazida (EMA), como el PI, son moléculas cargadas positivamente, que

excluyen células vivas o con apoptosis temprana, por lo que tiñen las células que han perdido la integridad de la membrana plasmática, es decir, células necróticas y apoptóticas tardías. Estos fluorocromos se intercalan con los ácidos nucleicos tras la exposición a la luz visible (Riedy et al., 1991). El fluorocromo de ADN denominado Hoechst 33342 (HO342), a diferencia del PI, no excluye las células vivas o apoptóticas. La captación de HO342, combinado con exclusión de PI, ayuda a diferenciar las células necróticas de las apoptóticas tardías. Otros fluorocromos, tales como SYTO-16 y LCS-751, también se pueden utilizar para la detección de células apoptoticas/necróticas por citometría de flujo (Belloc et al., 1994).

En la tabla 8 se observan ensayos que determinan procesos de apopotosis y necrosis causados por micotoxinas de *Alternaria* mediante marcadores fluorescentes.

Tabla 8: Ensayos de apoptosis y necrosis con micotoxinas de *Alternaria.*

CÉLULAS	MICOTOXINA	ENSAYO	FLUOROCROMO	CONDICIONES	RESULTADOS	REFERENCIA
HCT116	AOH	Muerte celular	FITC- Anexina V/ PI	50 μM; 24 h	Células pre-apoptóticas aumentan un 25% y post-apoptóticas un 15%. La apoptosis se caracterizó por un aumento de células en fase Sub G1.	Bensassi et al., 2012
		Actividad de las caspasas 3 y 9	DEVD/pNA	5-50 μM; 0-20 min	La actividad de la caspasa 9 aumenta dependiendo del tiempo y la concentración tras 6 h de exposición. La actividad de la caspasa 3, disminuye tras 24 h de exposición.	
		Regulación de p53 (por Western Blot)		5-50 μM; 6, 16 y 24 h	Western Blot: El tratamiento con AOH activa la p53 dependiendo del tiempo. El nivel de la p53 aumentó 170% respecto al control a las 16 y 24 h.	
		Proteína Bax	DiOC	5-50 μM; 6, 16 y 24 h	La proteína Bax favorece la apertura de PTP.	

CÉLULAS	MICOTOXINA	ENSAYO	FLUOROCROMO	CONDICIONES	RESULTADOS	REFERENCIA
HCT116	AOH AME	Ensayo PTP	Calceína /cobalto	10-200 μM; AOH: 25μM AME: 25μM AOH+AME: 25/25 μM; 24 h	AOH y AME causan apoptosis mediante la inducción del PTP y MMP. No se observa necrosis.	Bensassi et al., 2015
RAW 264.7	AOH	Muerte celular	PI	15, 30 y 60 μM; 6, 24 y 48 h.	A 60 μM, el 20% de las células son necróticas tras de 48 h de exposición. A 30 y 60 μM, el 6% las células son apoptóticas tras 48 h de exposición.	Solhaug et al., 2012
		Apoptosis	Anti-histona H_3 y anticuerpo [EPR996Y]	15 y 30 μM; de 4 a 48 h	El AOH reduce el número de células mitóticas a 30 μM	Solhaug et al., 2013

Abreviaturas:AME: alternariol monometil-eter; AOH: alternarioI; DEVD:N-acetil-L-α-aspartil-L- α-glutamil-N-(2-carboxil-1-formiletil)-L-valinamida; Bax: familia de proteínas pro-apoptótica; Bcl-2: familia de proteínas anti-apoptótica; N-acetil-L-α-aspartil-L-α-glutamil-N-(2-carboxil-1-formiletil)-L-valinamida; DiOC: yoduro de 3,3'-dihexiloxacarbocianina; FITC: isotiocianato fluoresceína; HCT116: células de carcinoma de colon humano; MMP: Potencial de membrana mitocondrial; PI: yoduro de propidio; pNA: p-nitroanalina; PTP: Transición de la permeabilidad mitocondrial; p53: proteína supresora de tumores; RAW 264.7: macrófagos de roedor.

1.2.7 Daño al ADN

La integridad genética de los seres vivos puede verse influenciada, entre otros factores, por la exposición a agentes exógenos (productos químicos, agentes genotóxicos, tratamientos médicos, cambio climático) o endógenos (polimorfismos) (Zalacaín et al., 2005).

Un agente genotóxico es aquel compuesto de naturaleza química, biológica o física que puede inducir, directa o indirectamente, alteraciones en el material genético de los seres vivos con el consiguiente bloqueo de la replicación así, como la aparición de mutaciones que derivarían en patologías y/o cambios en las características de dichos organismos (Croce, 2008).

El daño al ADN se puede determinar por distintas técnicas, entre las que se encuentran, el estudio de micronúcleos (MN), el intercambio de cromátidas hermanas (ICH) y el ensayo del cometa.

Los MN son fragmentos acéntricos de cromosomas o cromosomas enteros, que han quedado retrasados durante el proceso de división celular. Estos se visualizan durante el periodo interfásico de la célula como unos núcleos pequeños distintos del principal. Los MN reflejan eventos clastogénicos o aneugénicos (Bonassi et al., 2003). Las ICH se basan en el principio de replicación semiconservativa del ADN y marcaje radioactivo. Se utiliza el análogo de base bromodeoxiuridina (BrdU) durante dos ciclos de la célula en estudio. El contenido de BrdU en las hebras del ADN hace posible que, al teñir las células, puedan diferenciarse ambas cromátidas y de esta forma, visualizar el intercambio que entre ellas exista (Venegas et al., 1998). El ensayo del cometa determina roturas de la doble cadena de ADN por electroforesis en campo pulsado a un pH alcalino. En esta técnica, las células se sumergen en un gel de agarosa donde son lisadas

por detergentes y sales y así liberan el ADN. Durante la electroforesis a pH alcalino, el ADN dañado o los fragmentos del ADN migran a una velocidad diferente del resto del material nuclear formando colas en el "cometa"; cuanto más larga sea esta porción, mayor será el daño ocasionado al ADN. Las diferencias de los patrones de migración se miden cuantitativamente de acuerdo a la ecuación: TM=TL x ADN en la cola, donde TM es la mediana de la cola y TL es la longitud de la cola, es decir, la distancia entre el centro de la cabeza del cometa y el final de la cola del cometa.

En la Tabla 9 se observan los resultados que producen las micotoxinas de *Alternaria* sobre el ADN celular mediantes las técnicas descritas.

Tabla 9: Ensayos *in vitro* sobre daño a nivel nuclear producido por micotoxinas de *Alternaria.*

CÉLULAS	MICOTOXINA	ENSAYO	FLUOROCROMO	CONDICIONES	RESULTADOS	REFERENCIA
Caco-2 HCT 116 HepG2 V79	ATXII ATXI	Ensayo del cometa	PI	1 μM; 1, 5h	Mayores roturas en ATX II que en ATX I. No se observan diferencias entre las líneas celulares	Fleck et al., 2014
HT29 A431	AOH AME ALT Isoaltenueno	Ensayo del cometa	PI	0-100 μM; 1h	1 μM de AOH y AME aumentan la rotura del ADN en las células HT29 y A431. ALT e isoaltenueno no afectan al ADN.	Fehr et al., 2009
		Ensayo de tinción del ADN	Hoechst 33258	0-25 μM; 1 h	AOH compite con Hoechst 33258 en la unión al ADN, EC_{50} =8.1 ± 1.2 μM. ALT e isoaltenueno no compiten con Hoechst 33258 para la unión al ADN.	

CÉLULAS	MICOTOXINA	ENSAYO	FLUOROCROMO	CONDICIONES	RESULTADOS	REFERENCIA
HT29	AOH AME	Ensayo del cometa	PI	0.1-50 μM; 1-3 h	No hay daño al ADN	Tiessen et al., 2013
HT29	AOH AME TeA	Ensayo del cometa	PI	TeA: 0.2-200 μM; 1h AOH y AME: 0.1-50 μM; 1 h	El TeA no produce roturas de ADN 50 μM de AOH y AME: Incremento del 5% en la intensidad de la cola	Schwarz et al., 2012
RAW 264.7	AOH	Ensayo del cometa	SYBR	30 μM; 2 y 24 h	Daño a nivel del ADN.	Solhaug et al., 2012
V79, HepG2, HT-29	AOH AME	Ensayo del cometa	Hoechst 33258	1- 50 μM; 1 y 24 h	Ambas micotoxinas producen roturas en el ADN a partir de 5 μM, tras 1 h de exposición. A las 24 h, solo en las células HepG2 se produjo un aumento en las roturas de ADN.	Pfeiffer et al., 2007 (2)

CÉLULAS	MICOTOXINA	ENSAYO	FLUOROCROMO	CONDICIONES	RESULTADOS	REFERENCIA
V79	ATX II AOH AME	Ensayo del cometa	Hoechst 33258	ATX II: 0-0.75 μM AOH: 0-20 μM AME: 0-40 μM; 1.5 h	ATX II induce roturas en el ADN desde 0.1 μM.AOH induce roturas en el ADN desde 5 hasta 20 μM y AME desde 0.5 hasta 20 μM.	Fleck et al., 2012

Abreviaturas: ALT: altenueno; AME: alternariol monometil-eter; AOH: alternariol; ATX I: altertoxina I; ATX II: altertoxina II; A431: Células de carcinoma epidérmico; Caco-2: Células de adenocarcinoma de colon humano; HCT116: células de carcinoma de colon humano; HT29: células de carcinoma de colon humano; HepG2: células de hígado humano; PI: yoduro de propidio; RAW 264.7: macrófagos de roedor; SYRB: cianinas asimétricas (fluoróforos 4S); TeA: ácido tenuazoíco; V79: células de pulmón de hámster chino.

1.2.8 Alteración endocrina

Los disruptores endocrinos (ED) o disruptores hormonales se definen como cualquier producto químico que pueda interactuar directa o indirectamente con el sistema endocrino y que, posteriormente, provoca efecto sobre este sistema actuando directamente en órganos y tejidos (EFSA, 2010). Los ED mimetizan y antagonizan la acción de las hormonas, alteran su patrón de síntesis y metabolismo, y modulan los niveles de los receptores correspondientes, pudiendo ocasionar alteraciones en la salud de los individuos afectadas (Diamanti-Kandarakis et al., 2009).

Los ED actúan a dosis muy bajas, presentan distintos mecanismos de actuación y comprenden un gran número de sustancias con estructuras químicas muy diferentes (UNEP y WHO, 2013). Numerosas sustancias presentes en el medio ambiente y en los alimentos presentan actividad endocrina o tienen la capacidad para unirse a los receptores biológicos e interferir con la hormona y sus propios receptores. Se reconoce la presencia de estrógenos naturales como los fitoestrógenos, metabolitos vegetales o las micotoxinas. Actualmente, se están realizando estudios con diferentes micotoxinas (PAT, ENN B, DON, T-2, HT-2 y OTA) entre las cuales, cabe destacar la ZEA y sus metabolitos (α-ZOL y β-ZOL) por su gran actividad estrogénica (Frizzell et al., 2011). El AOH también se ha estudiado como posible ED, observándose que el AOH es una micotoxina estrogénica débil que tiene la capacidad de interferir con la vía de la esteroidogénesis (Frizzell et al., 2013). Igualmente, se están llevando a cabo estudios sobre otras micotoxinas, especialmente las más prevalentes en los alimentos como la BEA y la FB1.

La Organización para la Cooperación y el Desarrollo Económico (OCDE) revisa el desarrollo de directrices para la detección y el ensayo de los ED. Los

ensayos revisados comprenden cinco niveles. El nivel 1 se refiere a si existe información o si hay ausencia de la misma. Los niveles 2 y 3 se relacionan, respectivamente, con ensayos *in vitro* e *in vivo*, que aportan datos sobre el mecanismo endocrino seleccionado. El nivel 4 se corresponde con ensayos *in vivo* que proporcionan datos sobre efectos adversos del sistema endocrino y las diferentes variables relevantes. Por último, el nivel 5 se refiere a ensayos *in vivo que* proporcionan datos más completos sobre los efectos adversos en el sistema endocrino con criterios de valoración más extensos en el organismo.

Para corroborar si una micotoxina actúa como ED y estudiar su mecanismo de acción, existen 3 protocolos *in vitro*. En el protocolo OECD 457, se establece el ensayo de transactivación BG1Luc del receptor de estrógeno (BG1Luc ER TA), el cual es utilizado para la identificación de los receptores de estrógeno agonistas y antagonistas y se incluye en el nivel 2 de "ensayos *in vitro* que proporcionan datos sobre el mecanismo endocrino seleccionado".

El protocolo OECD 456 que describe un "screening" *in vitro* para efectos químicos sobre la esteroidogénesis, específicamente la producción de 17ß-estradiol (E2) y testosterona. La línea celular de carcinoma adreno-H295R humano, que se utiliza para el ensayo, expresa los genes que codifican para todas las enzimas clave para la esteroidogénesis. En último lugar, el protocolo OECD 455, proporciona la metodología de transactivación transfectadas de forma estable para detectar receptor de estrógeno agonistas y antagonistas (ensayos de ER TA). Se compone de los métodos de prueba mecánica y funcional similar para la identificación de agonistas y antagonistas de los receptores de estrógenos (Willemsen et al., 2004; Conolly et al., 2011; OECD, 2012 a,b; Frizzell et al., 2014). Dicho ensayo *in vitro* es utilizado en este estudio para evaluar la expresión de

genes específicos regulados por receptores nucleares específicos, tales como los receptores estrogénicos (OECD, 2012b).

En la T**abla 10** se observan ensayos relacionados con efectos de disrupción endocrina en cultivos celulares expuestos a micotoxinas producidas por hongos de los géneros *Penicillium*, *Fusarium* y *Alternaria*.

Tabla 10. Ensayos de alteración endocrina con micotoxinas

MICOTOXINA	CÉLULAS	ENSAYO	CONDICIONES	RESULTADOS	REFERENCIA
AOH	RGA	RGA (unión receptor-hormona)	0.1–1000 ng/ml; 48 h	Agonista: respuesta estrogénica débil. Antagonista: en los receptores de andrógenos, glucocorticoides y progesterona.	Frizzell et al., 2013
	H295R	Estereidogénesis		La exposición a 1000 ng/ml aumentó la producción de estradiol y progesterona.	
DON T-2 HT-2	RGA	RGA(unión receptor-hormona)	DON: 0.1–1000 ng/ml T-2: 0.0005–5 ng/ml HT-2: 0.005–50 ng/ml 48 h	Agonista: No hay respuesta para ninguna micotoxina. Antagonismo: en todos los receptores, estrógenos, andrógenos, progesterona y glucocorticoides a las concentraciones más altas ensayadas.	Ndossi et al., 2012
	H295R	Estereidogénesis		DON: disminuye la producción de estradiol, testosterona y cortisol al aumentar la concentración. T-2: disminuyó la producción de testosterona a todas las concentraciones y la de cortisol a la concentración más elevada. HT-2: Estradiol y progesterona disminuyen a 50 ng/ml.	

MICOTOXINA	CÉLULAS	ENSAYO	CONDICIONES	RESULTADOS	REFERENCIA
ENN B	RGA	RGA(unión receptor-hormona)	0.00156-15.6 µM; 48 h	Agonista: No hay respuesta. Antagonista: en los receptores de estrógenos, andrógenos, y progesterona.	Kalayou et al., 2015
	H295R LC	Estereidogénesis		Aumento la producción de progesterona, testosterona y cortisol en las células RGA. Disminución de estradiol y testosterona en las células LC	
OTA	RGA H295R	RGA(unión receptor-hormona)	0.25–2500 ng/ml; 48 h	Agonista: No hay respuesta. Antagonismo: Se observa en los receptores de estrógenos, andrógenos, corticoides y progesterona.	Frizzell et al., 2013
		Estereidogénesis	0.1–1000 ng/ml; 48 h	La exposición a 1000 ng/ml aumentó 3 veces la producción de estradiol respecto al control.	

MICOTOXINA	CÉLULAS	ENSAYO	CONDICIONES	RESULTADOS	REFERENCIA
PAT	RGA H295R	RGA(unión receptor-hormona) Estereidogénesis	0.52–5000ng/ml µM; 48 h	Agonista: No hay respuesta. Antagonismo: en los receptores de estrógenos, andrógenos, y progesterona. 5000 ng/ml de PAT aumentó 6 veces la producción de glucocorticoides y 2 veces la de esteroides. 500 ng/ml aumentó la producción de progesteronay la disminución de la testosterona.	Frizzell et al., 2014
ZEA β-ZOL α-ZOL	RGA H295R	RGA (unión receptor-hormona) Estereidogénesis	0.0005-5000ng/ml; 48 h 0.01, 0.1, 1, 10, 100 µM; 48 h	Agonista: Se observó respuesta estrogénica. La intensidad sigue el siguiente orden β-ZOL >ZEA> α-ZOL. Antagonismo: Se observó en los receptores androgénicos expuestos a β-ZOL, ZEA y α-ZOL. ZEA, β-ZOL, α-ZOL aumentaron la producción de las hormonas progesterona, estradiol, testosterona y cortisol.	Frizzell et al., 2011

Abreviaturas: AOH: Alternariol; DON: Deoxinivalenol; ENN B: Eniatina B; HT-2: Toxina HT-2; H295R: células de carcinoma adrenocortical humano; LC: células porcinas neonatales de Leyding; OTA: Ocratoxina A; PAT: Patulina; RGA: células de glándula mamaria humana transformada con el gen de la luciferasa bajo el control de un promotor inducible a hormonas esteroides; T-2: Toxina T-2; ZEA: Zearalenona, β-ZOL: β-Zearalenol; α-ZOL: α-Zearalenol.

1.2.9 Defensa antioxidante

Las células producen continuamente radicales libres y ROS, como parte de los procesos metabólicos. Para contrarrestar los efectos perjudiciales que tienen lugar en la célula, éstas han desarrollado mecanismos de protección física y, los más importantes, mecanismos de defensa antioxidante.

Se considera antioxidante aquella sustancia que en concentraciones muy pequeñas comparadas con las de un sustrato oxidable, disminuye o evita su oxidación (Krinsky et al., 1992). Existen mecanismos antioxidantes enzimáticos y no enzimáticos. Los antioxidantes enzimáticos de primera línea están representados por las enzimas superóxido dismutasa (SOD), catalasa (CAT) y glutatatión peroxidasas (GP_X). Entre los sistemas no enzimáticos y considerados de segunda línea encontramos el glutatión (GSH), vitaminas y polifenoles. Dichos antioxidantes son esenciales pues actúan como cofactores de las enzimas antioxidantes (Hicks et al., 2006).

1.2.9.1 Sistema de defensa intracelular

Un método para determinar el contenido de GSH es el descrito por Hissin y Hift (1967). Este método fluorimétrico está basado en la reacción del *o*-ftaldehído (OPT) con el GSH a pH=8. La especificidad del GSH dependiente del pH hace al fluoróforo OPT un reactivo ideal para diferenciar entre GSH y GSSG. La N-etilmaleimida (NEM) previene la oxidación del GSH y se adiciona para determinar GSSG siempre que el procedimiento no requiera GR (ya que NEM es un potente inhibidor de esta enzima). En la Tabla 11 se muestra el resultado de la actividad enzimática de GST y los niveles de GSH, del único ensayo obtenido tras la exposición de cultivos celulares a micotoxinas *Alternaria*.

Tabla 11. Actividad de la GST y niveles de GSH en células expuestas a micotoxinas de *Alternaria.*

CÉLULAS	MICOTOXINA	ENZIMA	CONDICIONES	RESULTADOS	REFERENCIA
HT29	AOH	GHS GST	0.5-50 μM; 1 y 24 h	GHS: Tras 1h de exposición, disminuyen los niveles en un 26% (10 μM) y un 19% (50 μM). Tras 24 h de incubación, no se detectaron cambios. GST: Incrementa la actividad solo a 50 μM	Tiessen et al., 2013

Abreviaturas:AOH: alternariol; GHS: glutatión;GST: glutatión-S-transferasa; HT29:células de adenocarcinoma de colon humano.

1.2.9.2 Antioxidantes de la dieta

La dieta contiene gran variedad de compuestos (vitaminas, polifenoles, etc.) con actividades antioxidantes o que pueden disminuir el deterioro funcional orgánico originado por un exceso de estrés oxidativo (Romero-Alvira et al., 1990). Numerosos estudios demuestran que los antioxidantes disminuyen el estrés oxidativo dependiendo del tipo de compuesto y la concentración en la que se encuentra (Wang et al., 2012; Lombardini et al., 2012).

Los polifenoles son compuestos bioactivos con capacidad antioxidante. Se han descrito más de 8.000 polifenoles distintos que, generalmente, se diferencian en 2 grupos: los flavonoides (antocianinas, flavonoles, flavonas y flavononas) y los no flavonoides (los ácidos y alcoholes fenólicos, estilbenos y lignanos). Dentro de los flavonoides, en el grupo de los flavonoles, está presente la quercetina (Quer) que se encuentra generalmente como *O* - glicósidos y en menor proporción como *C* – glicósidos. Entre las principales propiedades de la quercetina destaca su poder de eliminación de los radicales libres, ejerciendo un papel citoprotector frente al daño celular. La quercetina elimina aniones superóxidos, radicales hidróxidos, peróxidos lipídicos o hidroperóxidos (Martinez-Florez et al., 2002). En el grupo de los no flavonoides, dentro de los alcoholes fenólicos, se encuentran el tirosol y la oleuropeina, los cuales protegen las células frente al daño debido a la oxidación. Ambos compuestos tienen buena biodisponibilidad y gran poder antioxidante (Bock et al., 2013).

Los polifenoles se encuentran distribuidos ampliamente en muchas especies vegetales (como semillas de uva, manzana, cacao, frutas, semillas, aceites, etc.) y en bebidas como el vino tinto y blanco (Lombardi et al., 2012). También están presentes en los frutos secos y en algunas semillas de leguminosas

(Vinson et al., 2005; Chang et al., 2016). Los polifenoles son antioxidantes, pero también presentan efecto antiinflamatorio, antiagregante plaquetario, antitrombótico e, incluso hipolipemiante (Barcelos et al. 2011; Quiñones et al., 2012). Actualmente, no hay estudios de polifenoles expuestos a micotoxinas de *Alternaria*. Sin embargo, hay estudios en células expuestas a micotoxinas de *Fusarium* y *Penicillium* en los que polifenoles como la quercetina y resveratrol protegen a las células frente a ENNs (Lombardi et al., 2012), BEA (Mallebrera et al., 2015), AFB1 (Choi et al., 2010; Barcelos et al., 2011) y CIT (Chen et al., 2009).

Las grasas monoinsaturadas como el aceite de oliva virgen extra (AOVE) son más resistentes a la oxidación que las grasas saturadas y poliinsaturadas. Los principales antioxidantes del AOVE son compuestos fenólicos lipofílicos y fenólicos hidrofílicos. Entre los compuestos lipofílicos se encuentran el tocoferol y los tocotrienoles, que se pueden encontrar en otros aceites vegetales (Bulotta et al., 2011). Los compuestos hidrofílicos como el tirosol o la oleopeina solo están presentes en el AOVE (Boskou, 1996; Gioffi et al., 2010).

Por otra parte, en gran número de plantas y leguminosas, se encuentran las saponinas, glicósidos triterpénicos unidos a mono u oligosacáridos. Las soyasaponinas, saponinas del grupo B, son las más comunes en semillas y, a su vez, la soyasaponina I (SS-I) es la más abundante (Vila-Donat et al., 2014). Estudios recientes han demostrado que la SS-I disminuye el contenido de colesterol en el plasma de animales de laboratorio y podría esperarse un efecto similar en la dieta humana. Además, posee actividad anticarcinógena, hepatoprotectora y antioxidante (Kang et al., 2010; Yang et al., 2011). Investigaciones recientes estudian el efecto protector de hojas de plantas como el *aloe vera* o la hoja de llantén, las cuales poseen contenido significativo de saponinas, frente a lesiones gástricas. En estos estudios se observó un importante efecto citoprotector en la

lesión producida en la mucosa gástrica, llegando a recuperar el tejido en un 80% (Arce et al., 2007; Dávalos et al., 2008). Teniendo en cuenta los estudios recientes que demuestran el poder inhibitorio de los antioxidantes de la dieta y su efecto citoprotector, resulta de interés conocer el poder citoprotector de estos en células Caco-2 frente a micotoxinas como el AOH, ya que tanto la micotoxina como los antioxidantes se encuentran presentes, y de manera simultánea, en la dieta humana.

1.2.10 Bioacesibilidad y Biodisponibilidad

Los alimentos son la principal vía de contacto de las micotoxinas. La evaluación de la exposición de las micotoxinas se lleva a cabo considerando la biodisponibilidad oral, es decir, la fracción de una concentración ingerida que alcanza el compartimento central (sangre) desde el tracto gastrointestinal (Wragg et al., 2007; Caussy, 2003). El intestino tiene un papel muy importante en la toxicidad de los xenobioticos, incluidas las micotoxinas. Para poder evaluar la biodisponibilidad oral, primero se evalua la bioaccesibilidad oral, definida como la fracción soluble en el tracto gastrointestinal que está disponible para su absorción (Intawongse y Dean, 2006). Para ello, se utilizan principalmente los modelos estáticos porque son más fáciles de construir, de mantener y utilizar que los modelos de digestión dinámicos. En los modelos estáticos *in vitro* se realiza una exposición secuencial para simular la digestión en diferentes compartimentos (boca, estómago e intestino o combinaciones de los mismos). Las condiciones fisiológicas humanas como pH, enzimas gástricas e intestinales, temperatura y tiempos de residencia en cada compartimento son emuladas durante la aplicación de estos métodos (Versantvoort et al., 2005).

En la actualidad, está muy extendido el empleo de modelos de digestión *in vitro* para evaluar el riesgo asociado a la exposición oral de las micotoxinas, como

la FB1 (Azaiez et al., 2013), toxinas HT2 y T-2 (Angelis et al., 2014), BEA (Meca et al., 2012 a; Mallebrera et al., 2013), ZEA (Ramos et al., 1996) y ENNs (Prosperini et al., 2012). Sin embargo, hasta la fecha, no se disponen de estudios de bioaccesibilidad con micotoxinas de *Alternaria*.

Por otra parte, las células Caco-2, modelo de epitelio intestinal, han sido aceptadas como un ensayo estándar de predicción de la permeabilidad intestinal (Grajek y Olejnik, 2004). En los últimos años, un subclon de la línea celular Caco-2, la Caco-2 TC7 (FDA, 2000), ha sido seleccionado como modelo alternativo a las células Caco-2 para la evaluación de la biodisponibilidad (Gres et al., 1998). Este clon celular posee una población más homogénea y tiene desarrolladas las funciones más representativas de los enterocitos, teniendo así mayor capacidad para desarrollar uniones intracelulares, microvellosidades en el lado apical, transportadores de membrana típicos de intestino, enzimas metabólicas (mayor actividad del citocromo 3A) y de secreción propias del cepillo (mayor hidrolasa asociada a la actividad de la sacarasa-isomaltasa) (Turco et al., 2011).

Para la diferenciación, las células Caco-2 TC7 se siembran en un sistema de dos compartimentos (Transwells), en la parte basolateral (BL) se sitúa el medio de cultivo y en la parte apical (AP), donde se encuentra el soporte microporoso de policarbonato, se siembran las células. El proceso de diferenciación celular espontáneo se completa en dos o tres semanas. Las células diferenciadas expresan las características de un enterocito maduro (Figura 12). Transcurrido ese periodo, se estudia el paso de la sustancia y sus metabolitos desde la zona AP hacia la zona BL, a través de la capa epitelial de células diferenciadas.

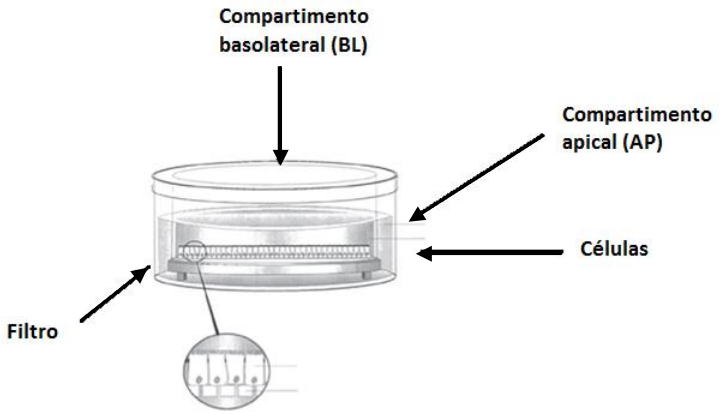

Figura 12. Representación gráfica de un sistema Transwell.
Imagen modificada de Prosperini et al., 2012.

Las células Caco-2 y Caco-2 TC7 han sido ampliamente utilizadas para realizar estudios de biodisponibilidad y transporte de micotoxinas (Tabla 12). Por otra parte, las células Caco-2 también se han utilizado para evaluar la biodisponibilidad duodenal y colónica.

Para simular la biodisponibilidad duodenal, las células Caco-2 se cultivaron con mucina en simbiosis con algunas bacterias características del tracto gastrointestinal (*Lactobacillus animalis* CECT 4060T, *L. casei* CECT 4180*, L. casei rhamnosus* CECT 278T, *L. plantarum* CECT 220*, L. rhuminis* CECT 4061T, *L.casei* CECT 277, *Bifidobacterium breve* CECT 4839T, *B.adolescentes* CECT 5781T y *B.bifidum* CECT 870T, *Corynebacterium vitaeruminis* CECT 537, *Streptococcus faecalis* CECT 407, *Eubacterium crispatus* CECT 4840 y *Saccharomyces cerevisiae* CECT 1324). Estas células forman una monocapa homogénea de células caliciformes con numerosas vacuolas de moco acumuladas en el citoplasma de la región apical de las células y algunas microvellosidades apicales que no llegan a formar un cepillo maduro (Meca et al., 2012b, Prosperini et al., 2012).

Tabla 12 Ensayos de biodisponibilidad con micotoxinas.

CÉLULAS	MICOTOXINA	CONDICIONES	RESULTADOS	REFERENCIA
Caco-2	FUS BEA	1.5 y 3 µM; 1, 2, 3 y 4 h	A las 4 h la biodisponibilidad oscila entre: BEA: 54.3 y 50.1 %. FUS: 83.2 y 80.2%.	Prosperini et al.,2012
	EN A EN A1 EN B EN B1	1.5 y 3 µM; 1, 2, 3 y 4 h	A las 4 h la biodisponibilidad oscila entre: EN A: 70.8 y 50.7 %. EN A1: 64.2 y 73.8%. EN B: 64.0 y 59.0%. EN B1: 55.2 y 66.1%.	Meca et al., 2012b
	AOH AME	20 µmol/l; 1-6 h	A las 6 h la biodisponibilidad oscila entre: AOH: 9.6% AME:11.9 %	Burkhardt et al, 2009
	DON	0.16 - 7.5 µg/ml, 3 h	La absorción media en la dirección AP-BL fue de 13,8, mientras que la secreción BL- AP fue 10.5%.	Sergent et al., 2006

CÉLULAS	MICOTOXINA	CONDICIONES	RESULTADOS	REFERENCIA
	ZEA	10–200 µM; 8 h	La secreción media de ZEA en la dirección AP-BL aumentó de 368 ± 43 pmol/cm^2/h hasta 2.749 ± 228 pmol/cm^2/h. La tasa de secreción BL-AP aumentó de 137 ± 37 pmol/cm^2/h hasta 2020 ± 184 pmol/cm^2/h	Videmann et al., 2008
	AFB1	150 µM; 1, 24, 48 y 72 h	A las 24, 48 y 72 h la biodisponibilidad es del 38.7 %.	Gratz et al., 2007
Caco-2/TC7	AFM1	1,000–10,000 ng/kg; 48 h	Se encuentra más de 70% de la micotoxina en el compartimiento AP	Caloni et al, 2006
	OTA	40 µM – 1 mM; 48 h	después de 48 h: Aumento significativo de la permeabilidad a concentraciones igual o superior a 100µM.	Ranaldi et al., 2007

Abreviaturas: AFB1: Aflatoxina B1; AFM1: Aflatoxina M1**;** AME: Alternariol monometil-éter; AOH: Alternariol; AP: Compartimento apical; BEA: Beauvericina; BL: Compartimento basolateral; Caco-2: Células de carcinoma de colon humano; Caco-2/TC7: Subclon de las Caco-2; DON: Deoxinivalenol; ENN A, A1, B, B1: Eniatina A, A1, B, B1; FUS: Fusaproliferina; OTA: Ocratoxina A; ZEA: Zearalenona.

1.2.11 Detoxificación/Descontaminación

La importancia adquirida en los últimos años por la contaminación de micotoxinas en los alimentos se debe a que son tóxicas en cantidades muy pequeñas. Son contaminantes naturales de los alimentos, no habiéndose desarrollado hasta el momento suficientes medidas de prevención para controlar la aparición de micotoxinas en los mismos. Además, las micotoxinas son contaminantes de alimentos de consumo masivo, como el trigo y el maíz, y producen pérdidas de alimentos de origen proteico debido a la mortalidad y morbilidad que ocasiona en los animales intoxicados por éstas.

La contaminación por micotoxinas puede producirse a lo largo de toda la cadena alimentaria desde la producción en el campo hasta el almacenamiento en el hogar. El mejor método para controlar la contaminación es la prevención; sin embargo, si la contaminación ya existe, se debe eliminar la toxina presente o provocar su degradación a compuestos no tóxicos o menos tóxicos que el original (Kabat et al., 2006).

Por tanto, la descontaminación se refiere a los métodos por los cuales las micotoxinas son eliminadas o neutralizadas de los alimentos, mientras que la detoxificación son los procedimientos para reducir o eliminar las propiedades tóxicas de las micotoxinas. El método ideal de descontaminación o detoxificación debe ser fácil de usar, económico, no tiene que formar compuestos más tóxicos que la micotoxina original y no debe alterar las propiedades nutricionales ni organolépticas de los alimentos (Soriano, 2007).

Se pueden diferenciar distintos métodos para la detoxificación de micotoxinas: métodos naturales, métodos físicos (radiaciones y calor), métodos microbiológicos y métodos químicos (fungicidas, uso de solventes y usos de

agentes químicos). Dentro de los agentes químicos, destacan los agentes absorbentes. Así, la inhibición de la absorción de la micotoxina en el tracto gastrointestinal es una de las estrategias empleadas de prevención, ya que estos agentes son capaces de disminuir la bioaccesibilidad y, por tanto, la biodisponibilidad de las micotoxinas (Galvano et al., 2001).

Los glucosinolatos (GL) o glucósidos bociogénicos son unos componentes que están presentes en la mayoría de plantas de la familia de las crucíferas (*Brassicaceae o Brasicáceas*), a la que pertenece la col, la rúcula, la coliflor, el repollo morado, la mostaza, la colza, los rabanitos o los nabos. Químicamente, los GL son compuestos orgánicos (heterósidos) azufrados, responsables del sabor picante y amargo característico de estas plantas. El daño al tejido de la planta conduce a la hidrólisis de los GL y a la producción de numerosos compuestos biológicamente activos, incluyendo los isotiocianatos (ITC), tiocianatos y nitritos (Borges et al., 2014).

Los ITC tienen varias actividades biológicas que incluyen la defensa de plantas frente a insectos e infecciones microbianas (Mansour et al., 2012); beneficios para la salud humana por sus propiedades quimiopreventivas y anti-angiogénicas y podrían ser utilizados como conservantes de alimentos naturales (Fimognari, et al., 2012;Borges et al., 2014). El isotiocianato de alilo (AITC) es uno de los ITC más comunes y se ha descrito como potente antimicrobiano (Luciano, 2009). Diversos estudios han demostrado su capacidad para reaccionar con micotoxinas como la BEA (Meca et al., 2012a) y las FBs (Azaiez et al., 2013) en soluciones tamponadas y en matrices de alimentos. El AITC es capaz de reaccionar con las dos micotoxinas, formando aductos y reduciendo su toxicidad.

2.OBJETIVOS

OBJECTIVES

2. OBJETIVOS

El **objetivo general** de la presente Tesis Doctoral es la evaluación *in vitro* de los efectos producidos por micotoxinas de *Alternaria* y *Fusarium* en células de mamífero. Para lograr este propósito se han planteado los siguientes **objetivos específicos**:

1. Evaluación de la citotoxicidad individual del alternariol, alternariol monometil éter, beauvericina deoxinivalenol, eniatina B, fumonisina B1, zearalenona y α-zearalenol en células de adenocarcinoma de colon humano (Caco-2) y evaluación de la citotoxicidad entre mezclas de las micotoxinas alternariol, eniatina B y dexonivalenol y entre alternariol y alternariol monometil éter en células Caco-2.

2. Estudio de la bioaccesibilidad, del transporte transepitelial del alternariol, zearalenona y α-zearalenol y su biodisponibilidad relativa usando la línea celular Caco-2 TC7.

3. Evaluación del estrés oxidativo mediante la determinación de la generación de especies reactivas de oxígeno y peroxidación lipídica, tras la exposición de alternariol en células Caco-2.

4. Determinación del ciclo celular, apoptosis/necrosis, potencial de membrana y daño a nivel del ADN, tras la exposición de alternariol en células Caco-2.

5. Determinación de la alteración endocrina de la beauvericina y fumonisina B1 en células RGA.

6. Evaluación de la defensa antioxidante intracelular y antioxidante de la dieta en células Caco-2 expuestas a alternariol y alternariol monometil éter.

7. Utilización de isotiocianato de alilo como sistema de detoxificación de zearalenona y α-zearalenol en alimentos.

2. OBJETIVES

The **overall objective** of this study was the *in vitro* evaluation of the effects produced by *Fusarium* and *Alternaria* mycotoxins in mammalian cells. To achieve this aim, the following **specific objectives** were proposed:

1. Assessment of individual cytotoxicity of alternariol, alternariol monomethyl ether, deoxynivalenol, beauvericin, enniatin B, fumonisin B1, zearalenone and α-zearalenol in Caco-2 cells and interaction effects of alternariol, eniatin B and dexonivalenol and between alternariol and alternariol monomethyl ether in Caco-2 cells.

2. Evaluation of oxidative stress by determination of reactive oxygen species and lipid peroxidation generation after exposure of alternariol in Caco-2 cells.

3. Determination of cell cycle, apoptosis / necrosis, membrane potential and DNA damage after exposure of alternariol in Caco-2 cells.

4. *In vitro* bioaccessibility and biodisponibility of alternariol, zearalenona and α-zearalenol in Caco-2/TC7 cells.

5. Determination of endocrine alteration effect of beauvericin and fumonisin B1 in RGA cells.

6. Evaluation of intracellular antioxidant and antioxidant defense of diet in Caco-2 cells exposed to alternariol and alternariol Monomethyl Ether.

7. Use of allyl isothiocyanate as a system of detoxification of zearalenone and α-zearalenol in food.

3.RESULTADOS

RESULTS

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3.1 Efectos tóxicos de alternariol por ensayos *in vitro*: revisión

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Resumen

Las micotoxinas son metabolitos secundarios producidos por hongos del genero *Aspergillus*, *Penicillium*, *Fusarium* y *Alternaria*. Las micotoxinas más abundantes son aflatoxinas (*Aspergillus*), ocratoxina A (*Penicillium*), fumonisinas, zearalenona, deoxinivalenol (*Fusarium*) y alternariol (*Alternaria*). De las especies de *Alternaria*, A. alternata es la especie productora más importante de micotoxinas. Todas están consideradas como contaminantes tóxicos que están presentes en alimentos de consumo diario. Esta revisión se centra en estudios *in vitro* relacionados con la respuesta y citotoxicidad a la micotoxina de *Alternaria*, alternariol (AOH). Para ello, se ha realizado una búsqueda bibliográfica de los artículos de AOH disponible en bases de datos como: Pubmed, Scopus, Science Direct y Current Contents en los últimos catorce años. Así pues, el objetivo de la revisión bibliográfica es evaluar los efectos de AOH investigados mediante ensayos *in vitro*.

Abstract

Toxic effects of alternariol by *in vitro* assays: a review. Mycotoxins are secondary metabolites produced by genera fungus of: *Aspergillus, Penicillium*, *Fusarium* and *Alternaria*. The most frequent mycotoxins are: aflatoxins (*Aspergillus*), ochratoxin A (*Penicillium*), fumonisins, zearalenone, deoxynivalenol (*Fusarium*) and alternariol (*Alternaria*). Among all *Alternaria* spp, A. Alternata is the most producer mycotoxin. All mycotoxins are considered toxic contaminants present in food of daily consumption. This review is based on *in vitro* studies where response and toxicity in cells of *Alternaria* mycotoxin, alternariol (AOH) have been carried out. In this sense a bibliographic search of AOH papers available

in on-line data bases such as: Pubmed, Scopus, Science Direct and Current Contents in the last fourteen years, have been collected. The main objective of this bibliographic review is to evaluate the AOH effects detected in *in vitro* assays.

1. INTRODUCCIÓN

Las micotoxinas son metabolitos secundarios producido por hongos del genero *Aspergillus*, *Penicillium*, *Fusarium* y *Alternaria*. La micotoxinas más abundantes (Tabla 1) son aflatoxinas (*Aspergillus*), ocratoxina A (*Penicillium)*, fumonisinas, zearalenona, tricotecenos (*Fusarium*) y alternariol (*Alternaria*). En la actualidad se conocen un total de 400 micotoxinas diferentes [1]. La micotoxinas son muy difíciles de eliminar y/o de erradicar a lo largo de toda la cadena alimentaria [2]. Los climas cálidos proporcionan las condiciones adecuadas e idóneas para conseguir el crecimiento de hongos de los géneros mencionados y la síntesis de las micotoxinas. La producción de micotoxinas puede verse favorecida por condiciones ecológicas adecuadas alcanzadas durante la recolecta, secado, manejo, embalaje, transporte y almacenamiento de los alimentos contaminados con hongos, tales como humedad, temperatura y actividad del agua del alimento [3,4].

Todas las micotoxinas están consideradas como contaminantes tóxicos que están presentes en alimentos de consumo diario. De acuerdo con la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) aproximadamente un 25% de la producción mundial de cereales está contaminada de micotoxinas; además, especias, hierbas para infusiones, fruta, frutos secos, semillas y productos derivados de estos, están contaminados también por micotoxinas [4-8].

Las micotoxinas provocan efectos tóxicos crónicos muy diversos en animales y humanos como: hepatotoxicidad, genotoxicidad, nefrotoxicidad,

neurotoxicidad, efectos sobre la reproducción, immunotoxicidad, etc. En la Tabla 2 se recogen las micotoxinas más comunes presentes en alimentos y sus efectos tóxicos. La toxicidad puede variar dentro de un mismo grupo estructural de micotoxinas y el efecto lo puede producir tanto la misma micotoxina como los metabolitos derivados de ella [2].

Debido a la toxicidad que las micotoxinas presentan y a su presencia en alimentos, la Comisión Europea ha establecido límites máximos que se recogen en los Reglamentos 1881/2006 del 19 de Diciembre de 2006 [9] y 1126/2007 de 28 Septiembre de 2007 [10]. Existen niveles máximos para aflatoxinas, ocratoxina A, patulina y micotoxinas de *Fusarium* (zearalenona, fumnosina, deoxinivalenol, y toxinas T-2 y HT-2) [9,10] que van de 0,5 µg/kg para ocratoxina A (en cereales para alimentos infantiles) a 4000 µg/kg para fumonisinas (en maíz sin procesar). No obstante, existen micotoxinas que todavía no presentan niveles legislados, como es el caso de las micotoxinas del genero *Alternaria*; de ahí, la importancia de estudiar este tipo de micotoxinas.

Las especies de *Alternaria* son los patógenos post-cosecha más comunes presentes en frutas (melones, manzanas, moras, fresas, frambuesas, uvas, pasas, naranjas, limones, mandarinas, nueces y aceitunas) capaces de producir diferentes micotoxinas. Debido a su estructura química, las micotoxinas producidas por *Alternaria* se pueden dividir en: i) dibenzo-α-pironas (alternariol (AOH), alternariol metil éter (AME) y altenuene (ALT)), ii) ácido tetrámico: ácido tenuazonico (TeA), iii) quinonas perilenas (altertoxinas I-III ATX I-III), iv) toxinas alternata (AAL-TA1, AAL-TA2, AAL-TB1, AAL-TB2); v) otras estructuras como los tetrapéptidos cíclicos: Tentoxina (TEN) [4]. La estructura química de algunas de estas micotoxinas se recoge en la Figura 1.

Micotoxina	Acrónimo	Hongo
Aflatoxinas B1, B2, G1,G2	AFB1, AFB2, AFG1, AFG2	*Aspergillus flavi*
Alternariol	AOH	*Alternaria alternata*
Alternariol monometil eter	AME	*A. alternata, A. solani*
Ácido tenuazonico	TeA	*A. alternata*
Altertoxinas (I-III)	ATX (I-III)	*A. tenuissima*
Altenuene	ALT	*A. alternata*
Ocratoxina A	OTA	*Aspergillus circumdati, A. nigri, Penicillium verrucosum, P. nordicum*
Fumonisinas B1, B2	FB1, FB2	*Fusarium liseola*
Deoxinivalenol	DON	*F. graminearum, F. culmorum, F. cerealis*
Zearalenona	ZEN	*F. graminearum, F. culmorum, F. cerealis, F. incarnatum, F. equiseti, F. verticillioides*

Tabla 1. *Micotoxinas principales y hongo productor (modificado de [4])*

Micotoxina	Efectos tóxicos	Mecanismos de acción celulares y moleculares
Aflatoxina	Hepatotoxicidad Genotoxicidad Inmunomodulación	Formación de aductos con ADN Peroxidación lipídica Bioactivación del Cit-P_{450}; Formación de glucuronatos
Ocratoxin A	Nefrotoxicidad Genotoxicidad Inmunomodulación	Efecto en la síntesis de proteínas Inhibición en la producción de ATP Detoxificación por peptidasas
Patulina	Nefrotoxicidad Mutagénesis (*in vitro*)	Inhibición enzimática indirecta
Tricotecenos (Toxina T-2, DON)	Hepatotoxicidad Inmunomodulación Toxicidad en piel	Efecto en la síntesis proteínas Inducción de apoptosis en células progenitoras hematopoyéticas y células inmunitarias Alteraciones de las inmunoglobulinas
Zearalenona	Fertilidad y reproducción	Unión a receptores de estrógenos; Bioactivación por reductasas; Formación de glucuronatos
Fumonisina B1	Daño en el sistema nervioso central Hepatotoxicidad Genotoxicidad Inmunomodulación	Inhibición de la síntesis de ceramida Alteración del ratio esfinganina/esfingosina Alteración del ciclo celular

Tabla 2. *Principales micotoxinas presentes en alimentos y sus efectos tóxicos (modificado de [2]).*

Figura 1. Estructura de las principales micotoxinas producidas por *Alternaria*: i) dibenzo-α-pironas (alternariol (AOH), alternariol metil éter (AME) y altenuene (ALT)), ii) ácido tetrámico: ácido tenuazonico (TeA), iii) quinonas perilenas (altertoxinas I-III ATX I- III).

Es frecuente encontrar los ingredientes principales para la elaboración de alimentos así como el propio alimento, contaminados con más de una especie de hongo o con más de una micotoxina. La importancia de la co-presencia de micotoxinas reside en las alteraciones de los efectos tóxicos que se pueden producir en la combinación de estas, con respecto a la toxicidad de las micotoxinas individuales; ya que se han descrito efectos de sinergismo, adición y/o antagonismo [1-13].

Con el fin de evaluar el riesgo tóxico de compuestos en humanos se deben aplicar diferentes estudios de toxicidad en animales. Sin embargo, debido al número de animales necesarios y por motivos éticos y científicos, se han establecido modelos *in vitro* que permiten trabajar con cultivos celulares y obtener datos de efectos tóxicos del compuesto ensayado.

Los datos que recoge la bibliografía de las micotoxinas de *Alternaria* reflejan la capacidad que tienen para producir efectos tóxicos tanto agudos como crónicos. Concretamente para la micotoxina AOH, se cree que tiene propiedades carcinógenas y mutágenas, aunque hasta la fecha no se han llevado a cabo ensayos in vivo [14], tampoco hay datos de absorción, distribución y excreción [15]; sin embargo, tras la realización de ensayos *in vitro* se ha visto que AOH se hidroxila fácilmente con el ácido glucurónido y el sulfato dando lugar a metabolitos conjugados; que es un potente genotóxico, citotóxico, que altera la proliferación celular y las cadenas de ADN, entre otras. Dada la ausencia de legislación para AOH y la toxicidad que recogen los estudios científicos, se hace necesaria la recopilación de todos los efectos tóxicos conocidos hasta la actualidad. Para ello, se ha realizado una búsqueda bibliográfica de los artículos de AOH disponible en bases de datos como: Pubmed, Scopus, Science Direct y Current Contents en los últimos catorce años. El objetivo de la siguiente revisión bibliográfica es evaluar los efectos de AOH recogidos de ensayos *in vitro*.

ENSAYOS *IN VITRO* CON ALTERNARIOL

El número de estudios *in vitro* llevados a cabo con AOH en líneas celulares es escaso. A continuación se muestran las líneas celulares sobre las que se han desarrollado ensayos *in vitro* con AOH.

1 Células de adenocarcinoma de colon: HT-29 y Caco-2

La composición del epitelio intestinal y las interacciones de los diferentes tipos de células *in vivo* presentan características complejas. Las líneas celulares intestinales humanas Caco-2 y HT-29 aisladas de adenocarcinoma de colon humano, son las más utilizadas en ensayos *in vitro* para estudios de adhesión y de procesos de transporte epitelial [16,17]. La línea celular Caco-2 forma mono capas polarizadas en cultivos celulares y se diferencia a células muy similares a los enterocitos del epitelio intestinal [18,19]. Las células HT-29 de morfología epitelial,

no están diferenciadas, son heterogéneas y contienen una proporción muy pequeña (i.e. < 5%) de células secretoras de mucosa y de enterocitos (células de las microvellosidades) y por tanto pueden obtenerse poblaciones diferenciadas bajo condiciones específicas de estrés metabólico [20].

Las células Caco-2 son las más utilizadas y las más caracterizada aunque presenta algunas limitaciones y/o ventajas según el compuesto a estudiar y por lo tanto condicionan el estudio de toxicidad que se vaya a llevar a cabo, como son: i) baja permeabilidad para compuestos hidrofílicos que atravesarían el epitelio por vía acuosa, debido a la uniformidad que presenta la monocapa lo que la hace más parecida a células de colon que a tejido intestinal; ii) están constituidas únicamente de células de absorción, mientras que el epitelio intestinal está formado por una mezcla de enterocitos y otras células como: caliciformes, endocrinas, células M,… siendo las células caliciformes el segundo tipo de célula más abundante (encargadas de producir la secreción de moco); iii) la sobre expresión de la P-glicoproteína (aunque en controversia), encargada de aumentar la secreción de moco y en consecuencia disminuir la permeabilidad para la absorción de sustancias; y iv) permeabilidad reducida para aquellos compuestos que se absorben mediados por un transportador, lo que pone de manifiesto el origen colónico de esta línea celular.

2 Células de pulmón de hámster chino: V79

La línea celular V79 fue desarrollada por Ford and Yerganian en 1958 desde tejido pulmonar de hámster chino (macho).

Las células V79 han sido ampliamente utilizadas para estudios de mutagenicidad y de reparación del ADN. Son células inmortales, tiene un ciclo celular corto y mutan fácilmente dando lugar a líneas estables aunque deficientes en enzimas de reparación del ADN, y de las funciones relacionadas con la respuesta al daño del ADN. Las células V79 no expresan la proteína p53 y

no muestran la inducción de la respuesta producida por el gen MDM2 al daño del ADN. Todas estas propiedades ha hecho que sean células muy útiles para desarrollar determinados ensayos celulares, pero también han hecho plantear preguntas acerca de la generalización de los resultados que con ellas se obtienen. Tanto es así, que ensayos de mutagénesis y relacionados con daño y reparación del ADN llevados a cabo con estas células se aconseja que sean interpretados con cautela debido a la interrupción/alteración de las vías normales de respuesta al daño del ADN que presentan.

3 Células de adenocarcinoma hepático: HepG2

Los hepatocitos primarios y la línea de hepatocarcinama, HepG2, son los modelos *in vitro* ideales para llevar a cabos estudios de reacciones de biotransformación que tienen lugar en el hígado; y son excelentes para estudios toxicológicos y farmacológicos. Mantiene tanto la morfología como las funciones específicas del hígado durante largos periodos de tiempo [21,22].

Son células fáciles de mantener y reproducen el sistema humano. Aunque son poco adecuadas para predecir/estudiar el metabolismo tal y como ocurre *in vivo* o en cultivos primarios, ya que la expresión de las enzimas encargadas de la metabolización de compuestos es distinta. Sin embargo, aunque las células HepG2 no tienen enzimas específicas del hígado, un aumento de la expresión de estas aumenta la sensibilidad genotóxica de las células HepG2. La mayoría de los autores describen la expresión del citocromo P-450, perteneciente a la ruta de metabolización del hígado de fase I, pero ignoran la expresión de enzimas pertenecientes a las de fase II, las cuales son importantes para las reacciones de destoxicación y activación de muchos xenbióticos [23].

4. Otras líneas celulares

A continuación se exponen características de líneas celulares sobre las que se han llevado a cabo ensayos de toxicidad del AOH en menor proporción, como son las células: MCF-7, A431, MLC, Ishikawa, H295R, HCT116, Hepa-1 y RAW 264.7.

Las células de cáncer de mama MCF-7, se caracterizan por tener abundante factor de transcripción *p53 (*wild-type: mutaciones y cadenas cortas), el cual es capaz de activar determinados genes mediante la unión a secuencias de ADN específicas. Las toposiomerasas controlan, mantienen y modifican las estructuras y la topología del ADN durante los procesos de replicación y traslación del material genético mediante la formación de un complejo de topoisomerasa-ADN. La topoisomerasa I interacciona directamente con la proteína *p53* supresora de tumores de forma inespecífica (wild- type); esta interacción dependerá del estado en que se encuentre el factor *p53*, de hecho en las células MCF-7 la asociación/interacción está estrictamente condicionada con la estructura espacial y temporal que presente el factor *p53*, y solamente ocurre durante periodos muy cortos de estrés genotóxico [24,25]. Por otra parte las células de cáncer de piel (epidermis) A431 se caracterizan por tener factor *p53* no funcional y niveles elevados de receptor de factor de crecimiento epidérmico (EGFR), que interfieren en el control de procesos de crecimiento, proliferación y apoptosis. Principalmente se utilizan para estudios de ciclo celular y para la señalización de rutas celulares de cáncer.

Ambos tipos de células, MCF-7 y A431 se han utilizado para estudiar la actividad de AOH sobre las topoisomerasas; bien para estudiar su efecto sobre la actividad catalítica (células MCF-7) o bien para estudiar la estabilidad del complejo topoisomerasa II-ADN (células A431). Las células de linfoma de ratón (MLC) se utilizan para determinar el efecto mutagénico del AOH, con el estudio de la

inducción de mutaciones en el locus de timidina kinasa (TK) de estas células. Las células Ishikawa se utilizan, para estudiar los efectos tóxicos sobre la reproducción, ya que estas células (de adenocarcinoma endometrial humano) contienen gran cantidad de receptores de estrógenos y de progestágenos. Las células de carcinoma adrenocortical humano H295R, se utilizan para ensayos de esteroidogenesis (disrupción endocrina), ya que contienen todas las enzimas implicadas en este proceso; además son capaces de producir hormonas esteroideas humanas y permiten evaluar alteraciones en la expresión génica y en la producción de esteroides sexuales. Las células para ensayos de genes de estrógenos RGAs inducibles de hormonas esteroides, que se generan a partir de glándulas mamarias por inclusión de un gen de luciferasa [26]. Las células de carcinoma de colon HCT116, se han utilizado para estudios de efecto tóxico en intestino y la ruta de muerte celular, por ser la primera línea de contacto del tóxico tras una intoxicación alimentaria. Las células de cáncer hepático de ratón Hepa-1 se caracterizan por tener la ruta del AhR (receptor aril hidrocarburo) intacta (1c1c7) o deficiente (1c1c4 o 1c1c12) lo que les confiere diferente capacidad metabólica; por tanto se utilizan para estudios de metabolismo (glucuronación). Y las células de macrófagos de roedores murinos RAW 264.7, caracterizadas por tener una respuesta en el DNA funcional y la proteína p53, se han utilizado para estudios efectos en el ciclo celular a través de daños en el ADN.

Las líneas celulares en los efectos tóxicos de alternariol

Los efectos tóxicos producidos por AOH en las diferentes líneas celulares, así como el tipo ensayo, tiempo y dosis estudiada, se recogen en la tabla 3.

1. AOH en células Caco-2 y HT-29

Los ensayos llevados a cabo en células HT-29 para el estudio de los efectos producidos por AOH son muy diversos: proliferación celular, Western Blot, estrés oxidativo, glutatión, Cometa (lesión del ADN),…; y de igual modo la dosis, que va

desde 0,1 a 100 µM; y el tiempo de exposición: de 20 min a 24 h. Los efectos del AOH para este tipo de células se recogen a continuación. Tiessen y col, 2013 [27] recoge que: i) a concentraciones elevadas de AOH se reduce la proliferación celular, aumenta la actividad enzimática de GST y se incrementa del estrés oxidativo; coincidiendo con la sobreexpresión de tres genes relacionados con la respuesta antioxidante; mientras que ii) a las concentraciones y tiempos de exposición más bajos, los niveles de GSH disminuyeron llegando incluso a no ser detectables (**Tabla 3**).

En cuanto a la lesión del ADN, realizado con el ensayo Cometa, este sólo fue observable a concentraciones ≥1 µM y tiempo de exposición de 1h; resultados que coinciden con los publicados previamente en 2012 [28] con el mismo tipo de ensayo. Este daño se ha visto que está relacionado con la viabilidad celular, la cual se vio afectada principalmente a tiempos de exposición prolongados (≥24h); no obstante, a tiempos bajos de exposición (1h) ésta se mantiene con valores >85% y sin alteración de los niveles de la lactato deshidrogenasa (LDH) [29].

Recientemente Fernández-Blanco y col. [30] han estudiado los efectos que AOH produce en la proliferación celular, estrés oxidativo (especies reactivas de oxígeno (ROS) y lipoperoxidación lipídica (LPO)) y actividad enzimática (catalasa (CAT) y superóxido dismutasa (SOD)). Los resultados permiten concluir que a tiempos prolongados de exposición (≥48 h) se produce una disminución del número de células a medida que aumenta la concentración; mientras que a tiempos cortos de exposición (24 h) se produjo un aumento de ROS, LPO y de la actividad enzimática de SOD. La actividad de la CAT disminuyó a concentraciones > 15 µM.

Por otra parte, la generación de metabolitos tras la exposición a AOH se llevó a cabo tanto en células HT-29 [31] como en células Caco-2 [32]. Ambos estudios coinciden en que los metabolitos conjugados con ácido glucurónico

(glucuronidación) son los más abundantes y los que se producen instantáneamente; incluso para la concentración y tiempo de exposición más bajo estudiado (20 µM durante 1 h). Las posiciones de conjugación por glucuronación del AOH se produjeron en los grupos hidroxilo del carbono 3 y 9, mientras que con sulfato se produjo el carbono 3. Por otra parte, se realizó un estudio de "liberación de AOH" desde el glucuronato generado mediante la adición simultanea de sulfato aril-beta-glucuronidasa, en células HT-29 observándose que la liberación de éste se producía en forma concentración dependiente [31] (Tabla 3). Así, la capacidad que posee AOH de producir daño celular se ve disminuida por la elevada capacidad que tanto las células HT-29 como las Caco-2 presentan para eliminar la micotoxina y los metabolitos generados a través de reacciones de glucuronidación.

Se estudió también el paso a través de la membranas apical o basal del AOH, simulada con la placas de Millicell®, y se observó que las formas conjugadas eran capaces de atravesar ambas membranas, mientras que la forma libre se acumula en el compartimento basolateral; concluyendo que la absorción a nivel intestinal de AOH era rápida y amplia [32].

2. AOH en células V79

En las células V79 principalmente se han llevado a cabo cuatro ensayos para el estudio de los efectos tóxicos producidos por AOH: daño al ADN, alteración del ciclo celular, genotoxicidad y mutagenicidad.

Los daños en las cadenas de ADN en las células V79 tras exposición a AOH durante tiempos cortos (1h) se produjo en forma concentración dependiente [31,33]. Teniendo en cuenta que estas células no presentan enzimas y por tanto no producen reacciones de metabolización (hidroxilación y de glucuronidación), no se generan metabolitos [31] y en consecuencia la alteración de la viabilidad

celular se debe únicamente al AOH en concentraciones elevadas (≥ 30 µM) [34] (Tabla 3).

Tras el estudio de la distribución del ciclo celular, se observó una alteración de las fases del ciclo tras la exposición a AOH, mostrándose una acumulación de células en la fase G2/M de forma concentración dependiente (32% a 10 µM y 62.6% a 30 µM) [33-35]. Esta acumulación también se observó en la fase S a medida que aumenta el tiempo de exposición; hecho que se asoció a la acumulación de células en la fase G2/M a tiempos inferiores [35].

La inducción de mincronucleos (MN) se utilizó para estudios de genotoxicidad. Los resultados demostraron la capacidad genotóxica de AOH mediante el aumento los MN en tiempo y concentración dependiente [35] (Tabla 3). Brugger y col. [34] y Fleck y col. [33] estudiaron la capacidad de producir mutaciones en el gen del locus de la hypoxantina-guanina fosforiltransferasa (HPRT) tras varios periodos de exposición (1, 3 y 6 días). Los resultados demostraron que se producían mutaciones en los genes de este locus, concluyéndose que el AOH tiene actividad mutagénica [33,34].

Tabla 3. Línea celular, dosis, tiempo de exposición, ensayo y efecto obtenido en ensayos *in vitro* llevados a cabo con la micotoxina Alternariol (AOH).

Línea celular	Dosis Tiempo de exposición	Ensayo	Efecto	Ref.
HT-29	0.1-50 µM a) 24h b) 3h c) 1h d) 20 min, 1 y 3h e) 1 y 24h f) 1 y 3h	A) SRB (a) B) Proliferación celular (WST-1) (b) C) DCF (c) D) Wetern Blot (contenido de Nrf2) (d) E) RT- PCR (Nrf2/ARE para tres genes) (b) F) GSH/GSSG (e) G) Actividad de GST (e) H) Cometa (f)	A) A la concentración más elevada de AOH (50 µM) se produjo una reducción de las proteínas celulares (10 - 15 %). B) La actividad mitocondrial no se vio afectada. C) AOH produjo estrés oxidativo (5.5-veces más que el control) D)A los 20 min: no hubo efecto A 1 y 3h: AOH aumento los niveles de la proteína nuclear Nrf2 E)AOH produjo aumento de tres genes (GSTA1, GSTA2 y γGCL) de forma concentración dependiente F) A 1h: los niveles de GSH total disminuyeron a 10 y 50 µM A 24h: los niveles de GSH total no se detectaron G)At 1h: AOH no afecto la actividad de la GST At 24h: AOH aumentó la actividad de GST (los niveles más altos a 50 µM) H)AOH no aumento el daño en el ADN *Tras la exposición de las células HT-29 a AOH: se produjo un aumento de la expresión de GST, indicando una activación de los mecanismos de defensa, una modulación del estado redox y efectos sobre la vía de señalización de Nrf2/ARE*	[27]
HT-29	0.1 - 50 µM 1h	A) Cometa B) DCF	A) AOH posee acción moderada sobre la rotura de las cadenas de ADN. B) AOH aumento la señal de DCF hasta 2.5 veces a 50 µM, poniendo de manifiesto los efectos redox de los niveles intracelulares	[28]
Caco-2	a) 20 µmol/l 1, 2 y 3h b) 10, 20, 30, 40 µmol/L 2h	A) Deteccion de metabolitos (a) B)Absorción con placas de 24-pocillos Millicell® plates (medidas a intervalos de 30 min) (b)	A) Glucuronidación de AOH en el grupo hidroxilo del C-3 y C-9; conjugación con sulfato en el grupo hidroxilo del C-3 B) Las formas conjugadas de AOH atravesaron las membranas apical y basal; el AOH libre alcanzó el compartimento basolateral y por tanto se facilita su llegada a la sangre de la vena porta	[32]
HT-29 **A431**	a) 0.1 - 50 µM b) 0 - 100 µM c) 0 - 25 µM 1h	A) Viabilidad y citotoxicidad (a): -Tripan Blue y -LDH B) Cometa (b) C) Ensayo alcalino con Hoechst 33258 (1µM) como ligando de unión (c)	A) La viabilidad celular (> 85%) se mantuvo a lo largo de todo el ensayo. AOH no afectó la liberación de LDH. B) AOH produjo un aumento de la rotura de las cadenas de ADN tras 1h de tratamiento y a una concentración ≥ 1 µM C) AOH compitió con Hoechst 33258 por unirse al ADN con un valor de EC_{50} de 8.1 ± 1.2 µM	[29]
A431	0 - 100 µM 1h	Ensayo de depleción de immunobanda	AOH actúa como veneno para las topoisomerasas celulares, especialmente para la isoforma Top.IIα	[29]
V79	0 - 20 µM a) 24h b) 1.5h	A) Ensayo de HPRT (1, 3 y 6 días) (a) B) Ciclo celular (CyStain DNA Protein 2, Partec) (a) C) Ensayo alcalino con Hoechst 33258 (b)	A) AOH produce un aumento dependiente de la concentración de la frecuencia de mutaciones B) AOH afecta al ciclo celular de las células V79 produciendo una acumulación estas en la fase G2/M (32%, 10 µM). C) AOH produjo rotura de las cadenas de ADN	[33]
V79 **HT-29** **HepG2**	a) 1 - 50 µM 1 y 24h b) 1 - 25 µM 24h	A) Ensayo alcalino con Hoechst 33258 (a) B) Conjugación de AOH (a)	A) A 1h: aumentó la rotura de las cadenas de ADN de forma concentración - dependiente (de 5 a 50 µM). A 24h: sólo para las células HepG2 se produjo un aumento de la rotura de las cadenas de ADN B) Las células HT-29: -produjeron reacción de conjugación (glucuronidación), mientras que un 75 % de las HepG2 no lo hicieron (a 1 y 24h) -fueron mucho más activas en la conversión de AOH conjugado que las HepG2	[31]
		C) Actividad de glucuronidación (2mM of UDPGA) (b)	-aumentaron el AOH libre cuando se trataron con β-glucuronidasa/aril sulfato en forma concentración dependiente. C) Las células HT-29 tienen mucha mayor actividad de reacción de glucuronidación que las otras líneas celulares estudiadas. *La reacción de conjugación por glucuronidación evita/reduce la capacidad de AOH de producir la rotura de las cadenas de ADN.*	
V79 **MLC**	0 - 30 µM 24h	A) Citotoxicidad B) Ensayo de HPRT (1, 3 y 6 días en células V79) Ensayo TK (1, 2 y 3 días, en células MLC) C) Ciclo celular (CyStain DNA Protein 2, Partec)	A) Disminución de la viabilidad celular en forma concentración-dependiente tras el tratamiento con AOH por encima de 30 µM (V79, 35%) o 20 µM (MLC, 69%). B) AOH produjo mutaciones en el locus HPRT (células V79) y en el locus TK (células MLC) confirmando la actividad mutagénica del AOH. C) Aumento de las células en la fase G2/M en forma concentración dependiente tanto para las células V79 (62.6%, 30 µM) como para las MLC (37.1%, 20 µM).	[34]
Ishikawa **V79**	<u>Células Ishikawa:</u> 0 – 10 µM a) 48 y 72h b) 48h <u>Células V79:</u> 0-50 µM c) 6h d) 6, 14 y 24h	**Ensayo de estrogenecidad:** A) ALP (con y sin: E2 o ICI182.780). Medida cada 10 min durante 1h. (sólo en células Ishiwaka) (a) B) RT- PCR (para mRNA de la expresión de ALP) (sólo en células Ishiwaka) (a) C) Proliferación y distribución del ciclo celular (CyStain DNA Protein 2, Partec). (a y c)	A) AOH provocó la inducción de ALP con una EC_{50} = 2.7 ± 1.7 µM; 10000-veces más débil que E2; esta inducción se inhibió tras el tratamiento simultaneo con IC182.780. No se observó una reducción de ALP tras el tratamiento simultaneo con E2 10nM. B) Misma expresión de mRNA de ALP para el tratamiento con AOH o con E2; mientras que esta se inhibió con el tratamiento simultaneo de AOH con ICI. C) La <u>proliferación</u> de las *células Ishikawa* disminuyo de 48 a 72h (50%) tras el tratamiento con AOH; en consecuencia se produjo una inhibición de la proliferación celular más que citotoxicidad. Estos efectos se correlacionaron con los efectos observados en el ciclo celular. Para las *células V79* no se observaron modificaciones en la proliferación celular ni a las concentraciones más elevadas	[35]
		Ensayos de genotoxicidad D) Inducción de MN: MN neg. y MN pos. (DAPI: 1 µg/mL). (b y d)	(25 and 50 µM). En cuanto al <u>ciclo celular</u>, el tratamiento con AOH produjo una disminución de células en la fase G1/G0 de forma concentración dependiente y aumento en la fase S y G2/M debido a la acumulación de células en G2 en los dos tipos de células. Sólo para las células V79, a 10 y 25 µM, las células en las fases G2/M y S aumentaron con el tiempo de exposición, debido a un arresto (acumulación) en la fase G2 (a 6h) y en la fase S (a las 14 y 24h). D) En las células Ishikawa, AOH aumento la suma total de MN neg. + MN pos. a las concentraciones más altas estudiadas; mientras que en las células V79, este aumento se obtuvo de forma tiempo y concentración dependiente. *AOH disminuyo la actividad de ALP debido a una disminución de la proliferación celular.*	

MCF-7	0 - 200 µM 1h	D) Topoisomerasa I -Ensayo de relajación -Ensayo de ruptura E) Topoisomerasa II - Ensayo de decatenation -Ensayo de ruptura	D) AOH inhibe la actividad catalítica de la Top.I a 50 µM AOH mejora la proporción de ADN plásmido circular en forma dependiente de la concentración: AOH actúa *in vitro* como veneno para la topoisomerasa I E)AOH inhibe la actividad catalítica de -Top.IIα a conc. ≥ 25 µM -Top.IIβ a conc. ≥ 100 µM AOH estabiliza: Top.IIα-ADN intermediario a conc. ≥ 10 µM Top.IIβ-ADN intermediario a conc. ≥ 50 µM AOH tiene menor efecto sobre Top.IIβ	[29]
H295R RGAs*	a) 0.1 - 1000 ng/ml b) 50 - 10000 ng/ml c) 25 - 5000 ng/ml 24 y 48h d) 3.87 µM a 48h	A) Citotoxicidad: -Alamar Blue (células H295R) (a) - MTT (células RGAs) (b) B) **Esteroidogenesis** - Test agonista (b) - Ensayo genético – Test antagonista (c)	A) La viabilidad de las células H295R no varió significativamente a las concentraciones estudiadas; mientras para las células RGAs esta variación se produjo a las concentraciones más altas estudiadas. B) AOH produjo un efecto agonista a las concentraciones más altas, probablemente relacionado con el efecto citotóxico AOH produjo un efecto de sinergismo en la unión de la progesterona al receptor progestágeno.	[35]
		C) Producción de hormonas (a): progesterona, estradiol, testosterona y cortisol D) RT-qPCR - expresión genética (d)	C) AOH aumentó la producción de progesterona y estradiol D) AOH fue capaz de regular la expresión ocho de los dieciséis genes analizados	

ARE: elementos de respuesta antioxidante; Nrf-2: factor -2 nuclear; SRB: ensayo de sulforodamine B; HPRT: hipoxantina-guanina fosforiltransferasa; RT-PCR: PCR tiempo real; MLC: células de linfoma de ratón L5178Y tk+/-; TK: timidina kinasa; ALP: ensayo de las fosfatasas alcalinas; E2: radio labeled 17β-estradiol (inductor de ALP); ICI182.780: receptor antagonista estrogénico de elevada afinidad; MN: micronucleos; MN neg.: micronucleos negativos: fragmentos de cromosomas acéntricos ; MN pos.: micronucleos positive: cromosomas enteros; H295R: carcinoma adenocrotical humano; *RGAs: líneas celulares para ensayos de genes.

3. AOH en células HepG2

Actualmente, un único artículo recoge la capacidad del AOH para producir alteraciones/daño en las cadenas de ADN de las células HepG2 [31]. Estos autores observaron que las células HepG2 tienen una mayor sensibilidad a la alteración de las cadenas de DNA que la que producen otras líneas celulares como HT-29 o V79. Además ofrecen mayor resistencia a producir reacción de conjugación incluso a tiempos de exposición prolongados (24h); probablemente debido a la inespecificidad enzimática que esta células poseen (Tabla 3).

4. Efectos de AOH en otras líneas celulares

Con el fin de estudiar si los efectos de AOH sobre las cadenas de ADN están relacionados con interferencias en la actividad catalítica de las topoisomerasas I y II (Top. I y Top. II con sus isoformas: Top.IIα y Top.IIβ), se llevó a cabo un estudio sobre las células MCF-7 [29]. Los resultados demostraron que AOH inhibe la actividad catalítica de ambas topoisomerasas, aunque este efecto era más acusado para la Top. I (conc. ≥ 50 µM) que para las Top.II (α y β) y para la Top. IIα más que para la Top. IIβ. Estos resultados se corroboraron mediante un ensayo de

depleción de inmunobanda con Western Blot de las topoisomerasas en células A431 [29].

El efecto muta génico y sobre la reproducción del AOH se ha llevado a cabo con células MCL e Ishikawa. El AOH demostró que en células MCL tiene actividad mutagénica sobre el locus TK a dosis ≥10 µM y tiempos de exposición ≥24h: estos efectos se relacionaron con la alteración en la distribución del ciclo celular, ya que se produjo un aumento de células en la fase G2/M del ciclo con el aumento de la concentración (37% para 20 µM) (Brugger y col., 2006). Sobre la reproducción, se observó una disminución de la proliferación de las células Ishikawa desde las 48 a las 72h de exposición, que estuvo correlacionado con las alteraciones observadas en la distribución del ciclo celular; la proporción de células en la fase G_1/G_O que disminuyó con el aumento de la concentración, mientras que la proporción de células en la fase S y G_2/M aumentaron [35].

Los efectos de citotoxicidad por AOH en las células RGAs sólo se produjeron a las concentraciones más altas (≥2500 ng/mL), mientras que este efecto no se observó para las H295R. En los ensayos de esteroidogénesis, para las mismas condiciones y para los dos tipos de células, AOH produjo un efecto agonista y de sinergismo con la progesterona, aumentó la producción de hormonas (progesterona y estradiol) y aumento la expresión génica de aquellos genes implicados en la esteroidogénesis [36].

Los estudios sobre la ruta de muerte celular de AOH con células HCT116 muestran que esta se produce por apoptosis dependiente de la actividad de la caspasa-9, en forma dosis dependiente, a través de la apertura de los PTP, y produciendo indirectamente la activación y despolarización del MMP [37]. La producción de ROS por AOH se asoció a las alteraciones mitocondriales provocadas por AOH, mientras que la sobrexpresión de p53 y aumento de la

actividad de la caspasa-3 se debió a la apoptosis producida por AOH. Por otra parte, el ensayo de AOH con células HCT116-Bax-KO, deficientes en la proteína pro-apoptótica Bax, refleja la capacidad protectora de esta proteína en las alteraciones mitocondriales producidas por AOH [37].

Estudios de metabolización con diferentes tipos de células Hepa-1 (Hepa-1c1c4, Hepa-1c1c7, Hepa-1c1c12) tras la exposición a AOH demuestran que la actividad enzimática de Cit- P450 disminuye [38]. Por otra parte, el metabolito mayoritario (no oxidativo) detectado fue el 4 - hidroxilado de AOH aunque sólo en las células Hepa-1c1c12. La actividad enzimática más baja de la catecol-O-metiltransferasa se obtuvo en las células Hepa-1c1c7, mientras que la más alta, para la UDP-glucuronosiltransferasa, en las células Hepa-1c1c4. En general se observó que las formas conjugadas de AOH aumentan con el aumento del tiempo de exposición. Y por último, el ciclo celular de estas líneas celulares tratadas con AOH se detuvo en la fase G2/M, aunque sólo para las células Hepa-1c1c7 y Hepa-c1c12 [38].

Los efectos tóxicos del AOH sobre el ciclo celular a través de daños en el ADN se han estudiado en las células RAW 264.7. Los resultados revelan que a dosis bajas (15 y 30 µM) se reducía la proliferación celular, mientras a que a dosis altas (60 µM) se producía muerte celular por necrosis con aumento de las células en la fase G2/M para todas las dosis y tiempos ensayados, efectos que coincidieron con la de otros autores. Se observó también un aumento de ROS, lo que produjo en consecuencia despolarización del MMP así como un aumento en las proteínas involucradas en la reparación y generación del ADN (histona H2AX fosforilada, Chk-1 y Chk-2) [39]. En un estudio posterior llevado a cabo por el mismo grupo de autores, se estudiaron alteraciones implicadas en el efecto de AOH en el ciclo celular y que se van produciendo en los diferentes estadios de afectación del ciclo celular [40]. Así se ensayaron los efectos en la fluidez de

membrana, se determinaron proteínas intracelulares (Histona H3 como marcador mitótico y el complejo ciclina B-cdc2) y gangliósidos implicados en la organización de las bases lipídicas (GM1). Los resultados demostraron que tras la exposición a AOH las células mitóticas disminuyeron, los niveles de proteínas implicadas en el complejo ciclina B-cdc2 aumentaron, al igual que la fluidez de la membrana [40].

Conclusiones

Los ensayos *in vitro* recogidos en la bibliografía con la micotoxina AOH ponen de manifiesto la capacidad que tiene de producir reducción en la proliferación y viabilidad celular, aumento de la actividad enzimática de GST y SOD, disminución de la actividad de la CAT, acumulación de ROS, inducción de LPO, generación de metabolitos conjugados con ácido glucurónico, detención del ciclo celular en la fase S, lo que produce una acumulación en la fase G2/M, inhibición catalítica de las topoisomerasas, daños en el ADN, aumento de la esteroidogenesis y capacidad genotóxica y mutagénica.

En resumen, los resultados obtenidos revelan la capacidad de AOH de provocar efectos tóxicos a corto y largo plazo, y por tanto la especial atención que hay que poner a esta micotoxina, así como la necesidad de legislar sus niveles en alimentos. Por otra parte, dada la presencia simultánea de sustancias tóxicas en un alimento, el planteamiento de futuros ensayos *in vitro* relacionadas con la acción del AOH debería de ir encaminado al estudio de la alteración de estos efectos tóxicos mediante combinaciones dobles, triples o cuádruples de micotoxinas.

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3.2 Interaction effects of Enniatin B, Deoxinivalenol and Alternariol in Caco-2 cells.

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Abstract

Enniatin B (ENN B), deoxinivalenol (DON) and alternariol (AOH) are secondary metabolites of filamentous fungi. These mycotoxins are contaminants of vegetables and cereals. They are cytotoxic and their effects are enhanced by their mixtures. The objectives of this study were to compare the cytotoxicity of ENN B, DON and AOH alone or in combination in human adenocarcinoma (Caco-2) cells and to evaluate the type of interactions of mycotoxin mixtures by the isobologram analysis. Cells were treated with concentrations ranging from 1.85 to 90 µM (AOH) and from 0.312 to 10 µM (for ENN B and DON), individually and in combination of two and three mycotoxins (from 1.85 to 30 µM for AOH and from 0.312 to 5 µM for ENN B and DON). The relation ratios between the mixtures DON + ENN B was 1:1; AOH + DON and ENN B + AOH was 1:6, and for the tertiary combination DON, ENN B and AOH 1:1:6. The IC_{50} value of ENN B and DON were 3.87 and 5.54 µM, respectively. No IC_{50} values were obtained for the AOH at any time tested in Caco-2 cells. With the isobologram the type of interaction between mycotoxin was evaluated. Synergistic, antagonistic and addictive effect was observed for the combination studied depending on the concentration affected. Mycotoxins combinations reduce cellular viability in the following increasing order: (DON+ ENN B)> (ENN B+AOH)> (DON+AOH)> (DON+AOH +ENN B).

1. INTRODUCTION

Mycotoxins are a group of diverse chemical compounds produced from secondary metabolism of moulds (filamentous fungi). They are mainly produced by five genera: *Aspergillus, Penicillium, Fusarium, Alternaria* and *Claviceps* (Gutleb et al., 2002). Many mycotoxins are stable under normal food processing conditions and can therefore, be present not only in food and feed but also in

processed products (Lauren and Smith, 2001). Cereal grains, like many other agricultural commodities, can be contaminated by mycotoxins which may colonize the various substrates at all stages of the production chain (Malachova et al., 2011). About 20% of the crops grown in the European Union for foods and animal feeds contain measureable amounts of mycotoxins (Gutleb et al., 2002). Several mycotoxins have been characterized as food contaminants with a possible health risk, where deoxinivalenol (DON), enniatin B (ENN B) and alternariol (AOH) are included (EFSA, 2011).

DON, also known as vomitoxin, is the most prevalent type B trichothecene mycotoxin worldwide. Cereal grain is infected with DON mycotoxin in the fied or during storage by *Fusarium* spp (Savard et al., 2015). It can cause deleterious health effects like anorexia, weight loss, malnutrition, endocrine dysfunction and immune alterations (Ndossi et al., 2012, Yang et al., 2014). DON is reported to inhibit cell proliferation, RNA and DNA synthesis, and induce apoptosis partly due to inhibition of protein synthesis (Rotter et al., 1996).

ENN B is also a fungal metabolite produced by several *Fusarium species* (Ivanova et al., 2006). It has become an issue of high concern for human and animal health during the last decade, because of its potential toxicity, probably linked to its ionophoric activities (Prosperini et al., 2014). ENN B is described as phytotoxins, with a wide range of toxicological effects, such as antibacterial, antifungal, insecticidal, phytotoxic and cytotoxic properties (Kamyar et al., 2004). ENN B cytotoxicity involved early reactive oxygen species (ROS) generation that induced lipid peroxidation (LPO), apoptosis and necrosis via the mitochondrial pathway (Prosperini et al., 2013). Moreover, it produced adrenal endocrine toxicity (Kalayou et al., 2015).

AOH is the main mycotoxin produced by *Alternaria alternata*. AOH has been found in considerable concentrations in a wide variety of grains and grain-

based products, vegetables, fruits, and oilseeds (Marin et al., 2013). However no risk assessment studies about *Alternaria* mycotoxins in food and feed has been established to date (EFSA, 2011). AOH has cytotoxic properties and inhibit cellular proliferation interfering with the cell cycle (Lehmann et al., 2006; Tiemann et al., 2009; Fernández-Blanco et al., 2014). It has been associated with DNA damage (Solhaug et al., 2012; Fernández-Blanco et al., 2015) and acts as an endocrine disruptor (Frizzell et al., 2013).

Mixtures of toxins can frequently be present in foods, however due to the complications of determining their combined toxic effects, mechanisms of action, etc., legal limits of exposure are determined for single compounds. The Commission Regulation (EC) 1881/2006 and its amendments set maximum levels for certain contaminants in foodstuff as for certain mycotoxins including some *Fusarium* toxins (EC, 2006). However, these maximum levels have been established taking into account only the presence of individual mycotoxins. For this reason, it is necessary to consider toxicological ‘‘mixture’’ effects derived from mycotoxins to implementing human risk assessment (Speijers and Speijers, 2004).

The aim of this study is to determine the joint action of DON, ENN B and AOH and to assess the nature of toxicological interactions among them. For this purpose, interactions between two or three mycotoxins combination was studied. Interactions were evaluated with the isobologram analysis method. This method has been applied to determine addition, synergism and antagonism effects, produced by mycotoxin combinations (Chou and Talalay, 2006). In addition, taking into consideration that the major route to mycotoxins is via oral, human colon adenocarcinoma (Caco-2) cells were used in this study.

2. MATERIALS AND METHODS

2.1 Reagents

The reagent grade chemicals and cell culture components used, Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, HEPES, trypsin/EDTA solutions, non-essential amino acids (NEAA) and phosphate buffer saline (PBS). Thiazolyl blue tetrazolium bromide (approx. 98%; M2128; MTT). AOH (258.23 g/mol; purity >96%) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). ENN B (639.82 g/mol; purity >95 %) and DON (296.35 g/mol; purity >98 %) were purchased from Sigma–Aldrich (St. Louis MO, USA). Dimethyl sulfoxide (DMSO) and methanol (MeOH) were obtained from Fisher Scientific (Madrid, Spain). Fetal calf serum (FCS) were from Gibco by Thermo Fisher Scientific). Deionised water (resistivity <18 MΩ cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Stock solution of AOH was prepared in DMSO and stock solutions of ENNB and DON were prepared in MeOH and maintained at -20 ºC. The final AOH, ENN B and DON concentrations tested were achieved by dilutions in the culture medium. The final DMSO, MeOH or mixture of both concentration in the culture medium was ≤ 1% (v/v).

2.2 Cell culture and AOH, ENB and DON treatment

The mycotoxin problem in public health is longstanding and all humans and animals are at risk for mycotoxin exposure. People are mainly exposed via the ingestion of contaminated foods (Pestka et al., 2007).

Caco-2 cells (ATCC HTB-37) were maintained in DMEM medium supplemented with 25 mM HEPES buffer, 1% (v/v) of NEAA, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) FCS inactivated. Incubation conditions were pH 7.4, 37°C under 5% CO_2 and 95% air atmosphere at constant humidity. Culture medium was changed every two days. Absence of mycoplasma was

checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St Louis Mo. USA). Cells were plated in 96-well tissue culture plates at a density of $3x10^4$ cells/well to perform the experiment. Six concentrations for ENN B and DON (0.3125–10 µM) and seven concentrations for AOH (1.875–90 µM) were assayed. For the binary and tertiary combinations, the concentrations ranged from 0.3125 to 5 µM for ENN B and DON and from 1.875 to 30 µM for AOH. The relation ratios between the mixtures were, DON and ENN B 1:1; AOH + DON and ENNB + AOH 1:6, and for the tertiary combination DON, ENNB and AOH 1:1:6.

The basal cytotoxicity endpoint selected was the tetrazolium salt reduction (MTT). MTT was measured according to Ruiz et al. (2006). For each mycotoxin from stock solutions, serial dilutions in supplemented medium were prepared. Culture medium without DON, ENN B and AOH and with 1% MeOH or DMSO was used as control.

After cell confluence was achieved, the mycotoxin concentrations were added to each well and plates were incubated at 37 ºC for 24, 48 and 72 h. After, medium containing DON, ENN B and AOH was removed, cells were washed with PBS and 50 µM MTT were added. The absorbance was measured after 4 h at 570 nm on an ELISA plate reader Multiscan EX (Thermo scientific, MA, USA). Mean inhibition concentration (IC_{50}) values were calculated from full dose–response curves.

2.3. Experimental design and assessment of effect of mycotoxin combinations.

The isobologram analysis was used to determine the type of interaction that occurs when DON, ENN B and AOH are in combination. The type of interaction is described by the median- effect/combination index (CI)-isobologram equation by Chou (2006), and Chou and Talatay (1984).

The isobologram analysis involves plotting the dose–effect curves for each compound and its combinations in multiple diluted concentrations by using the median effect equation:

$$fa/fu = (D/D_m)^m$$

Where D is the concentration of a product, D_m is the median-effect dose (e.g., IC_{50}, EC_{50}, or LD_{50}) that inhibits the cells under study by 50%, *fa* is the fraction affected by concentration D (e.g., percentage inhibition/100), *fu* is the unaffected fraction (therefore, *fa* = 1 - *fu*), and m is the coefficient signifying the shape of the dose-effect relationship, where *m* = 1, *m* > 1, and *m* < 1 indicate hyperbolic, sigmoidal, and negative sigmoidal dose-effect curve, respectively (Chou and Talalay, 1984). Therefore, the method takes into account both the potency (D_m) and shape (*m*) parameters.

Chou and Talalay (1984) introduced the term combination index (CI) x for quantification of synergism or antagonism for *n* compounds, and the general equation for *n*-compound combination at *x*% inhibition becomes:

$$^{n}(\mathrm{CI})_x = \sum_{J=1}^{n} (D_j/D_x)_j = \frac{(D_x)_{1-n}\{[D]_j \sum_{j=1}^{n}[D]\}^{1/mj}}{(D_m)_j\{(\mathrm{fax})_j/[1-(\mathrm{fax})_j]\}}$$

Where $^{n}(CI)_x$ is the combination index for n compounds (e.g., mycotoxins) at *x*% inhibition (e.g., proliferation inhibition); $(D_x)_{1-n}$ is the sum of the concentration of n compounds that exerts *x*% inhibition in combination, $\{[D_j]/ \sum^{n}_{1} [D]\}$ is the proportionality of the concentration of each of n compounds that exerts *x*% inhibition in combination; and $(D_m)_j \{(fax)_j/[1-(fax)_j]\}^{1/mj}$ is the concentration of each compound alone that exerts *x*% inhibition. From this equation, CI <1, =1, and >1 indicates synergism, additive effect and antagonism,

respectively. The types of interactions produced by DON, ENN B and AOH combinations were assessed by isobologram analysis using CalcuSyn software version 2.1. (Biosoft, Cambridge, UK, 1996–2007).

2.4 Statistical analysis

Statistical analysis of data was carried out using SPSS version 22 (SPSS, Chicago, IL, USA), statistical software package. Data were expressed as mean ± SD of three independent experiments. The statistical analysis of the results was performed by Student's t-test for paired samples. Differences between groups were analyzed statistically with one-way ANOVA followed by the Tukey HDS *post-hoc* test for multiple comparisons. The level of $p \leq 0.05$ (*), was considered statistically significant.

3. RESULTS

3.1. Cytotoxicity of individual mycotoxins

The cytotoxic effect of ENN B, DON and AOH in Caco-2 cells were evaluated by the MTT assay at 24, 48 and 72 h of exposure. The dose–response curves of individual mycotoxin in Caco-2 cells are shown in Figure 1. At 24 h, ENN B decreased cell viability from 2.5 to 10 µM, whereas at 48 and 72 h of exposure ENN B decreased the viability from 1.25 to 10 µM. However, ENN B at concentrations ranging from 0.3125 to 0.625 µM after 24 h of incubation produces some stimulations of the mitochondrial function generating significatives differences with the control. The IC_{50} value of ENN B was 3.87 µM after 72 h (Fig. 1a). At 24 and 72 h, DON exhibited a reduction of viability from 1.25 to 10 µM. After 48 h of exposure, DON decreased cell viability from 0.312 to 10 µM. The IC_{50} value of DON was 5.54 µM after 72 h (Fig. 1b). AOH did not decrease the viability at any of the concentrations tested at 24 h. Nevertheless, after 48 and 72 h, AOH reduces cell viability from 60 to 90 µM (Fig.1c). No IC_{50} values were obtained for AOH. The results clearly showed that the three

mycotoxins reduce cell viability in a time-, and concentration dependent manner. As observed in Figure 1, AOH showed the less cytotoxic effect on Caco-2 cells at the three times of exposure, followed by DON and ENN B that resulted to be the most cytotoxic. ENN B, DON and AOH individually decrease cell viability from 29% to 93%, from 43% to 57% and from 14% to 26%, respectively.

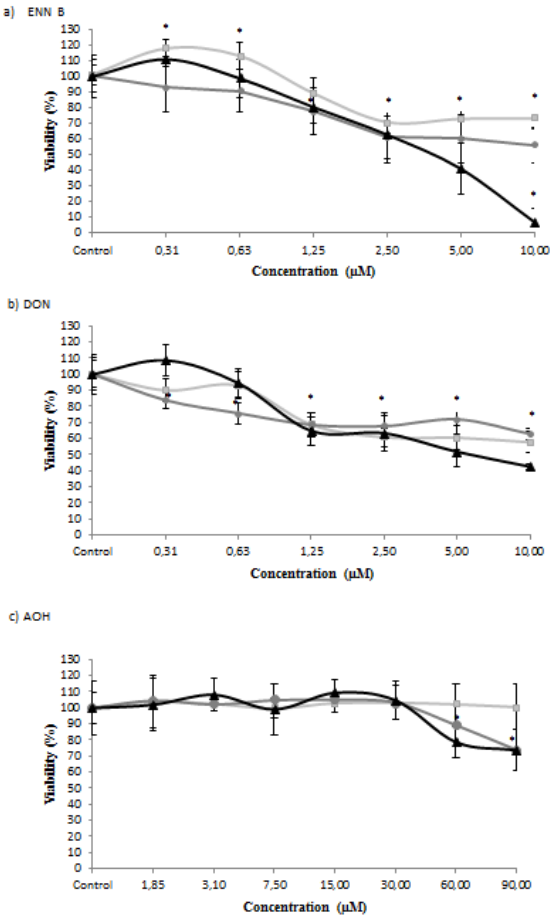


Figure 1. Dose response curve of a) ENN B , b) DON and c) AOH on Caco-2 cells after 24h (–■–) , 48 (–●–) and 72 h (–▲–) of exposure. All values are expressed as mean ± SD of 3 replicates. $p \leq 0.05$ (*) represents significant differences from control values.

3.2. Cytotoxicity of binary and tertiary mixture of mycotoxins

Figures 2-5 show the viability (%) – concentration (µM) plot of binary and tertiary mycotoxin combinations in Caco-2 cells at 24, 48 and 72 h of exposure, compared to the concentration–response curve of each mycotoxin when applied individually in the same assay. Considerable differences in binary mycotoxin combination compared to each myxotoxin taken individually can be observed. The mixtures lead a significant decrease of Caco-2 cell viability. Figure 2a shows significant differences in binary mycotoxin combination for DON and ENN B after 24 h of exposure. The binary combination decrease cell viability by 11% and 15% more than ENN B and DON assayed individually. After 48 and 72 h, it can observe significatives differences between DON and the mixture (DON+ENN B) at 5 µM, where the mixture decrease the viability in a 21% and 15% compared to DON alone, respectively (Figs 2b and 2c). No differences were observed between ENN B and the mixture.

Moreover, ENN B and AOH in combination (Fig. 3) showed a reduction from 38% to 66%. At highest concentrations in all times tested, it can observe a significatives differences between AOH and the mixture. The higher reduction of cell viability between AOH and the mixture was from 41%, 57% and 71% after 24, 48 and 72 h of exposure, respectively. No differences were observed between ENN B and the mixture.

Otherwise, at 24 h, DON is much more effective decreasing cell viability itself than AOH, where DON alone reduced cell viability about 40%, respect to 32% of the mixture (Fig. 4a). Nevertheless, after 48 and 72 h, the reduction in the mixture was much higher than DON individual exposure, being from 54% to 64% and from 28% to 48%, respectively (Fig 4b and 4c). At higher concentrations in all times tested, we can observe significative differences between AOH and the

mixture. The higher reduction of cell viability between AOH and the mixture was 35%, 57% and 67% after 24, 48 and 72 h of exposure, respectively (Fig.4).

Tertiary combinations lead to an important decrease of Caco-2 cell viability as compared to AOH taken individually (Fig. 5). Nevertheless, tertiary combinations produces a smaller decrease in cell viability compared to binary mixture combinations. Mycotoxins combinations reduce cellular viability in the following decreasing order: (DON+AOH +ENN B) < (DON+ AOH) < (ENN B+AOH) < (DON+ ENN B).

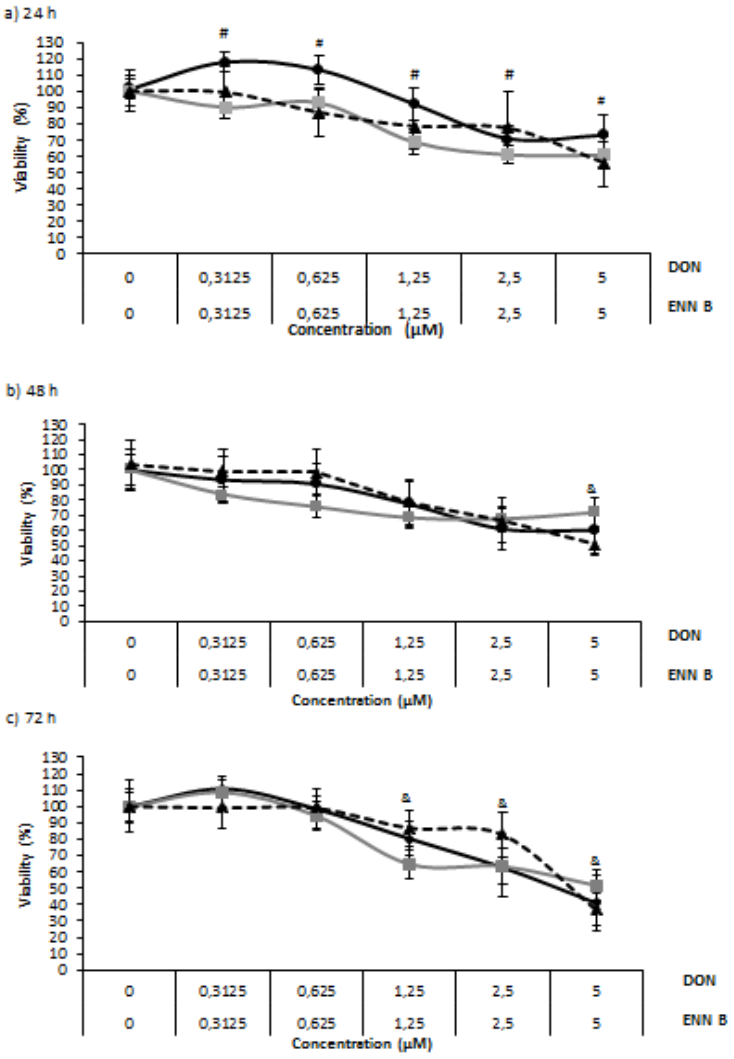


Figure 2. Cytotoxicity effects of individual ENN B (–● –), DON (–■–) and the binary combination ENN B+DON (--▲--) after a) 24 h, b) 48 h and c) 72 h. All values are expressed as mean ± SD (n=3). When binary mixture concentrations are 0.3125, 0.625, 1.25, 2.5 and 5 µM it means that concentrations of ENN B and DON in its combination are 0.1562, 0.3125, 1.25 and 2.5 µM respectively. $p \leq 0.05$ (#) represents significant differences from DON and ENN B, $p \leq 0.05$ (&) represents significant differences from DON and the mixture.

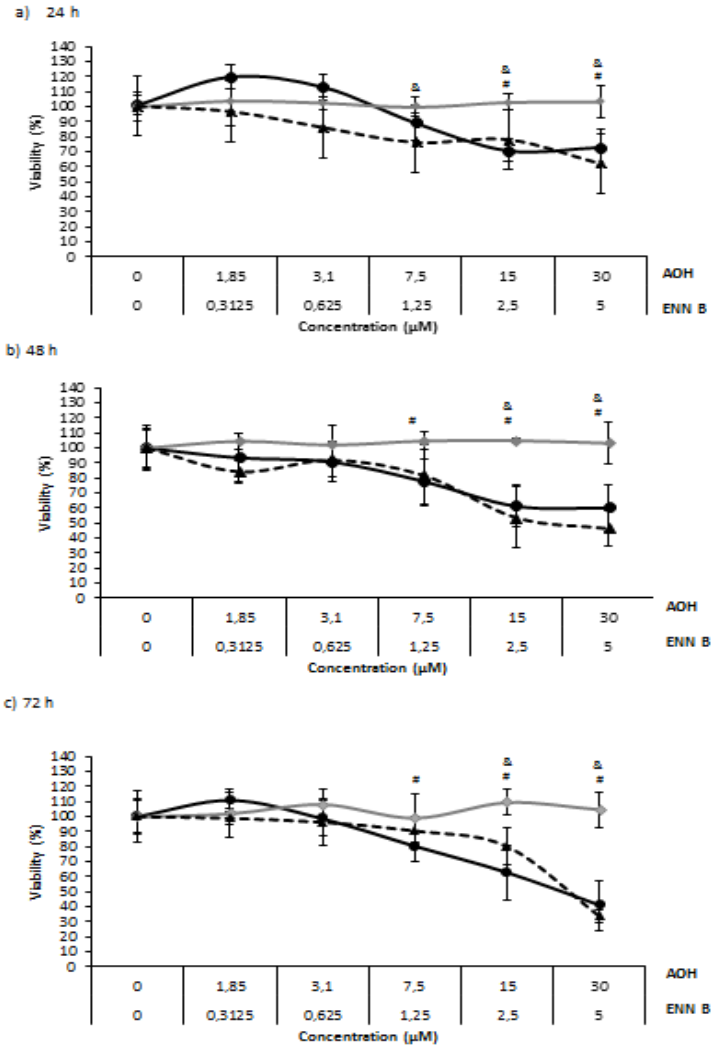


Figure 3. Cytotoxicity effects of individual ENN B (–● –), AOH (–♦–) and the binary combination ENN B+AOH (--▲--) after a) 24 h, b) 48 h and c) 72 h. All values are expressed as mean ± SD (n=3). When binary mixture concentrations are 0.3125, 0.625, 1.25, 2.5 and 5 µM ENN B and 1.85, 3.75, 7.5, 15 and 30 µM AOH, it means that concentrations of ENN B and AOH in its combination is 0.1562, 0.3125, 1.25 and 2.5 µM, and 0.925, 1.85, 3.75, 7.5 and 15 µM respectively. $p \leq 0.05$ (#) represents significant differences from AOH and ENN B, $p \leq 0.05$ (&) represents significant differences from AOH and the mixture.

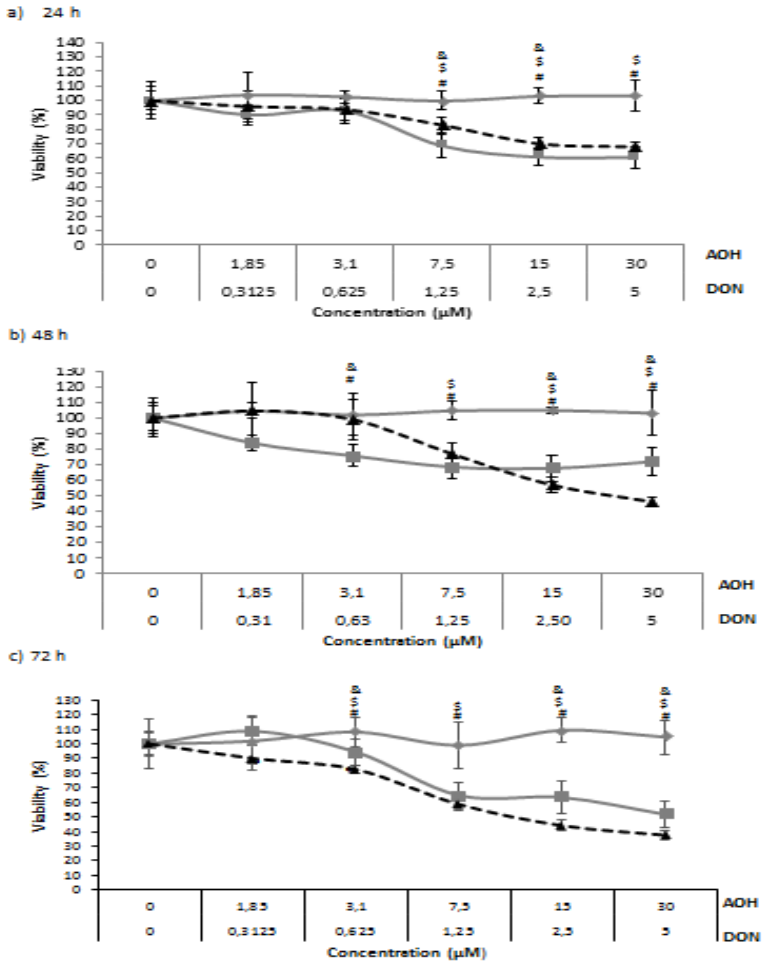


Figure 4. Cytotoxicity effects of individual DON (–■–), AOH (–♦–) and the binary combination DON+AOH (--▲--) after a) 24 h, b) 48 h and c) 72 h. All values are expressed as mean ± SD (n=3). When binary mixture concentrations are 0.3125, 0.625, 1.25, 2.5 and 5 µM DON and 1.85, 3.75, 7.5, 15 and 30 µM AOH, it means that concentrations of DON and AOH in its combination is 0.1562, 0.3125, 1.25 and 2.5 µM, and 0.925, 1.85, 3.75, 7.5 and 15 µM respectively. $p \leq 0.05$ (#) represents significant differences from AOH and DON, $p \leq 0.05$ (&) represents significant differences from DON and the mixture, $p \leq 0.05$ ($) represents significant differences from AOH and the mixture.

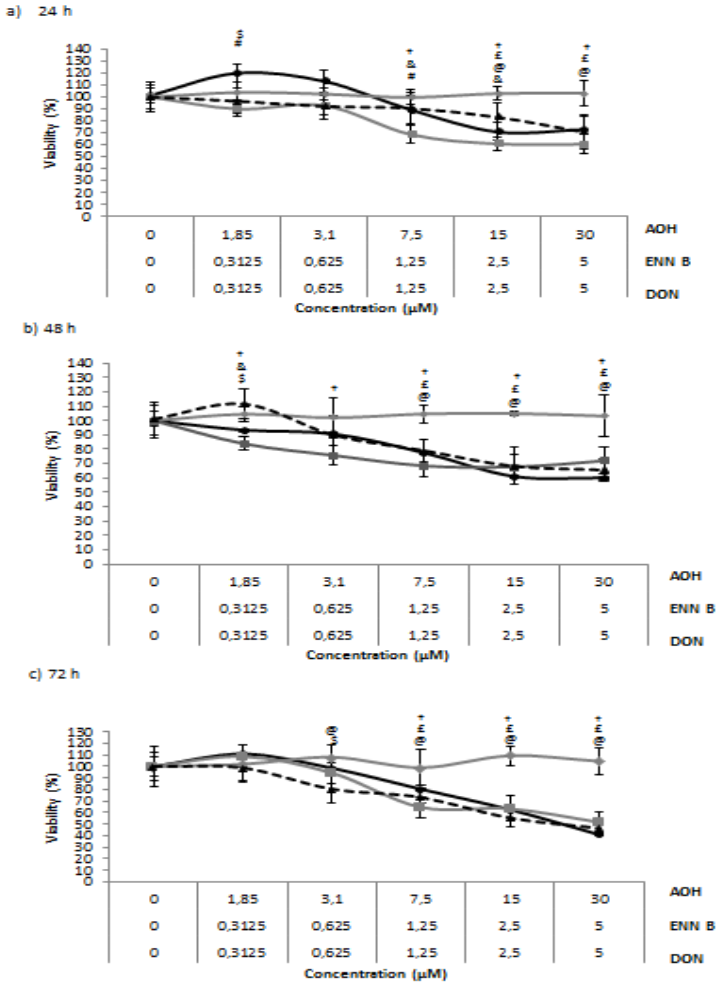
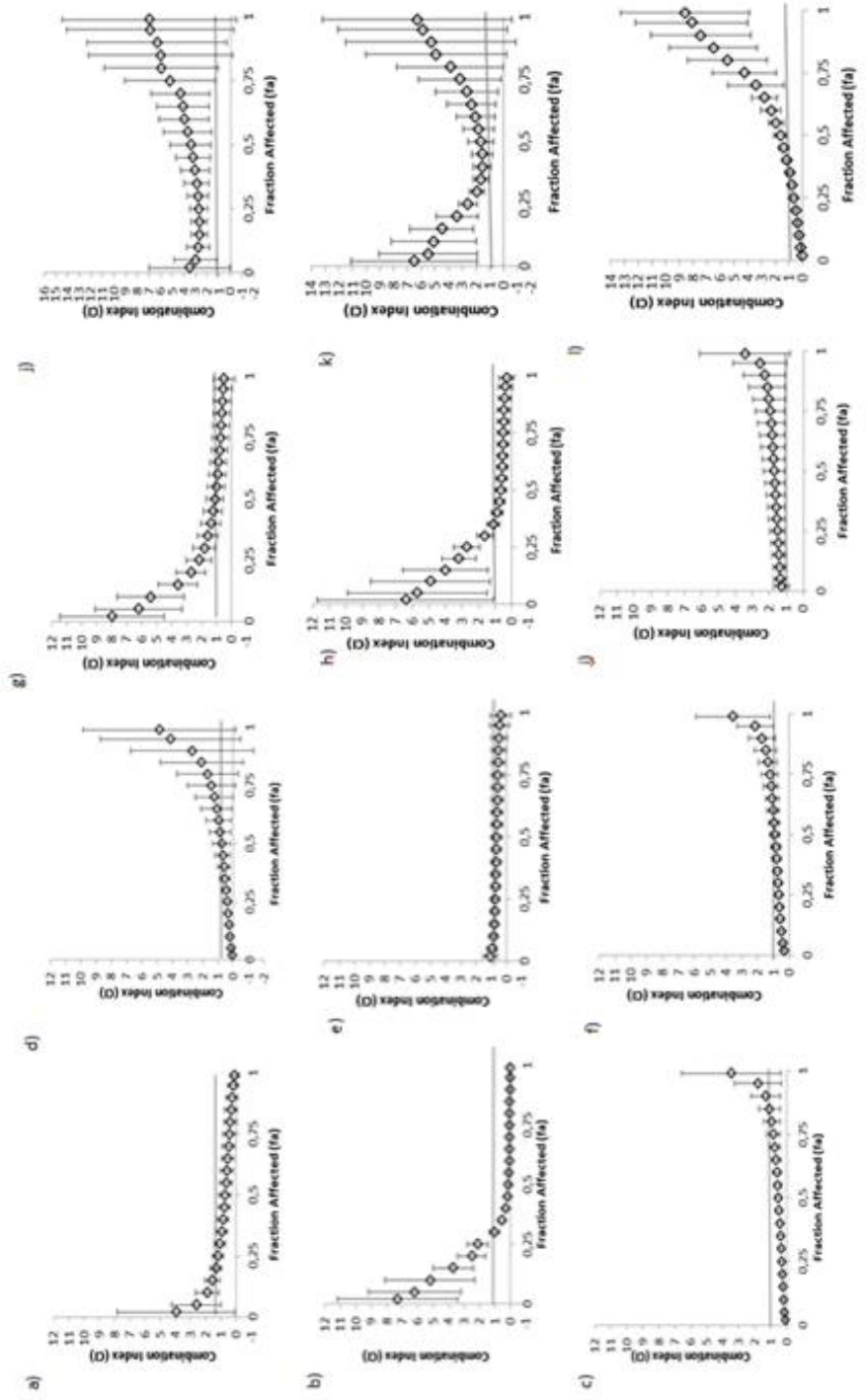


Figure 5. Cytotoxicity effects of individual ENN B (–● –), AOH (–♦–), DON(–■–) and the tertiary combination DON+AOH+ ENN B (--▲--) after a) 24 h, b) 48 h and c) 72 h. All values are expressed as mean ± SD (n=3). When tertiary mixture concentrations are 0.3125, 0.625, 1.25, 2.5 and 5 µM DON and ENN B; and 1.85, 3.75, 7.5, 15 and 30 µM AOH, it means that concentrations of DON, ENN B and AOH in its combination is 0.104, 0.208, 0.416, 0.833, 1.66 µM, and 0.616, 1.25, 2.50, 5 and 10 µM respectively. $p \leq 0.05$ (#) represents significant differences compared DON and ENN B, $p \leq 0.05$ (&) represents significant differences from DON and the mixture, $p \leq 0.05$ ($) represents significant differences from ENN B and the mixture, $p \leq 0.05$ (@) represents significant differences from AOH and the mixture, $p \leq 0.05$ (£) represents significant differences from AOH and ENN B, $p \leq 0.05$ (†) represents significant differences from AOH and DON.

3.3. Interaction of mycotoxin combinations

In order to investigate the type of interaction between ENN B, DON and AOH in their combinations, the isobologram method has been applied. Results are summarized in Table 1. Figure 6 shows the fa–CI plots of mycotoxin interactions after 24, 48 and 72 h of exposure. The binary (AOH + DON and AOH + ENN B) mixtures show mainly synergism effect (Fig. 6a to 6f). When DON was in the mixtures moderate antagonism was observed (Figs. 6g to 6h).

Figure 6. Combination index (CI)/fractional effect curve as described by Chou and Talalay model for Caco-2 cells exposed to DON, ENN B and AOH binary and tertiary combinations. Each point represents the CI ± SD at a fractional effect (fa) by computer simulation from fa = 0.10 to 0.95 as determined in our experiments. The dotted line indicates additive, the area under the dotted line synergism, and the area above of the dotted line antagonism. Caco-2 cells were exposed with DON+AOH (a) 24 h (b) 48 h (c) 72 h; ENN B + AOH (d) 24 h (e) 48 h (f) 72 h; DON+ ENN B (g) 24 h, (h) 48 h. (i) 72h; and DON + ENN B + AOH (j) 24 h, (k) 48 h, (l) 72 h. Binary DON + ENN B combinations were at molar ratio of 1:1 (equimolar proportion), binary DON + AOH, ENN B+ AOH combinations were at molar ratio of 1:6 (equimolar proportion). Tertiary DON+ ENN B + AOH combination was at molar ratio of 1:1:6 (equimolar proportion).


a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
k)
l)
Combination Index (CI)
Fraction Affected (fa)

Mycotoxin	Time (h)	Dose–effects parameters				CI values							
		Dm (μM)	m	r	IC_{10}	IC_{25}		IC_{50}		IC_{75}		IC_{90}	
DON	24 h	11.23	0.68	0.93									
	48 h	52.78	0.28	0.93									
	72 h	5.03	1.87	0.91									
ENN B	24 h	9.20	1.99	0.90									
	48 h	6.90	1.52	0.90									
	72 h	5.09	3.97	0.92									
AOH	24 h	1.96[a]	0.11	0.99									
	48 h	360.50	1.52	0.91									
	72 h	284.34	1.60	0.90									
DON + ENN B	24 h	5.59	2.20	0.93	5.39±2.24	Ant	2.17±0.83	Ant	1.11±0.59	Add	0.74±0.55	Add	0.60±0.56	Add
	48 h	4.05	1.89	0.95	4.87±3.28	Ant	2.73±0.78	Ant	0.67±0.33	Add	0.51±0.35	Syn	0.44±0.35	Syn
	72 h	4.33	1.98	0.96	1.41±0.36	Ant	1.54±0.45	Ant	1.71±0.61	Ant	1.96±0.87	Ant	2.28±1.24	Ant
DON + AOH	24 h	8.26	0.96	0.97	1.90±0.97	Add	1.18±0.36	Add	0.67±0.41	Add	0.46±0.42	Syn	0.28±0.40	Syn
	48 h	4.05	1.89	0.95	6.83±5.26	Ant	2.13±0.67	Ant	0.14±0.09	Syn	0.06±0.06	Syn	0.05±0.06	Syn
	72 h	2.38	1.04	0.98	0.21±0.07	Syn	0.33±0.10	Syn	0.52±0.20	Syn	0.83±0.43	Add	1.3±0.90	Add
AOH+ ENN B	24 h	7.21	0.94	0.92	0.23±0.10	Syn	0.42±0.21	Syn	0.79±0.57	Add	1.46±1.55	Add	2.71±4.04	Add
	48 h	4.05	1.89	0.95	0.87±0.27	Add	0.75±0.27	Add	0.65±0.31	Syn	0.57±0.37	Syn	0.50±0.41	Syn
	72 h	4.20	1.77	0.99	0.51±0.13	Syn	0.68±0.20	Syn	0.91±0.32	Add	1.25±0.51	Add	1.72±0.83	Add
AOH + ENN B+ DON	24 h	17.31	0.78	0.98	1.06±0.80	Add	2.74±0.75	Ant	3.42±1.75	Ant	5.62±4.52	Ant	11.21±12.67	Add
	48 h	9.25	0.74	0.95	0.48±0.93	Add	2.61±0.69	Ant	1.67±0.92	Add	3.20±3.00	Add	6.77±9.60	Add
	72 h	3.91	0.79	0.99	0.24±0.36	Syn	0.64±0.15	Syn	1.63±0.44	Ant	4.23±1.63	Ant	11.44±6.02	Ant

The parameters m, Dm and r are the antilog of x-intercept, the slope and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose–effect curve, the potency (IC_{50}), and conformity of the data to the mass-action law, respectively (Chou and Talalay, 1984; Chou, 2006). Dm and m values are used for calculating the CI values (CI <1, =1, and >1 indicate synergism (Syn), additive effect (Add), and antagonism (Ant), respectively. IC_{25}, IC_{50}, IC_{75} and IC_{90}, are the doses required to inhibit proliferation 25%, 50%, 75% and 90%, respectively. IC_{25}, IC_{50}, IC_{75}, IC_{90} and the dose–effect parameters were generated on line by using Computer software CalcuSyn

Table 1. Dose–effect relationship parameters and mean combination index (CI) values (as a function of fractional inhibition of proliferation) of binary and tertiary combinations of DON, ENN B and AOH on Caco-2 cells.

4. DISCUSSION

In the last decade, scientific and regulatory concern over the effects and risks of chemical and natural mixtures has increased (Pape-Lindstrom and Lydy. 1997). The number of studies on the occurrence of mycotoxins in European foodstuffs have been increased. Mycotoxins are ubiquitously present in food and feed raw materials throughout Europe where maximum contamination stages exceeding the EU maximum levels (Rafai et al., 2000). Moreover, considering the fact that mycotoxigenic fungi are usually capable of producing more than one mycotoxin and that food and feed raw materials are commonly infected with various fungal species at a time, studying the occurrence of any single mycotoxin provides incomplete information about the risk associated with diet (Bottalico et al., 1995). Thus, even if only a very limited number of mycotoxins are analysed, and the evidence of possible additive or synergistic interaction of co-occurring mycotoxins were established, maximum levels should not only be set for each mycotoxin individually but also for particularly concerning combinations which would provide more data on the impact of mycotoxin combinations on humans and animal health (Klaric, Segvic, 2012). Grenier and Oswald, (2011) reviewed 112 publications on toxicological interactions of mycotoxins *in vivo*. They found that most of the studies reported synergistic or additive interactions regarding adverse effects on animal performance. When it comes to other parameters, especially biochemical ones, results were more variable, ranging from synergistic to antagonistic for the same toxin combination. Nevertheless, although fulfilment with EU regulations is usually high, continuous monitoring is needed in order to avoid negative impacts on human and animal health and performance due to elevated contamination levels (Klaric, Segvic, 2012). Over the last few years researchers have used cell cultures to study the mechanisms of mycotoxin action and to predict the effect of mycotoxin mixtures. These i*n vitro* systems are

particularly useful for studying the interaction between low-molecular-weight compounds such as mycotoxins and their mixtures (Ruiz et al., 2011a, 2011 b; Klaric, 2012; Lu et al., 2013; Prosperini et al., 2014; Tatay et al., 2014).

In this study, the mixture of DON, ENN B and AOH was evaluated due to the high incidence that has been found in cereals. DON was detected around 60% in cereal and around 95% in maize samples (Rafai et al., 2000). ENN B was detected in 73% and in 82% of cereal based products in Czech Republic (Malachova et al., 2011) and in Spanish Markets (Meca et al., 2010). AOH was found in cereals, fruits and vegetables, where the highest concentration of AOH detected in sunflower was 1200 µg/kg (Pozzi et al., 2005). The range of concentrations tested in this study was selected depending on the concentrations found in cereals, as reported in literature, taking into consideration the maximum levels established in cereals for DON and ENN B and considering the European consumption of cereals (EC, 2007). In this way, it was possible to calculate the approximately ingestion estimated. Nevertheless, as AOH is still not legislated and is not available the estimate the daily intake (TDI), the data on AOH occurrence in feed and agricultural commodities in Europe were used. AOH was found in a range from 6.3 to 1840 µg/kg of tested samples (EFSA, 2011). In all cases, the bioavailability of each mycotoxin was also considered.

The results obtained shows that Caco-2 cells were more sensitive to ENN B than DON and, DON was more cytotoxic than AOH (Fig.1). Literature data frequently report the cytotoxicity associated with exposure of ENN B to different types of cells. Prosperini et al., (2014) detected an IC_{50} of 11.7 ± 2.4 µM in Caco-2 cells by the MTT assay. Moreover, in HepG2 (hepatocellular carcinoma) and C6 (rat glioma) cells, the IC_{50} values were approximately about 10 µM (Wätjen et al. 2014). Similar results to our study were reported in MRC-5 (human fibroblast) cells where was detected an IC_{50} value of 3.6 µM determined by BrdU

(Bromodeoxyuridine) assay (Ivanova et al., 2006), and an IC_{50} of 9.8 µM when the Alamar Blue assay was used (Ivanova, et al., 2006). In H4IIE (rat hepatoma) cells an IC_{50} of 2.5 µM was observed (Wätjen et al. 2014). In addition, the behaviour characterized by low-concentration stimulation and high concentration inhibition in cells exposed to ENN B has been previously detected by others authors (Meca et al., 2011; Lombardi et al., 2012; Prosperini et al., 2014). It may be a hormetic effect, due to at low concentrations it was produced the opposite effect that at higher concentrations. On the other hand, in our experiments, DON evidenced comparable values than those obtained in literature. In addition, the IC_{50} values obtained in MRC-5 cells by the NR (Neutral red, 3.6 µM) and BrdU (1.7–8.9 µM) assays were similar to the IC_{50} values obtained in Hep G2 cells using the BrdU (3.3–3.7 µM) assay (Ivanova et al., 2006). Otherwise, Nielsen et al. (2009) obtained an IC_{50} values from 0.6 to 4.9 µM depending on the cell line tested. Also, the same author determined the cytotoxic activity of DON on Caco-2 cells (IC_{50} of 1.0 µM) and on a HUVEC (primary culture of human endothelial) cells (IC_{50} of 4.5 µM) at 72 h of exposure by WST-1 (Cell Proliferation Reagent) test. In addition, Ruiz et al., (2011a, 2011b) observed an IC_{50} of 1.83 µM and 5.05 µM after 72 h of exposure in CHO-K1 (hamster ovarian) and Vero (mammalian kidney epithelial) cells, respectively. AOH showed cytotoxic effects after 24 h of exposure on Caco-2 cells. Similar results were obtained previously (Fernández-Blanco et al., 2014; Vila-Donat et al., 2015), where AOH reduced cell proliferation in a dose- and time-dependent manner, but not IC_{50} was obtained. These results were in agreement with Wollenahupt et al. (2008) and Tiemann et al. (2009) who do not obtained IC_{50} values in the concentration ranged tested. On the other hand, Tiessen et al. (2013) determined a decreased on cell viability about 15% at 50 µM AOH after 24 h of incubation, by SRB (sulforhodamine B) assay in human colon HT29 cells and Brugger et al. (2006) observed reduction in the number of cells incubated with

AOH to be approximately 35% (with 30 µM in Chinese hamster V79 cells) and 69% (with 20 µM; mixed lymphocyte culture MLC cells) after 24 h of incubation. The results obtained with the three mycotoxins tested individually demonstrated that there are slightly differences in susceptibility between cell lines and the time of exposure.

Data from literature about cytotoxic effects on mycotoxin mixtures differs about the type of cells exposed and, mainly in the method for analysing dose–effect relationships in cell culture. Thus, there are no studies about the cytotoxicity of AOH, DON and ENN B in combination. In the higher concentrations of DON+ ENN B combination is observed that this mixture decreases more the viability than DON alone after 48 and 72 h (Fig. 2b and 2c). The same behaviour was reported by Ruiz et al., (2011) when the mixture BEA+ DON was studied in the Vero cells. This effect may be comparable with our results due to BEA and ENN B have the same capacity of establishing channels with the cells, increasing membrane permeability and therefore to be able to cross it and to produce toxic effects (Tedjiotsop et al., 2010). Wan et al., (2013) evaluated the cytotoxicity of a mixture composed by DON, Nivalenol (NIV), Zearalenone (ZEA) and Fumonisin B1 (FB1) in intestinal epithelial (IECs) cells and observed that for mixtures containing DON there were no increases in overall cytotoxicity of such mixtures, compared to the individual DON. The same model of action happens in our mixtures, where the mixture is no more cytotoxic than DON alone after 24 h (DON+ENN B; Fig. 2a). DON was significative different respect to the mixture, due to it higher toxicity (DON+AOH; Fig. 4, DON+ENN B+AOH; Fig. 5) at 24 h. However, after 48 and 72 h of exposure, the pattern changes being the binary mixture (DON + AOH) more effective in reducing Caco-2 cells viability. Similarly, mixture with DON, such as DON + FB1, DON + BEA and DON + ZEA, the mixtures were more cytotoxic than the mycotoxins tested alone (Kouadio et al., 2007; Ficheux, Sibiril and Parent-

Massin, 2012). The development of predictive models for mycotoxin based on combination data is a valuable tool to estimate the true risk of harmfulness (Klaric et al., 2012). Many studies of mycotoxins interactions were carried out with the isobologram method (Bernhoft et al., 2004; Luongo et al., 2008; Ruiz et al., 2011a; Ruiz et al., 2011b; Lu et al., 2013; Prosperini et al., 2014; Tatay et al.,2014). In general, in this study, it was demonstrated that combinations of two or three mycotoxins interacted to produce alterations in toxic response over cell viability. The interactions between binary and tertiary combinations produced by DON, ENN B and AOH mixtures revealed that the pattern was not uniform along the fraction affected (Fig. 6; Table 1). Synergism and slight additive effect was observed at the higher fraction affected in DON + AOH and ENN B + AOH combinations. All mixtures containing AOH were more cytotoxic than AOH assayed individually due to the other mycotoxin in the combination (ENN B or DON) were more cytotoxic than AOH. On the contrary, the binary (DON + ENN B) and tertiary (DON + ENN B + AOH) combinations produce an antagonism effect. This effect may be due to a competition with the same receptor site. Taking into account that a synergism effect was observed in the binary combinations (AOH + DON and AOH + ENN B) and tertiary combination at 72 h of exposure and at low values of fa, it may be produced because mycotoxin increases the activity of other mycotoxin in the mixture or because mycotoxins act on different receptors of the same route. It is difficult to give an explanation to the interactions which not follow the same pattern because the combination index method only allows quantitative determination of synergism or antagonism and don't give information about the mechanism by which it occurs (Ince et al. 1999; Cheng, 2002; Chen et al., 2014).

In conclusion, CI-isobologram method provides a more accurate prediction of the interaction between mycotoxins in combination. The synergistic

behaviour for some of the mixtures may indicate a potential risk associated to the co-occurrence of mycotoxins in food, which it also indicates that the evaluation of the data from a single mycotoxin when exists the possibility of an additive behaviour can lead to an underestimation. Thus, these findings should be taken into account since toxic effects are enhanced by DON, ENN B and AOH combinations whose co-occurrence is in fact naturally encountered. Further research needs to be completed to enable an appropriate assessment of health risk effects in foodstuff.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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3.3 Oxidative stress of alternariol in Caco-2 cells

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Abstract

Alternariol (AOH) is a mycotoxin produced by fungus *Alternaria*. It is found in a wide variety of fruits and cereals products. AOH is able to damage human health. The aim of this study was to evaluate the cytotoxicity of AOH in human colon adenocarcinoma (Caco-2) cells. Moreover, some events related to oxidative stress were evaluated: reactive oxygen species (ROS) generated by oxidation of 2',7'-dichlorodihydrofluorescein diacetate; peroxidation of lipid (LPO) by malodialdehide (MDA) production; and antioxidant enzymatic capability of catalase (CAT) and superoxide dismutase (SOD). Cytotoxicity of AOH (from 3.125 to 100 µM) was determined during 24, 48 and 72 h of exposure by different endpoints. AOH decreased cell viability by MTT, NR and PC assays. However, no IC_{50} values were obtained by any of the assays tested. AOH induced a strong oxidative stress in Caco-2 cells by generation of ROS production and LPO associated with a rise in the SOD activity at all concentration tested. ROS increased 1.2-fold respect to the control and MDA production ranged from 130% to 250% compared to control. Our results demonstrated that in spite of AOH showed cytotoxic effect on Caco-2 cells at the highest concentration tested, oxidative stress by LPO and ROS was observed at all concentrations assayed. This could cause an injury and be hazardous to health.

1. INTRODUCTION

Mycotoxins are toxic secondary metabolites of fungi including the genus *Alternaria*. In the early 1990s, around 70 metabolites were isolated and characterized from *Alternaria*. Since then, at least 270 metabolites of *Alternaria sp* have been characterized. These bioactive compound with different chemical structure also exhibit different biological activities and functions (Ostry, 2008; Lou

et al., 2013). Moreover, some of these metabolites can be produced by more than one species and even from other fungal genera.

Alternaria fungi are ubiquitous in the environment as well as their mycotoxins. The most widespread metabolite of *A. alternate* is alternariol (AOH). The AOH is structurally characterized as a dibenzopyranone (Fig. 1). Mycotoxins from *A. alternate* have been found among of a widely type of foodstuffs.

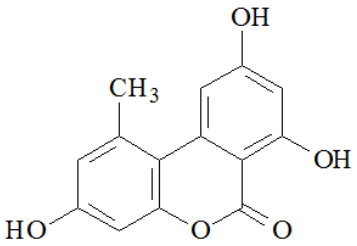

Figure.1 Chemical structure of AOH

According to the EFSA, concentrations from 6.3 to 1840 µg of AOH per kg were found in agricultural commodity samples (maximum found in sunflower seeds) purchased from European countries (EFSA, 2011). Vegetables, oilseed and fruits are known as susceptible commodities to be contaminated with *Alternaria* mycotoxins, (Patriarca et al., 2007; Pavón et al., 2012; Van de Perre et al., 2014). The total daily *per capita* consumption of vegetables in Spain is at approximately 424 g, oil crops (mainly sunflower seed and olives) account for approximately 66.3 g / person and apple (including fruit juice) is 33.69 g per person (http://faostat3.fao.org/faostat-gateway/go/to/home/E). Therefore, AOH contaminated products are consumed regularly by the majority of the population. However, no risk assessment studies about *Alternaria* mycotoxins in food and feed has been established to date (EFSA, 2011).

Among secondary metabolites produced by *Alternaria* fungi, AOH and alternariol 9-methyl ether represent the main toxic metabolites (Brase et al., 2009). These mycotoxins in food and feed are able to injure humans and animals. However, current knowledge concerning toxic effects of AOH is still very limited. The hydroxylation of AOH leads reactive oxygen species (ROS) production (Pfeiffer et al., 2007). An excess of ROS can lead oxidative stress, which has been associated with lipid peroxidation (LPO); Ferrer et al., 2009). LPO is started by active oxygen species attracting unsaturated fatty acids, and is propagated by a chain reaction cycle involving lipids, peroxy radicals and lipid hydro peroxides. (Kayali and Tarhan. 2003).

The toxic propagation cycle of LPO is broken by both enzymatic and non-enzymatic defense systems, which defend the cells against this oxygen toxicity, either minimizing production of ROS, or by eliminating those generated. To maintain the intracellular ROS at normal levels, the increased intracellular antioxidative enzymes protect them from oxidative damage (He et al., 2011). Superoxide dismutase (SOD) catalyzes dismutation of superoxide anion, producing molecular oxygen and hydrogen peroxide (H_2O_2), which is efficiently removed to water and dioxygen by catalase (CAT) (Martins and English. 2014).

AOH also causes cells cycle arrest and apoptosis of cells (Soulhaug et al., 2012). Moreover, its biological activities and functions include effects on inhibitory cell proliferation (Tiemann et al., 2009; Bensassi et al., 2012), inhibition of topoisomerase I and II (Fehr et al., 2009) and impairment of DNA integrity in Chinese hamster fibroblast (V79), human hepatic cancer (HepG2), human colon adenocarcinoma (HT29) and human lung cancer (A431) cells (Fleck et al., 2012). It was also demonstrated its genotoxic and hard clastogen activities in mammalian carcinoma cells (Liu et al., 1992) as well as oestrogenic effects (Lehmann et al., 2006). However, inconsistent results were obtained for AOH in bacterial

mutagenicity assays (Fleck et al., 2012). In China and South Africa population the AOH exposure has been linked to esophageal tumor (Fleck et al., 2012; Ostry, 2008).

The aim of this work was to determine the effect of AOH on cell viability during 24, 48 and 72 h of incubation. Furthermore, the role of AOH on the oxidative stress was studied. For this purpose, ROS generation, MDA production and antioxidant enzymes after increasing concentrations of AOH were monitored in human colon adenocarcinoma (Caco-2) cells. These cells were selected in this study, considering that ingestion of food contaminated with AOH is the main exposure route for animal and humans.

2. MATERIALS AND METHODS

2.1 Reagents

The reagent grade chemicals and cell culture components used, Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, HEPES, tetrazolium bromide (MTT), non-essential aminoacids (NEAA), phosphate buffer saline (PBS), pyruvate, trizma base, Triton X-100, glucose, dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), deferoxamine mesylate salt (DFA), di-ter-butyl-methylphenol (BHT), 1,1,3,3 tetramethoxipropan, 2´,7´-dichlorodihydrofluorescein diacetate (H_2-DCFDA), , hydrogen peroxide (H_2O_2), ethylenediaminetetraacetic acid (EDTA), sodium azide and AOH (258.23 g/mol purity >96%) were from Sigma Chemical Co. (St Louis, MO, USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Deionised water (resistivity <18 MΩ cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were of standard laboratory grade.

Stock solutions of AOH was prepared in DMSO and maintained at -18°C. The final AOH concentrations tested were achieved by adding the culture medium. The final DMSO concentration in medium was $\leq$ 1% (v/v). SOD activity

was determined by RANSOD kit (Randox Labs., County Antrim, UK) and total protein content (µg/mL) was determined by Bradford method (Bio-Rad DC Protein Assay (catalogue number 500-0116) http://www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf).

2.2 Cell culture

Caco-2 cells (ATCC HTB-37) were maintained in DMEM medium supplemented with 25 mM HEPES buffer, 1% (v/v) of NEAA, 100U/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) FCS inactivated. Incubation conditions were pH 7.4, 37°C under 5% CO_2 and 95% air atmosphere at constant humidity. Culture medium was changed every two days. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St Louis Mo. USA).

2.3 *In vitro* cytotoxicity assays

The tetrazolium bromide (MTT), Neutral Red (NR) and Protein content (PC) assays were performed to determine cell proliferation. MTT assay is based on the ability of viable cells to metabolize the tetrazolium salt to a formazan blue colorless in the mitochondria; in contrast NR accumulates this dye in lysosomes of viable cells and PC gives data about cell damage in independence of the toxic mechanism involved. The NR and MTT assays were performed as described by Ruiz et al. (2006) with some modifications and PC assay was analysed according to the procedure given by Pichardo et al., (2007). Caco-2 cells were cultured in 96-well tissue culture plates by adding 200 µL/well of a suspension of $3x10^4$ cells/well. After cells reached 90% confluence the culture medium was replaced with fresh medium containing the serial dilution of AOH ranging from 3.125 to 100 µM.

For NR assay, after 24, 48 and 72 h of incubation with different AOH concentrations (serial dilution=2), medium containing AOH was removed and 200

µL of freshly prepared NR solution (50 µg/mL) pre-warmed to 37°C was added to each well and all plate returned to the incubator at 37°C for 3 h. The cells were washed once with PBS and fixed with formaldehyde–$CaCl_2$ solution, and then extracted by adding acetic acid–ethanol solution. Plates were gentle shaking for 5 min so that complete dissolution was achieved before measured absorbance at 540 nm with an automatic ELISA plate reader Multiscan EX (Thermo scientific, MA, USA).

For MTT assay, after 24, 48 and 72 h of exposure with serial concentrations of AOH, each well received 200 µL of fresh medium containing 50 µL of MTT. After 4 h of incubation (37 °C in darkness), the resulting formazan was solubilised in DMSO. The absorbance was measured at 570 nm using an automatic ELISA plate reader MultiscanEx (Thermo Scientific, MA, USA).

PC was analyses after 24, 48 and 72h on the same 96-well culture plates in which NR assay was performed. First, the plates were washed with PBS and each well received 200 µL of NaOH. After 2h of incubation at 37°C, 170 µL of NaOH was removed of each well and 180 µL of Coomassie Brillant Blue were added in each well. After 30 min of incubation at room temperature, the absorbance was measured at 620 nm using an automatic ELISA plate reader MultiscanEx (Thermo Scientific, MA, USA).

For MTT, NR and PC assays, cell viability was expressed in percent relative to control cells (1% DMSO). Mean inhibition concentration (IC_{50}) values were calculated from full dose-response curve.

2.4 Lipid peroxidation assay

AOH concentrations to determine oxidative stress by LPO and intracellular ROS production were selected according to the previous cytotoxic assays carried out. AOH concentrations tested were 15, 30 and 60 µM. All of them resulted to be

nontoxic to Caco-2 cells and below the IC_{50} values obtained by the 3 cytotoxicity assays performed (Fig. 2).

Thiobarbituric acid reactive substance (TBARS) assay was selected for evaluating the LPO. TBARS determine the production of toxic aldehyde resulting from oxidative fatty acyl degradation, the malondialdehyde (MDA), which is a biomarker used to prove that LPO has occurred.

TBARS assay was developed according to Ferrer et al (2009) with slight modifications. Briefly: 4.8 x 10^5 cells/well were seeded in six-well plates. After cells achieved the 90% confluence, cells were treated with 15, 30 and 60 µM of AOH for 24h. Then, the medium was removed and cells were washed with PBS, and homogenized in 150 mM NaH_2PO_4 pH 7.4 and lysate with the polytron Ultra-Turrax T8 IKA®-WERKE. Immediately, cells were exposed to 0.5% TBA, 1.5 mM DFA and 3.75% BHT, under acid condition, and boiled (100 ºC in water bath for 20 min). Immediately, the samples were placed on ice for 5 min and centrifuged (4000 rpm/15 min). The absorbance was measured at 532 nm. Determinations were performed in four independent experiments. Results were expressed as ng of MDA/mg of protein measured by the Bradford method.

2.5 Intracellular reactive oxygen species by H_2- DCFDA

Fluorescence probes are used to determine ROS generation in real-time monitors. The fluorescent probe H_2-DCFDA detect intracellular oxidants very quickly, directly and sensible. H_2-DCFDA is non-fluorescent until it is hydrolysed by intracellular esterase and readily oxidized to the highly fluorescent DCF in the presence of ROS. ROS generation was monitored according to Ruiz-Leal and George (2004) with slight modification. Briefly: 3x10^4cells/well were seeded in 96-well black culture microplate. Once the cells reach 90% confluence, the culture medium was replaced and 20 µM H_2-DCFDA/well was added and maintain for 20 min. Thereafter, the medium with H_2-DCFDA was removed and cells were washed

with PBS. Cells were exposed to medium with 1% DMSO (control) or medium with AOH concentrations (15, 30 or 60 µM). Increases in fluorescence were measured at intervals up to 120 min at excitation and emission wavelengths of 485 and 535 nm, respectively. Twenty four replicates were performed. Results are expressed as increase in fluorescence respect to control.

2.6 Superoxide dismutase activity

To determine scavenging procedures in Caco-2 cells exposed to AOH, the CAT and SOD activities were determined at the concentrations previously selected. For both assays, 4.8 x 10^5 cells/well were seeded in six-well plates. After cells achieved the 90% confluence, cells were treated with 15, 30 and 60 µM of AOH for 24 h. Then, the medium was removed and cells were homogenized in 0.02 M NaH_2PO_4 pH 7.4 and lysate with the polytron Ultra-Turrax T8 IKA®-WERKE.

The SOD destroys the free radical superoxide by converting it to peroxide. SOD activity was determined with Ransod kit (Randox Laboratories, United Kingdom) adapting for 1.5 mL cuvettes. SOD activity was monitored at 505 nm during 3 min at 37 °C in a thermo circulation of Perkin Elmer UV/Vis spectrometer Lambda 2 version 5.1. SOD enzymatic activity was expressed as Units of SOD per mg protein.

2.7 Catalase Activity

The CAT function is to protect cells from the toxic effect of H_2O_2 produced in various redox reactions of normal aerobic metabolism or because of many xenobiotics can be oxidants or reductants, catalysing its decomposition into oxygen and water. The CAT activity was measured according to Espin et al (2014) with slight modifications. Briefly: 50 µL of the homogenized cell suspension was mixed with 950 µL of 0.05M NaH_2PO_4 and 500 µL of 0.03M of H_2O_2. The kinetics of enzymatic decomposition of H_2O_2 was determined as absorbance decrements at 240 nm for 2 min with a spectrophotometer (superaquarious CECIL CE 9500). CAT

enzymatic activity was calculated by using the molar absorptivity of H_2O_2 (43.6 mM^{-1} cm^{-1}) and expressed as µmol of H_2O_2/min/mg of protein. Determinations were performed in quadruplicate.

2.8 Determination of total protein

The protein content was determined by the Bio-Rad DC Protein Assay, catalog number 500-0116 (www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf). Protein concentration (µg/mL) was measured at 690nm.

2.9 Statistical analysis

Statistical analysis of data was carried out using SPSS version 19 (SPSS, Chicago, IL, USA), statistical software package. Data were expressed as mean ± SD of different independent experiments. The statistical analysis of the results was performed by Student's t-test for paired samples. Differences between groups were analyzed statistically with one-way ANOVA followed by the Turkey HDS *post-hoc* test for multiple comparisons. The level of $p \leq 0.05$ was considered statistically significant.

3. RESULTS

3.1 Cytotoxicity

AOH affects the number of viable Caco-2 cells by MTT assay after 48 and 72 h of exposure. AOH showed a significant reduction ($p \leq 0.05$) on Caco-2 cells proliferation from 50 up to 100 µM and from 12.5 up to 100 µM concentration, respectively (Fig. 2a). Respect to the NR assay, after 48 and 72 h of exposure, AOH showed a decrease in cell proliferation at 100 µM concentration (Fig. 2b). In the results obtained by PC assays, AOH decreased cell proliferation from 3.125 to 100 µM after 48h. Nevertheless, after 72 h of exposure AOH indicated a significant reduction in cell proliferation at 50 and 100 µM (Fig. 2c). Otherwise, AOH did not affect the number of viable Caco-2 cells by MTT, NR and PC assay after 24 h of exposure at any of the concentration tested (3.125-100 µM). The higher inhibition

in cell proliferation was 40%, 30% and 45% when compared to their own controls by MTT, NR and PC assay, respectively. No IC_{50} values were obtained for AOH after any of the times of exposure.

On the other hand, AOH at concentrations ranging from 3.125 to 50 µM after 24 h of incubation produces some stimulations of the mitochondrial function teaching 150% compared to control (Fig. 2a). Similar stimulation, but up to 12.5 and 6.25 µM AOH, also observed with NR and PC assays at 24 h of incubation (Fig. 2b and 2c, respectively).

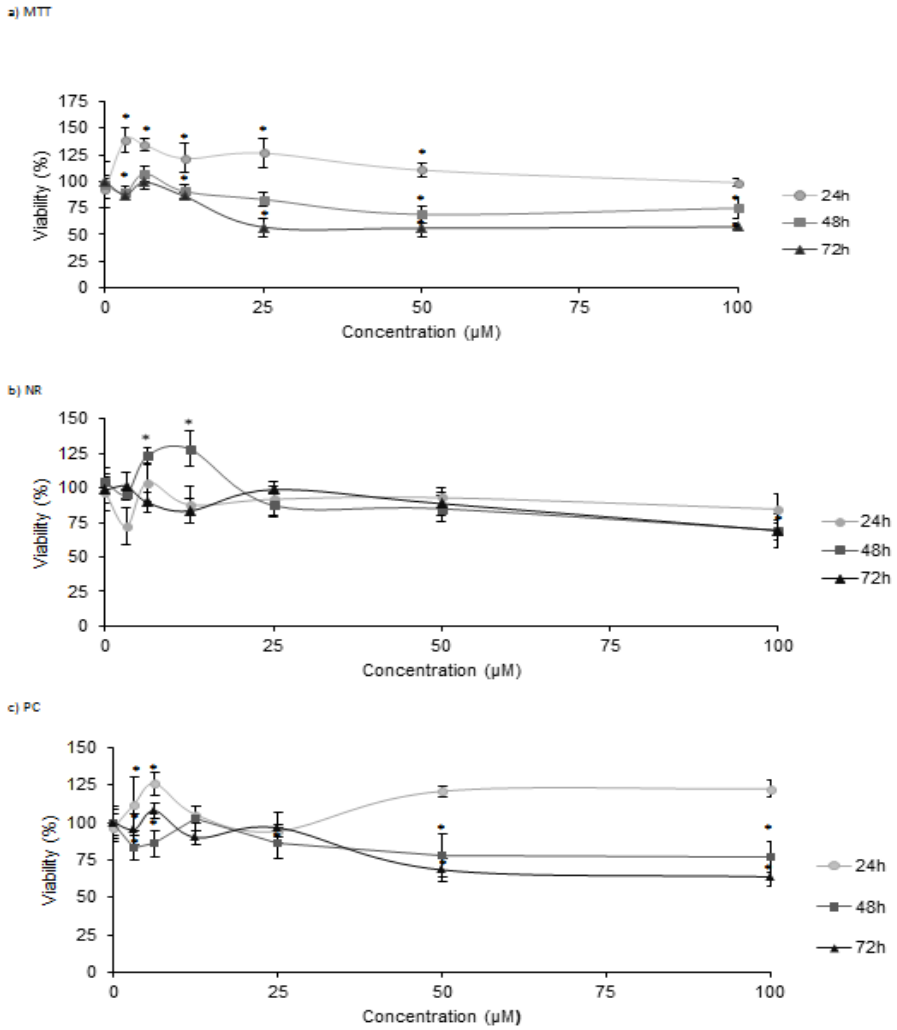

Figure.2 Effects of the mycotoxin AOH (from 3.125 to 100 µM) on the viability of Caco-2 cells by a) MTT, b) NR and c) PC assays. Cells were incubated for 24 (●), 48 (■) and 72 (▲) h in the absence (control) or presence of different concentration of AOH. Results are mean ± SD of the three independent experiments. (*) $p \leq 0$, 05, indicates significant differences from the control.

3.2 Lipid peroxidation

The results obtained in this study, shown that AOH increased significantly the production of MDA in a concentration dependent manner (Fig. 3). The increase in the MDA levels ranged from 130% (30 µM) to 250% (60 µM) respect to control. Moreover, significant difference between 15 and 30 µM (approx. 65%) and between 15 and 60 µM (approx. 135%) AOH were observed (Fig. 3). No differences between 30 and 60 µM of AOH were observed related to MDA production in Caco-2 cells at 24 h of exposure.

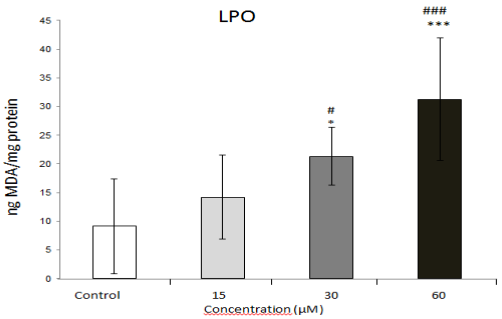

Figure.3 LPO as measured by MDA production in Caco-2 cells incubated for 24 h with 15, 30 and 60 µM of AOH. Results are expressed as mean ±SD in ng of MDA/mg of protein measured by Bradford method. (*) $p \leq 0.05$, (***) $p \leq 0.000$ significantly different from control. (#) $p \leq 0.05$, (###) $p \leq 0.000$ significant differences from 15 µM of AOH.

3.3 Intracellular reactive oxygen species determined by H2-DCFDA

To evaluate the role of ROS generation in AOH-mediated cytotoxic effect in Caco-2 cells, the production of the fluorescent DCF (resulting from DCFH oxidation) in response to 15, 30 and 60 µM AOH were analyzed. As observed in Figure 4, the increase of ROS production was produced in Caco-2 cells at all concentration of AOH tested. The highest relative intensity of fluorescence in

Caco-2 cells was observed at the early stage of AOH exposure (0 min) to about 1.4-folds of control. However, ROS production did not exceed 1.2-folds of control between 5 and 120 min interval (Fig. 4).

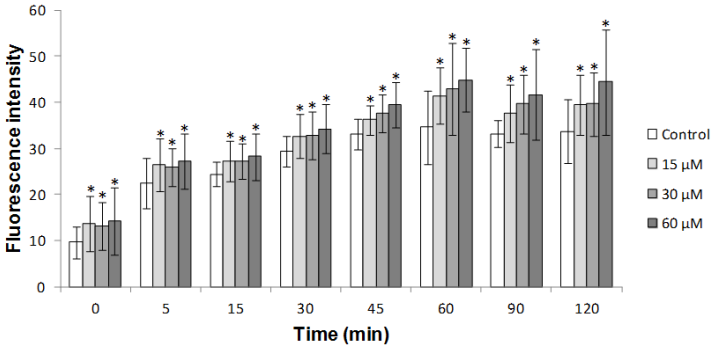

Figure.4 Time dependence of ROS-induced fluorescence in Caco-2 cells exposed to AOH. H2-DCFDA was added to Caco-2 cells and left for 20 min and then exposed to 15, 30 and 60 µM of AOH. Fluorescence of oxidized DCF was followed by emission and excitation wavelengths of 535 nm and 485 nm, respectively. Values are mean ± SD of 24 replicate. (*) $p \leq 0.05$, significantly different from the control.

3.4 Catalase

The H_2O_2 scavenging activity generated after AOH exposure was evaluated by CAT activity. Differences were noticed in the CAT activity in Caco-2 cells after AOH exposure at 24 h. The CAT activity decreased significantly from 53% (30 µM) to 56% (60 µM) respect to the control. However, no differences after 15 µM of AOH incubation were observed in Caco-2 cells (Fig. 5).

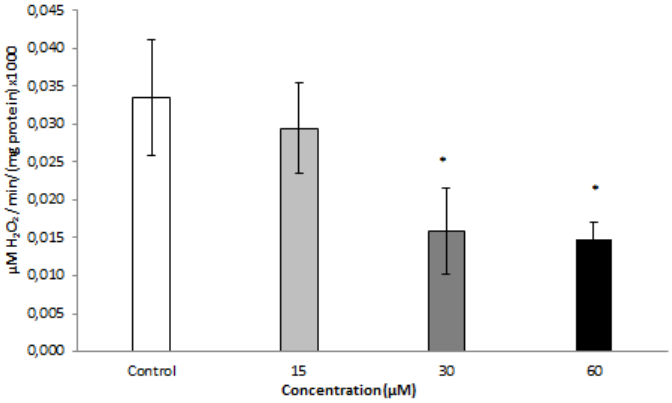

Figure.5 Measurement of CAT enzymatic activity in Caco-2 cells exposed to 15, 30 and 60 µM of AOH for 24h. Values are mean ± SD of four independent experiments. (*) $p \leq 0, 05$, significantly different from the control.

3.5 Superoxide dismutase

The protective effect from SOD was observed in cells treated with all concentration of AOH. The results obtained in this study, shown that AOH increased significantly the SOD activity in a concentration dependent manner. The increased was 67 % (15 µM), 74 % (30 µM) and 93 % (60 µM) when compared to the control (Fig. 6).

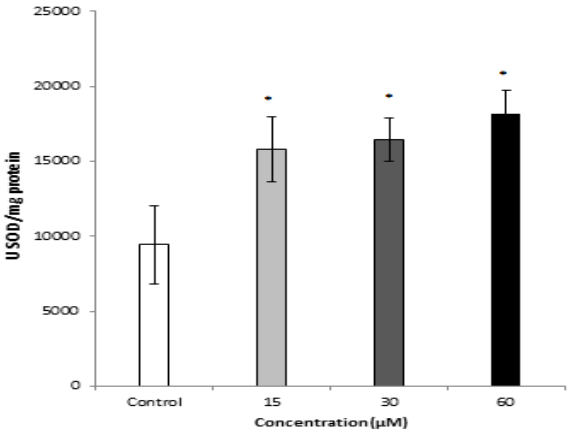

Figure.6 Effects of AOH on SOD activity. Caco-2 cells were treated for 24 h with 15, 30 and 60 µM of AOH. Values are mean ± SD of four independent experiments. (*) $p \leq 0, 05$, significantly different from the control.

4. DISCUSSION

The present study demonstrated that AOH reduced cell proliferation in Caco-2 cells in a dose and time-dependent manner. According to the results obtained, AOH was more sensible to the mitochondrial metabolism through succinate dehydrogenase activity (MTT assay) than lysosome functionality (NR assay) and PC content. This results are in accordance with observations obtained by Wollenhaupt et al. (2008), demonstrating a decreased of the metabolic activities of endometrial cells incubated with concentrations from 0.39 to 12.5 µM AOH during 24 h. Similar results were obtained with AOH (concentration 0.8-25.6 µM) in granuloma cells (Tiemann et al., 2009) after 24 h of exposure. Neither Wollenhaupt et al. (2008) nor Tiemann et al. (2009) obtained IC_{50} value for AOH in the concentration range tested. Tissen et al. (2013) and Fehr et al. (2009) assayed higher AOH concentrations in human HT29 and A431 cells by SRB, WST-1 and

trypan blue exclusion assays. They found similar results to our study, because the highest concentration tested (50 µM) reduced up to 15% cell viability, but IC_{50} values were not reached. In contrast, other study demonstrated higher cell death after AOH exposure. Solhaug et al. (2012) suggested that AOH (0-60 µM) resulted in a reduction of viable RAW 264.7 cells with an EC_{50} of 49.65 µM and 78.01 µM by the NR and Alarm Blue assay after 24h, respectively. Bensassi et al (2012) showed that AOH induced an IC_{50} value about 65 µM in HCT116 cells by the FDA assay. In the same way, Brugger et al (2006) and Lehmann et al (2006) observed reduction in the number of cells treated with up to 30 µM of AOH to approximately 35% (V79 cells, 24 h of incubation), 69% (MLC cells, 24 h of incubation) and more than 50% for cells treated with 10 µM of AOH (V79 cells, 72 h of incubation). In addition, the behaviour characterized by low-concentration stimulation and high concentration inhibition in the Caco-2 cells exposed to AOH has been previously detected in other cell line exposed to mycotoxins (Meca et al, 2011; Lombardi et al, 2012; Prosperini et al, 2014).

Cytotoxicity of AOH may occur via LPO and resulting impairment in cellular viability. The degree of the imbalance between the concentrations of ROS, MDA levels and the antioxidative defense mechanisms of the cell which control the intracellular response to regulate the oxidative stress. In Caco-2 cells, AOH is able to induce ROS production (Fig. 4) demonstrating its implication in cell toxicity. These findings are consistent with those of Tiessen et al., (2013) and Schwarz et al., (2012) showing that from 10 to 50 µM, AOH induced an increase in ROS generation in HT-29 cells about 2.5-fold higher compared to the control. As previously demonstrated, oxidative stress produced by mycotoxins corresponded with ROS generation (Ferrer et al., 2009; Meca et al., 2010; Prosperini et al., 2013). The different species of ROS have various abilities to produce deleterious effects in the cell. The superoxide anion, $O_2^{\bullet-}$ is capable of producing H_2O_2, which

can cause damage or to produce the most toxic ROS, the hydroxyl radical $OH^{\bullet}$. Bensassi et al. (2012) carried out different assays to determine the predominant ROS product generated after 50 µM AOH exposures in HCT116 cells. They found that AOH did not induce a significant H_2O_2 production. Nevertheless, mitochondrial $O_2^{\bullet-}$ levels increased 4-folds in the presence of AOH, being the mainly ROS product generated in response to AOH exposure in HCT116 cells. The $O_2^{\bullet-}$ radical is not extremely reactive, it can act both as a reductant and an oxidant. Moreover, in cells, $O_2^{\bullet-}$ is effectively removed by SOD enzymes. In this way, Solhaugh et al (2012) found that H_2O_2 production increased after 30 min exposure to 30 µM AOH; whereas increased $O_2^{\bullet-}$ was evidenced after 24 h. On the other hand, oxidation reactions have the highest propensity to produce reactive metabolites. Cytochrome P450 (CYP) 1A1 is one of the most important isoenzymes responsible for the oxidation of xenobiotic (Seubert and Zeldin, 2008). Schreck et al. (2012) were not capable to detect ROS production in mouse hepatoma Hepa 1c1c7 cells after 40 µM AOH during 24 h of incubation. But, hydroxylation of AOH leads to catechols or hydroquinones, which may produce oxidative stress. These data were in line with Fehr et al (2009) and Solhaguh and collaborators (2012) which suggested that the increased ROS in AOH treated cells could be due to the products from cellular AOH-metabolism. They demonstrated that aromatic hydroxylation of AOH may generate both reactive catechols as well as hydroquinone. However, they postulated that metabolites undergo redox cycling resulting in the generation of ROS. However, Schreck and co-workers hypothesize those predominant cytotoxic effects of AOH in hepatoma cells is due to the high reduction in cell number, indicating that there is no CYP 1A1 dependent formation of ROS via redox cycling (Schreck et al. 2012). Moreover, according to Ferrer et al. (2009), ROS production is correlated with the increasing of MDA production, indicating an enhancement of LPO. AOH interacts with

membrane lipids which have been evidenced by the accumulation of MDA. This statement agrees with the results obtained in this study, because of at the higher concentration of AOH tested, an increase in MDA levels, and ROS generation was produce. MDA levels in association with ROS generation play a role in the cellular damage. Severals authors reported that some mycotoxins can cause cell membrane damage through the increase of LPO. Koaudio et al., 2007 showed that the exposure of Fumonisin B1 (FB1), Zearalenone (ZEA) and Deoxynivalelol (DON) to 10 µM of concentration of each mycotoxin affects the functionality of Caco-2 cell mitochondria inducing LPO and MDA production after 24 h of exposure. This data are consistent with those of Mary et al., (2012) who suggested that the exposure of spleen mononuclear cells (SMC) to Aflatoxin B1 and FB1 for 48 h produced a significant MDA formation relative to the control. Moreover, similar results were obtained by Yang et al., (2014) who observed that DON in a range of concentration of 6.25 to 50 ng/ml caused an increase of MDA in human peripheral blood lymphocytes after 6, 12 and 24 h of exposure.

The measurement of the oxidative stress can be complicated due to the complexity of the antioxidant defense system, such as the antioxidant enzymes SOD and CAT. These enzymes quickly reduce products generate in cells which cause oxidative stress. Wijeratne et al., (2005) observed that Caco-2 cells exhibit small but significantly different antioxidant profiles with increasing time of culturing. Although CAT has been studied over many years, there are conflicting reports regarding their protective role on H_2O_2 challenge (He et al., 2011). These findings are consistent with those of Matés (2000) who suggested that CAT is not essential from some cells type under normal conditions; it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells. Moreover, an overdone of H_2O_2 production can stimulate or block CAT activity, which can upregulate depending on nutrient status of the growth medium, which

is the more critical parameter controlling the outcoming of H_2O_2 production. In this study, the exposure to AOH did not induce an increase in CAT activity. However, SOD activity was stimulated after AOH incubation. It can be assumed that Glutathione Peroxidase (GPx) will be more active than CAT in removing H_2O_2. In other study developed by our research group, this fact has been confirmed (data not shown). Our results are in accordance with Dinu et al., (2011) who was capable to detect an elevated level of LPO in the exposure of Hek-293 cells with 5 µM of DON and simultaneously the CAT activity was significantly decreased and GPx activity increased after 12 h of exposure. Furthermore, Ramyaa et al., (2014) observed an increase of LPO levels and concurrently a decreased in CAT activity after exposure to 10 µM of OTA in HepG2 cells. The CAT activity going down may be a result of the accumulation of $O_2^{\bullet-}$ radical observed particularity after 30 and 60 µM of AOH, as previously demonstrated (Kono and Fridovich, 1982). On the other hand, the variation of $O_2^{\bullet-}$ radical levels, about 1.2-fold after 15, 30 and 60 µM compared to control values can be correlated to the modulation in SOD activity, which has a significant increase after 15, 30 and 60 µM AOH exposure. Similar data were obtained by He et al., (2011) and Theumer et al., (2010) who found an increased in SOD activity caused by T-2 toxins, FB1 and AFB1 in GPCs and SMC cells during 48 h of incubation. Dinu et al., (2011) detected the same effects in Hek-293 cells exposed to 5 µM of DON.

In conclusion, AOH produce changes in the oxidative stress markers and antioxidant defense in Caco-2 cells in dose- and time- dependent manner. The imbalance of ROS metabolism, leading to ROS accumulation and LPO, could ultimately generate cell death.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgement

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3.4 Oxidative DNA damage and disturbance of antioxidant capacity by alternariol in Caco-2 cells

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Abstract

Oxidative stress occurs as a consequence of an imbalance between the prooxidant/antioxidant systems, causing an increase of intracellular generation of reactive oxygen species. Alternariol (AOH), a mycotoxin produced by *Alternaria sp*, can alter the action of–glutathione (GSH) and the enzymes involved in the redox system, causing damage to cellular macromolecules such as DNA. The aims of this work were to determine the induction of oxidative stress by the antioxidant defenses imbalance in relation to glutathione (GSH), glutathione reductase (GR), glutathione transferase (GST), glutathione peroxidase (GPx) levels and DNA damage in Caco-2 cells derived from adenocarcinoma human colon. Oxidative stress by AOH was confirmed by alteration of GSH levels and the antioxidant defense system after 15, 30 and 60 µM AOH exposure during 24 h. GSH levels significantly decreased by 43% after treatment with 60 µM AOH compared to the control. The activity of GPx and GR was reduced by 30% and 23%, respectively after 60 µM AOH. The GST activity was significantly increased (approximately 22%) with 30 µM AOH, while 60 µM AOH decreased it by 30% in comparison to the control. Analysis of DNA damage was performed using the Comet assay after 24 h, where the % of DNA in tail increased from 70% to 85% compared the control.

1. INTRODUCTION

Alternaria alternata is a fungus found to occur in a wide variety of fruit, vegetables (Pavon et al., 2012; Yogendrarajah et al., 2014), cereals and seed (Ostry, 2008). Moreover, It was isolated the fungus of Alternaria alternata from 46% of sunflower seed samples in Brazil where the highest concentration of AOH in sunflower found was a 1200 µg/kg (Pozzi et al., 2005). Alternaria toxins known

as dibenzopyrones derivates include alternariol (AOH), alternariol monomethyl ether (AME) and altenue (ALT). AOH is the most predominantly occurring of these three mycotoxins in food. AOH is rapidly absorbed as aglycone. It undergoes cytochrome-mediated oxidative metabolism in liver and is conjugated with glucuronic acid and sulfate (Burkhardt et al., 2009). Thus, AOH and its conjugates through bile can reach the duodenum and to be distributed by blood anywhere in the body and to provoke toxic effects. AOH exposure is of concern to public health due to its toxigenic properties. It has been demonstrated that AOH inhibited metabolic activity and cellular proliferation of porcine granulosa cells (Tiemann et al., 2009), and inhibited cell proliferation by interfering with the cell cycle in Chinese hamster V79 cells (Lehmann et al., 2006). AOH has been associated with DNA damaging effects (Solhaug et al., 2012), acts as a potential endocrine disruptor (Lehmann et al., 2006, Tiemann et al., 2009 and Frizzell et al., 2013) and is also mutagenic in the low micromolar range (Brugger et al., 2006). The etiology relevance of AOH in human esophageal cancer has been demonstrated (Bensassi et al., 2012). However, the underlying mechanisms of action have not yet been fully elucidated (Lehmann et al., 2006; Fehr et al 2009).

Oxidative stress is a term used to describe an imbalance favoring prooxidants and/or disfavoring antioxidants. Reactive oxygen species (ROS) levels dramatically increase under certain pathological conditions and due to the effect of some environmental factors. They are unstable and may attack and damage vital components of the cell, such as polyunsaturated fatty acids, proteins and DNA what can result in cell membrane damage, alterations in membrane fluidity, permeability and DNA damage.

Activation of the antioxidative system occurs in order to maintain the intracellular ROS at normal levels and protect from oxidative damage (He et al., 2011). The antioxidant system includes protective mechanisms by glutathione

(GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). GSH, a tripeptide containing a free thiol group, is a nonenzymatic antioxidant that plays a crucial role in antioxidant defense and detoxification of xenobiotics as well as their metabolites. Consequently, it is considered an important and efficient antioxidant by maintaining cells in a reduced condition and protecting the organs and tissues against oxidative damage (Golli- Bennour and Bacha, 2011). The GP_X takes part in the transformation of ROS, catalyzing the reduction of peroxide or lipoperoxide. Under oxidative stress conditions, GSH is oxidized to GSSG (oxidized form of glutathione) by GPx, and GSSG is reversed to GSH when glutathione reductase (GR) catalyzes the reaction. GSH is the predominant form, and GSSG content is less than 1–1.2% of GSH (Lu et al., 2009). Glutathione-S-transferase (GST) is another antioxidant detoxifying enzyme, which acts by conjugating GSH to electrophilic centers in many toxic oxidised products to form nontoxic products (Gu et al., 2013).

In a recent study, we found that AOH produces lipid peroxidation (LPO) and ROS in mammalian cells (Fernández-Blanco et al., 2014). However, information regarding oxidative stress and antioxidant systems in cell lines exposed to AOH are scarce. Understanding the mode of action of AOH is essential to predict any harmful effects on human health when AOH contaminates food commodities. This study evaluates DNA damage following AOH exposure in Caco-2 cells, and the effects of AOH on GSH levels and antioxidant enzymes activities, including GP_X, GST and GR.

2. MATERIALS AND METHODS

2.1 Reagents

The reagent grade chemicals and cell culture components used, Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, HEPES, trypsin/EDTA solutions, non-essential amino acids (NEAA),

ethylenediaminetetraacetic acid (EDTA), phosphate buffer saline (PBS), sodium pyruvate, t-octylphenoxypolyethoxyethanol (Triton X-100), GSH, GSSG, GR, beta-nicotinamide adenine dinucleotide phosphate (β-NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), o-phtaldialdehyde (OPT), benzo(α)pyrene, dimethyl sulfoxide (DMSO), propidium iodide (PI), sodium azide (NaN_3), hydrogen peroxide (H_2O_2), tris hydroxymethyl aminomethane (Tris), Low Melting Point Agarose (LMA), agarose precoated and AOH (258.23 g/mol; purity >96%) were from Sigma Chemical Co. (St Louis, MO, USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Deionised water (resistivity <18 MΩ cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). BEA were from Sigma Chemical Co. (St. Louis, MO, USA).

Stock solutions of AOH were prepared in DMSO and maintained at +4°C. The final AOH concentrations tested were achieved by dilution in the culture medium. The final DMSO concentration in medium was $\leq$ 1% (v/v).

Total protein content (µg/mL) was determined by Bradford method (Bio-Rad DC Protein Assay (catalogue number 500-0116). Protein concentration (µg/mL) was measured at 690nm.

2.2 Cell culture and AOH exposures

Caco-2 cells (ATCC HTB-37) were maintained in DMEM medium supplemented with 25 mM HEPES buffer, 1% (v/v) of NEAA, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) FCS inactivated. Incubation conditions were pH 7.4, 37°C under 5% CO_2 and 95% air atmosphere at constant humidity. Culture medium was changed every two days. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St Louis Mo. USA).

AOH concentrations for the following assays were selected using data previously obtained in the MTT, NR and PC assays previously determined

(Fernández-Blanco et al., 2014). These results showed that AOH did not decrease cell proliferation on Caco-2 cells after 24 h of exposures at any of the concentrations tested (3.125-100 µM). Thus, the AOH concentrations select in this study to determine the GSH levels, enzymatic activities and DNA damage were 15, 30 and 60 µM.

AOH concentrations were selected considering that AOH is a food chemical. Thus, dietary exposure assessment takes into consideration the occurrence and concentrations of the AOH in the diet, the consumption patterns of the foods containing the AOH and the likelihood of consumers eating large amounts of the foods in question (high consumers). Usually a range of intake or exposure estimates will be provided (e.g. for average consumers and for high consumers), and estimates may be broken down by subgroup of the population (e.g. infants, children, adults). To do the calculations, data provided by EFSA (2011) and Food Balance Sheets of FAOSTAT (2011) were applied.

For GSH levels assay and antioxidant enzymes activities, 4×10^5 cells/well were seeded in six-well culture plates. Once confluent, the culture medium was removed and 3 mL of medium with 15, 30 or 60 µM AOH were added for 24 h. Following incubation, the medium was removed and cells washed twice with PBS and then homogenized in 0.5 mL of 20 mM Tris and 0.1% Triton.

2.3 Determination of glutathione

Determination of GSH was assayed by adapting a method described previously by Maran et al., (2009). Briefly, 10 µL of each homogenized cell sample were placed, in 96 well black tissue culture plates, with 200 µL GSH buffer (pH 8.0) and 10 µL of the OPT solution, mixed and incubated in darkness at room temperature for 15 min. Concentration of GSH was determined using a microplate reader (WallaceVictor^2, 1420 Multilaber Counter, PerkinElmer, Turku, Finland)

with excitation and emission wavelengths of 345 and 425 nm, respectively. GSH levels were expressed in µg/mg protein.

2.4 Determination of GP_X and GR activities

GPx activity was assayed spectrophotometrically using H_2O_2 as substrate for the Se-dependent peroxidase activity of GP_X by following oxidation of NADPH at 340 nm during the first 2 min in a coupled enzymatic reaction with GR as described by Maran et al., (2009). In 1.0 mL final volume, the reaction mixture contained 500 µL of 0.1M phosphate buffer (pH 7.5, 1 mM EDTA and 2mM NaN_3, 0.1% TritonX-100), 250 µL of ultrapure water, 100 µL of 20 mM GSH, 20 µL of 0.2 mM NADPH, 2.5 U freshly prepared GR and 50 µL of 5 mM H_2O_2. Fifty microliter of cell extract was added to the reaction mixture. One unit of GPx will reduce 1 µmol of GSSG per min at pH 7.5. Assays were conducted at 25 ºC during 2 min in a thermocirculator of Perkin Elmer UV/vis spectrometer Lambda 2 version 5.1.

GR activity was determined by following the oxidation of NADPH to NADP during the reduction of GSSG. GR was assayed by adapting the method described previously by Maran et al., (2009). In 1.0 mL final volume, the reaction mixture contained 800 µL of 0.1M phosphate buffer (pH 7 with 5 mM EDTA), 10 µL of 10 mM NADPH in phosphate buffer and 90 µL of cell extracts. Reaction was started started by the addition of 100 µL GSSG. The decrease in absorbance was monitored at 340 nm during 4 min at 25 ºC in a thermocirculator of Perkin Elmer UV/Vis spectrometer Lambda 2 version 5.1.

GR and GP_X enzymatic activities were calculated by using a molar absorptivity of NADPH (6.22 $mM^{-1}cm^{-1}$) and expressed as nmol of NADPH oxidized/min/mg of protein.

2.5 Determination of GST activity

GST activity was determined by measuring the increase in absorbance at 340 nm on conjugation of CDNB with GSH by the method of Maran et al., (2009) with slight modifications. The reaction mixture contained in a final volume of 1.0 mL : 825 µL sodium phosphate buffer 0.1 M (pH 6.5 with 0.5 Mm EDTA and 0.1% Triton X-100), 25 µL of 50 Mm CDNB dissolved in ethanol, 100 µL of 25 Mm of GSH and 50 µL of cell extract. Enzymatic activity was assayed at 25 ºC in a thermocirculator of Perkin Elmer UV/Vis spectrometer Lambda 2 version 5.1 during 3 min. GST activity was expressed as mol of product formed/min/mg of protein using a molar absorptivity of CDNB (9.6 Mm^{-1} cm^{-1}).

2.6 Alkaline comet assay (pH>13)

The induction of DNA strand breaks was determined using the alkaline comet assay (pH>13), according to the method described previously by Prosperini et al (2013). Briefly, Caco-2 cells (4.8 x 10^5cells/well) were seeded in 6-well plates. Once 90% confluent, cells were treated with AOH (15, 30 and 60 µM) for 24 h. Subsequently, the cells were suspended in pre-warmed LMA (0.5% PBS; 37°C) and 80 µL of the suspension rapidly transferred to agarose precoated slides (1% PBS) and covered with a coverslip (24x36 mm). After gelling for 10 min at 0°C, the coverslip was gently removed and a second layer of 80 µL LMA was added and the gelling step was repeated. The slides were placed in a tank filled with lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 250 mM NaOH, 10% DMSO and 1% Triton X-100, freshly added) for 30 min at 0 °C. The lysis solution was then removed, washed with neutralization buffer (0.4 M Tris, pH 7.5) and incubated in fresh electrophoresis buffer (300 mM NaOH, 1 mM Na-EDTA) for 20 min at room temperature to allow the DNA to unwind. Electrophoresis was then carried out at room temperature in fresh electrophoresis buffer for 40 min (25 V, 300 mA). After electrophoresis, slides were gently washed once for 5 min in fresh neutralization buffer. After drying overnight at 4 °C, slides were stained with 500 µL of PI (20

µg/mL) and covered with a coverslip. Slides were visualized under a fluorescence microscope (NIKON Eclipse E800), equipped with camera (NIKON DXM1200F) to capture imagines. A minimum of 50 randomly selected individual cells were selected and analyzed by using the Automatic Comet Assay by TriTek CometScore™ freeware (http://autocomet.com/index.php?id=cometscore). The DNA damage in Caco-2 cells was expressed as a percentage of the total DNA content in the tail based on the total fluorescence and single cells with damaged DNA appeared as comets. Comets were classified according to the tail moment (TM), calculated according to the following equation: TM = TL x Tail DNA (%) where TL is the tail length, i.e. the distance (L) between the center of the comet head and the end of the comet tail.

2.7 Statistical analysis

Statistical analysis of data was carried out using SPSS version 19 (SPSS, Chicago, IL, USA), statistical software package. All determinations were performed in quadruplicate. Data were expressed as mean ± SD of three independent experiments. The statistical analysis of the results was performed by Student's t-test for paired samples. Differences between groups were analyzed statistically with one-way ANOVA followed by the Tukey HDS *post-hoc* test for multiple comparisons. The level of $p \leq 0.05$ (*), $p \leq 0.01$(**), $p \leq 0.001$ (***) was considered statistically significant.

3. RESULTS

3.1 Glutathione determination

The alteration of GSH content was measured after exposure to 15, 30 and 60 µM AOH in Caco-2 cells for 24 h. No noticeable changes in the intracellular GSH content were observed after exposure of 15 and 30 µM AOH (Fig. 1). However, after exposure to 60 µM AOH the GSH levels produced a marked decreased ($p \leq$ 0.01) respect to the control cells. The GSH content was diminished by about 43 % compared to the control (Fig. 1).

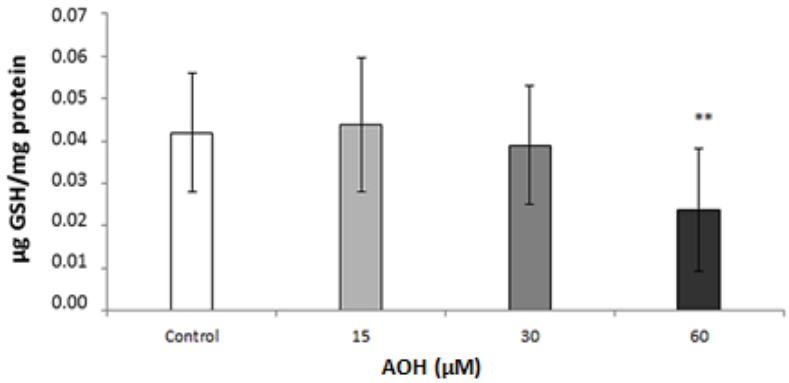

Figure 1. GSH levels in Caco-2 cells exposed to AOH (15, 30 and 60 µM) for 24 h. Data are expressed as mean values ± SD. $p \leq 0, 01$(**) indicates a significant difference from the control.

3.2 Enzymatic activities

The effects produced by 15, 30 and 60 µM AOH on the enzymatic activities involved in GSH metabolism are shown in Figures 2 to 4. After 24 h AOH exposure in Caco-2 cells, the GP_X and GR activities did not present significant differences at 15 and 30 µM concentrations with respect to the control cells (Figs. 2 and 3). However, after exposure to 60 µM AOH, the GP_X and GR activities decreased significantly ($p \leq 0.05$) approximately 30% and 23% respect to the control, respectively (Figs. 2 and 3).

No changes in GST activity were recorded after 15 µM AOH exposure in Caco-2 cells (Fig. 4). However, after exposure to 30 µM AOH the GST activity was significantly increased ($p \leq 0.05$) approximately 22% with respect to the control. While treatment with 60 µM AOH significantly decreased ($p \leq 0.01$) the GST activity by 30% with respect to the control (Fig. 4).

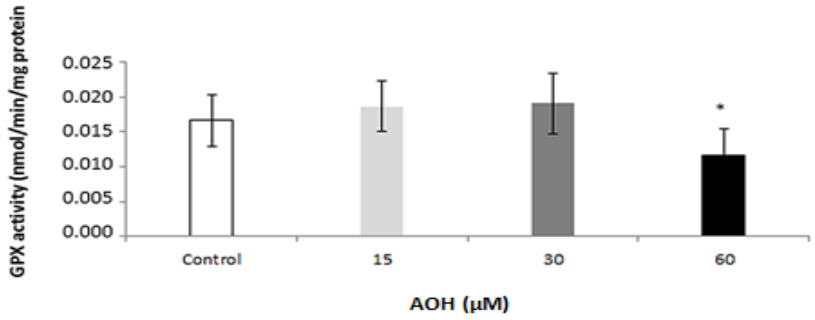

Figure 2. Effects of AOH (15, 30 and 60 µM) on GPx activity in Caco-2 cells following exposure for 24 h. Data are expressed as mean values ± SD. $p \leq 0, 05$(*) indicates a significant difference from the control.

Fig. 3

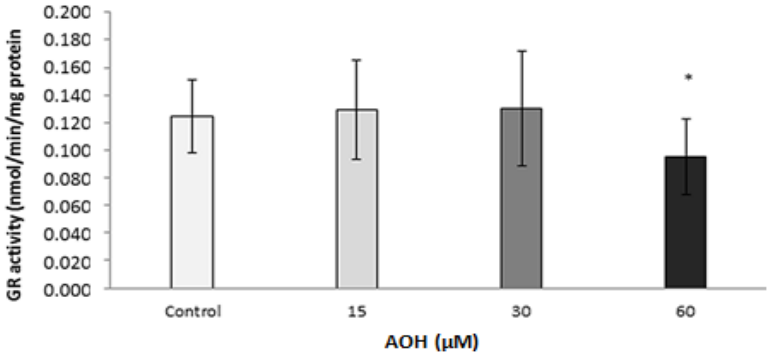

Figure 3. GR activity in Caco-2 cells after exposure to AOH (15, 30 and 60 μM) exposure for 24 h. Data is expressed as mean values ± SD. $p \leq 0, 05$(*) indicates a significant difference from the control.

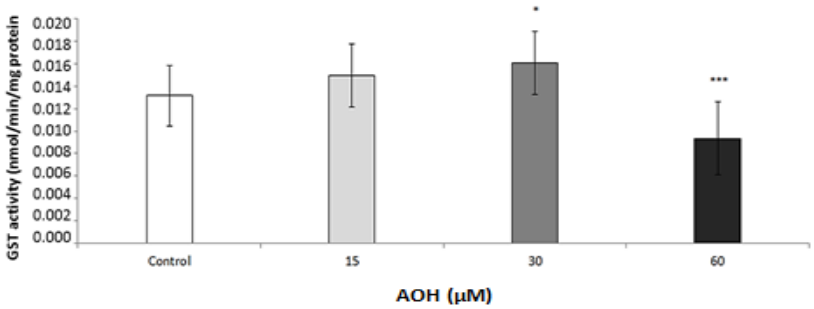

Figure 4. GST activity in Caco-2 cells after exposure to AOH (15, 30 and 60 μM) for 24 h. Data are expressed as mean values ± SD. $p \leq 0, 05$(*) and $p \leq 0, 001$(***) indicate significant differences from the control.

3.3 Alkaline Comet Assay

The single cells with damaged DNA appeared as comets (Fig. 5) whose tail moment (TM) were assessed using the Automatic Comet Assay by TriTek CometScore™. The cells tested were categorized into four grade of damage: class A (no damage, TM =0), class B (minimal or low damage, TM: 0–5), class C (mid damage, TM: 5–60) and class D (high damage, TM > 60), which correspond to the control, 15, 30 and 60 µM AOH, respectively.

Figure 6 shows the % of DNA in the tail when Caco-2 cells were exposed to AOH for 24 h. 20 µM benzo(α)pyrene was used as positive control (data not shown). Results obtained indicated that after this time of exposure all concentration tested (15, 30 and 60 µM) produced a significant increase in the % of DNA in the tail compared to the control. The percentage of DNA in the tail significantly increased by 70%, 78% and 85% after 15, 30 and 60 µM AOH exposure, respectively, compared to the control.

Tukey's multiple comparison indicates that the % DNA in tail was significantly higher ($p \leq 0.05$) after treatment of Caco-2 cells with 60 µM AOH when compared to the 15 µM AOH treatment (Fig. 6).

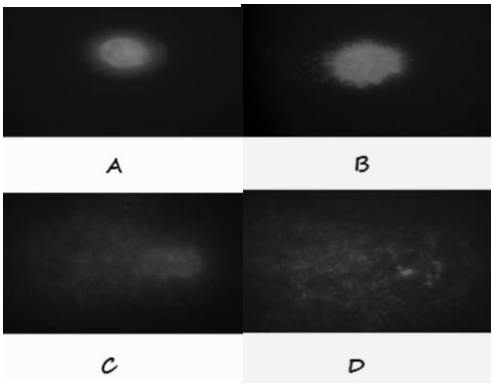

Figure 5. Images of comets with a) control; and increasing amounts of AOH b) 15 µM c) 30 µM and d) 60 µM.

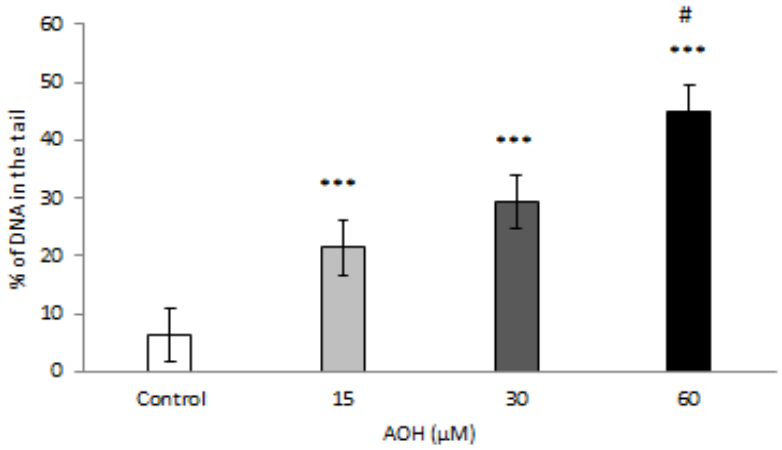

Figure 6. The % of DNA in the tail on Caco-2 cells after exposure to 15, 30 and 60 µM AOH. $p \leq 0.001$ (***) significantly different from the control. $p \leq 0.05$ (#) significant differences from 15 µM AOH.

4. DISCUSSION

According to Burkhardt et al. (2009) AOH suffer oxidative metabolism producing by cytochrome P450. Cytochrome P450 is an enzyme related to ROS production. Thus, ROS could be produced by AOH which may permeate into cell nuclei and induce oxidative DNA damage and may also react with membranes (lipids), proteins, and other components to initiate/cause cellular damage and degeneration. To evaluated DNA damage of AOH and its influence on the GSH levels and antioxidant enzymes activities, Caco-2 cells were selected in this study, considering that ingestion of food contaminated with AOH is the main exposure route for animal and humans. In this study, an increase in DNA damage after AOH exposure on Caco-2 cells was observed after 24 h of incubation. The DNA damage,

evidenced by the percentage of DNA in the tail of the comet assay was substantially increased when the concentration of AOH increased. This finding clearly indicates than AOH is a clastogenic compound able to induce DNA strand breaks in Caco-2 cells. Similar results were observed by Fleck et al., (2012) and Solhaug et al., (2012) who demonstrated that 5, 10 and 20 µM AOH on V79 cells and 30 µM AOH on RAW 264.7 cells induces DNA strand breaks after 24h of incubation, respectively. The oxidative DNA damage of AOH can be attributed to the epoxide group enabling formation of covalent adducts with DNA (Solhaug et al., 2012). Furthermore, Fehr et al (2009) demonstrated the affinity of AOH to the minor groove of DNA, resulting from its planar aromatic structure, which may contribute to the DNA-damaging properties of the AOH. Similar results were obtained by Solhaug et al., (2012) who evidenced an increase in oxidative DNA damage following AOH exposure. However, they demonstrated that DNA damage produced by AOH was closely connected to the elevated ROS levels and cellular pathways related to genomic integrity, like cell cycle checkpoints, apoptosis and induction of mitochondrial damage. Moreover, these effects were confirmed by Fehr et al (2009) in HT29 cells, which found increasing tail intensities after treatment with $\geq$ 1 µM. Significant difference was also seen at 10 µM AOH (Schwarz et al., 2012). However, Tiessen et al., (2013) exposed that after 50 µM AOH incubation no significant DNA damage on HT29 cells was observed. Moreover, they suggested that GSH plays a role in the detoxification of AOH and protection against DNA damage.

GSH is the major endogenous antioxidant produced by cells (Yang et al., 2014). The GSH participates directly in the neutralization of free radicals and reactive oxygen species and is involved in determining cell fate decisions, such as proliferation and apoptosis (Dalton et al., 2004). Multiple ROS spike events may damage or destroy the antioxidant GSH and induce several changes at the cellular

level. Excessive production of ROS leads to oxidative stress, loss of cell function and damage to cellular components such as lipids by means of LPO. Early *in vitro* studies developed in our laboratory demonstrated that AOH induced ROS and LPO in Caco-2 cells (Fernández-Blanco et al., 2014). Similarly, Tiessen et al., (2013) and Schwarz et al., (2012) demonstrated similar AOH effects in other cells lines. In this study, GSH depletion was observed in Caco-2 cells after 24 h of incubation with 60 µM AOH. This finding suggests that GSH is involved in the cellular defense mechanisms in response to the exposure to this mycotoxin in Caco-2 cells. Similar results were obtained by Tiessen et al., (2013) who showed that GSH levels decreased (from 19% to 26%) in a concentration dependent manner in HT29 cells after exposure to 10-50 µM AOH for 1 h. Furthermore, parallel results were observed with mycotoxins from other genera of fungi (Penicillium*, Aspergillus* and Fusarium), like deoxynivalenol (DON) in human peripheral blood lymphocytes (PBL) (Yang et al., 2014) and Hek-293 (Dinu et al., 2011); Beauvericin (BEA) in Caco-2 cells (Prosperini et al., 2013) and CHO-K1 cells (Mallebrera et al.,2014); Zearalenone (ZEA) in HepG2 cells (Hassen et al., 2007); Ochratoxin (OTA) in LLC-PK1 cells (Schaaf et al., 2002) and T-2 toxin in GPC_S cells (He et al., 2011).

At normal physiological conditions, GSSG is reduced to GSH by GR at expense of NADPH, thereby forming a redox cycle. The decrease in GR activities indicates an impaired reduction from GSSG to GSH. Moreover, at 60 µM AOH a decrease in intracellular GSH levels is observed. Thus, these effects can lead to an oxidative imbalance in the GSH cycle and consequently contribute to the producing oxidative process. On the other hand, GST, an enzyme related to GSH, participates in the defense against oxidative stress. It has the ability to detoxify products that cause cellular toxicity. In addition, GST enzymes are shown to respond to toxin exposure, catalyzing the conjugation of electrophilic compounds to GSH (LaCourse et al., 2009). A reduction of GST activity may be related to a

reduction of this protein, to a catalytic inhibition due to high ROS levels or to depletion on the GSH availability. Tiessen et al., (2013) also suggested that the increase in the GST activity might result from metabolites, which may generate catechols or hydroquinones and the consequent formation of ROS. In this study, it was observed that low concentrations AOH showed a stimulation of GST enzymatic activities, suggesting that GST acts by detoxifying AOH in Caco-2 cells; however, in spite of this, higher concentrations AOH decreased the ability of GST to detoxify AOH possibly due to saturation of enzyme activity. Moreover, Tiessen et al., (2013), demonstrated that AOH only achieved significant levels of GST after 50 µM AOH exposures. This data demonstrated the inactivation of GST following exposure to higher concentrations and may suggest that induction of the antioxidant response pathway may depend on the exposure duration. Furthermore, as an increase of ROS and LPO levels after exposure of 60 µM AOH in Caco-2 cells has been demonstrated (Fernández-Blanco et al., 2014) this could also contribute to the disruption of the proper functioning of antioxidant enzymes such as GST activity in the Caco-2 cells.

The enzymatic antioxidant system is the first and best line of defense against free radicals. It consists of three main enzymes involved in chain to selectively disable free radicals: SOD, CAT and GPx. The GPx enzyme works in tandem with CAT to scavenge excess of H_2O_2. The GPx activity significantly decreased after 60 µM AOH in Caco-2 cells. This result suggests that 60 µM AOH saturates this enzymes activity. The decrease in GPx activity may have resulted in accumulation of the superoxide anion as observed particularity after 60 µM of AOH treatment in Caco-2 cells. On the other hand, generation of superoxides can be destroyed by SOD. In our previously study (Fernández-Blanco et al., 2014), an increase in SOD activity was observed after 15, 30 and 60 µM AOH exposure. This increase suggests that CAT is not eliminating the overproduction of H_2O_2 due to

saturation or blockage; SOD decreases the products generated in cells which cause oxidative stress. Thus, according to the data obtained in our laboratory previously (Fernandez-Blanco et al., 2014) related to the detoxifying activities of SOD and CAT and the data obtained in this work, we may confirm that the GST and SOD are more efficient than CAT and GPx removing the H_2O_2 in the Caco-2 cells after exposure to AOH.

Taken together, our data provide that AOH produced DNA damaged and an increase of the GST activity in Caco-2 cells. The GST activities inhibit the oxidative degradation, reacting with ROS and free radicals produced by AOH. Therefore, to the best of our knowledge, GSH and GST play a vital role in protecting Caco-2 cells against oxidative stress produced by AOH.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgement

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3.5 Alternariol induce toxicity via cell death and mitochondrial damage in Caco-2 cells.

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Abstract

Alternariol (AOH), a mycotoxin produced by *Alternaria sp*, appears as food contaminant in fruit, vegetables and cereal products. Its toxicity has been demonstrated, but the mechanisms involved have not been elucidated yet. In this study, the pathways triggered by AOH and degradation products generated in Caco-2 cells were evaluated. Cells were exposed to AOH sub-cytotoxic concentrations of 15, 30 and 60 μM. Cell cycle disruption, the induction of apoptosis/necrosis and changes in mitochondrial membrane potential (Δψm) after 24 and 48 h was asses by flow cytometry. Also, AOH and its degradation products were evaluated after 24 and 48 h by high-performance liquid chromatography with tandem mass spectrometric (LC-MS / MS) to detect and quantify its levels. Cell cycle was significantly decreased at G1 phase and increased at S and G2/M phase at the time of exposure. AOH induced necrosis, apoptosis/necrosis and loss of Δψm in a dose and time-dependent manner. The concentrations of AOH quantified in the culture media exposed to AOH decreased as the exposure time was increased. In conclusion, AOH caused cytotoxic effects supported by blocking cell cycle, decreasing cell proliferation and increasing apoptosis*/necrosis cells.*

1. INTRODUCTION

Molds of the genus *Alternaria* have been reported as potencial contaminants of variety of food and feed. Because the mold grows at low temperature, *Alternaria* spoilage may also occur in cereals, fruits and vegetables kept under refrigeration (Solfrizzo et al., 2004). Thereby *Alternaria* ssp. are able to produce mycotoxins under a wide range of environmental conditions. Moreover, literature of *Alternaria* mycotoxins shows that a wide range of *Alternaria*

metabolites have been described although relatively few have been fully characterized. Alternariol (AOH) is the major *Alternaria* mycotoxins and is a member of the dibenzo-alfa-pyrone group.

Previous work have demonstrated that AOH is cytotoxic and produces oxidative stress (Solhaug et al., 2012; Wollenhaup et al., 2008; Fernández-Blanco et al., 2014, Vila-Donat et al., 2015) and the prevention of AOH-induced cytotoxic effects caused by enzymes (Tiessen et al., 2013; Fernández-Blanco et al., 2014) or antioxidants (Chiesi et al., 2015). Moreover, genotoxic AOH effects on mammalian cells have also demonstrated (Fehr et al., 2009; Brugger et al., 2006; Fernández-Blanco et al., 2015).

On the other hand, it has been suggested that consumption of food infested by *Alternaria* fungi may be associated with an increasing incidence of esophageal cancer in certain areas of China (Liu et al., 1992). But the underlying mechanisms of action have not been fully clarified yet (Lehmann et al., 2006; Fehr et al., 2009). Few information about metabolism of AOH is available. Since all products of aromatic hydroxylation of AOH are catechols or hydroquinones, which can create reactive semiquinones and quinones or undergo redox cycle, its inactivation by methylation or glucuronidation/ sulfation is considerated as a possible toxicological product (Pfeiffer et al., 2007a; Burkhardt et al., 2009). Moreover, AOH is susceptible to cytochrome P450 (CYP)-mediated hydroxylation *in vitro*. The catechol metabolites of AOH were found to be O-methylated by catechol-O-methyl transferase (COMT) under cell-free conditions (Burkhardt et al., 2011). Knowledge of the toxicity and disposition of the AOH metabolites is compulsory for a better understanding of the health risks caused by these *Alternaria* toxins.

However, very few data about biochemical mechanisms in mammalian cells are available about AOH. There is a widespread interest in the cellular

mechanisms used to deal with the disruption in homeostasis (Bensassi et al., 2012). Two main mechanisms responsible for the coordinate stimulation of toxicants induced cell death have been identified; apoptosis, a programmed cell death, characterized by cell reduction and condensation/fragmentation of nuclear DNA, which is strictly distinct from necrosis, in which damage leads to plasma membrane rupture and inflammatory responses (Krysko et al., 2008). The mitochondria appears to play a crucial role as a central regulator of the intrinsic apoptotic pathway originating from death signals initiators inside the cell such as DNA damage, oxidative stress, as well as stimuli induced by various toxicants (Wang et al., 2010). Mitochondrial membrane permeabilization (MMP) can be mediated by the opening of the permeability transition pore (PTP), which initiates the loss of ionic homeostasis (Bensassi et al., 2012). MMP is an important indicator of cellular viability, as it reflects the pumping of hydrogen ions across the internal membrane during the process of electron transport and oxidative phosphorylation. Early in the course of apoptosis, mitochondria undergo metabolic changes that can potentially alter the enzymatic reactions essential for mitochondrial function, as alkalinisation of the mitochondria matrix (Tsiper et al., 2012). In addition, cells have various cell cycle checkpoints that ensure that individual phases of the cells cycle are not initiated unless conditions are favourable and previous phases have been successfully completed (Bartek et al., 2004).

So, in this study, the aims were determinate whether the cytotoxic effects of AOH can be related to the disturbance of the cell cycle progression, the mechanism implicated in cell death and changes in the mitochondrial membrane potential ($\Delta\psi$) using flow cytometer. The detection and quantification of AOH in the medium was evaluated using LC-MS-MS.

2. MATERIALS AND METHODS

2.1 Reagents

The reagent grade chemicals and cell culture components used, Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, HEPES, tetrazolium bromide (MTT), non-essential aminoacids (NEAA), phosphate buffer saline (PBS), pyruvate, trizma base, propidium iodide (PI), RNase A, glucose, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), sodium azide and AOH (258.23 g/mol; purity >96%) were from Sigma Chemical Co. (St Louis, MO, USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Deionised water (resistivity <18 MΩ cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were of standard laboratory grade from Sigma–Aldrich (St. Louis MO, USA). Stock solutions of AOH was prepared in DMSO and maintained at -4°C. The final AOH concentrations tested were achieved by adding the culture medium. The final DMSO concentration in medium was ≤ 1% (v/v). On the other hand, for LC-MS/MS determination, standard stock solutions and standard calibration curves of AOH were prepared in MeOH and MeOH: H_2O (70:30, v/v), respectively.

2.2 Cell culture

AOH is mainly exposed via the ingestion of contaminated foods. Thus, taking into consideration that the major route to mycotoxins is via oral, human colon adenocarcinoma (Caco-2) cells were used in this study. Caco-2 cells (ATCC HTB-37) were maintained in DMEM medium supplemented with 25 mM HEPES buffer, 1% (v/v) of NEAA, 100U/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) FCS inactivated. Incubation conditions were pH 7.4, 37°C under 5% CO_2 and 95% air atmosphere at constant humidity. Culture medium was changed every two days. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St Louis Mo. USA).

2.3 Exposure to AOH

The cells were treated with AOH at 15, 30 and 60 µM for 24 and 48 h. The range of concentrations tested in this study was selected depending on the concentrations found in feed and agricultural commodities in Europe, where AOH was found in a range from 6.3 to 1840 µg/kg of tested samples (EFSA Panel on Contaminants in the Food Chain (EFSA, 2011).

AOH concentrations were selected considering the bioavailability and the previous data obtained in our laboratory in Caco-2 cells. The concentrations of AOH used for this study (15, 30 and 60 µM) were lower than the inhibitory concentration 90 (IC_{90}) obtained by previously MTT, Neutral Red (NR) and Protein Content (PC) assays (Fernández-Blanco et al., 2014).

2.4 Cell proliferation and cell cycle distribution by propidium iodide (PI) staining

Cell proliferation an cell cycle distribution assays were carried out by using Vindelov´s PI staining solution 0.01 M Tris base, 7.5 x 10^{-5} M PI, 0.1% tergitol-type NP-40, 10 mg (700 U/L) RNase A and 10 mM NaCl. PI is a standard flow cytometry probe used to distinguish viable from non-viable cells. PI is a DNA intercalating agent that stains only cells in the late phases of cell death when the integrity of both, cellular and nuclear membranes is lost (Chang et al., 2010; Le Drean et al., 2005; Van Engeland et al., 2010).

For this purpose, Caco-2 cells were grown in 6-well plates for 24 h previous to incubation with AOH. Afterwards, for determing the number of cells and cell cycle distribution, medium was removed; cells were trypsinized and incubated at 37ºC for 30 min with 860 µL of fresh medium containing 29 ng/mL of Vindelov´s PI staining solution. Cell cycle analysis was carried out as described by Prosperini et al. (2013) by rectangular fitting (CYLCHRED or MODIFIT software, Beckton Dickinson, Milan, Italy) using 1024 channels which produced histograms with a single G_1 peak at channel 200 when DNA was diploid, an S-peak between

channels 200 and 400 when DNA was replicating, a G_2/M peak at channels 400 when DNA was tetraploid and a Sub-G1 peak (debris´ peak), between 100 and 200 when DNA was hypodiploid or damaged. The small coefficient of variation (CV) obtained in this study was the result of the high resolution achieved by proper alignment. Four independent experiments were performed for each AOH treatment. At least 10,000 cells were analyzed for each sample.

No time course of the cell cycle distribution was determined in Caco-2 cells, and the induction of an irreversible cell cycle arrest in G_2/M and S-phase or the synchronization of the Caco-2 cell population by a temporary cell cycle arrest earlier in the course of the experiment was not distinguished.

2.5 Measurement of necrosis-apoptosis by Annexin V FITC-PI

Cell death through necrosis and/or apoptosis was evaluated with fluorescein isiothiocynate (FITC)-labelled Annexin-V simultaneously with PI dye exclusion (Apoptest TM-FITC, Nexins Research, The Netherlands). Annexin-V- FITC measures phospatidylserine (PS) exposure on the cell surface. The Caco-2 cells (10^6 cells/mL) after incubation with AOH at 24 and 48 h were resuspended in 400 µL of Annexin-V-binding buffer containing 10 mM HEPES-NaOH (pH 7.4), 135 mM NaCl, and 2.5 mM $CaCl_2$. They were kept on ice during 25 min in darkness with 1.25 ng/mL Annexin-V- FITC and 10 µg/mL PI dyes to bind at early phases of apoptosis to PS in the presence of Ca^{2+}. Positioning of quadrants on AnnexinV-FITC/PI dot plots was performed as described by Prosperini et al., (2013).

The cells were analyzed by FACSCalibur flow cytometer (Beckton-Dickinson, Italy) operated to detect green (FL-1, 530 nm) and orange-red fluorescence (FL-2, 585 nm) emitted by FITC and PI dyes, respectively. Since a double labelling was applied to the cells, compensation between FL1 and FL2 was used to reduce the artefactual effects of spectral overlap between these channels.

Four independent experiments were performed for each AOH treatment. At least 10,000 cells were analysed for each sample.

2.6 Measurement of mitochondrial membrane potential

During apoptosis, MMP is lost; in consequence changes produced in MMP were measured. Cell death could be induced by a mitochondria-dependent apoptotic process which includes opening of mitochondrial permeability transition complex pore (PTPC) and mitochondrial transmembrane potential; increase in ROS production and other factors (Bouaziz et al., 2011). Changes in MMP, after AOH exposure (15, 30 and 60 µM) were evaluated using tetramethyl rhodamine methyl ester (TMRM) and carbocyanine monomer nucleic acid (To-Pro-3)® double labelling as described by Tsiper et al. (2012). Cells were seeded at a density of 75,000 cells/mL onto 6 well/plate and after reach confluence; cells were exposed with AOH at different concentrations (15, 30 and 60 µM) for 24 and 48 h. After exposure, the cells were trypsinized, resuspended in 400 µL DMEM medium, transferred in single tubes and incubated with 140 nM TMRM and 160 nM ToPro-3® for 30 min at 37 °C in the presence of 20 µg/mL verapamil to block activity of the multidrug resistance pump and allow efficient and uniform dye loading. Tubes were placed in a water bath a 37 °C and kept in darkness. After that, cells were analysed using a FACSCalibur flow cytometer (Beckton-Dickinson, Italy). Four independent experiments were performed for each AOH treatment. At least 10,000 cells were measured for each sample.

2.7 AOH extraction in culture media

To determine the concentration of the mycotoxin exposed to cell culture, extraction procedure of AOH and its degradation products in the culture media was carried out following Juan et al. (2014) method, with several modifications. Briefly: 1.6 ± 0.05 mL of culture media was collected into 2 mL Eppendorf Safe-Lock Microcentrifuge Tube, transferred into a polypropylene tube by using a

Pasteur pipette, 2 mL of ethyl acetate added, and shakes during 2 min with an Ika T18 basic Ultra-Turrax (Staufen, Germany). Afterward, the mixture was centrifuged at 3500 rpm for 5 min at 4 °C (Centrifuge 5810R, Eppendorf, Germany) and, the supernatant phase was collected in a new polypropylene tube. The process was repeated three times with 2 mL of ethyl acetate each time. Finally, the total volume obtained (aprox. 6 mL) was evaporated to dryness at 45 °C in a N_2 stream with a TurboVap-LV (Zymark, Allschwil, Switzerland) and then re-dissolved in 1 mL of Methanol/H_2O mixture (70:30, v/v) by vortexing vigorously (15 s), before transferred into a vial for LC-MS/MS injection. The injection volume was 20 µL.

2.8 Determination of AOH by LC-MS/MS

The analysis was performed using a LC-QTRAP system, consisting in a LC Agilent 1200 using a binary pump and an automatic injector, and coupled to a 3200 QTRAP ® AB SCIEX (Applied Biosystems, Foster City, CA) equipped with a Turbo-V™ source (ESI) interface. The chromatographic separation of the analytes was conducted at 25 °C with a reverse phase analytical column Gemini-NX ® C_{18} (3 µM, 150 x 2 mm ID) and a guard-column C_{18} (3 µM; 4 x2 mm, ID) from Phenomenex (Madrid, Spain). Mobile phase was a time programmed gradient using methanol (0.1% formic acid and 5mM ammonium formiate) as phase A, and water (0.1% formic acid and 5 mM ammonium formiate) as phase B. The following gradient was used: equilibration during 2 min at 10% A at 0.25 mL/min, 10-80 % A in 3 min at 0.25 mL/min, 80% A for 1 min at 0.25 mL/min, 80-90% A in 2 min, 90% A for 6 min at 0.25 mL/min, 90-100% A in 3 min at 0.25 mL/min, 100% for 1 min at 0.35 mL/min, 100-50% in 3 min at 0.4 mL/min, return to initial conditions in 2 min and maintain during 2 min. Total run time was 21 min. The injection volume was 20 µL.

Regarding the mycotoxin analysis, a 3200 QTRAP® System AB SCIEX (Applied Biosystems, Ontario, Canada) was used in function as triple quadrupole mass spectrometry detector (MS/MS). The Turbo-V™ source was used in positive mode to analyze the AOH with the following settings for Source/Gas Parameters: vacuum gauge (10e-5 Torr) 2.7, curtain gas (CUR) 20, ionspray voltage (IS) 5500, source temperature (TEM) 450 ºC, ion source gas 1 (GS1) and ion source gas 2 (GS2) 50. Therefore, in this study, optimization of the MS/MS parameters was performed using AOH standard. The precursor ion (Q1) of the mycotoxin was confirmed in product ion (Q3) scan mode. As shown in Table 1, a protonated molecule was observed as the base peak ion in the mass spectra of the analyzed mycotoxin. Thus, this ion was selected as Q1 for the mycotoxin. The optimization of Q3 and its collision energy was performed in the product ion scan mode. The multiple reaction monitoring (MRM) transitions in positive ion mode for the compound, the optimal decluttering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision cell exit potential (CXP), and collision energies (CE) were also optimized. The EP was 10 V. The final selection values of CE and CXP are shown in Table 1. Data acquisition and processing were performed using Analyst® software version 1.5.2.

Table 1. Optimized ESI (+) MS/MS conditions.

Analite	Rt (min)	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)	CXP	CEP
AOH	8.5	259 $[M+H]^+$	128 Q 184 q	39	65 42	3	16

Q1: Parent ions; Q3: Product ions; DP: Declustering Potential; CE: Collision Energy; Rt: Retention time; CXP: Colision cell Exit Potential; CEP: Collision Energy Potential.

2.9 Statistical analysis

Statistical analysis of data were carried out using IBM SPSS Statistic version 19.0 (SPSS, Chicago, Il, USA) statistical software package. Data were expressed as mean ± SD of four independent experiments. The statistical analysis of the results was performed by student´s T-test for paired samples. Differences between groups were analyzed statistically with ANOVA followed by the Turkey HDS post hoc test for multiple comparisons. The level of $p \leq 0.05$ was considered statistically significant.

3. RESULTS

3.1 Cell proliferation and cycle analysis

Flow cytometry was used to determine cell proliferation by cell cycle analysis with PI staining. As shown in Figure 1, after 24 and 48 h of exposure to 15, 30 and 60 µM AOH, a significant percentage of cell reduction in G1 phase accompanied by an increase in the percentage (%) of number of Caco-2 cells of S and G2/M phases was observed, as compared to the control. After 24 h of exposure (Fig.1a), the percentage of Caco-2 cells in the G1 phase decreased until 34.0% ± 1.9, 32.4% ± 2.1 and 27% ± 2.1 after 15, 30 and 60 µM AOH, respectively as compared to the control (50.6% ± 1.6). For S phase increased at 15 and 30 µM AOH, the percentage of cells were about 27.3% ± 1.1 and 26.9% ± 1.7, respectively, as compared to the control (21.1% ± 1.7), with no changes at 60 µM; similarly, for G2/M phase number of cells increased until 31.2% ± 0.8 and 32.7% ± 1.4, respectively, as compared to the control (24.1% ± 1.5). At 60 µM G2/M phase decreases at 22.6% ± 2.7 (Fig. 1a). Similar effects were obtained after 48 h of exposure to 15, 30 and 60 µM AOH: a reduction of G1 phase (28.1% ± 1.8, 20.3% ± 1.6 and 28.3%± 1.3) was observed compared to the control (42.7% ± 0.8) and an increase in S phase at all concentration assayed (32.6% ± 0.9, 35.4% ± 1.5 and 27.6% ± 0.8) was obtained compared to the control (24.0%± 0.6) (Fig.1b). An increase in cells in G2/M phase was obtained only at 30 µM of AOH with a cell percentage of 37.9% ± 3.9, while at 15 and 60 µM a decrease of 27.3%± 0.2 and 25.9%± 2.1 was obtained respectively, respect to control (28.1% ± 1.8) (Fig. 1b).

The percentage of cells in SubG1 phase remained unchanged after 24 h of exposure; while a marked increase was observed after 48 h of exposure. As compared to the control (1.3% ± 1.8). This increase was ranged from 3.9%± 1.9 to 6.7% ± 2.8. An example of the histograms representative of the control and 60 µM AOH after 48 h of exposure can be observed in the Figure 2.

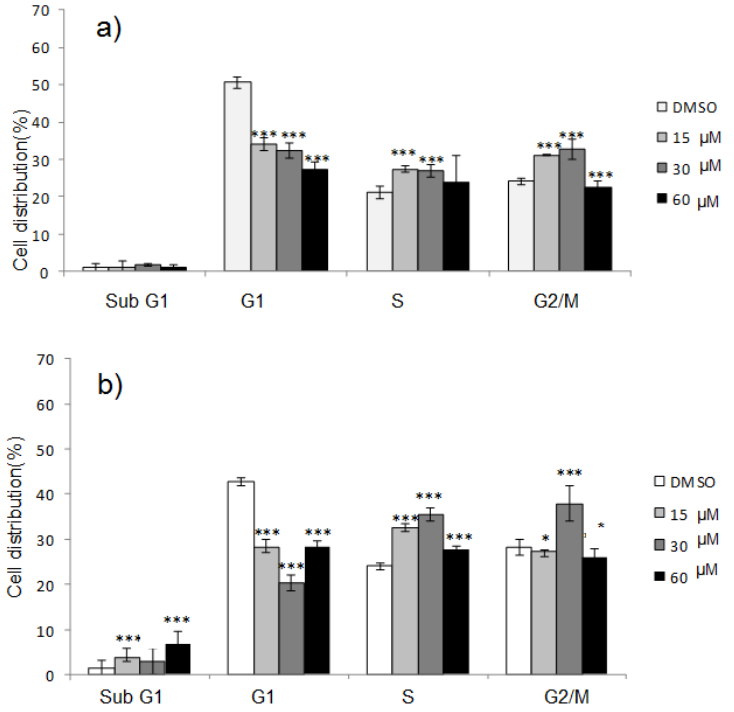

Figure 1. Analysis of cell cycle distribution of Caco-2 cells after exposure to different concentrations (15, 30 and 60 µM) AOH for 24 (a) and 48 h (b). Data are the mean ± SD (n=4). *$p \leq 0.05$, **$p \leq 0.01$ and *** $p \leq 0.001$ compared to control.

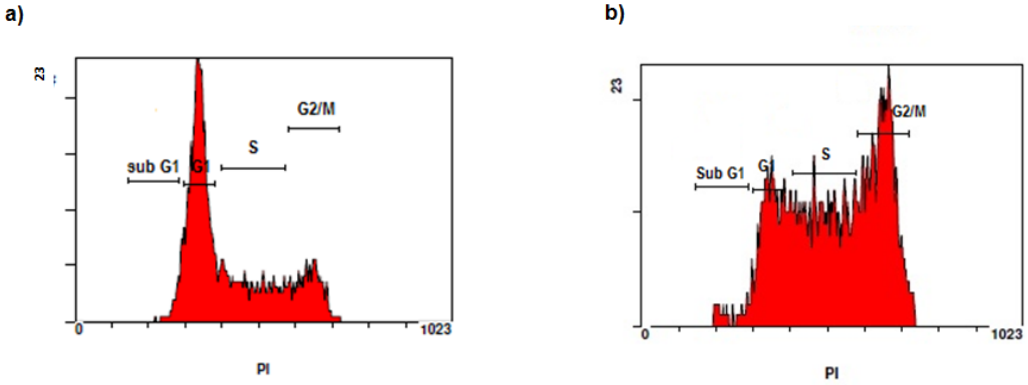

Figure 2. Cells were treated with AOH, stained with PI and subjected to cell cycle analysis by flow cytometer. The histograms are representative of Caco-2 cells treated with control (a) and 30 µM AOH (b) for 48 h. (n=4). *$p \leq 0.05$, **$p \leq 0.01$ and *** $p \leq 0.001$ compared to control.

3.2 Necrosis-apoptosis analysis

To determine whether the observed decline on cell proliferation was related to AOH exposure in Caco-2 cells, the early apoptotic, apoptotic/necrotic and/or necrotic cells induced by the studied concentrations (15, 30 and 60 µM AOH) was assayed (Figure 3). Proportion of apoptotic/necrotic and necrotic cells was obtained at 15, 30 and 60 µM either at 24 and 48 h (Figs. 3a and b); however, early apoptotic cells proportion were not produced at 24 and 48 h indicating that under the studied conditions, early apoptotic pathway is not activated in Caco-2 cells (Data not shown).

Figure 3a shows apoptosis/necrosis analysis in Caco-2 cell line. Cells treated with 15, 30 and 60 µM AOH during 24 and 48 h, resulted in an increase of this process respect to the control (Fig. 3a). The increase for both times ranges from 7.4% ± 1.6 to 27.8% ± 5.6 respect to the control (1.6% ± 0.8 to 1.8 % ± 1.2). The higher increase was obtained at 60 µM at both times studied.

Cells treatment using AOH at any studied concentration (15, 30 or 60 µM), during 24 and 48 h resulted in an increase of Caco-2 cells necrotic rate comparing to control (Fig. 3b). This increase was detected in 27.5% ± 2.4, 59.0% ± 3.6 and 73.5% ± 2.2 at 24 h respect to the control (2.3% ± 0.6) for 15, 30 and 60 µM, respectively; and at 48 h the increase detected was similar with 11.5% ± 0.9, 22.5% ± 1.10, and 34% ± 4.5 respectively, comparing to the control (1.1% ± 0.1).

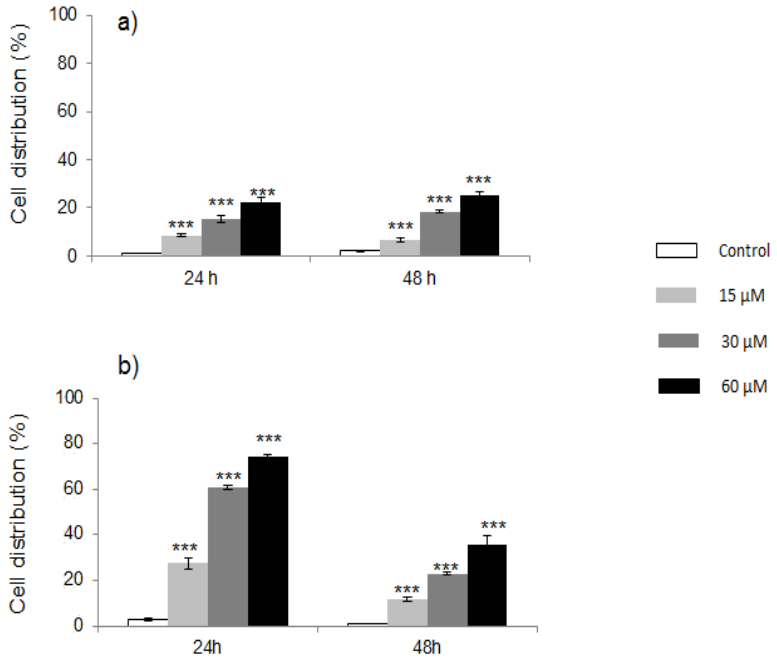

Figure 3. Analysis of apoptosis/necrosis induction in Caco-2 cell after exposure to different concentrations (15, 30 and 60 µM) AOH, and different incubation periods (24 and 48 h). Cells were stained with Annexin-FITC and PI to distinguish a) apoptotic/necrotic and b) necrotic cells. Data are the mean ± SD (n=4). *$p \leq 0.05$, **$p \leq 0.001$ and *** $p \leq 0.001$ compared to control.

3.3 Mitochondria membrane potential analysis

To evaluate the role of mitochondria in AOH-induced apoptosis, the ability of AOH to modify the $\Delta\psi$ was investigated. A double staining with TMRM and ToPro-3® for studying perturbation in MMP was carried out. TMRM is a mitochondrial-specific and voltage-dependent dye that indicates mitochondria depolarization start. ToPro-3® allows identifying if affected cells are progressing to death through apoptosis (Lee et al., 2006; Banjerdpongchai et al., 2010).

AOH caused loss of MMP in Caco-2 cells (Figure 4). It must be taken into account that ToPro-3® cells staining allow identification of cells subpopulations with altered membrane permeability and associated loss of membrane integrity

and subsequently cells death. A reduction of the MMP leads to the release of TMRM from mitochondria and decrease in fluorescence intensity (Lee et al., 2006). Alterations in MMP were concluded from the decrease proportion cells of TMRM+ and increase of ToPro-3® positive cells compared to controls.

At 24 h, TMRM increased signal was approx. 30% when Caco-2 cells were exposed to 30 and 60 µM AOH; while at 48 h the increase was observed at all concentration tested (Fig 4a).

At 48 h, Caco-2 cells treated with 30 and 60 µM of AOH, showed a significant loss of MMP which it is related with increase of ToPro-3 signal (Fig. 4b). It revealed an increase in apoptosis/necrosis of dead cells for Caco-2 cells exposed to same AOH concentrations (Fig. 3). At 24 h no loss of MMP was detected.

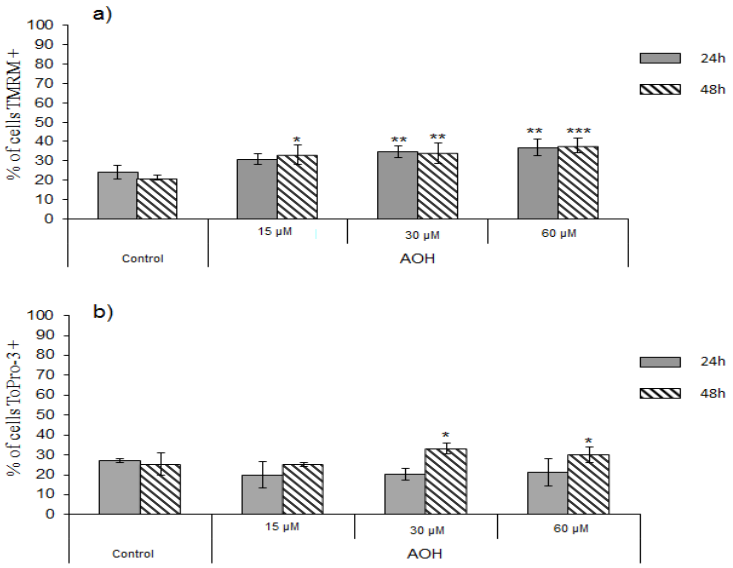

Figure 4. Changes in the MMP in Caco-2 cells by double staining signal TMRM-ToPro-3® after exposure to different concentrations (15, 30 and 60 µM) AOH, and different incubation periods (24 and 48 h). Figure shows a) percentage of Caco-2 cells TMRM positive signal treated with AOH; and b) percentage of Caco-2 cells ToPro-3® positive signal treated with AOH. Data are the mean ± SD (n=4). *$p \leq 0.05$, **$p \leq 0.01$ and *** $p \leq 0.001$ compared to control.

3.4 Determination of AOH by LC–MS/MS.

LC–MS/MS method was validated previously to use for the analysis. Recovery experiments were conducted at two different levels one 2 times the Limit of Quantification (LQ) and the other one 10 times the LQ. In both cases, the extraction procedure evidenced a recovery above 88%. Regarding to the linearity by regression coefficient of calibration curves showed a correlation coefficient (r^2) higher than 0.992. Sensitivity was evaluated by Limit of Detection (LD) and LQ values. The LD was estimated from growth medium (DMEM medium), spiked with decreasing concentrations of the mycotoxins. The response of the mycotoxin peak was equal to 3 times the response of the growth medium. Once evaluated, three samples were spiked at the estimated levels and extracted according to the proposed procedure. The LD and LQ evidenced for AOH were 3 and 7 ng/mL, respectively.

LC-MS/MS was used to calculate the amount of mycotoxin remaining in the growth medium after treatment with different concentration. Three concentrations of AOH (15, 30 and 60 µM), were diluted in DMEM medium and maintained during 24 and 48 h, and after that, the growth medium were collected and analysed.

Figure 5 represents the amount of AOH remaining in growth medium after each treatment, which is related with the concentration added initially. As can be observed in Figure 5, the concentration in the growth medium oscillated by time of exposure between 14.6 and 9.4 µM, between 28.8 and 8.9 µM and between 49.6 and 44.5 µM AOH after addition of 15, 30 and 60 µM, respectively. So the quantity of AOH in the media decrease as time of exposure is increased, being the higher reduction at 48 h and 30 µM AOH (67.0%).

Fig. 6 shows the LC-MS/MS chromatogram of the AOH (retention time, RT, 16.42 min) in the growth medium at 48 h 15 µM AOH solution. As it is indicated,

the intensity of AOH in the chromatograms decrease as time of exposure is increased.

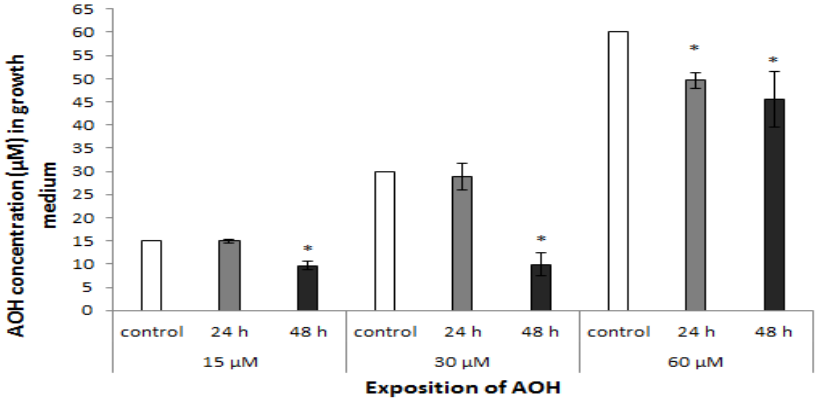

Figure 5. Concentration of AOH in the growth medium after exposition of 15, 30 and 60 µM AOH at 24 and 48 h.

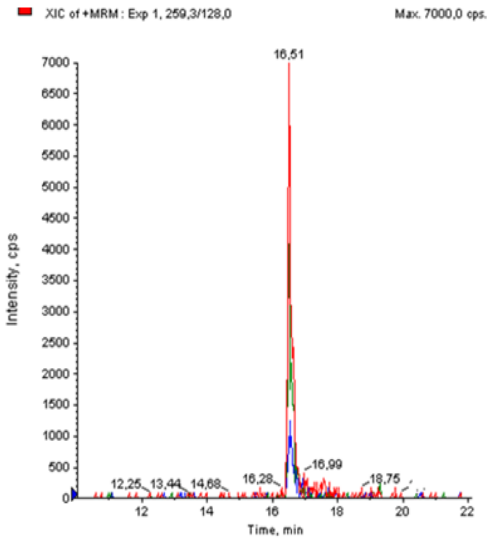

Figure 6. Total ion chromatogram of 15 µM AOH from growth medium at 24 and 48 h of incubation by LC-MS/MS.

4. DISCUSSION

AOH is known to induced reduction in cell viability and cell proliferation (Wollenahupt et al. 2008; Fernández-Blanco et al.,2014; Chiesi et al., 2015; Vila-Donat et al., 2015) DNA damage (Lehmann et al., 2006; Fernández-Blanco et al., 2015) and increased steroidogenesis (Frizzelli et al., 2013). Moreover, AOH seems to be mutagenic (Fleck et al., 2012), and acts as poison of topoisomerase II-alpha-isoform (Fehr et al., 2009) in mammalian cells. Moreover, we previously reported that AOH causes oxidative stress, lipid peroxidation and produces changes in the antioxidant defense system in Caco-2 cell (Fernández-Blanco et al., 2014; Chiesi et al., 2015; Vila-Donat et al., 2015).

In this study, it has been observed that AOH is capable to disturb the normal progression of proliferating cell. Moreover, after 24 h of exposure, AOH led to move the cell cycle distribution, getting visible as increased proportion of cells in the G2/M phase, while cells in the G1 phase were diminished. On the other hand, after 48 h of exposure, AOH produced an arrest of cell cycle in G2/M and it induced a significant increase in the S-phase. When DNA damage is irreparable, checkpoints eliminate such potentially hazardous cells by permanent cell-cycle arrest or cell death. The induction of cell cycle arrest in G2/M and/or in S-phase is a common response of most eukaryotic cells to a genotoxic challenge (Shackelford et al., 1999). Furthermore, the reduction in G1 is still maintained through all time of AOH exposure as well as the increase in Sub G1 phase that increased after 48 h of exposure. G1 phase is a period when cells make critical decisions about their destiny, including the optional requirement to replicate DNA and complete the cell division cycle. In unstressed cells, this requirement to replicate DNA and divide seems irreversible until the next G1 phase. Importantly, the checkpoints alarmed by genotoxic stress can delay cell-cycle progression even

when cells have already passed this restriction point. Thus, depending on the nature of DNA damage, the cell cycle can maintain of the G1 block (Bartek et al., 2001). Our results are in accordance with most of the previous findings in mouse lymphoma L5178Y tk+/-, Chinese hamster, Ishikawa cells lines and macrophages RAW 264.7. (Brugger et al., 2006; Lehmann et al., 2006; Fleck et al., 2012; Solhaug et al., 2012). Although an accumulation of cells in G1 has also been reported in endometrial cells (Wollenhaupt et al., 2008). Further, Solhaug et al. (2013) have reported that AOH leads the induction of signalling cascades including chk1 and chk2 mammalian kinases with a subsequent activation of p53. The activation of these proteins is involved in cell cycle arrest and are well known to be an important mediator of the G2/M DNA damage checkpoint. If DNA damage is not repair cells can undergo apoptosis or necrosis. Moreover, we have previously demonstrated that AOH can exert toxicological effects by means of ROS-dependent pathway (Fernández-Blanco et al., 2014) and DNA damage (Fernández-Blanco et al., 2015).

The apoptotic/necrotic and necrotic property of AOH are still maintained at all concentrations and exposure time, whereas the early apoptosis is not induced at the concentration tested (Figure.3). In the same manner, Soulhaug et al., 2012 notify that after 48 h of exposure at 60 µM AOH, an increase of necrotic RAW 264.7 cells was observed. This finding could support the induction of apoptosis/necrosis and necrosis via the mitochondrial pathway. At the stage of apoptosis/necrosis or following traumatic cell damage leading to necrosis, cells are no longer able to maintain the integrity of their membrane. Thus, the loss of balance between the intracellular and extracellular medium characterizes the ultimate stage in the cell death process (Berghe et al., 2010).

Supporting this hypothesis, MMP is considered to be a central event and the point-of-no-return in apoptosis (Galluzzi et al., 2006). In this study, after exposure of AOH, cells showed a significant alteration of Δψ in a time-depedent manner revealed by ToPro-3 signal (Figure 4b). An increase in TMRM signal may due to the amount of cells in S phase and the population of cells in apoptosis/necrosis (Figure 4a). These results suggest that the mitochondrial pathway plays an important role in AOH-induced apoptosis. Moreover, Bensassi et al. (2012) showed that AOH induced the opening of PTPC in human colon adenocarcinoma (HCT116) cells after exposure of 50 µM AOH during 24 h. The PTPC opening result in dissipation of the Δψ, disruption of the outer mitochondrial membrane and subsequent release of proteins such as the cytochrome c from the intermembrane space. On the other hand, oxidative stress is known to induce PTPC opening in mammalian mitochondria. Thereby, ROS modulate PTPC opening by oxidizing different sites, two of which are thiol groups (-SH), located in the matrix site (Viannello et al., 2012). It might be explained by the oxygen radicals produced during mitochondrial respiration which suggests a possible correlation between oxidative stress and mitochondrial activity; this hypothesis has been previously corroborate in our laboratory because of an increased in cytotoxicity by MTT and oxidative stress produced by ROS generation and LPO production in Caco-2 cells after AOH exposure has been recently demonstrated (Fernández-Blanco et al., 2014; Chiesi et al., 2015).

A toxicology screening of AOH degradation in mammalian cell culture like Caco-2 cells could provide detailed information of their elimination and potential toxic effects. To detect change of AOH, the growth medium exposed this mycotoxin was analysed by the LC–MS/MS. The results showed that the concentrations of AOH quantified in the growth medium exposed to 15, 30 and 60 µM decreased as the exposure time increased. A decrease in the amount of AOH remaining in the growth medium indicated either absorption through the membrane cell or degradation of the AOH itself in the culture media. The quantification and identification of AOH or its degradation products through the LC-MS/MS in the growth medium allows us to know the AOH absorption by cells or its degradation. The amount of AOH in the growth medium varies depending on the concentration added and the exposure time. The percentage of AOH in the medium was between 83% and 97% and between 33% and 76% after 24 and 48 h, respectively. These results may indicate that as the time of exposure increase, higher diffusion of AOH through the membrane cells is produced or bigger degradation occurs. However, even at the longest exposure time a large amount of AOH remains in the medium. This fact could indicate that as the concentration of AOH increases in the medium, the diffusion through the cell membrane is saturated, decreasing the absorption process. Moreover, the AOH in the growth medium may also decrease due to a degradation process.

Others authors conducted *in vitro* tests providing supplementary information, Pfeiffer et al., (2007b) suggested that microsomes from rats, pigs and humans are able to hydroxylate AOH at each conceivable position of the molecule

and confirm that the four major oxidative metabolites of AOH are catechols. In addition, Pfeiffer et al., (2007a) demonstrated that 50 µM AOH are complete conjugated in human colon adenocarcinoma (HT29) cells at 24 h. In the same way, Burkhardt et al., (2011) have demonstrated that AOH hydroxylated easily with glucuronide and sulphate acid leading to conjugated metabolites in precision-cut rat liver slices. However, these authors demonstrated the formation of glucuronides and sulphates of AOH in cultured human Caco-2 cells by incubation of the aglycones with rat liver cytosol in the presence of enzymes as 3'-Phosphoadenosine-5'-phosphosulfate (PAPS) and with β-glucuronidase type B-1 from bovine liver (Burkhardt et al., 2009). So, further studies in *in vitro* cell culture related these aspects must be developed to contribute to their achievement.

In summary, the results of this study show the block in G2/M phase of the cell cycle due to a checkpoint activated after DNA damage. AOH induced initially apoptosis/necrosis and then necrosis in Caco-2 cells. Moreover, relative correlation with MMP results and the death through apoptosis/necrosis to end up in necrosis. The AOH and its degradation products cause toxic effects in cells lines, so maybe it could be hazardous in short and long term in humans, and therefore special attention must be put to this mycotoxin for further information. To better understanding how mycotoxins exert cytotoxic effects in cells, more knowledge about bioaccessibility, biodisponibility and metabolic pathways are needed.

Conflict of interest

The authors declare that there are no conflicts of interest.

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3.6 Role of quercetin on Caco-2 cells against cytotoxic effects of alternariol and monomethyl ether.

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Abstract

Molds of the genus Alternaria have been reported as contaminants of a variety of food and feed. *Alternaria* toxins such like alternariol (AOH) and its naturally occurring monomethylether (AME) produce cytotoxicity and oxidative stress in cell culture.

On the other hand, it has been proved that natural polyphenols have antioxidant effect. Quercetin (Quer) is a polyphenol present in berries and other commodities which exhibits these effects. The aims were to evaluate the cytotoxicity of AOH, AME and the binary combination of them, and the cytoprotective effect of Quer exposed simultaneously with AOH, AME and the mycotoxin mixture in human adenocarcinoma (Caco-2) cells. The cytotoxicity and the cytoprotective effect were determined by the MTT test after 24 and 48 h of exposure and interactions were evaluated with the isobologram analysis method. Cell viability decreased after 48 h of AOH and AME exposures, being the binary combination more cytotoxic, causing a synergism effect. No cytoprotective effect of Quer against AOH and AME was observed when exposed simultaneously in Caco-2 cells. The cytoprotective effect of Quer against mycotoxins (AOH, AME or other different which could present higher cytotoxic effect) depends on the concentration, the presence and the interaction between the compounds in food.

1. INTRODUCTION

Mycotoxins are low-molecular weight natural products mainly produced by mycelial structure of filamentous fungi of genera *Fusarium, Penicillium, Aspergillius, Claviceps* and *Alternaria* (Hussein and Brasel, 2001). Contamination can take place also after the harvest and storage, wherefore a wide variety of

foods can be affected. They represent a threat to food safety and risks for disease in humans and animals consuming these foods (Ostry, 2008; Chiesi et al., 2015).

Molds of the genus *Alternaria* have been reported as potential contaminants of a variety of food and feed (EFSA, 2011; Ostry, 2008). They can generate, under appropriate conditions, such as low temperatures and low water activity a wide range of metabolites, some of which are potent mycotoxins (Woody and Chu, 1992). Alternariol (AOH) and alternariol-9-O-methyl ether (AME) (Figures 1a and b) are usually considered as major mycotoxins produced by *Alternaria* (Fleck et al., 2014).

The toxic effects of AOH and AME are wide-ranging. Recent studies have shown that AOH and AME are able to induce cells cycle arrest, apoptosis of cells and DNA damaging effects (Fernandez-Blanco et al. 2015; Bensassi et al. 2011; Pfeiffer et al. 2007; Lehmann et al. 2006) and to inhibit topoisomerase I and II under cell-free conditions (Fehr et al. 2009). It has been shown that AOH produce cytotoxic effects in mammalian cells by causing ROS generation and LPO production which results in impairment of cellular viability (Chiesi et al., 2015; Vila-Donat et al., 2015). Moreover AOH induces the antioxidant enzymatic defences as well as the GSH protective mechanism in cell cultures (Fernandez-Blanco et al. 2014; Tiessen et al. 2013). Furthermore the genotoxic activity of AOH in mammalian systems has been demonstrated (Pfeiffer et al. 2007), as well as its estrogenic potential (Lehmann et al. 2006).

Polyphenols are widely distributed in fresh fruits, berries, black tee, red wine, purple grape juice, medicinal herbals, daily nutrition supplements, etc. (Lombardi et al., 2012). Quercetin (Quer), is a bioactive phytochemical that belongs to a large group of polyphenolic flavonoid substances (Barcelos et al. 2011) (Figure 1c). Quer (3, 3', 4', 5, 7-pentahydroxyflavone) is one of the most abundant flavonoids in the human diet (Manach et al., 1999), being the major

polyphenol ingested daily by dietary intake (20–100 mg) (You et al. 2010). This regular dietary intake of Quer is associated with numerous potential health benefits, because of its various biological effects: antioxidant, antitumoral, anti-inflammatory, antiplatelet, and vasoprotector activities (Barcelos et al. 2011). It is also associated with cytoprotective effects and can inhibit oxidative stress (Lombardi et al., 2012). The antioxidant and pro-oxidant activity of Quer has been extensively investigated as a result of contradictory findings about its ability to protect mammalian cells from cytotoxicity. Therefore, studies on the level of security are of great importance Robaszkiewicz et al (2007); Choi et al. (2010).

Natural occurrences of AOH, AME, and other *Alternaria* micotoxins have been reported in food commodities (Scot et al., 2012). In addition, the co-occurrence of several mycotoxins in the same sample is of great interest because their combination could lead to hazardous effects in health, since their possible interaction in humans and animals (Ruiz et al., 2011).On the other hand, taking into account that in a standard diet can be found a whole of mycotoxins and antioxidants, the aims of this study were to investigate the cytotoxic effects of the AOH and AME and to assess the nature of toxicological interactions among them. Moreover, to evaluate the cytoprotection of Quer when it is simultaneously exposed with AOH or AME or their mixtures. For this purpose, AOH, AME and Quer were tested individually and in two- and three- combinations. The method of combination index (CI)-isobologram equation was applied to determine the type of interactions among the AOH and AME. Considering that the major route to mycotoxins is via oral, Caco-2 cells, from intestinal origin, were used in this study.

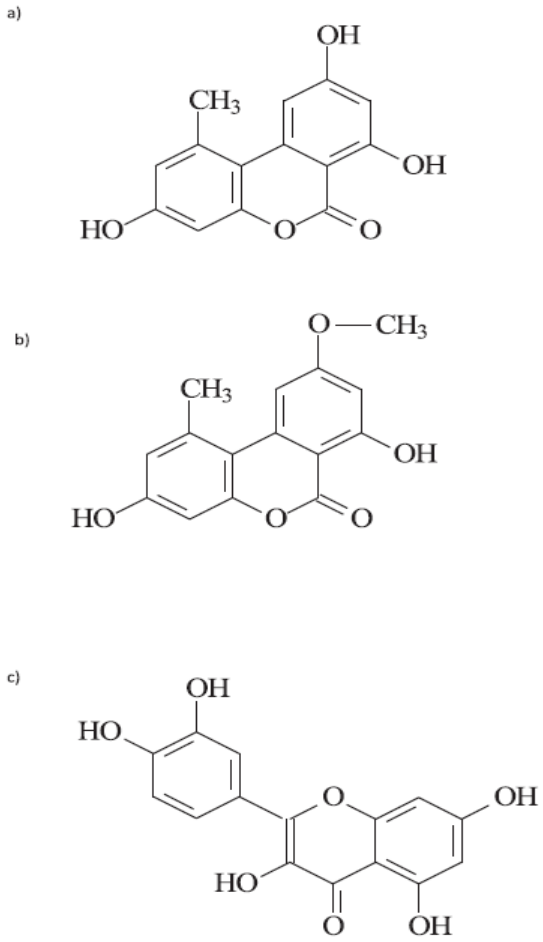

Figure 1. Chemical structure of a) AOH b) AME and c) Quer.

2. MATERIALS AND METHODS

2.1 REAGENTS

The reagent grade chemicals and cell culture components used, Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, HEPES, trypsin/EDTA solutions, non-essential amino acids (NEAA), phosphate buffer saline (PBS), Quer (338.27 g/mol; purity >98%), Dimethyl sulfoxide (DMSO) and

thiazolyl blue tetrazolium bromide (approx. 98%; M2128-10G) for MTT assay were from Sigma Chemical Co (St Louis, MO, USA). AOH (258.23 g/mol; purity >96%) and AME (272.25 g/mol >96%) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Deionised water (resistivity <18 MΩ cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

All stocks solutions were prepared in DMSO. AOH and Quer were maintained at -4°C and AME at -18ºC. The final AOH, AME and Quer concentrations tested were achieved by dilutions in the culture medium. The final DMSO concentration in medium was ≤ 1% (v/v).

2.2 Cell culture and AOH, AME and Quer treatment

Caco-2 cells (ATCC HTB-37) were maintained in DMEM medium supplemented with 25 mM HEPES buffer, 1% (v/v) of NEAA, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) FCS inactivated. Incubation conditions were pH 7.4, 37°C under 5% CO2 and 95% air atmosphere at constant humidity. Culture medium was changed every two days. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St Louis Mo. USA). Cells were plated in 96-well tissue culture plates at a density of 3x 104 cells/well to perform the experiment for individual exposure. Six concentrations for AOH, AME and Quer (from 3.125 to 100 µM) were assayed (Fig. 2). Similar range of concentration was selected for the binary combination AOH + AME, the concentration ranged from 3.125 to 30 µM (Fig. 6). AOH and AME concentrations for the binary combination were selected using individual data obtained in the study. For binary AME+ Quer or AOH + Quer combinations, the concentration selected ranged from 1.56 to 100 µM (1:1). For the tertiary combination, AOH+ AME + Quer the concentration selected ranged from 1.56 to 50 µM (0.5:0.5:1).

DMEM medium without AOH, AME or Quer and with 1% DMSO was used as control. After 24 h medium was removed and replaced with fresh medium containing the concentration previously described and incubated at 37 ºC for 24 or 48 h. The basal cytotoxicity endpoint selected was the tetrazolium salt reduction (MTT). MTT was measured according to Ruiz et al. (2006). Medium containing AOH, AME or Quer or the different mixtures selected were removed, cells were washed with PBS and 50 µM MTT were added. The absorbance was measured after 4 h at 570 nm on an ELISA plate reader Multiscan EX (Thermo scientific, MA, USA). Mean inhibition concentration (IC50) values were calculated from full dose–response curves.

2.3 Experimental design and assessment of effect of the combination.

The isobologram analysis was used to determine the type of interaction that occurs when AOH, AME or Quer are in combination. The type of interaction is described by the median- effect/combination index (CI)-isobologram equation by Chou (2006), and Chou and Talatay (1984). The isobologram analysis involves plotting the dose–effect curves for each compound and its combinations in multiple diluted concentrations by using the median effect equation:

$$fa/fu = (D/D_m)^m$$

Where D is the concentration of a product, D_m is the median-effect dose (e.g., IC_{50}, EC_{50}, or LD_{50}) that inhibits the cells under study by 50%, *fa* is the fraction affected by concentration D (e.g., percentage inhibition/100), *fu* is the unaffected fraction (therefore, *fa* = 1 - *fu*), and m is the coefficient signifying the shape of the dose-effect relationship, where *m* = 1, *m* > 1, and *m* < 1 indicate hyperbolic, sigmoidal, and negative sigmoidal dose-effect curve, respectively (Chou and Talalay, 1984). Therefore, the method takes into account both the potency (D_m) and shape (*m*) parameters.

The mediam-effect equation for a single compound can be extended to multiple mycotoxins. And the equation becomes:

$$\left[(fa)_{1,2}/(fu)_{1,2}\right]^{1/m} = D_1/(D_m)_1 + D_2/(D_m)_2 + (D)_1(D)_2/(D_m)_1(D_m)_2$$

When two compounds are combined and subjected to several dilutions, the combined mixture of the two compounds behaves as the third compound for the dose-effect relationship. Thus, y= log $[(fa)_{1,2}/(fu)_{1,2}]$ versus x = log $[(D)_1+(D)_2]$ will give $m_{1,2}$, $(D_m)_{1,2}$, and $r_{1,2}$ values.

Chou and Talalay (1984) introduced the term combination index (CI)x for quantification of synergism or antagonism for two compounds:

$$\begin{aligned} \mathrm{CI} &= \mathrm{D}_1/(\mathrm{D_x})_1 + \mathrm{D}_2/(\mathrm{D_x})_2 \\ &= (\mathrm{D})_1/(\mathrm{D_m})_1[fa/(1-fa)]^{1/\mathrm{m1}} \\ &\quad + (\mathrm{D})_2/(\mathrm{D_m})_2[fa/(1-fa)]^{1/\mathrm{m2}} \end{aligned}$$

Where in the denominator $(D_x)_1$ is for D_1 "alone" that inhibits a system *x*%, and $(D_x)_2$ is for D_2 "alone" that inhibits a system *x*%. And, the general equation for *n*-compound combination at *x*% inhibition becomes:

$${}^n(\mathrm{CI})_x = \sum_{J=1}^{n} (D_j/D_x)_j = \frac{(D_x)_{1-n}\{[D]_j \sum_{J=1}^{n}[D]\}^{1/mj}}{(D_m)_j\{(\mathrm{fax})_j/[1-(\mathrm{fax})_j]\}}$$

Where $^{n}(CI)_x$ is the combination index for n compounds (e.g., mycotoxins) at *x*% inhibition (e.g., proliferation inhibition); $(D_x)_{1-n}$ is the sum of the concentration of n compounds that exerts *x*% inhibition in combination, $\{[D_j]/\sum^{n}_{1} [D]\}$ is the proportionality of the concentration of each of n compounds that exerts *x*% inhibition in combination; and $(D_m)_j$ $\{(fax)_j/[1-(fax)_j]\}^{1/mj}$ is the concentration of each compound alone that exerts *x*% inhibition. From this equation, CI <1, =1, and >1 indicates synergism, additive effect and antagonism, respectively. The types of interactions produced by AOH, AME and Quer combinations were assessed by isobologram analysis using CalcuSyn software version 2.1. (Biosoft, Cambridge, UK, 1996–2007).

2.4 Statistical analysis

Statistical analysis of data was carried out using SPSS version 22 (SPSS, Chicago, IL, USA), statistical software package. Data were expressed as mean ± SD of three independent experiments. The statistical analysis of the data was performed by Student's t-test for paired samples. Differences between groups were analyzed statistically with one-way ANOVA followed by the Tukey HDS post-hoc test for multiple comparisons. The level of $p \leq 0.05$ was considered statistically significant.

3. RESULTS

3.1 Influence of AOH, AME and Quer on cell viability

The cytotoxic effect of AOH, AME and Quer in Caco-2 cells were evaluated by the MTT assay at 24 and 48 h (Fig. 2). At 24 h, AOH and AME did not decrease the number of viable Caco-2 cells at any of the concentration tested (3.125 - 100 µM) (Figs. 2a and 2b). In contrast, at 48 h, AOH reduced the number of viable Caco-2 cells and shows a significant reduction ($p \leq 0.05$) to 50 up and 100 µM (Fig.

2a). AME affects the number of viable Caco-2 cells after 48 h of exposure in a similar pattern than AOH and shows a significant dose-dependent inhibition ($p \leq 0.01$) of cell viability at concentrations ranging from 25 up to 100 µM (Fig. 2b). The results showed that both mycotoxins reduce cell viability in a time-, and concentration dependent manner, being the highest inhibition in cell proliferation of 30% (50 µM) for both mycotoxins compared to their own controls. On the other hand, AOH generated a stimulations of the mitochondrial function (3.125 - 50 µM) after 24 h of exposure, which was observed because of cell proliferation increased up to 50 % compared to the control (Fig. 2a). Quer had no influence on the number of viable Caco-2 cells after 24 and 48 h of exposure, except for the highest concentration tested (100 µM), which significantly ($p \leq 0.01$) decreased cell viability (Fig. 2c). This is due to the fact that antioxidants, at high concentrations, can evolve prooxidant effects. No IC50 values were obtained for AOH, AME and Quer at any time tested.

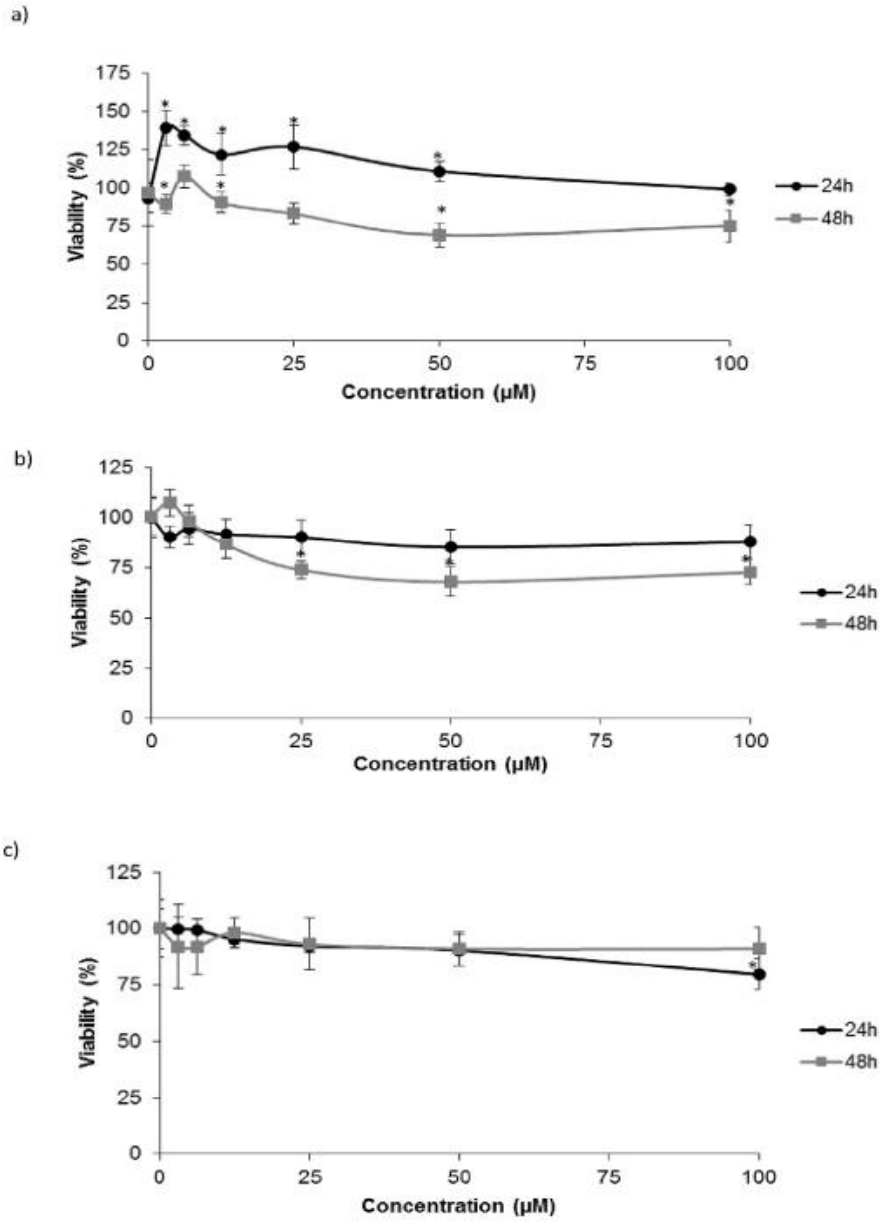

Figure 2. Dose response curve of a) AOH, b) AME and c) Quer on Caco-2 cells after 24(•) and 48 (▪) h of exposure. Results of 3 independent experiments. All values are expressed as mean ± SD. $p \leq 0.05$ (*) represents significant differences as compared to control values.

3.2 Interaction of AOH and AME combination

Figure 3 shows the viability (%) – concentration (μM) plot of binary AOH + AME combinations in Caco-2 cells at 24 and 48 h of exposure, compared to the concentration–response curve of each mycotoxin when applied individually in the same assay. Considerable differences in binary mycotoxin combination compared to each myxotoxin taken individually can be observed. At 24 h, the mycotoxin combination shows a significant reduction in cell proliferation at a concentration of 15 μM ($p \leq 0.05$) and 30 μM ($p \leq 0.01$) respect to AME tested alone. The higher reduction (26% for the combination respect to AME tested alone) was observed at 30 μM (Fig. 3a). However, when the combination was compared with AOH tested alone, higher cytotoxic effect was observed at all concentrations tested (from 3.125 to 30 μM). The higher reduction was 61% at 30 μM. (Fig 3a). At 48 h, AOH + AME combination shows a significant dose-dependent inhibition of cell viability at concentrations ranging from 7.5 ($p \leq 0.05$) up to 30 μM ($p \leq 0.001$; Fig. 3b). The cell proliferation reduction ranged from 18% to 25% for AME and from 29% to 32% for AOH. No IC50 values were obtained for AOH + AME at any time tested.

In order to investigate the type of interaction between AOH and AME combinations, the CI-isobologram method has been applied. Results are summarized in Table 1. Figure 4 shows the fa–CI plot of mycotoxin interactions after 24 and 48 h of exposure. After 24 h, synergistic effect between AOH and AME was observed (Fig. 4a). After 48 h, synergistic effect at small fraction affected (fa) was observed, which turn into addition effect at higher fa.

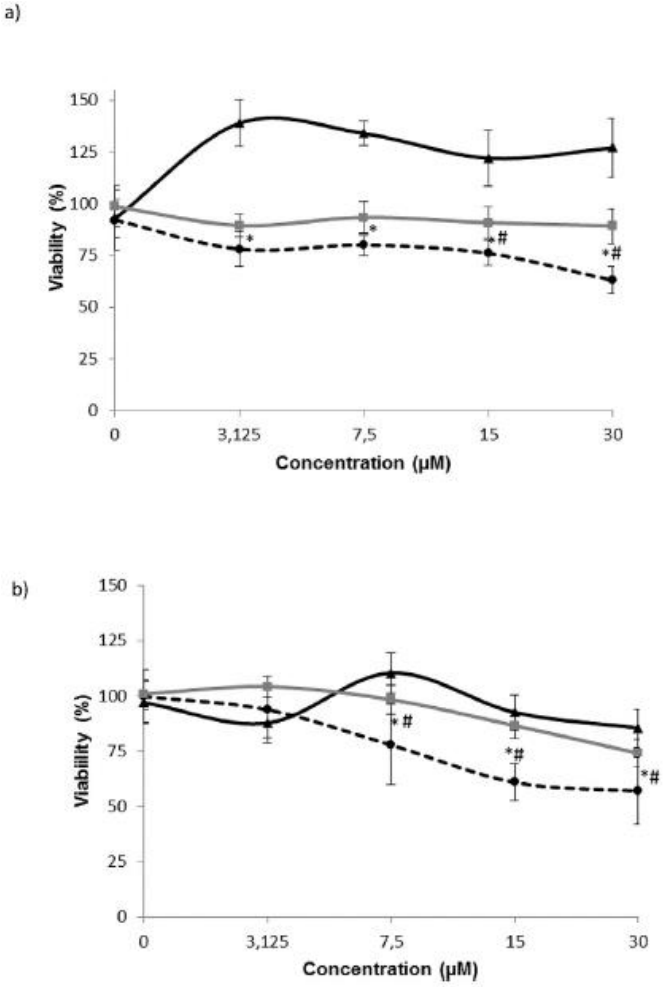

Figure. 3. Cytotoxicity effects of individual AOH (▲), AME (▪) and the binary combination AOH + AME (•) after a) 24 h and b) 48 h. AOH þ AME at molar ration of 1:1. Results of 3 independent experiments. All values are expressed as mean ± SD. p ≥ 0.05 (*) represent significant differences between the mixture and the AOH tested alone. p ≥ 0.05 (#) represents significant differences between the mixture and AME tested alone.

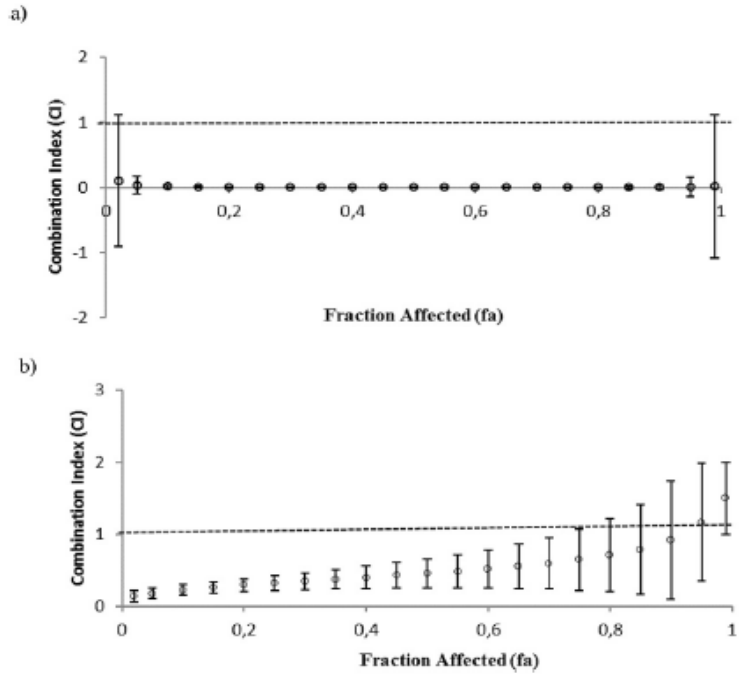

Figure 4. Combination index (CI)/fractional effect curve as described by Chou and Talalay model in Caco-2 cells exposed to AOH and AME binary combinations. Each point represents the CI ± SD at a fractional effect as determined in our experiments. The dotted line (CI = 1) indicates additivity, the area under the dotted line synergism, and the area above of the dotted line antagonism. Caco-2 cells were exposed during 24 (a) and 48 (b) h with AOH + AME at molar ratio of 1:1 equimolar proportion).

Table 1. Dose-effect relationship parameters and mean combination index (CI) values (as a function inhibition of proliferation) of binary combinations of AOH and AME on Caco-2 cells by MTT assay.

Micotoxin	Time (h)	*Dm* (μM)	*m*	*r*	CI values			
					CI_{25}	CI_{50}	CI_{75}	CI_{90}
AOH	24 h	8.915×10^5	0.52	0.9375				
	48 h	278.36	1.67	0.9159				
AME	24 h	1.567×10^5	0.25	0.9149				
	48 h	84.67	1.76	0.9230				
AOH + AME	24 h	226.97	0.35	0.8897	0.00 ± 0.01Syn	0.00 ± 0.01 Syn	0.00 ± 0.01Syn	0.00 ± 0.04Syn
	48 h	29.32	1.11	0.9586	0.32 ± 0.10 Syn	0.45 ± 0.21 Syn	0.64 ± 0.42Add	0.91 ± 0.83 Add

3.3 Cytoprotective effect of Quer

In order to study the cytoprotective effect of Quer, Caco-2 cells were exposed to the binary (AOH + Quer and AME + Quer) and tertiary (AOH + AME + Quer) combinations (Figures 5 to 7). Micotoxins and Quer were exposed at the same concentration range (3.125 to 100 μM) for 24 and 48 h (Figs. 3 and 4). As shown in Fig. 5a, the combination of AOH + Quer decreased cell viability respect to AOH tested alone (from 3.125 to 100 μM). After 48 h of exposure, similar cytotoxicity effect was observed with the combination respect to AOH tested individually (Fig. 5b). Thus, Quer only increased the cytotoxicity of AOH when is tested in combination at 24 h of exposure.

The combination of AME + Quer (Fig. 6), showed similar effect as observed when AME was incubated individually. There was no significant impact on the cell viability observed after 24 or 48 h of exposure at any of the protect Caco-2 cells against AME toxicity.

Regarding tertiary (AOH concentration tested (3.125 - 100 μM). Therefore, Quer did not + AME + Quer) combinations, significant decrease in cell viability after 24 and 48 h of exposure was observed (Fig. 7). At 24 h, the highest reduction in cell proliferation ($p \leq 0.001$) was 47% for the tertiary combination compared to AOH tested individually. No differences were observed between AME tested alone and the tertiary combination (Fig. 7a). At 48 h, significant

differences were observed; being 26% the reduction in cell proliferation between AME and the combination at 3.125 µM and 12% of reduction in cell proliferation was observed between AOH and the combination at 3.125 and 50 µM (Fig.7b). Quer didn't show any cytoprotective effect when tested simultaneously with AOH + AME at any of the concentration (3.125 - 50 µM) tested (Fig.7). Due to Quer does not protect Caco-2 cells from AOH or AME exposure, the type of interaction between there is not shown.

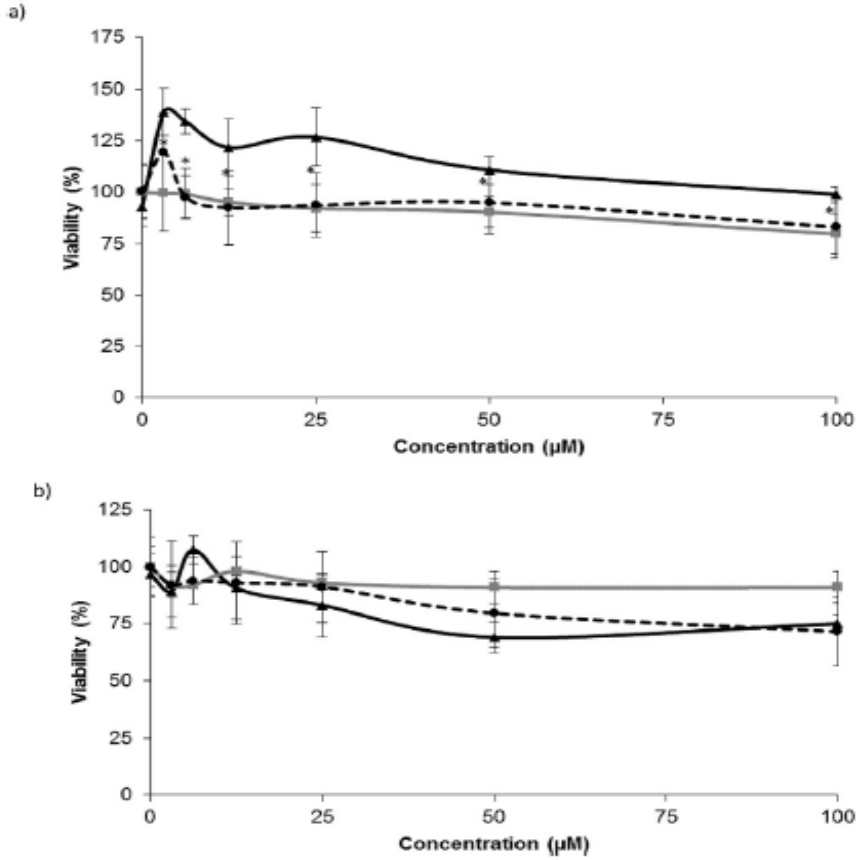

Figure 5. Cytotoxicity effects of individual AOH (▲), Quer (▪) and the binary combination AOH + Quer (•) after a) 24 h and b) 48 h. AOH +Quer at molar ration of 1:1. Results of 3 independent experiments. All values are expressed as mean ± SD. p ≥ 0.05 (*) represent significant difference between the binary combination and AOH tested alone.

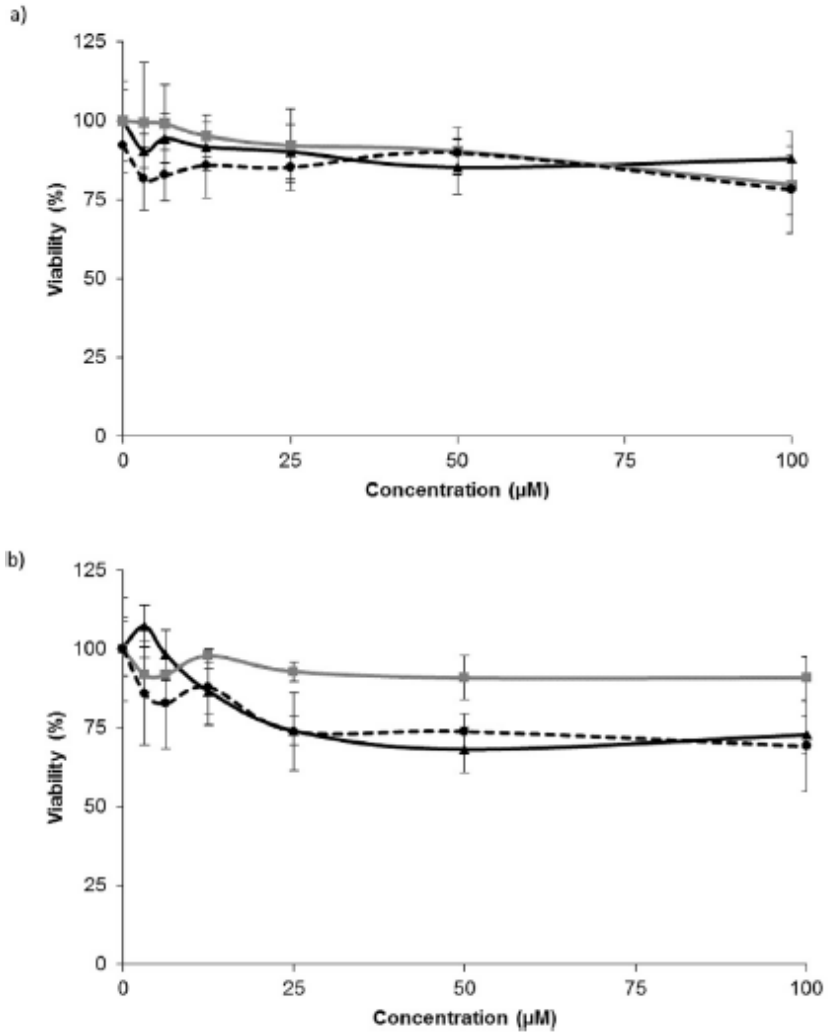

Figure 6. Cytotoxicity effects of individual AME (▲), Quer (▪) and the binary combination AME +Quer (•) after a) 24 h and b) 48 h. AME + Quer at molar ration of 1:1. Results of 3 independent experiments. All values are expressed as mean ± SD. $p \geq 0.05$ (*) represent significant difference between the binary combination and AME tested alone.

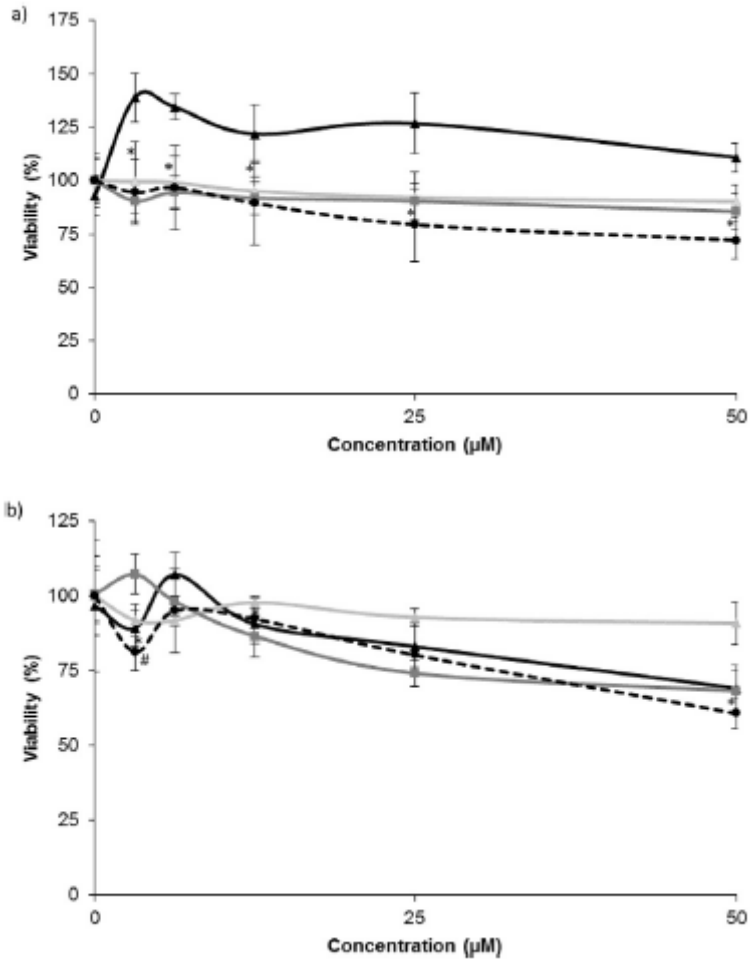

Figure 7. Cytotoxicity effects of individual AOH (▲), AME (▪), Quer (▲) and the tertiary combination AOH + AME + Quer (•) after a) 24 h and b) 48 h. AOH + AME + Quer at molar ration of 0.5:0.5:1. Results of 3 independent experiments. All values are expressed as mean ± SD. $p \geq 0.05$ (*) represents significant differences between the tertiary combination and the AOH tested alone. $p \geq 0.05$ (#) represents significant differences between the tertiary combination and the AME tested alone.

4. DISCUSSION

The consumption of fruits and vegetables in Europe and Spain is increasing due to the beneficial properties containing. In Spain is considering the vegetable intake in 47 kg/week/capita (FAO, 2011). Nevertheless, fruits and vegetables that are ingesting may contain *Alternaria* mycotoxins, which are some of the most common pathogens of postharvest (Troncoso-Rojas et al., 2009). These micotoxins are often found in combination, because of their natural co-occurrence (Bensassi et al., 2015). Moreover, AOH is expected to be extensively and rapidly absorbed from the intestinal lumen and reach the portal blood (Burkhardt et al., 2009). Although, intestinal absorption of AME appears to be poorer. Moreover, AME carry two phenolic hydroxyl groups which makes it candidate for the formation of conjugated metabolites (Hildebrand et al., 2015). Therefore, considering mycotoxin occurrence in feed and agricultural commodities (Serrano et al., 2012), it is very likely, that humans and animals are always exposed to mixtures rather than to individual compounds. For this reason, the present study evaluated the cytotoxic effects of AOH, AME, their combination and the cytoprotective effect of Quer against the two micotoxins on Caco-2 cells.

AOH and AME inhibited Caco-2 cells growth in a time-dependent manner showing a significant reduction on cells proliferation to 50 and 100 µM and from 25 up to 100 µM concentration, respectively (Fig.2). These results are in accordance with observations obtained by Juan-Garcia et al., (2015); Fernández-Blanco et al., (2014) and Tiessen et al. (2013) whose studied the cytotoxicity of AOH in HepG2(human hepatocarinoma), Caco-2 and HT-29 (human colorectal carcinoma) cells in a range of 0-100 µM concentration. Moreover, these authors neither obtained IC50 for AOH. The same pattern of AME cytotoxicity was reported by Tiemann et al., (2009) and Tiessen et al., (2013) in P4 (ovarian cells) and HT-29 cells where IC50 values were not reached. On the contrary, an IC_{50}

values of about 65 µM and 120 µM for AOH and AME was obtained in HCT116 (human colorectal carcinoma) cells by the FDA assay by Bensassi et al. (2011, and 2012).

Numerous data obtained with AOH and AME in different cell lines showed that both mycotoxins have the ability to induce oxidative stress and others cell disturbances (Chiesi et al., 2015; Fernández-Blanco et al., 2014; Tiessen et al., 2013; Bensassi et al., 2011; 2012). In contrary, there is a lack of knowledge about the possible synergistic effects of AOH and AME when they are in combination. When AOH+AME combination was evaluated, higher cytotoxicity effect was observed (Fig. 3). Our results are in concordance with Da Motta and Valente Soares (2000) who suggested a synergism effect between AOH and AME whereas Bensassi et al., (2015) who study the combination of AOH and AME (25 µM; 1:1) during 48 h observed additive effect. To corroborate the type of interaction, the isobologram method was applied in this study among AOH + AME and synergistic effect was observed. However, the nature of interactions produced by the mycotoxins combination was not uniform along the fa value (from IC25 to IC90). At 48 h, synergism predominated at low fa values but at the highest ones, interactions became additive though the combination tested. The same effects were showed in the study of Tatay et al., 2014, where zearalenone (ZEA), α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) where studied in binary combinations. Bouslimi et al. (2008) and Prospeini et al., 2014 studied the combination of *Penicillium* and Fusarium mycotoxins where ocratoxin A (OTA) in combination with citrinina (CTN) and Enniatins (A, A1, B and B1) in combination showed synergistic effect. Therefore, the toxicity of mycotoxins in combination cannot be predicted accurately by the individual effect of each mycotoxin.

Respect the cytoprotectic effect of Quer, it is important to consider that Quer acts as an antioxidant and a free-radical scavenger (Durgo et al, 2007;

Pietta., 2000). However, there is also evidence of its prooxidative action (Durgo et al, 2007; Lombardi et al., 2012). Sahu and Washington (1991) and Soria et al (2010) have found that the resultant prooxidant properties of Quer are responsible for its *in vitro* mutagenic and cytotoxic effects. Quer antioxidant and prooxidant effects largely relates to its dose in a given biological system. In this study, the highest concentration of Quer (100 µM) decreased cell viability. This result corresponds to observations by Robaszkiewicz et al (2007) and Lombardi el al. (2012), who obtained a significant decrease of cell viability at 50 µM and 100 µM Quer on A549 (human alveolar basal epithelial) and CHO-K1(Chinese hamster ovary) cells, respectively. In addition, Barcelos et al. (2011) proved the cytotoxic and cytoprotective effect of Quer exposed individually or in combination with aflatoxin B1 (AFB1) in HepG2 cells. They observed that Quer at higher concentrations (50 µg/mL and 100 µg/mL) acts as a prooxidant increasing the intracellular ROS production. Matsuo et al. (2005) showed similar cytotoxic effects for Quer (0 - 500 µM) in TIG-1 (human lung embryonic fibroblast) and HUVE (umbilical vein endothelial) cells. Nevertheless, lower doses than 100 µM caused neither cytotoxic nor cytoprotection effect when Caco-2 cells were exposed to AOH and AME in this study. The same result was observed with others mycotoxins by Hundhausen et al. (2005), and Sergent et al. (2005) who studied the effect of Quer on HepG2 and Caco-2 cells exposed to OTA; and both authors proved that Quer shows no cytoprotective effect, when cell lines were exposed to OTA. Contradictory to our results, Robaszkiewicz et al (2007) and Lombardi et al (2012) demonstrated that low concentrations of Quer stimulated cell proliferation in A549 and CHO-K1 cells, respectively. Moreover, when Barcelos et al 2011 exposed low concentrations of Quer and AFB1 on HepG2 cells, the combination was efficient reactive oxygen species (ROS) scavengers and protects HepG2 from AFB1-induced DNA strand breaks and proves that Quer could modulate CYP

isoenzymes, which are known to be involved in mycotoxin activation. Likewise, Choi et al. (2010) observed that Quer and derivatives could diminish oxidative stress, GSH depletion, and lipid peroxidation induced by AFB1 in HepG2 cells *in vitro*, while these effects could be not correlated their antioxidant activity.

In conclusion, cell viability depends on a balance between prooxidant and antioxidant compounds. High levels of antioxidants may correspond to polyphenols in food and dietary supplements which help prevent oxidative damage. According to the result obtained, AOH and AME have a slight cytotoxic effect on Caco-2 cells at the highest concentrations tested after 24 and 48 h of exposure. And, Quer at the concentration tested did not show stimulation of cell proliferation neither cytoprotection. Therefore, when Quer was simultaneously exposed in Caco-2 cells with AOH, AME or AOH+AME not cytoprotective effect was evidenced. However, the cytoprotective effect could be evidenced as demonstrated previously by other authors in cell culture with other more cytotoxic mycotoxins. Thus, foodstuffs containing Quer or metabolites of it, could contribute to reduce the health risk that mycotoxins presents in diet can produce to humans. To understand better the cytoprotective effects of Quer, more research about bioavailability, bioaccessibility, and particularly about the mechanism of action of Quer are required.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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3.7 Alternariol-induced cytotoxicity in Caco-2 cells. Protective effect of the phenolic fraction from virgin olive oil.

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Abstract

The extra virgin olive oil (EVOO) has been associated to antioxidant effects. The mycotoxin alternariol (AOH) can contaminate olives. The aims of this work were to determine the cytotoxic effects and reactive oxygen species (ROS) produced by AOH, tyrosol and oleuropein (two polyphenols of olive oil) and a real EVOO extract in Caco-2 cells. The MTT assay and the ROS production by the H_2-DCFDA probe were used. Results demonstrated that AOH reduces cellular proliferation depending on concentration, whereas tyrosol and oleuropein did not (12.5e100 µM). The combination of AOH + oleuropein (50 µM) increased cell proliferation (24%) whereas, AOH + tyrosol decreased (47%) it. Besides, AOH increased ROS generation depending on time and concentration. Oleuropein + AOH decreased ROS production. However, 25 µM of tyrosol increased 1.2-fold the ROS production. Respect to the EVOO extract, cytoprotective effect (151%) was evidenced, even with the combination EVOO extract + AOH (15 %-55 % respect to cells exposed to AOH alone). ROS generation was signifcantly reduced compared to ROS generation produced by 25 µM of AOH alone. The phenolic antioxidant of EVOO decreases cytotoxicity and ROS production in Caco-2 cells exposed to AOH. Thus, polyphenols of EVOO could contribute to diminish the toxicological risk that mycotoxins can produce to humans.

1. INTRODUCTION

Mycotoxins are biologically active secondary fungal metabolites found as contaminants of food and feed. These compounds pose a risk for disease in human and animals (Ostry, 2008). Mycotoxins are mainly produced by genera *Fusarium*, *Penicillium*, Aspergillus, *Alternaria* and Claviceps. Alternariol (AOH), a diphenolic compound produced by *Alternaria* sp, is often found as contaminant in

fruit (including olives) and cereals products (Logrieco et al., 2003). It has been demonstrated that extracts of *Alternaria* alternata are genotoxic and mutagenic *in vitro* (Solhaug et al., 2012). Therefore, A. alternata has been implicated in an elevated incidence of oesophageal carcinogenesis (Liu et al., 1991). AOH at concentration of 15-30 mM almost completely blocked cell proliferation (Solhauget al., 2012).

Olives are often affected by *Alternaria* sp. particulary if the fruits remain for a long time on the soil after ripening (Logrieco et al., 2003). Olive oil is the main fatty component of the Mediterranean diet. Extra virgin olive oil (EVOO) is a vegetable oil which can be obtained directly from olive fruit using only mechanical extraction and which can be consumed without further treatments. Its chemical composition has been regulated by Commission Regulation EC No. 1989/2003. This oil exhibits numerous biological functions, which are beneficial for the healthiness of humans (Di Benedetto et al., 2007; Driss and El-Benna., 2010). It is characterized by high percentage of monounsaturated fatty acids as well as by its elevated content in antioxidant agents (Di Benedetto et al. 2007).

On the other hand, under certain conditions, antioxidants can act as pro-oxidants. The antioxidant or pro-oxidant activity intimately depends on antioxidant concentration. The consequences of pro-oxidant activity could be the possible damage to the biomolecules such as DNA, proteins and lipids, and the consequent cellular death (Bouayed and Bohn, 2010).

Several important biological properties (antioxidant, anti- infammatory, chemopreventive and anti-cancer) and the characteristic pungent and bitter tasty properties have been attributed to EVOO phenolic compounds (Servili et al., 2009). Olive polyphenols are recognized as potential nutraceutical targets for food and pharmaceutical industries (Obied et al., 2007). Moreover, the antitioxidants present in olive oil are able to scavenge free radicals and afford an

adequate protection against peroxidation (de la Lastra et al., 2001). The main antioxidants of EVOO are polyphenols rep- resented by lipophilic and hydrophilic phenolic compounds. The lipophilic phenolic compounds, among tocopherols and tocotrienols, can be found in other vegetable oils, but the hydrophilic phenolic compounds, including tyrosol or 2-(4-hydroxyphenyl) ethanol (p-HPEA) and oleuropein, two of the most representative antioxidants present in the EVOO, are not generally present in other oils and fats (Boskou, 1996). *In vitro* methods are widely used to determine the implication of intracellular reactive oxygen species (ROS) in the cytotoxic effect produced by mycotoxins (Ferrer et al., 2009; Prosperini et al., 2013a, b). The cytoprotection effect of polyphenols in cells exposed to mycotoxins was also tested by cell culture methods (Manna et al., 1997, 2000; Lombardi et al., 2012). Among the *in vitro* systems to assess bioavailability, in recent years, the use of cell cultures have been widely employed (Sambruy et al., 2001). During the past few years, Caco-2 cells monolayer (CCM) have been widely accepted by pharmaceutical companies and by regulatory authorities as a standard permeability-screening assay for prediction of drug intestinal permeability (Grajek and Olejnik, 2004). Caco-2 model is considered as a model to study passive drug or other toxic substances absorption across the intestinal epithelium due to the good correlation obtained between data on oral absorption in humans and the results in Caco-2 model (Artursson and Karlsson, 1991; Manyes et al., 2014; Meca et al., 2012; Prosperini et al., 2012).

In this study, the Caco-2 cells were selected to determine, a) the cytotoxic effects of AOH, b) cytotoxicity of tyrosol and leuropein, in order to consider the concentration which acts as an antioxidant, c) the cytotoxic effects of the binary combination of AOH and each antioxidant selected; d) ROS generation of AOH, tyrosol and oleuropein; e) ROS generation of the binary combinations of AOH plus tyrosol and AOH plus oleuropein. And !nally, the Caco-2 cells were used to

determine the antioxidant effect of a real extract of an EVOO sample obtained from a local producer in the Umbria region (Italy) after AOH exposure.

2. MATERIALS AND METHODS

2.1 Reagents

The reagent grade chemicals and cell culture components used, mainly Dulbecco's Modfied Eagle's Medium (DMEM), penicillin, streptomycin, trypsin/EDTA solution, HEPES, thiazolyl blue tetrazolium bromide (MTT), no essential aminoacids (NEAA), Phosphate Buffer Saline (PBS), Sorensen's glicine buffer, glucose, dimethyl sulfoxide (DMSO), dichlorodihydro"uorescein diacetate (H_2-DCFDA), tyrosol (138.16 g/mol; ≥98 % purity), oleuropein (540.51 g/ mol; ≥98 % purity) and AOH (258.2 g/mol; ≥98 % purity) were SigmaeAldrich products (Sigma Co., St. Louis, Mo., USA). Foetal calf serum (FCS) was from Cambrex Company (Belgium). Deionized water (<18 MU cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were of standard laboratory grade. Stock solutions of AOH, tyrosol and oleuropein were prepared in DMSO. Final concentration of AOH, tyrosol and oleuropein in the assay were achieved by adding the culture medium. The final DMSO concentration in the medium was ≥1 % (v/v). Control cultures were exposed to the equivalent concentration of DMSO.

2.2 Cell culture of Caco-2 cells and treatment

The Caco-2 (ATCC HTB-37) cells were cultured in monolayer in 9 cm^2 polystyrene tissue culture dishes with DMEM supplemented with 25 mM HEPES, 1% NEAA, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% heat inactivated FCS. Incubation conditions were pH 7.4, 37 º C and 5% CO_2 in a 95% relative humidity atmosphere. The cells were subcultivated after trypsinization (trypsin- EDTA) once or twice per week and resuspended in complete medium in a 1:3 split ratio. Cell were subculture routinely with only a small number of sub-

passages (<70 subcultures) in order to maintain the genetic homogeneity. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (SigmaeAldrich, St. Louis, MO, USA).

2.3. Determination of cell viability by the MTT assay

Caco-2 cells were cultured into 96-well tissue-culture plates by adding 200 ml/well of a suspension of 3 % 10^4 cells/ml. After cells reached 90% confluence, the culture medium was replaced and cells were exposed to 200 ml of i) fresh medium containing different concentrations of AOH (12.5, 25, 37.5, 50, 75 and 100 mM) plus a control during 24, 48 and 72 h and ii) fresh medium containing different concentrations of tyrosol or oleuropein (12.5, 25, 37.5, 50, 75 and 100 µM) plus a control during 24 h. For the binary combinations (AOH + oleuropein and AOH + tyrosol), the cells were exposed to oleuropein or tyrosol (concentrations: 25, 50 and 100 µM) during 24 h. Afterwards, the medium containing oleuropein or tyrosol was removed and cells were exposed to AOH (12.5, 25, 50 and 100 µM) for 24 h of incubation. For the binary combinations procedure, from now on it will call pre-treatment assay. The plates were incubated for 24 h at 37 ºC, and the cytotoxicity was determined by the MTT assay.

The MTT assay determines the viability of cells by the reduction of yellow soluble tetrazolium salt (MTT), only in the metabolically active cells, via a mitochondrial-dependent reaction to an insoluble purple formazan crystal. The MTT viability assay was performed as Ruiz et al. (2006). Briefy: after exposure of AOH, tyrosol, oleuropein or the binary combinations, the medium containing these com- pounds was removed and cells of each well received 200 ml fresh medium plus 50 ml of MTT. The plates were wrapped in foil and incubated for 4 h at 37 º C. After 4 h incubation, the medium contained the MTT was removed and the resulting formazan was solubilised in DMSO. The absorbance was measured

at 570 nm using an ELISA plate reader Multiscan EX (Thermo Scientific, MA, USA). The results were expressed in relative form to cell culture protein content in order to avoid misinterpretation due to the influence of the chemical tested on cell proliferation and detachment.

2.4 Determination of intracellular reactive oxygen species by H_2- DCFDA

Intracellular ROS production was monitored in Caco-2 cells by adding the H_2-DCFDA according to Ruiz-Leal and George (2004). In Brief: 3 % 10^4 cells/well were seeded in a 96-well black culture microplate. Once cells exhibited 90% cofluence, the culture medium was replaced and cells were loaded with 20 mM H2-DCFDA for 20 min and then, the medium with H_2-DCFDA was removed and washed with PBS before the addition of AOH (15, 30 and 60 µM), AOH + tyrosol, AOH + oleuropein or control (medium with 1% DMSO). For binary combinations, cells were simultaneously incubated with tyrosol or oleuropein (1, 2.5 and 25 µM) and AOH (15, 30 and 60 µM). H_2-DCFDA is non-fluorescent until it is hydrolysed by intracellular esterases and readily oxidized to the highly fluorescent DCF in the presence of ROS. Increases in fluorescence were measured at intervals up to 2 h at excitation and emission wave- lengths of 485 and 535 nm, respectively.

To determine oleuropein and tyrosol test concentrations, several considerations were taken into account. Tyrosol and oleuropein concentrations reported were: 11.9 mg/kg olive oil (Servili et al., 2009) and 11 mg/kg olive oil (Perri et al., 1999), respectively. Taking into consideration this value and considering that olive oil consumption in Spain is 11.5 kg/year (EFSA, 2011), it could be possible to calculate the daily intake of tyrosol and oleuropein, 0.375 mg/day and 0.346 mg/day, respectively. Finally, the daily intake of oleuropein and tyrosol corresponds to the concentration tested of 2.5 and 25 µM, respectively. Therefore, to determine ROS generation, 1, 2.5 and 25 µM of AOH were tested to verify if the concentration which corresponds to the daily intake could really

protect and for comparing them. Moreover, 1 µM oleuropein and tyrosol was selected considering worse situations, i.e. countries where the consumption of olive oil is less.

2.5 Preparation of a real extract of extra virgin olive oil

An EVOO is a complex matrix which contains a great variety of antioxidant components, so the analysis of the cytoprotective effect by the *in vitro* method was carried out. The application of *in vitro* method with Caco-2 cells is very remarkable because of human studies conducted on patients fed with olive oil polyphenols showed that ingested olive oil phenols are absorbed, supporting the evidence that absorption occurs in the small intestine (Covas et al., 2010; Corona et al., 2014).

The EVOO sample was obtained from a local producer in the Umbria region (Italy) and stored at room temperature until analysis. The EVOO extract was prepared according to Gutfinger, (1981), properly modified as follow: Two grams of oil were dissolved in 1 ml hexane and the solution was extracted three times with 2 ml portions of 60% aqueous methanol. The mixtures were shaken for 2 min and combined. The obtained extract was brought to dryness in a vacuum rotary evaporator at 65 ºC. The residue was dissolved in 1 ml DMSO and stored at -20 º C until it was used.

To determine if EVOO extract alters Caco-2 proliferation cells, the MTT assay was used as previously described. For the MTT assay, the following dilution series of the extract with medium was per- formed: 1, 1/2, 1/4, 1/6 and 1/8. To investigate as the antioxidant effect of EVOO extract can inhibit cytotoxic effect on AOH-induced in Caco-2 cells, the EVOO extract and its different dilutions were simultaneously incubated with AOH (25, 50 and 100 µM). Knowing that AOH produce ROS in Caco-2 cells, EVOO extract was checked to know if it could prevent ROS generation by AOH when they were simultaneously exposed in Caco-

2 cells. For this assay, only 25 µM of AOH was selected for the mixtures according to the maximum concentration of AOH found in oilseeds in Europe (2300 ng/g olive; Logrieco et al., 2003) and according to the total daily per capita consumption of olive oil in Europe (FAO, 2009; EFSA, 2011).

2.6 Statistical analysis.

Statistical analysis of data was carried out using SPSS version 19 (SPSS, Chicago, IL, USA), statistical software package. Data were expressed as mean ± SD of three independent experiments. The statistical analysis of the results was performed by Student's t-test for paired samples. Differences between groups were analysed statistically with one-way ANOVA followed by the Tukey HDS posthoc test for multiple comparison. The level of $p \geq 0.05$ was considered statistically significant.

3. RESULTS

3.1. Effect of AOH, oleuropein and tyrosol on Caco-2 cells viability

Fig. 1 shows the chemical structures of AOH, tyrosol and oleuropein. The cytotoxic effect of AOH, tyrosol and oleuropein on Caco- 2 cells is shown in Fig. 2. To determine the molar concentration of AOH, oleuropein and tyrosol that reached 50% inhibition of cellular proliferation (IC_{50}) under assay condition. The IC_{50} values were determined graphically from the dose-response curves. Fig. 2a shows that after 24 and 48 h of incubation the AOH significantly reduced cell viability (about 40%) at the higher concentrations tested (75 and 100 µM). Similar cellular viability reduction was observed at 72 h of incubation from 50 µM to 100 µM of AOH. Moreover, AOH did not show IC_{50} value in the range of concentrations tested and the time intervals assayed (Fig. 2a). However, morphological changes induced by AOH were reduction in the cell number, being these alterations more evident at 48 and 72 h after the exposure to AOH. However, no morphological studies were performed.

The cytotoxic effect of tyrosol and oleuropein on Caco-2 cells is shown in Fig. 2b. Not cytotoxic effect of oleuropein and tyrosol was observed. And no IC_{50} values were obtained for any of the two polyphenols assayed. On other hand, they produce some stimulations of the mitochondrial function reaching 157% and 126% compared to control, for oleuropein and tyrosol, respectively.

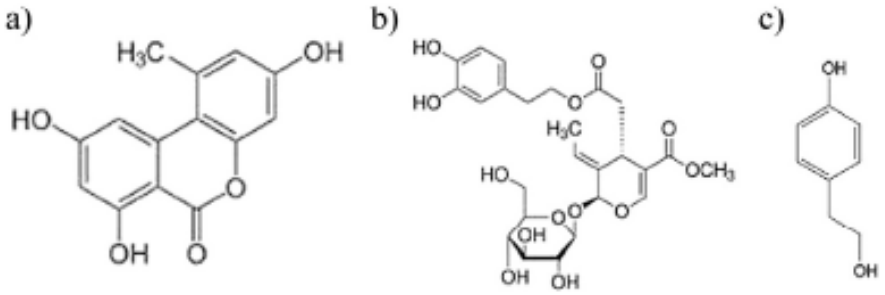

Figure 1. Chemical structure of a) alternariol, b) oleuropein and c) tyrosol

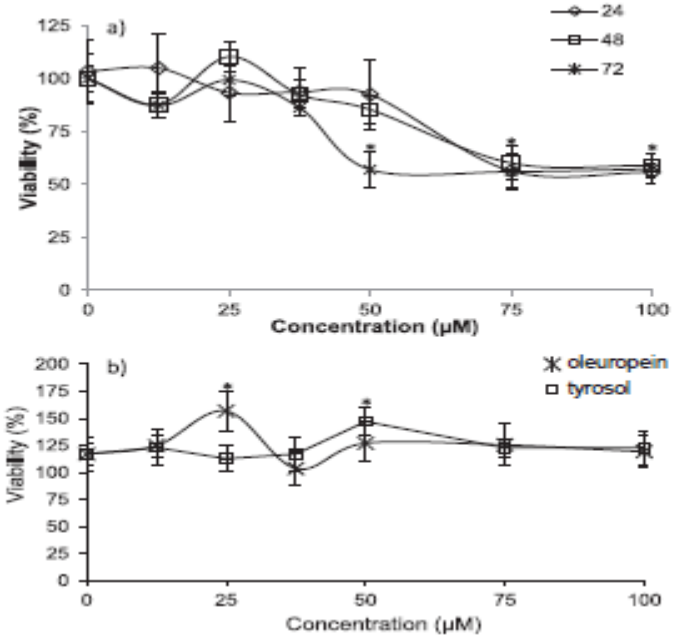

Figure 2. Cytotoxic effects of a) AOH and b) oleuropein and tyrosol in Caco-2 cells after 24 h of exposure by the MTT assay. Each point represents the mean value of at least three experiments. An asterisk indicates a significant difference from control value ($p \geq 0.05$).

3.2. Intracellular ROS production

The ability of AOH to produce ROS and consequent oxidative damage was also evaluated. To determine the changes in the redox status in response to AOH, Caco-2 cells were exposed to different concentrations of this mycotoxin from 0 to 120 min (Fig. 3). The increase of ROS production was time- and concentration dependent, when compared to the basal rate. These results indicated that high production of oxidizing species was produced immediately after AOH exposure.

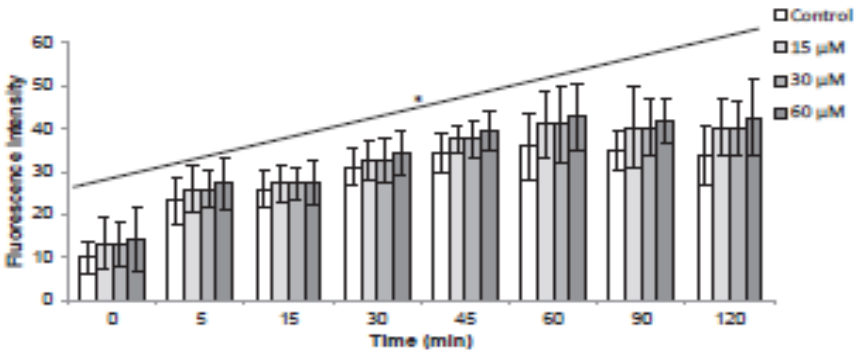

Figure 3. Time dependence of ROS-induced fluorescence in Caco-2 cells exposed to AOH. Caco-2 cells were loaded with H2-DCFDA for 20 min in 96-well plates (30,000 cells per well) and then exposed to AOH (15, 30 and 60 mM) or control (1% DMSO). Fluorescence of oxidized DCF was followed by emission at 535 nm and the excitation of 485 nm. Mean ± SD, 24 replicates. (*p ≥ 0.05) different significantly from the control.

3.3. Cytoprotection and antioxidant activity of tyrosol and oleuropein

To investigate whether oleuropein and tyrosol can inhibit cytotoxic effect AOH-induced in Caco-2 cells, fresh medium containing simultaneously the phenolic compound (concentration 25, 50 and 100 µM) and AOH (12.5, 25, 50 and 100 µM) plus a control (1% DMSO) were assayed. The effects of these binary

combinations on the Caco-2 cells proliferation using the MTT assay is showed in Fig. 4.

As shown in Fig. 4a after 24 h of incubation no significant differences were observed when AOH was simultaneously exposed with 25 µM oleuropein respect to AOH tested alone. However, when Caco-2 cells were simultaneously incubated with AOH and 50 µM oleuropein a slight cytoprotection (24%) was observed. Fifty mM oleuropein prevented cell damage induced by 100 µM of AOH respected AOH tested alone. However, 100 µM oleuropein incubated with 12.5 and 50 µM of AOH, decreased cell proliferation about 20% and 10%, respectively respect to AOH tested alone (Fig. 4a). In relation to tyrosol, no differences were observed when AOH and tyrosol were simultaneously exposed in Caco-2 cells over all concentration tested (Fig. 4b). Moreover, the lowest concentration of tyrosol simultaneously exposed with 100 µM AOH is not able to protect Caco-2 cells producing a significantly increase in the cytotoxic effect. With this binary combination, cell proliferation decrease about 47% respects to AOH tested alone (Fig. 4b). Even with this combination, an IC_{50} value of 70 µM was obtained (Fig. 4b).

On the other hand, to assay the capacity of oleuropein and tyrosol to protect Caco-2 cells from AOH-mediated oxidative injury, the cells were incubated in the presence of each phenolic com- pound (1, 2.5 and 25 µM concentration) simultaneously with AOH (Figs. 5 and 6). As can be observed in Fig. 5, after combinations of AOH (15, 30 and 60 µM) and oleuropein (1, 2.5 and 25 µM) not ROS were produced when compared to the basal rate. The oleuropein could serve as ROS scavenger. Similarly, the treatment of Caco-2 cells with AOH (15, 30 and 60 µM) and tyrosol (1 and 2.5 µM) prevented ROS production. However, when AOH (30 and 60 µM) was simultaneously exposed with 25 µM of tyrosol a slight production of ROS was produced at 5 min which is maintained up

to 120 min respect to 0 min of exposure. The highest relative intensity of fluorescence observed in Caco-2 cells was about 1.2-fold higher than the basal rate (Fig. 6c).

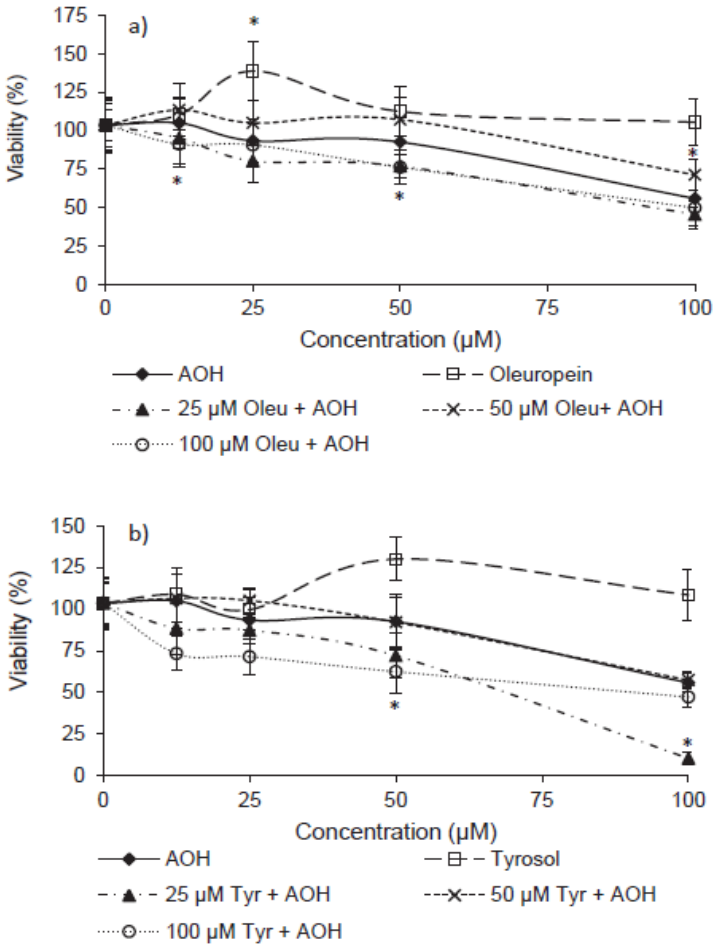

Figure 4. Protective effect of (a) oleuropein and (b) tyrosol in Caco-2 cells exposed to AOH (12.5, 25, 50 and 100 μM concentration): Cells were pre-treated with oleuropein and tyrosol (25, 50 and 100 μM) during 24 h. Thereafter, AOH was exposed during 24 h and viability of these cells was measured using the MTT assay. An asterisk indicates a significant difference from control value ($p \geq 0.05$).

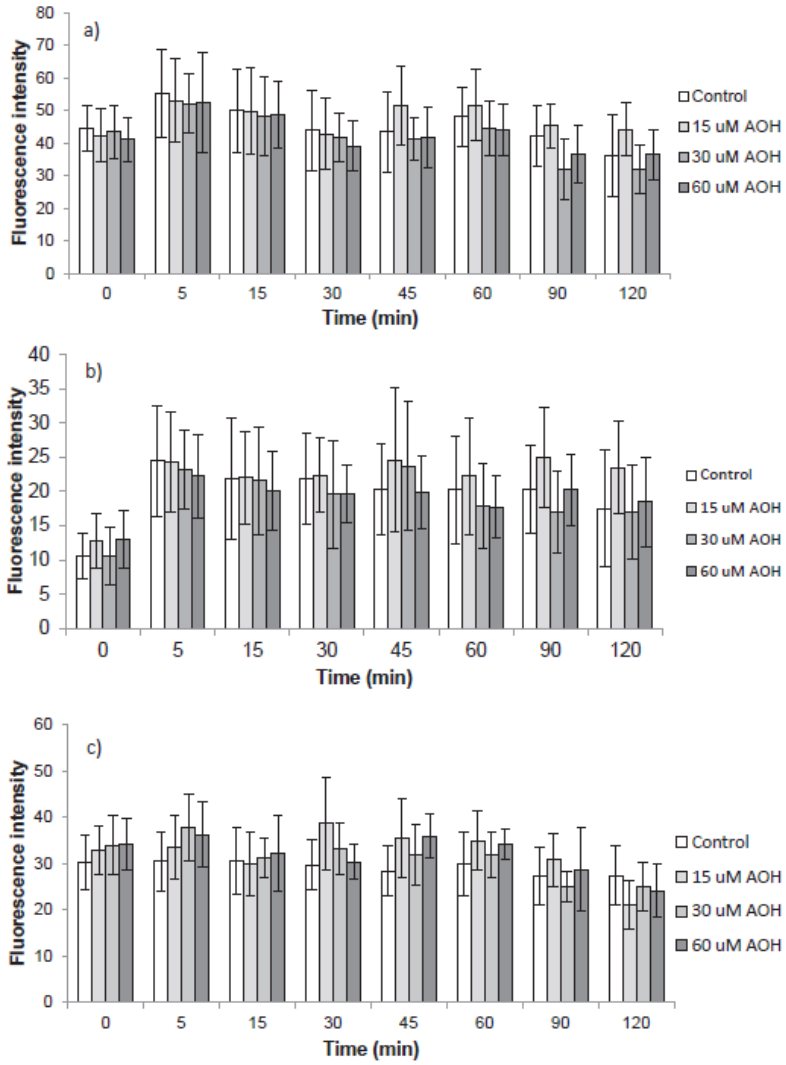

Figure 5. Time dependence of ROS-induced fluorescence in Caco-2 cells exposed to AOH (15, 30 and 60 µM) simultaneously with oleuropein a) 1 µM, b) 2.5 µM and c) 25 µM. Caco-2 cells were loaded with H_2-DCFDA for 20 min in 96-well plates (30,000 cells per well) and then exposed to the binary combination (AOH + oleuropein) or control (1% DMSO). AOH was tested alone at the same concentrations and time of exposure (see Fig. 3). Fluorescence of oxidized DCF was followed by emission at 535 nm and the excitation of 485 nm. Mean ± SD, 24 replicates. (*$p \geq 0.05$) different significantly from the control.

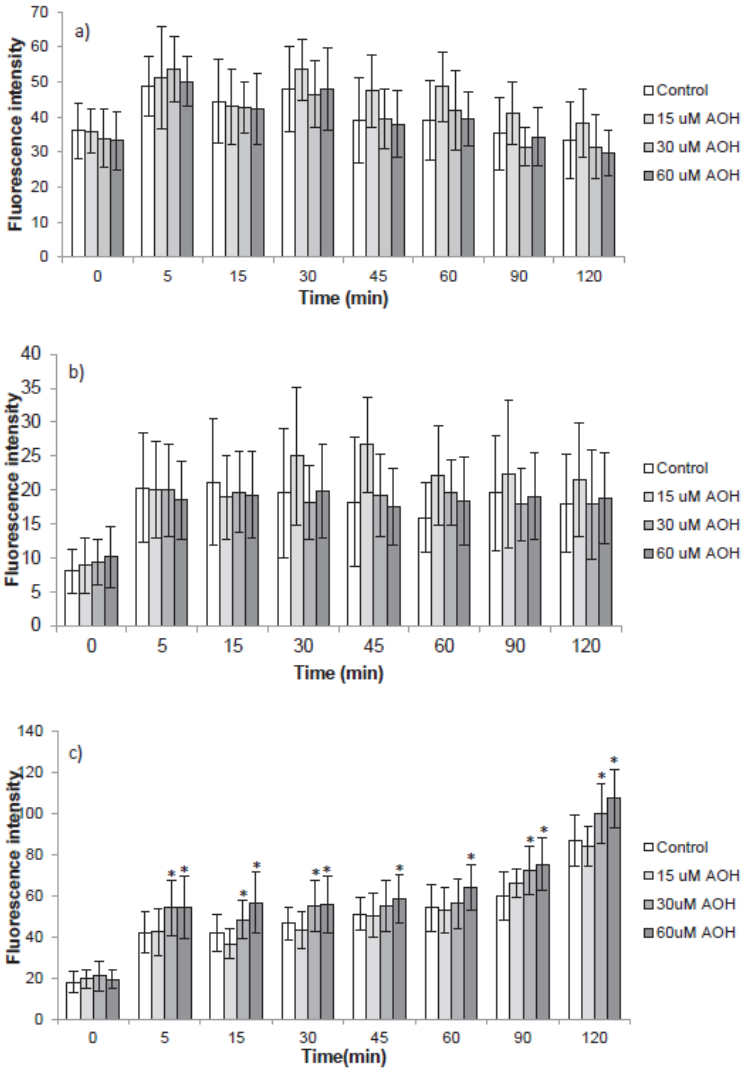

Figure 6. Time dependence of ROS-induced fluorescence in Caco-2 cells exposed to AOH (15, 30 and 60 µM) simultaneously with tyrosol a) 1 µM, b) 2.5 µM and c) 25 µM. Caco-2 cells were loaded with H_2-DCFDA for 20 min in 96-well plates (30,000 cells per well) and then exposed to the binary combination (AOH + tyrosol) or control (1% DMSO). AOH was tested alone at the same concentrations and time of exposure (see Fig. 3). Fluorescence of oxidized DCF was followed by emission at 535 nm and the excitation of 485 nm. Mean ± SD, 24 replicates. (*p ≥ 0.05) different significantly from the control.

3.4 Effect of a real extract of EVOO in Caco-2 cells exposed to AOH

Fig. 7 shows the effect of a real extract of EVOO on Caco-2 cells proliferation evaluated by the MTT assays over 24 h of exposure. As can be observed, cell viability (%) increased after all dilutions of the EVOO extract tested (Fig. 7); it means that cell proliferation increased more than the number of cells in control showing a protective effect of EVOO extract in Caco-2 cells. The highest protective effect reached 151% (extract dilutions 1/2, 1/4 and 1/6) versus control (100%; Fig. 7). The effect of the simultaneous combination of the EVOO extract (and its dilutions, ½, ¼, 1/6, 1/8) and AOH (25, 50 and 100 µM) are shown in Fig. 7. As shown in Fig. 7, when AOH and the EVOO extracts were simultaneously exposed in Caco-2 cells cytoprotective effect was observed respect to AOH tested alone. Simultaneous exposition of the EVOO extract and 25 or 50 µM of AOH increased (from 24 to 55%) cell proliferation respect AOH tested alone (Fig. 7). Smaller increase in cell proliferation was obtained with the mixtures of 100 µM of AOH plus EVOO extracts (Fig. 7). The combinations of 100 µM AOH plus EVOO extracts only increased significantly Caco-2 cell proliferation with ½-dilution extract (15%) and the EVOO extract without dilution (36%) respect to AOH tested alone (Fig. 7).

As shown in Fig. 8, the increase of ROS generation was produced in Caco-2 cells after 25 µM of AOH exposure. Twenty-five µM of AOH increased ROS generation in time dependent manner, when compared to the basal rate. Similar effect was observed when 25 µM of AOH was simultaneously exposed with 1/8 dilution EVOO extract (Fig. 8) from 45 up to 120 min of incubation. However, when 25 µM of AOH was simultaneously exposed with EVOO extract without dilution, ROS generation was signifcantly reduced (from 5 to 120 min) compared to ROS generation produced by 25 µM of AOH tested alone (Fig. 8). So, EVOO

extract could be considered as a potential protector against a condition of oxidative stress generated by AOH.

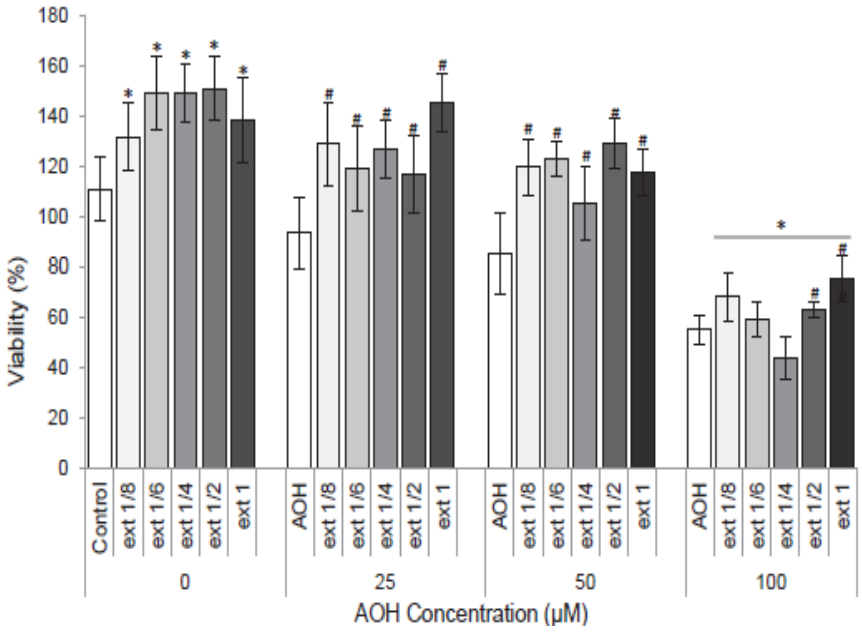

Figure 7. Protective effect of EVOO extract dilutions in Caco-2 cells exposed to AOH (25, 50 and 100 μM concentration). Cells were pre-treated with EVOO extract dilutions (1, 1/2, 1/4, 1/6 and 1/8) during 24 h. Thereafter, AOH was exposed during 24 h and viability of these cells was measured using the MTT assay. (*p ≥ 0.05) different significantly from the control. (#p ≥ 0.05) different significantly from each own EVOO extract without AOH treatment.

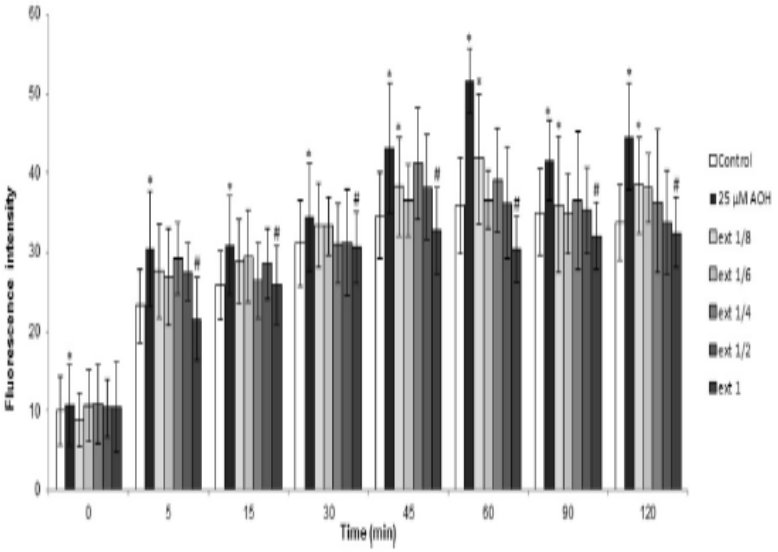

Figure 8. Time dependence of ROS-induced fluorescence in Caco-2 cells exposed simultaneously to 25 µM AOH and EVOO extract dilutions (1, 1/2, 1/4, 1/6 and 1/8). Caco-2 cells were loaded with H_2-DCFDA for 20 min in 96-well plates (30,000 cells per well) and then exposed to 25 µM AOH, extract dilutions+25 µM of AOH or control (1% DMSO). Fluorescence of oxidized DCF was followed by emission at 535 nm and the excitation of 485 nm. Mean ± SD, 24 replicates (*p≥0.05) different significantly from the control.

4. DISCUSSION

The resorcyclic lactone AOH represents one of the major secondary metabolites produced by *Alternaria* alternate. This mycotoxin can be found in foods and feeds and represents a health risk for humans and animals (EFSA, 2011). The present study investigated the cytotoxic effects of the AOH in Caco-2 cells. Our results demonstrated that AOH decrease cell viability and proliferation in Caco-2 cells at higher concentrations (>50 µM). However, there is a wide variation in sensitivity to *A. alternata* mycotoxins among cell lines depending on the tissue of origin and possible the degree of differentiation.

Wollenhaupt et al. (2008) demonstrated that AOH (from 3.12 to 12.5 µM) inhibits the metabolic activity of porcine endometrial cells as determined by the MTT assay. Tiemann et al. (2009) shown that the cell viability measured as the activity of mitochondrial dehydrogenases was more sensitive to toxic effects of AOH (from 1.6 to 12.8 µM) in porcine granuloma cells than the number of cells (relative to untreated controls), suggesting that the AOH inhibits metabolic activity and proliferation rather than to promote cell death. Similar results were obtained by Tiessen et al. (2013). They found that AOH (from 0.1 to 50 µM) did not produced IC_{50} in HT29 cells neither using the SRB assay nor the WST-1 assay after 24 h of incubation. However, about 10-15% cell viability reduction was observed at concentration of 10 µM or higher. Brugger et al. (2006) determined the number of viable cells with an electronic cell counter as a measure of cytotoxicity and proliferation. They found a concentration-dependent reduction of viable cells after AOH treatment with up to 30 µM (V79 cells) or 20 µM (MLC) AOH, which reduced the number of cells to approximately 35% and 69%, respectively. Bensassi et al. (2012) demonstrated that the human colon carcinoma (HCT116) cells are sensible to AOH. They demonstrated that AOH induced a decrease in HCT116 cell viability in a dose-dependent manner. Moreover, they found an IC_{50} value of 65 µM after 24 h of exposure.

Although cytotoxicity of AOH is low compared to other prominent mycotoxins, its relevance in the context of fruit (including olives) and cereal products has led to considerable interest in public health. On the other hand, virgin olive oil (VOO) is one of the components of the Mediterranean diet, which is considered an important source of phenolic compounds like hydroxytyrosol, tyrosol, oleuropein, ligstroside, lignans (Driss and El-Benna., 2010). The presence of potent antioxidant compounds in the VOO has contributed to the increasing interest of scientists to investigate the possible preventive effect

of the Mediterranean countries, associated high oil consumption, on chronic-degenerative diseases such as cardiovascular diseases and cancer. Antioxidants have been often associated with cytoprotective effects.

In this study, tyrosol and oleuropein do no decrease cell viability in small-intestinal epithelial Caco-2 cells. Bulotta et al. (2011) analysed the effect of oleuropein in two human breast cancer cells (T-47D and MCF-7 cells) during 72 h of incubation. They proved that oleuropein caused a dose-dependent inhibition of cells proliferation in T-47D cells, with a maximal reduction evidenced at 100 µM (aprox. 40%). Whereas, no alteration in MCF-7 cell viability was produced by oleuropein. Tyrosol is a simple phenol and oleuropein is a phenolic secoiridoid glycoside, and both of them identified in VOOs. Moreover, secoiridoids comprised about 50-70% of the total phenolic derivates (Gioffi et al., 2010; Bulotta et al., 2011). Once the absence of cytotoxic effect was demonstrated, the cytoprotection effect in cells treated with the AOH in concentration within the daily intake range was assessed. From all concentrations of tyrosol and oleuropein tested, only 50 µM of tyrosol prevented cell cytotoxicity (Fig. 4a) induced by AOH (increasing Caco-2 cells viability respect to AOH exposure) indicating that tyrosol suppresses the AOH-induced cytotoxicity. These findings proved that tyrosol was higher effective in cell cytoprotection than oleuropein. And, can be corroborated by Manna et al. (2000), whom demonstrated that the transport of tyrosol and other tyrosol derivates occurs via a passive diffusion mechanism. And, that uptake is linear in the 50-100 µM range at 37 ºC. Furthermore, discoveries of Di Benedetto et al. (2007) support this evidence because they evaluated the intracellular content of tyrosol with time, demonstrating that tyrosol was detected early and showed a time-dependent intracellular storage, doubling its concentration from 5 min to 18 h. However, oleuropein can to be absorbed in small intestine, albeit poorly. Therefore, to be highly absorbed oleuropein has to

be degraded into hydroxytyrosol as the major end product (Covas et al., 2010; Corona et al., 2014).

On the other hand, the AOH produces ROS at all concentrations tested leading to oxidative stress in Caco-2 cells. Oxidative stress produced by AOH might be one mechanism of the cytotoxicity of this mycotoxin. Correspondingly, Tiessen et al. (2013) found that AOH (10, 25 and 50 µM) enhances ROS production in HT29 cells incubated for 1 h. Analogously, Schwarz et al. (2012) demonstrated that the treatment of HT29 cells with AOH resulted in an increase of the DCF signal, up to 2.5-fold at 50 µM. This increase was significant from 10 µM of AOH. Bensassi et al. (2012) demonstrated that ROS produced in response to AOH in HCT116 cells appears to be mainly mitochondrial superoxide anion. On the other hand, Schreck et al. (2012) no significant differences in ROS-levels observed between untreated cells and cells treated with 20 and 40 µM of AOH and 1 or 2 h after incubation with H_2DCF-DA.

Tyrosol and oleuropein showed protective effect against the AOH-induced oxidative injury to the intestinal Caco-2 cells immediately after the addition of the two olive oil polyphenols (Figs. 5) and 6). Driss and El-Benna (2010) reported that hydroxytyrosol (structurally related phenol to tyrosol present in olive oil) can serve as scavenger of aqueous peroxyl radicals near the membrane surface, while oleuropein acts as a scavenger of chain-propagating lipid hydroxyl radicals within membranes. Bulotta et al. (2011) observed that exposure of T-47D and MCF-7 cells to H_2O_2 (250 µM) in presence of oleuropein (10 and 100 µM) reduced ROS generation respect to cells without oleuropein. Furthermore, Gioffi et al. (2010) demonstrated the moderate scavenging activity of oleuropein in Caco-2 cells, against superoxide anion, one of the most aggressive ROS products. Equal results were obtained by Di Benedetto et al. (2007) in J774 A.1 cells, where tyrosol was slightly effective at counteracting the early production of super- oxide anion

radical (within 12 h of exposure) and hydrogen peroxide (after 24 h). However, pre-treatment of J774 A.1 cells with tyrosol for 2 h and then removed from the culture medium before oxidant compound addition, appeared highly effective, because of complete inhibition of ROS rise (at 6 h of incubation). These results suggest that the effectiveness of tyrosol depend on its capability to penetrate the cells. Similarly to our results, they demonstrated that when cells were exposed to oleuropein (250 µmol/l) an increase in cell viability respect to the positive oxidant control (10 µmol/l of H_2O_2) was observed. So, oleuropein suppresses the H_2O_2-induced toxicity.

The antioxidant activity of EVOO extract was determined with the same procedure described previously. The EVOO extract showed cytoprotection in Caco-2 cells (Fig. 7). Isik et al. (2012) determined the effects of olive extracts on Caco-2 cell viability and proliferation. They found that 1/20 dilution extract reduce significantly the Caco-2 cells viability without cytotoxicity while low concentrations increased cell growth in a time-dependent manner. And, when the extracts were simultaneously exposed with AOH in Caco-2 cells, these cytoprotection was maintained at the lower AOH concentrations tested (Fig. 7). However, 100 µM of AOH blocks the cytoprotection function of the more diluted EVOO extract, showing no differences with the cytotoxic effect produced by AOH (Fig. 7). Similarly, EVOO extract (without dilution), which is the highest polyphenol-rich extract tested, showed to be a potent scavenger of ROS produced by AOH in Caco-2 cells (Fig. 8). The EVOO extract protected epithelial cell line Caco-2 cells from ROS- induced cytotoxicity. Thus, these findings demonstrated that EVOO extract possess antioxidant/free-radical scavenging proper- ties, which are very likely due to the presence of high contents of phenolic compounds. The results were in agreement with those of other authors whose revealed that olive oil phenolic compounds are effective scavengers of hydroxyl, superoxide and

peroxyl radicals (Gioffi et al., 2010; Driss and El-Benna, 2010; Sarria et al., 2012). Moreover, olive polyphenols are recognized as potential antioxidant additives for food, dietary supplements, functional foods and natural cosmetics as well as for pharmaceutical industries (Obied et al., 2007; Bulotta et al., 2011). In conclusion, the protective effect of cells against cytotoxic compounds depends on a balance between antioxidants and pro-oxidant components. Increased levels of antioxidants may respond to polyphenols in food commodities (as olives, olive oil, wine, etc.) and dietary supplements (oleuropein-rich diets, etc.) which have been studied extensively to improve antioxidant reserve and thereby help regulate oxidative damage. Our data also provide experimental support for the hypothesis of the key role played by phenolic antioxidant fraction of olive oil, thus contributing to the positive effect of olive oil in lowering the risk of ROS in Mediterranean diet.

The results obtained shown that olive oil phenols improve antioxidant defence system. Thus, food commodities containing polyphenols (specially, EVOO) could contribute to diminish the toxicological risk that natural contaminants in diet, as mycotoxins, can produce to humans.

Ethical statement

The authors have obeyed all the standards of ethical behaviour in relation to: Reporting standards; Data Access and Retention; Originality and Plagiarism; Multiple, Redundant or Concurrent Publication; Acknowledgement of Sources; Authorship of the Paper; Hazards and Human or Animal Subjects (not any used); Disclosure and Conflicts of Interest; Fundamental errors in published works.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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3.8 Effects of soyasaponin I and soyasaponins-rich extract on the Alternariol-induced cytotoxicity on Caco-2 cells.

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Abstract

Alternariol (AOH) is a mycotoxin produced by Alternaria spp. Soyasaponin I (Ss-I) is present naturally in legumes, and it has antioxidant properties. Cytotoxic and genotoxic effects of AOH have been demonstrated previously *in vitro*. In the present study, the cytotoxicity of AOH, Ss-I, and soyasaponins-rich extract from lentils was investigated; as well as, the cytoprotective effects of Ss-I and lentil extracts against AOH induced-cytotoxicity on Caco-2 cells. Cytotoxicity was carried out using MTT and PC assays (AOH: 3.125–100 μM, Ss-I: 3.125–50 μM, and lentil extracts: 1:0–1:32) during 24 h of exposure. Only AOH showed cytotoxic effect. The reduction in cell proliferation ranged from 25% to 47%. Simultaneous combination of Ss-I with AOH (1:1) increased cell proliferation (35%) compared to AOH tested alone. The Ss-I and extracts showed synergistic cytoprotective effects against cytotoxicity induced by AOH on Caco-2 cells. Food commodities containing Ss-I could contribute to diminish the toxicological risk that natural contaminant as AOH in diet can produce to humans.

1. INTRODUCTION

Saponins are triterpene glycosides that occur in a wide variety of plants. Group B of soyasaponins, the predominant form of saponins, are found principally in legume seeds. In particular, Soyasaponin I (Ss-I), a major constituent of group B of soyasaponins, has been shown to possess hypocholesterolemic, anticarcinogenic and hepatoprotective properties and antioxidant activity (Gurfinkel and Rao, 2003; Kang et al., 2010; Lee et al., 2005; Yang et al., 2011). In analytical work previously developed in our labs, Ss-I was identified in several kinds of legumes such as chickpeas, beans, peas, and lentils. The Ss-I content ranged from 220 to 907 mg kg−1 (Sagratini et al., 2009; Sagratini et al., 2013; Vila-

Donat et al., 2014). The Mediterranean basin is a large geographical region with a temperate climate and a diversified agricultural system that includes legumes, cereals, vegetables, fruits and oil crops. Among European countries, higher legume consumption is observed around the Mediterranean, with per capita daily consumption between 8 and 23 g (Bouchenak and Lamri-Senhadji, 2013). Moreover, lentils (legumes) are recognized by the European Union as Protected Geo- graphical Indication (PGI) in the Marche-Umbria regions from Italy (Sagratini et al., 2009). According to food balance sheets obtained by Food and Agriculture Organization of United Nations revised lastly in 2011, the total daily per capita consumption of legumes in Italy was 13 g (FAOSTAT, 2011).

On the other hand, pathogenic fungi may infect legumes and cause rotting or produce toxic secondary metabolites as mycotoxins in the colonized crop. Mycotoxins produced may persist in stored products and finally in the manufactured products. Thus mycotoxins have a significant economic impact and pose serious problems for human health (Ostry, 2008; Visconti et al., 1986). Alternariol (AOH) is the main mycotoxin produced by *Alternaria* alternata fungi, which represents a high mycotoxicological risk in harvested plants (Logrieco et al., 2003). In fact, AOH has been found in considerable concentrations in a wide variety of grains and grain-based products, vegetables, fruits, and oilseeds (Ostry, 2008; Scott, 2001). Even, AOH has been found in legumes such as soybean, and lentils (Ostry et al., 2004; Pavón Moreno et al., 2012). According to published data on mycotoxin occurrence in feed and agricultural commodities in Europe, AOH was found in a range from 6.3 to 1840 µg/kg of tested samples (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2011). Despite the incidence of AOH in food, there is neither European nor International legislation to date.

Related to toxicity of AOH, genotoxicity of AOH has been previously demonstrated as *in vitro* (Brugger et al., 2006; Fleck et al.,2012; Solhaug et al.,

2012) and as in vivo (Yekeler et al., 2001). AOH also produces oxidative stress by increasing ROS production and LPO generation in mammalian cells (Fernández-Blanco et al., 2014; Tiessen et al., 2013).

On the other hand the antioxidant properties of group B of soyasaponins have been studied with *in vitro* cells (Gurfinkel and Rao, 2003; Kang et al., 2010) and in animals sub- jects (Francis et al., 2002; Yang et al., 2011). However, to our best knowledge, no studies about cytotoxicity of Ss-I and lentil ex- tracts combined with AOH have been performed up to now. In the present work, the cytoprotective effect of Ss-I present in high concentrations in legumes against a natural contaminant, such as AOH, present in the food commodity has been investigated. For this purpose, Caco-2 cells derived from a human colon carcinoma were employed; since the oral intake of AOH through contaminated food is the main route of exposure for animals and humans. Moreover Caco-2 cells are considered target human cells of the digestive system. The *in vitro* total protein content (PC) and MTT assays are usually employed for determining cytotoxicity of toxic substances in a wide variety of mammalian cells (Pichardo et al., 2007; Ruiz et al., 2011). To assess the cytoprotective effects of Ss-I, and soyasaponins-rich extract, they were simultaneously exposed with AOH on Caco-2 cells. Finally, the interaction of Ss-I + AOH mixture was evaluated in order to find out whether they could interact among themselves and produce synergistic, additive or antagonistic effect.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

The reagent grade chemicals and cell culture components used, Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, HEPES, 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), non-essential

amino acids (NEAA), phosphate buffer saline (PBS), sodium pyruvate, dimethyl sulfoxide (DMSO) were from Sigma Chemical Co (St. Louis, MO, USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Deionized water (resistivity <18 MΩ cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other standards were of standard laboratory grade.

Pure standard of Ss-I (C48H78O18; molecular weight: 943.12; purity >97%) was purchased from Tauto Biotech Co (Zhangjiang High-Tech Park, Shanghai, P.R. China). Ss-I stability was checked routinely using HPLC-MS/MS Agilent 1290 Infinity Series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA, USA). AOH (C14H10O5; molecular weight 258.23 g/mol; purity >96%) was from Sigma Chemical Co. Stock solutions of Ss-I and AOH were prepared in DMSO and stored in glass- stoppered bottle at −18 °C. Final concentrations of Ss-I and AOH were achieved by adding the culture medium. The final DMSO concentration in medium was ≤1% (v/ v). Total protein content (µg/mL) was determined by Bradford Method (Bio-Rad DC Protein Assay (catalogue number 500-0116)) (http://www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf).

2.2 Cell culture and treatment

Caco-2 cells are continuous lines of heterogeneous human epithelial colorectal adenocarcinoma cells. Caco-2 cells from American Type Culture Collection (ATCC HTB-37) were grown in basic culture medium in tissue culture flasks. Cell cultures were kept at 37 °C, pH 7.4, in a 5% CO2/95% air atmosphere. These cells were routinely maintained in culture 75 cm2 plastic flasks in DMEM medium supplemented with 25 mM HEPES buffer (pH 7.4), 1% of NEAA, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% (v/v) FCS inactivated and 1 mM sodium pyruvate. Absence of mycoplasma was checked routinely using the mycoplasma stain kit (Sigma- Aldrich, St. Louis, MO, USA).

2.3 Lentil extracts preparation

Lentil extracts were prepared according to Vila-Donat et al. (2014) with some modifications. Summarized, 2.5 g of lentil samples provided by Fertitecnica (Colfiorito, Italy) were ground using a Taurus coffee grinder (Taurus group, Spain).

The obtained lentil flour was extracted for 3 h under magnetic stirring with 25 mL of a solution H2O/EtOH (70/30) at room temperature. The mixture was then filtered by paper filter and the ethanol was evaporated by Buchi Rotavapor R-200 (Postfach, Switzerland). The extracts were stored in glass-stoppered bottle at −18 °C. Lentil extracts dilutions were performed with ultrapure water. The final dilutions obtained were from 1:2 to 1:32 (v/v).

2.4 Cytotoxicity

Cytotoxicity was determined by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl- 2H-tetrazolium bromide (MTT) and total protein content (PC) assays. The MTT assay is based on the ability of viable cells to metabolize in the mitochondria the yellow tetrazolium salt to a formazan product (purple color). MTT assay was performed as described previously by Ruiz et al. (2006) with some modifications. Caco-2 cells were cultured in 96-well tissue culture plates by adding 200 µL/well of suspension of 3 × 104 cells/well. After cells reached 90% of confluence, culture medium was replaced with fresh medium containing five serial dilutions (serial dilution factor = 2) of AOH (3.125 to 100 µM), four serial dilutions of Ss-I (3.125 to 50 µM) or five serial dilutions of lentil extracts (1:0 to 1:32) and maintained during 24 h. After that, the medium was removed, the wells were washed with PBS and each one received 200 µL of fresh medium containing 50 µL of MTT. After 4 h of incubation (37 °C in darkness), the resulting formazan was solubilized with 200 µL of DMSO and 25 µL of Sorensen's glycine buffer. The absorbance was measured at 570 nm using an automatic ELISA plate reader MultiscanEx (Thermo Scientific, MA, USA).

The PC assay was carried out according to Pichardo et al. (2007). The PC gives data about cell damage independently of the toxic mechanism involved. PC was analyzed after 24 h in situ, in the same 96-well culture plates in which MTT took place. First the plates were washed with PBS, and 200 µL of NaOH were added in order to dissolve the proteins. After 2 h of incubation at 37 °C, 170 µL of NaOH were removed from each well and 180 µL of Coomassie Brilliant Blue were added. After 30 min of incubation at room temperature, the absorbance at 620 nm was measured using an automatic ELISA plate reader MultiscaEx (Thermo Scientific). Cell viability (for MTT and PC assays) was expressed in percent relative to control cells (1% DMSO for AOH and Ss-I or 1% EtOH for lentil extracts). Mean inhibition concentration (IC50) values were calculated from full dose–response curve.

2.5 Cytoprotective effects of Ss-I and soyasaponins rich-extracts

The effects of pre-incubation of Ss-I in Caco-2 cells during 24 h were evaluated. Briefly, cells were seed in medium with 6.25 µM of Ss-I. After 24 h, the medium was removed and replaced with fresh medium containing five serial dilutions of AOH (3.125–50 µM). After 24 h, the MTT assay was performed as previously described. This procedure is called "pre-treatment assay" throughout the manuscript.

To determine the interactive effect between AOH and Ss-I or lentil extract, the MTT and PC assays were used. Both, AOH + Ss-I or AOH + lentil extract were incubated at the same time during 24 h in Caco-2 cells. For the combination of AOH + Ss-I: once the cells reached 90% of confluence, the culture medium was replaced by fresh medium with a) AOH (3.125–25 µM) + Ss-I (3.125–25 µM) or b) 15 µM of AOH + 6.25 µM of Ss-I. After 24 h the MTT and PC content was measured. The concentrations appointed last test were selected considering the range of Ss-I intake (3–12 mg) according to food balance sheets obtained by

FAOSTAT (2011), and results obtained by Sagratini et al. (2013) and Vila-Donat et al. (2014), as well as, are related to a wide range of AOH occurrence in food according to results published by EFSA Panel on Contaminants in the Food Chain (CONTAM) (2011).

For testing AOH + lentil extracts combination: 15 µM of AOH were simultaneously incubated with lentil extract without dilution in Caco-2 cells during 24 h. For this assay lentil extract without dilution was selected according to previous cell proliferation assays that confirm, that lentil extracts (from 1:0 to 1:8) dilution resulted to be non-toxic. On the basis of the concentration–response function of individual AOH and Ss-I, predictions of effect concentration were calculated for mixtures containing Ss-I and AOH in a definite ratio (3.125–25 µM of each in a relation ratio 1:1). The AOH–Ss-I type of interaction is described by the median-effect/combination index (CI)-isobologram equation by Chou (2006), and Chou and Talatay (1984):

$$^{n}(\mathrm{CI})_x = \sum_{J=1}^{n} (D_j/D_x)_j = \frac{(D_x)_{1-n}\{[D]_j \sum_{J=1}^{n}[D]\}^{1/mj}}{(D_m)_j\{(\mathrm{fax})_j/[1-(\mathrm{fax})_j]\}}$$

where $^{n}(CI)_x$ is the combination index for n compounds (e.g., SSI and AOH) at *x%* inhibition; $(D_x)_{1-n}$ is the sum of the concentration of *n* compounds that exerts *x%* inhibition in combination, $\{[Dj]\ \sum^{n}[D]\}$ is the proportionality of the concentration of each of n compounds that exerts *x%* inhibition in combination and, $(Dm)_j\{(\mathrm{fax})_j/[1-(fax)_j]\}^{1/mj}$ is the concentration of each compound alone that exerts *x%* of inhibition. The CI < 1, =1, >1 indicates synergism, additive and

antagonism effect of the combination, respectively. The type of interaction produced by Ss-I + AOH combination was assessed by isobologram analysis using CalcuSyn software version 2.1. (Biosoft, Cambridge, UK, 1996–2007).

2.9 Statistical analysis

Statistical analysis of data was carried out using SPSS version 19 (SPSS, Chicago, IL, USA), statistical software package. Data were expressed as mean ± SD of different independent experiments. The statistical analysis of the results was performed by Student's t-test for paired samples. Differences between groups were analyzed statistically with one-way ANOVA followed by the Turkey HDS post-hoc test for multiple comparisons. The level $p \leq 0.05$ was considered statistically significant.

3. RESULTS

3.1 Cytotoxicity assay

The effects of AOH, Ss-I and lentil extracts in cell viability were evaluated by the MTT and PC assays after 24 h of exposure. Figure 1 shows the dose–response curve of Ss-I and AOH by both assays. The Ss-I did not affect cell proliferation with respect to control cells (Fig. 1a). As can be observed in Fig. 1b, AOH significantly ($p \leq 0.05$) decreased cell proliferation at concentrations over 50 μM(MTT assay) and over 25 μM (PC assay). The reduction in cell proliferation ranged from 27% to 47% and from 25% to 32% by the MTT and PC assays, respectively. As determined by MTT and PC after 24 h of exposure, Ss-I and AOH did not show IC50 values at the tested concentrations range.

Figure 2 shows cell proliferation of lentil extracts in Caco-2 cells by MTT and PC assays. The lentils extract not diluted (1:0) significantly ($p \leq 0.05$) increased (10%) cell proliferation by both assays. From 1:2 to 1:8 dilution extracts, cell viability was not affected. However, the 1:16 and 1:32 dilution extracts showed a

significant ($p \leq 0.05$) reduction in cell proliferation by MTT (approx. 13%) and by PC (approx. 11%) assays. Nevertheless, no IC50 values were obtained from lentil dilution extracts.

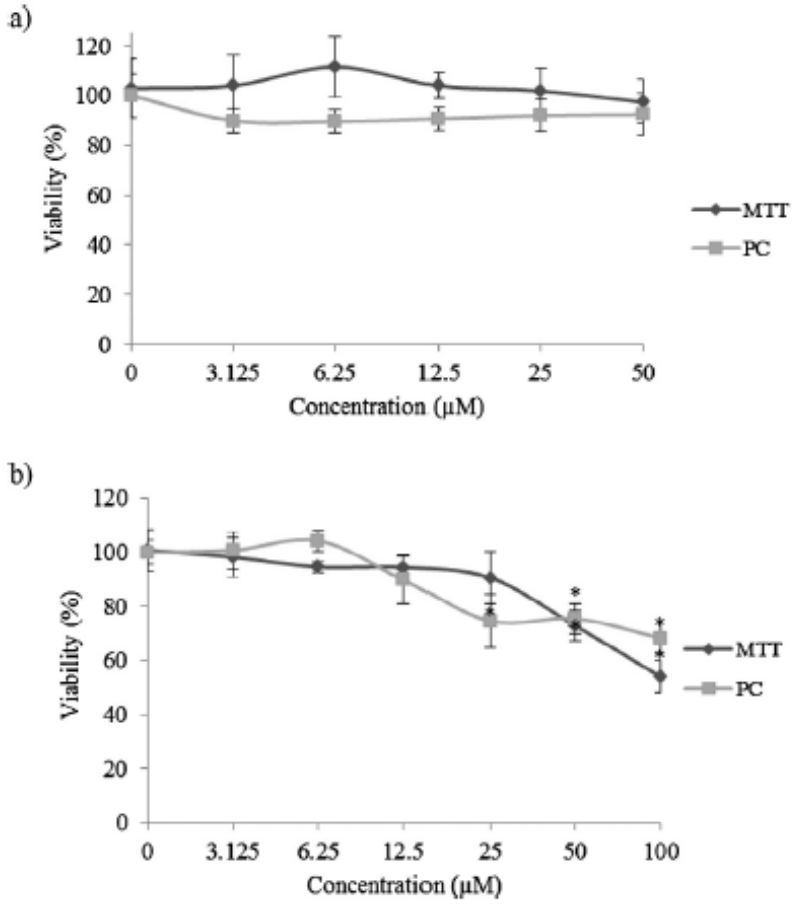

Figure 1. Concentration–effect curves of (a) Ss-I and (b) AOH in Caco-2 cells after 24 h of exposure by MTT and PC assays. All values are expressed as mean ± SD of 8 replicates. (*) $p \leq 0.05$ represents significant difference as compared to control values.

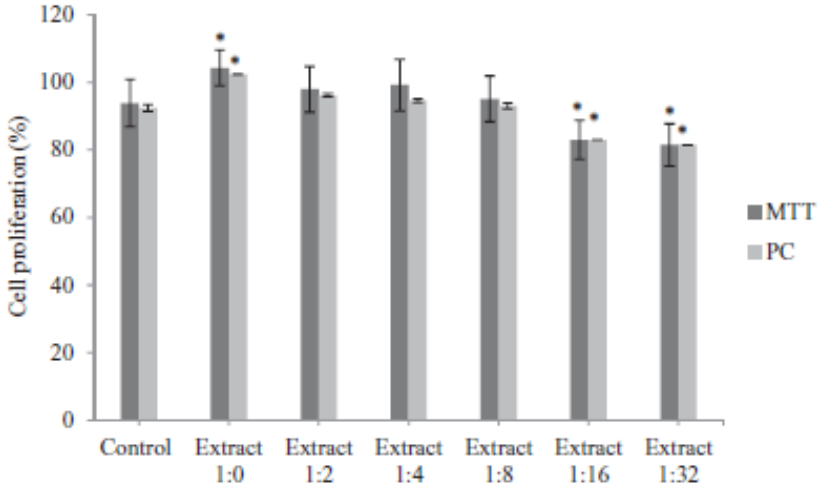

Figure 2. Lentil extracts–effect relationship in Caco-2 cells after 24 h of exposure by MTT and PC assays. Serial lentil extract dilutions (from 1:0 to 1:32) were obtained with distilled water. All values are expressed as mean ± SD of 8 replicates. (*) $p \leq 0.05$ represents significant difference as compared to control values.

3.2 Cytoprotective effects

Cytoprotective effect of Ss-I was determined by MTT and PC assays. Dose–response curves obtained with Ss-I pre-treatment assays were similar by MTT and PC assays. For that, only MTT graph was reported in Fig. 3. With Ss-I (6.25 µM) pre-treatment, Caco-2 cell viability increased at the higher AOH concentration (approx. 30%) compared to AOH tested alone (Fig. 3).

Figure 4 shows Ss-I simultaneously exposed with AOH (1:1 ratio). Figure 4 exhibits that Caco-2 cells increased ($p \leq 0.05$) cell proliferation from 6.25 µM of AOH + Ss-I with respect to cells exposed to AOH tested alone. Cell viability increased from 20 to 35% compared to AOH tested alone.

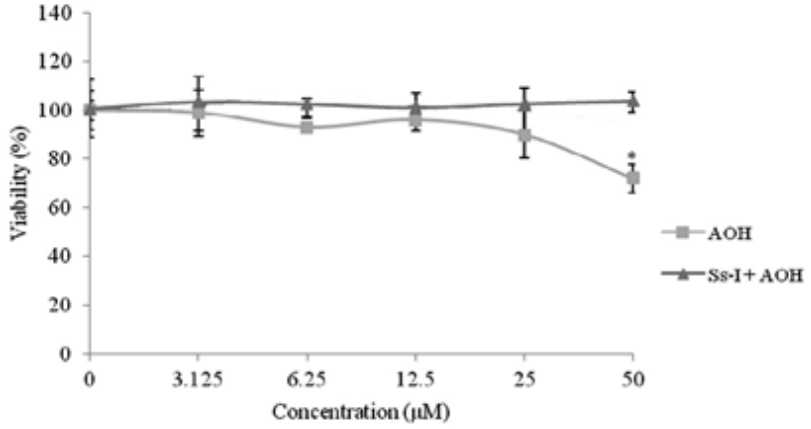

Figure 3. Concentration curves obtained after pre-treatment of Ss-I (6.25 µM) during 24 h, and subsequent addition of fresh medium with serial dilutions of AOH (3.125–50 µM) during 24 h in Caco-2 cells by MTT assay. All values are expressed as mean ± SD of 8 replicates. (*) $p \leq 0.05$ represents significant difference as compared to AOH tested alone.

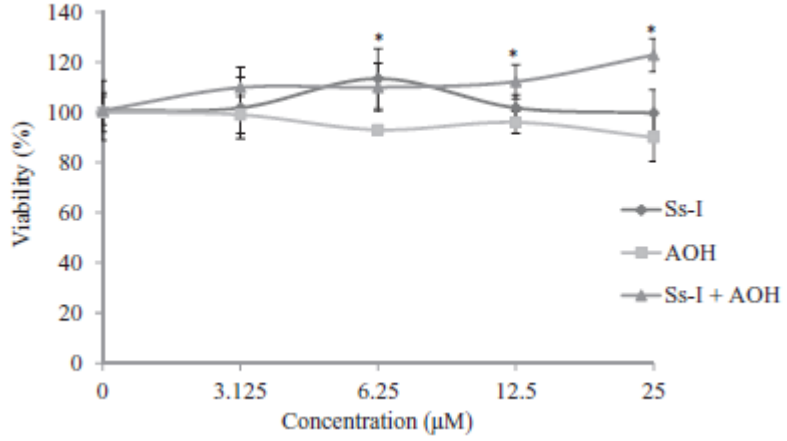

Figure 4. Concentration–effect curves obtained after simultaneous combination of Ss- I + AOH (1:1) in Caco-2 cells during 24 h of exposure by MTT assay. Both, Ss-I and AOH were tested from 3.125 to 25 µM. All values are expressed as mean ± SD of 8 replicates. (*) $p \leq 0.05$ represents significant difference as compared to AOH tested alone.

Figure 5 provides percentage of cell viability after simultaneous exposure of the mixture Ss-I (6.25 µM) + AOH (15 µM) in Caco-2 cells. The results show that this mixture did not affect cell proliferation.

Regarding cytoprotective effects of lentil extracts, Fig. 6 represents the viability (%) of simultaneous combination of AOH (15 µM) and lentil extract (without dilution) on Caco-2 cells by the MTT and PC assays. The results demonstrated that cell viability of the combination (lentil extracts + AOH) increased significantly ($p \leq 0.05$) over 30% with respect to AOH tested alone by both assays (Fig. 6).

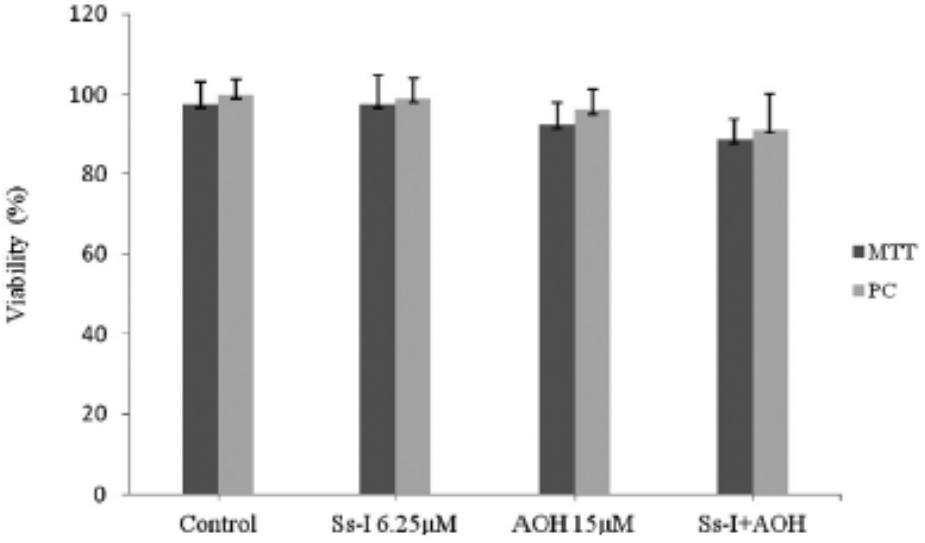

Figure 5. Cell viability (%) of simultaneous combination of AOH (15 µM) + Ss-I (6.25 µM) on Caco-2 cells during 24 h of exposure by the MTT and PC assays. All values are expressed as mean ± SD of 8 replicates.

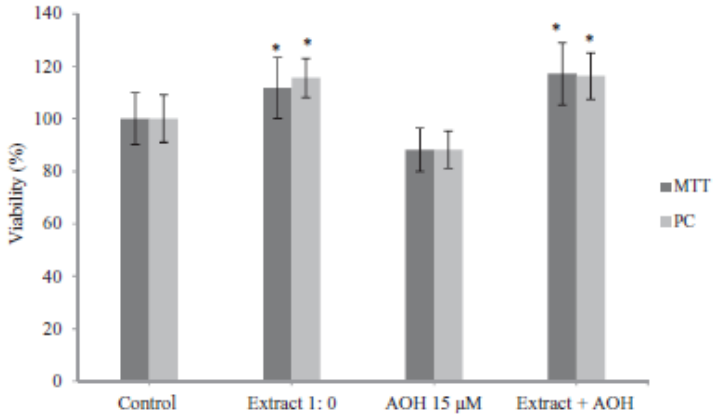

Figure 6. Cell viability (%) of simultaneous combination of AOH (15 µM) + lentil extract (1:0) on Caco-2 cells during 24 h of exposure by MTT and PC assays. All values are expressed as mean ± SD of 8 replicates. (*) $p \leq 0.05$ represents significant difference as compared to control values.

3.3 Ss-I and AOH interaction effect

In order to investigate the type of the interaction between Ss-I and AOH in combination (1:1 ratio), the CI-isobologram method was applied. The parameters Dm, m and r of the binary combination as well as CI values are shown in Table 1. The CI50, CI75 and CI90 are the doses required to inhibit proliferation at 50%, 75% and 90%, respectively.

These CI values were calculated automatically by the computer software CalcunSyn. The CI/fa curve for combination tested on Caco- cells is shown in Fig. 7. As assessed by CI-isobologram equation, synergistic effect (CI < 1) was produced by all the combinations tested (Table 1; Fig. 7). However, at the lower fraction affected CI values of 1.88 ± 0.59 indicated antagonism effect (Fig. 7).

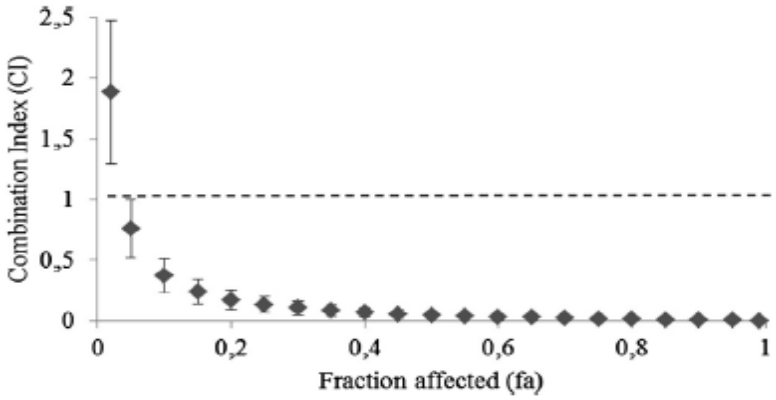

Figure 7. The CI/fa curve for binary combination of AOH + Ss-I (1:1) in Caco-2 cells after 24 h of exposure by MTT assay. Each point represents the CI at a dose effect as determined in our experiments. The dotted line indicates additive (CI = 1), the area under the dotted line synergy (CI < 1), and the area above of the dotted line antagonism (CI > 1).

Table 1 Dose–effect relationship parameters and mean combination index (CI) values of binary combination of Ss-I and AOH (1:1) in Caco-2 cells after 24 h of exposure by the MTT assay.

Compound	Dose-effects parameters			CI values		
	Dm (μM)	*m*	*r*	CI_{50}	CI_{75}	CI_{90}
Ss-I	67.46	2.42	0.9370			
AOH	81.99	1.67	0.9179			
Ss-I + AOH (1:1)	1.74	−2.29	0.9243	0.047 ± 0.031 Syn	0.017 ± 0.014 Syn	0.006 ± 0.006 Syn

The parameters m, Dm, and r the antilog of x-intercept, the slope nd the linea correlation coefficient of the median-effect plot, which signifies the shape of the dose effect curve, the potency (IC50), and the conformity of the data to the mass-action lav respectively (Chou, 2006; Chou and Talatay, 1984). Dm and m are used for calculating the (value (CI < 1, = 1, > 1) indicates synergism (Syn), additive effect (Add) and antagonism (Ant respectively. IC50, IC75, and IC90 are the doses required to inhibit proliferation 50, 75 an 90%, respectively. CalcuSyn Software provides automatically the IC50, IC75, IC 90 values.

4. DISCUSSION

AOH showed cytotoxic effects after 24 h of exposure on Caco-2 cells; at concentrations from 25 to 100 µM. Similar results were obtained by Tiessen et al. (2013), who determined the cytotoxicity effects of AOH after 24 h of incubation, by SRB assay in human colon HT29 cells. They found that cell viability decreased about 15% at 50 µM of AOH. In the same way, Fernández-Blanco et al. (2014) demonstrated that AOH reduced cell proliferation in a dose- and time-dependent manner on Caco-2 cells, and they did not find IC50 for AOH. Brugger et al. (2006) observed reduction in the number of cells incubated with AOH to be approximately 35% (with 30 µM; Chinese hamster V79 cells) and 69% (with 20 µM; MLC cells) after 24 h of incubation. Our results are in agreement with the results obtained by Fernández-Blanco et al. (2014) and Wollenahupt et al. (2008), as AOH resulted to be more sensitive to the mitochondrial metabolism through succinate dehydrogenase activity (MTT assay) than PC assay.

As expected, Ss-I and lentil extracts did not show cytotoxicity in Caco-2 cells. The results obtained in this work are consistent with those of other authors in which HT-29 colon cells were incubated with similar concentrations of Ss-I during 24 h and had no effect on cell inhibition (Gurfinkel and Rao, 2003; Sarkar and Li, 2003). Also Salyer et al. (2013) observed that after 24 h of incubation with Ss-I (0.3–0.9 mg/L) Caco-2 cell proliferation did not decrease, while cell growth decreased after 48 h or 72 h in a dose- and time dependent manner. But these concentrations are up to 18 times higher than the maximum concentration tested in our experiments, and even higher than the approximate intake of Ss-I for the Italian population previously estimated in section 2.5. However, biological activities of soyasaponins depend on the type of soyasaponin (chemical structure), as well as their polarity (glycosides or aglycones; being more active aglycones than their related glycosides), dose-time, and cell line tested (Podolak

et al., 2010). According to the study performed by Hu et al. (2004), the absorption of soyasaponins in the intestine might be enhanced when the Ss-I concentration is low. In fact, the relationship between permeability coefficients (Papp) values obtained fromCaco-2 cell model and human in in vivo intestinal absorption for a number of drugs, suggests that permeability coefficients for Ss-I is similar to some intestinal absorptions in humans. In our study, lower concentrations of Ss-I (6.25 µM) increased cell proliferation, with respect to the higher concentrations tested (Fig. 4).

On the other hand, no *in vitro* studies about cytoprotective effect of Ss-I or soyasaponins-rich extracts against toxicity induced by mycotoxins were found in literature. However, there are in vivo studies in which protective effects of soyasaponins have been studied. Yang et al. (2011) investigated the cytoprotective effects of soyasaponins rich-extracts from soybean, against acute alcohol-induced hepatotoxicity in mice. Their results indicated that supplementation with soyasaponins rich-extracts could restrain the hepatic damage after acute alcohol exposure by the activation of the hepatic antioxidant system and reversal LPO. Excessive oxidative stress produced by alcohol consumption can be repaired by antioxidant system initiated by soyasaponins rich-extract intake.

In the present work, cytoprotective effects when Ss-I (Fig. 4) or lentil extracts (Fig. 6) were exposed simultaneously with AOH were observed. Even, lentil extracts (1:0) (Fig. 6) demonstrated higher cytoprotective effect with respect to pure Ss-I, indicating that the components of extracts could interact synergistically, increasing protective effects. It is demonstrated that the interactions about the components of the mixtures in food can produce synergistic effects which improve their bioactive properties (Bouchenak and Lamri-Senhadji, 2013; Kang et al., 2010; Wang et al., 2011), probably due to other

functional lentil components, such as isoflavones and polyphenols (Oomah et al., 2011; Sarkar and Li, 2003).

Cytoprotective effects of Ss-I and extracts could be explained by antioxidant defense mechanism of soyasaponins against cytotoxicity induced by AOH. The cytotoxicity of AOH is often related to its ability to induce oxidative stress via enhanced LPO and ROS production (Tiessen et al., 2013). As seen previously, in the work of Yang et al. (2011) the excessive ROS initiates the LPO chain reactions, which produce lipid peroxyl radicals. However, soyasaponins may enhance its antioxidant capacity against oxidative stress. But more studies, in order to investigate oxidative stress produced by AOH when Ss-I or extracts are present, will be required to understand this. Otherwise, in vivo studies have demonstrated the lowering cholesterol effects of soyasaponins. Oakenfull et al. (1984), found that rats treated with soyasaponins decreased intestinal absorption of cholesterol by increasing the excretion of fecal bile acid and neutral sterols. Also, Lee et al. (2005) observed greater fecal of bile acids excretion and neutral sterols in hamsters fed with soyasaponins compared with those fed with casein. These data show that soyasaponins may inhibit intestinal reabsorption of bile acids. However, the mechanism mediating this effect is not investigated in this study. Nevertheless, the main mechanism responsible for the cholesterol-lowering effect of free and esterified plant sterols is the inhibition of intestinal cholesterol absorption. Different mechanisms such as competition between cholesterol and plant sterols for the solubilization in dietary mixed micelles, co-crystallization with cholesterol with the formation of insoluble mixed crystals during food lipolysis in the gastrointestinal tract, interference with the hydrolysis process by lipases and interference with transportmediated processes of cholesterol uptake have been proposed to contribute to the lowering of cholesterol concentrations by plant sterols (Trautwein et al., 2003).

On the other hand, soyasaponins are metabolized by the intestinal biota to release sugars and aglycones (Gurfinkel and Rao, 2003; Hu et al., 2004). The aglycone form of Ss-I, is soyasapogenol B. Soyasapogenol B is more bioactive than its glycoside and is a water insoluble substance (Hu et al., 2004). When compounds are not water-soluble, a crucial step in their luminal absorption is the solubilisation in the intestinal fluids and transport of solubilized compounds toward the border membrane. An alternative hypothesis assumes that Soyasapogenol-B and AOH, both of them with lipophilic properties, compete for its incorporation into dietary mixed micelles, as may occur with cholesterol. In consequence, when they are forming a complex, Ss-I could inhibit absorption of AOH, and therefore, cytoprotective effect of Ss-I increased with respect to AOH tested alone (Figs. 3, 4 and 6).

In conclusion, the cytoprotective effects of Ss-I and mostly of extracts against AOH induced-cytotoxicity on Caco-2 cells, have been demonstrated. Moreover, synergistic cytoprotective effects of Ss-I have been demonstrated. The cytoprotective effect depends on the concentration of Ss-I or extracts and AOH in the food commodity, mixing ratio, simultaneous presence of both compounds and interaction between them. So, legumes, in particular lentils, which present high concentrations of bioactive Ss-I could have a cytoprotective effects versus natural contaminants such as AOH that may be present in a wide range of agricultural commodities. Then consumption of legumes could contribute to prevent the toxicological risk to humans that AOH can produce. For better understanding about the cytoprotective effects of Ss-I, more knowledge about mechanism of actions, bioaccessibility and bioavailability of soyasaponins and AOH are required.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Short communication

3.9 Absorption and transepithelial alternariol transport in Caco-2/TC7 cells *in vitro*.

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Abstract

The bioavailability of the alternariol (AOH) mycotoxin utilizing an *in vitro* method, which allows the simulation of the small and large intestine tracts, has been studied. The Caco-2/TC7 cells grown alone or in symbiosis with several strains characteristics of the gastrointestinal tract, has permitted to simulate the duodenal and colonic intestinal compartments, respectively.

The addition of 15, 30 and 60 μM of AOH in the duodenal and colonic intestinal models shows a bioavailability of 18.1, 18.3 and 11.9 %, respectively. Total bioavailability considers the sum of duodenal and colonic bioavailabilities was 32.22 %.The transephitelial transport ranged from 10.1 % to 31.26 %.

Keywords: bioavailability, transepithelial transport, Caco-2 cells, LC-DAD, LC-MS.

1. INTRODUCTION

Mycotoxins are biologically active products formed as secondary metabolites by a few species of fungi, which easily colonize and contamined field crops with their toxins. Contamination can also take place after the harvest and storage, wherefore a wide variety of foods can be affected. Mycotoxin contamination represents a threat to food safety and risks for disease in humans and animals consuming these food and feed (Chiesi et al. 2015; Fernandez-Blanco et al. 2014; EFSA, 2011; Turnera et al. 2009).

The mainly producers of mycotoxins are the genera *Fusarium, Penecillium, Aspergillus, Alternaria* and *Claviceps*. Alternariol (AOH) represent the main toxic metabolite produced by the fungi of the genus *Alternaria* and can provoke serious health problems for humans and animals. The toxic effects of AOH are wide-ranging. The AOH produces cytotoxic effects in mammalian cells by causing ROS

generation and LPO production which results in impairment in cellular viability. Besides, AOH can provoke cells cycle arrest, apoptosis of cells and DNA damaging effects (Fernandez-Blanco et al. 2015; Pfeiffer et al. 2007; Lehmann et al. 2006). However, AOH induces the antioxidant enzymatic defenses as well as the GSH activity in cell cultures to reduce cytotoxic effects (Fernandez-Blanco et al. 2015; Juan-García et al. 2015; Fernandez-Blanco et al. 2014; Juan-García et al. 2013; Tiessen et al. 2013).

AOH has shown fetotoxic and teratogenic effects in animals as well as mutagenic and clastogenic effects in various *in vitro* systems (Fernández-Blanco et al., 2015; Juan-García et al. 2013; EFSA, 2011). Furthermore the genotoxic activity of AOH in *"in vitro*" mammalian systems has been demonstrated (Pfeiffer et al. 2007; Lehmann et al. 2006), as well as its estrogenic potential (Lehmann et al. 2006; Frizzell et al., 2013).

The bioavailability of the bioactive compounds in the colonic intestinal compartment depends on many factors. One of the most important factors is the presence of several microorganisms on the surface of the intestinal epithelium and the interaction that these microorganism have with the mycotoxins during the fermentation process.

The ability to adhere to the intestinal epithelium by the *Bifidobacteria* and *Lactobacillus* strains plays an important role in gut colonization; because these kinds of bacteria are responsible of the modulation of the immune system, reduce the attaching of the pathogenic bacterial strains as *Staphylooccus aureus*, *Listeria monocytogenes*, *Salmonella spp*., *Escherichia coli spp*., on the intestinal epithelium (Laparra et al., 2009). The bacterial strains of the *Bifidobacteria* and *Lactobacillus* family have a great capacity to adhere to the colonic epithelium cells interacting with several bioactive compounds presents in food (Wang et al., 2010).

Several *in vitro* methods have been developed to study the propensity of bacteria to adhere to the human intestinal epithelium and the influence that these bacteria have on the bioavailability of different compounds present in food (Laparra et al., 2009). One of the most employed approaches used to study the bioavailability consists in the Caco-2 and Caco-2/TC7 cell grown in symbiosis of several bacterial strains characteristic of the gastrointestinal tract.

On the internal side of the intestines there is a layer of enterocytes. Their make a polar structure of cells, with separate parts basolateral and apical. The upper part of the cell membrane creates the "brush border", which consists of numerous microvilli. A single enterocyte can create a brush border in the apical part, covered by numerous microvilli. After 2–3 weeks, with full confluence, it makes up a monolayer of highly polarized cells whose structure is typical of enterocytes (Grajek and Olejnik, 2004).

Caco-2 cells are the most commonly used. Caco-2 cells are human colonic adenocarcinoma cells that present the differentiation characters typical of the human intestinal cells and, for this reason are used *in vitro* tools for bioavailability and for investigating the mechanisms underlying the interaction between bacterial cells and the human gut *in vitro* (Perales et al., 2007; Laparra et al., 2009). Caco-2/TC7 clone was isolated from a late passage of the parental Caco-2 line and has shown to consist of a more homogeneous population with respect to the most representative functions of the small intestinal enterocytes, with more developed intercellular junctions (Turco et al., 2011).

About bioavailability of the AOH, no data are available; inversely many authors evaluated the bioavailability of other mycotoxins. In particular, Avantaggiato et al. (2004) studied the intestinal absorption of zearalenone (ZEA), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), ochratoxin A (OTA), deoxinivalenol (DON) and aflatoxin B_1 (AFB_1) using a laboratory system that simulate the metabolic

processes of the gastrointestinal tract of healthy pigs. Videmann et al. (2008) and Prosperini et al. (2012) studied the bioavailability and the transepithelial transport of ZEA and enniatins (ENs), in the Caco-2 cell system.

Considering the few data reflected in the literature, the aim of this study was to determine the bioavailability of AOH in Caco-2/TC7 cells by *in vitro* methods that permit to simulate the duodenal and small intestinal compartment.

2. MATERIALS AND METHODS

2.1. Materials

Acetonitrile, methanol and ethyl acetate were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MX cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. AOH was purchased by Sigma (Sigma Co., St Louis, USA). The reagent grade chemicals and cell culture components used, Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, amphotericin B, HEPES, non essential aminoacids (NEAA), Hank's Buffered Salt Solution (HBSS), phosphate buffer saline (PBS), Sorensen's glycine buffer, glucose and dimethyl sulfoxide (DMSO) were Sigma products (Sigma Co., St. Louis Mo. USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium).

2.2. Cells culture

Caco-2/TC7 cells from human epithelial colorectal adenocarcinoma were cultured in monolayer in 9 cm2 polystyrene tissue culture dishes with DMEM supplemented with 25 mM HEPES, 1% NEAA, 100 U/ml penicillin, 100 mg/ml streptomycin, 2.5 lg/ml amphotericin B, and 10% heat inactivated FCS. Incubation conditions were pH 7.4, 37 C and 5% CO2 in a 95% relative humidity atmosphere.

2.3. Caco-2/TC7 intestinal transport

Caco-2/TC7 cells were seeded at 25 10^4 cells/cm^2 on six-well Transwell Permeable Supports (Corning, NY, USA). Traswell insert (6-wells) were covered with a monolayer of Caco-2/TC7 cells at 37 C. The medium was changed every 2 days, and the cells were allowed to grow and differentiate up to 21 days after reaching confluence (Hilgers et al., 1990). After removal the growth medium from both sides of monolayers, the cells were preincubated at 37 C for 10 min with HBSS–HEPES buffer. For this purpose, in the 6 wells permeable supports 1.5 mL of HBSS–HEPES buffer were added in the apical compartment and 0.5 mL of HBSS–HEPES were added in the basolateral compartment.

The colonic bioavailability was calculated in Caco-2/TC7 mucina and bacteris (*Lactobacillus animalis CECT 4060T, L. casei CECT 4180, L. casei rhamnosus CECT 278T, L. plantarum CECT 220, L. rhuminis CECT 4061T, L.casei CECT 277, Bifidobacterium breve CECT 4839T, B. adolescentes CECT 5781T y B.bifidum CECT 870T, Corynebacterium vitaeruminis CECT 537, Streptococcus faecalis CECT 407, Eubacterium crispatus CECT 4840 y Saccharomyces cerevisiae CECT 1324*) of the gastrointestinal system were added to Caco-2/TC7 cells. After 1 h, bacteris were removed and cells were washed twice with PBS, followed by a final wash with HBSS (transport medium).

After medium removal, medium containing AOH in the concentration of 15, 30 and 60 µM was added in the apical compartment and natural medium to the basolateral compartment. The monolayers were incubated for 3 h at 37 C. For transport measurements, aliquots of incubation medium were taken from the basolateral compartment at specified times and samples were collected for immediate analysis by direct injection (20 µL) in the liquid chromatography (LC) apparatus. 2.

2.4. AOH extraction from apical and basolateral compartment

According to the method described by Ambrosino et al. (2004). In particular, 1 mL of each sample was dissolved in 3 mL of methanol and then filtered through 0.22 lm nylon filter purchased from Análisis Vínicos (Tomelloso, Spain). The samples were purified with a C18-E solid-phase extraction column (100 mg, Phenomenex, Torrance, CA) that had been preconditioned with 3 mL of methanol and 3 mL of water, and then eluted with 1 mL of methanol. The eluate was completely evaporated under nitrogen at 50 C and reconstituted with 100 lL of methanol. Then, 20 µL of each extract was injected in the LC apparatus.

2.5. Statistical analysis

All experiments were performed three times. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnet's multiple comparison tests. Differences were considered significant if $p \geq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Transepithelial transport and bioavailability

The Caco-2/TC7 cell monolayers were used in the design of a simulated *in vitro* gastrointestinal model in order to assess the absorption of AOH. The apical to basolateral transport of different concentrations of AOH was determined after 0, 1, 2 and 3 h.

AOH showed a duodenal transport of 17.88 %, 15.10 % and 12.96 % after 3 h of incubation with 15, 30 and 60 µM, respectively. In the apical compartment, 16.55 %, 18.18 % and 15.02 % of AOH were detected. AOH showed a colonic transport of 18.39, 21.55 and 10.87 % after 3 h of incubation with 15, 30 and 60 µM, respectively (Fig.1b). In the apical compartment, 24.62, 18.07 and 10.82 % of AOH were detected. AOH presented a similar profile among all concentrations studied, evidenced the highest absorption by Caco2-TC7 cells at 15 µM.

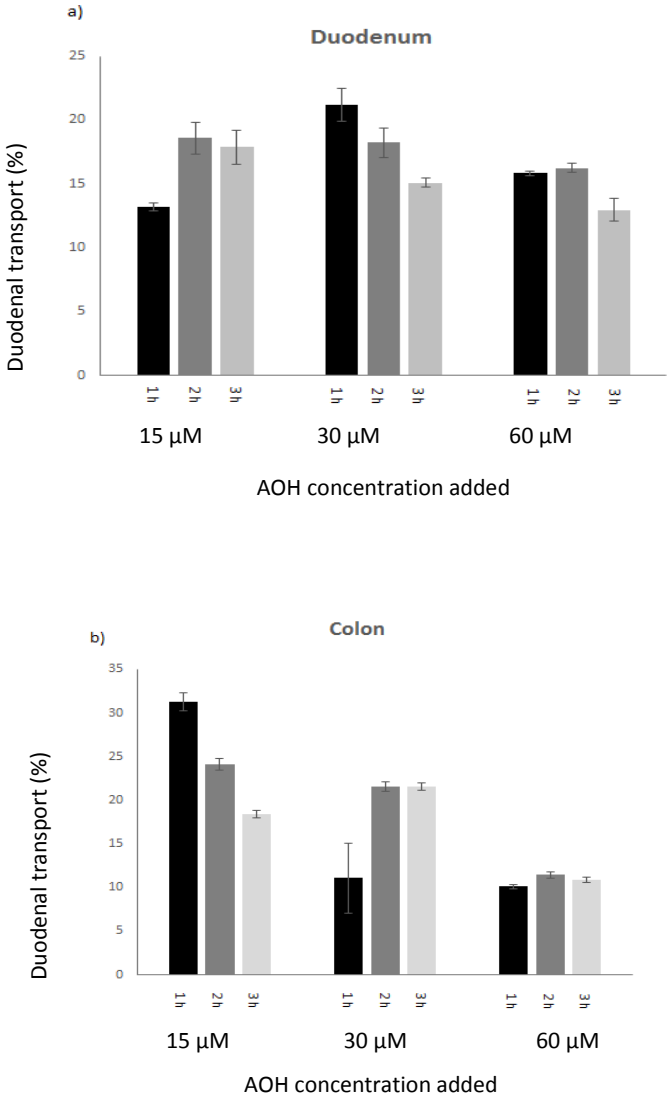

Figure 1. *In vitro* bioaccesibility assessment of AOH in a) duodenum and b) colon.

AOH is the most predominantly occurring of *Alternaria alternata* mycotoxins in food. It is known that AOH is rapidly absorbed as aglycone. It undergoes cytochrome-mediated oxidative metabolism in liver and is conjugated with glucuronic acid and sulfate (Burkhardt et al., 2009). Thus, AOH and its conjugates through bile can reach the duodenum and to be distributed by blood anywhere in the body and to provoke toxic effects. Burkhard et al., (2009) showed that the basolateral concentration of unconjugated AOH reach its higher level at 1.5–2 h after 10, 20, 30 and 40 µM AOH. Likewise that our study, where the high basolateral concentration was reach at 2 h with 17.7 % of AOH.

Taking into account the apparent permeability coefficients values of AOH observed in this study and the rapidly absorption of conjugate metabolites obtain by Burkhardt et al., (2009), AOH must be expected to be extensively absorbed from the intestinal lumen.

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3.10 An *in vitro* investigation on the cytotoxic and nuclear receptor transcriptional activity of the mycotoxins fumonisin B1 and beauvericin.

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Abstract

Fumonisin B1 (FB1) and beauvericin (BEA) are secondary metabolites of filamentous fungi, which under appropriate temperature and humidity conditions may develop on various foods and feeds. To date few studies have been performed to evaluate the toxicological and endocrine disrupting effects of FB1 and BEA. The present study makes use of various *in vitro* bioassays including; oestrogen, androgen, progestagen and glucocorticoid reporter gene assays (RGAs) for the study of nuclear receptor transcriptional activity, the thiazolyl blue tetrazolium bromide (MTT) assay to monitor cytotoxicity and high content analysis (HCA) for the detection of pre-lethal toxicity in the RGA and Caco-2 human colon adenocarcinoma cells.

At the receptor level, 0.001-10 □M BEA or FB1 did not induce any agonist responses in the RGAs. However at non-cytotoxic concentrations, an antagonistic effect was exhibited by FB1 on the androgen nuclear receptor transcriptional activity at 10 µM and BEA on the progestagen and glucocorticoid receptors at 1 µM. MTT analysis showed no decrease in cell viability at any concentration of FB1, whereas BEA showed a significant decrease in viability at 10 µM. HCA analysis confirmed that the reduction in the progestagen receptor transcriptional activity at 1 □M BEA was not due to pre-lethal toxicity. In addition, BEA (10 µM) induced significant toxicity in both the TM-Luc (progestagen responsive) and Caco-2 cells.

1. INTRODUCTION

Mycotoxins are secondary metabolites of filamentous fungi, which under appropriate temperature and humidity conditions may develop on various foods and feeds. They are mainly produced by fungi belonging to the genera *Aspergillus, Penicillium, Fusarium, Alternaria* and *Claviceps* (Fung et al., 2004). *Fusarium*

species are contaminants of wheat, maize, and other grains worldwide, capable of producing high levels of fumonisin mycotoxins. Fumonisin B1 (FB1) is the most prevalent of the fumonisins, accounting for approximately 70% of total fumonisins (Martins et al., 2012). Studies have also highlighted that *Fusarium* species can co-produce other mycotoxins such as Beauvericin (BEA) simultaneously (Dombrink-Kurtzman, 2003).

Total fumonisin concentrations in feed materials have been reported to vary from a few µg/kg to tens of mg/kg (EFSA, 2005). Dietary fumonisin estimates, by the Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO, 2001), indicate exposure levels ranging from 0.02-0.2 µg/kg in body weight (b.w.)/day, thus remaining below the Tolerable Daily Intake (TDI) of 2 µg/kg b.w./day as set in Europe by the Scientific Committee on Food (SCF, 2003). Nevertheless, a wide range of animal diseases and pathophysiological effects such as leukoencephalomalacia, porcine pulmonary oedema, liver and kidney toxicity and liver cancer, as well as human oesophageal carcinoma are associated with FB1 ingestion (Harrison et al., 1990; Kellerman et al., 1990; Gelderblom et al., 1997; Hussein et al., 2001). While the molecular mechanism of FB1 toxicity is poorly understood, it appears to be related to the deregulation of sphingolipid metabolism (Merrill et al., 2001).

BEA is predominantly found in cereal grains such as wheat, maize and rice (Serrano et al., 2012) as well as other matrices such as nuts and dried fruits (Tolosa et al., 2013). The mean dietary exposure to BEA varies from a minimum of 0.003 µg/kg b.w. /day to a maximum of 0.050 µg/kg b.w. /day (EFSA, 2014). However, the Panel on Contaminants in the Food Chain (CONTAM) concluded that there was insufficient data to establish a TDI or/and an acute reference dose (ARfD) for BEA in humans (EFSA, 2014). BEA possesses a wide range of biological activities. These substances are known as ionophores, forming a complex with

essential cations (Ca2+, Na+, K+), which increases ion permeability of biological membranes, therefore potentially affecting ionic homeostasis (Chen et al., 2006). Many mycotoxins such as ochratoxin A, patulin, alternariol and zearalenone have been found to possess endocrine disrupting capabilities (Frizzell et al., 2011, 2013a, 2013b and 2014).

Endocrine disruptors (EDs) include both natural and man-made substances that may interfere with the body's endocrine system by acting like endogenous hormones and inducing adverse developmental, reproductive, neurological and immune effects (IPCS, 2002). A few studies suggest that FB1 may act as a potential ED (Collins et al., 1998; Gbore, 2009). While there is not enough data to confirm that FB1 is a developmental or reproductive toxicant in animals or humans, Collins et al., (1998) reported that FB1 was toxic to maternal rats and the foetus at 15 mg/kg of feed consumption. In addition, Gbore (2009) reported that FB1 affected fertility in pigs by causing a delay in sexual maturity and poor sperm production and quality. There are no *in vivo* toxicological studies available on reproduction and developmental toxicity, neurotoxicity or carcinogenicity for BEA. However, it has been shown to be absorbed and rapidly metabolised to a range of uncharacterised metabolites as detected in the eggs of laying hens and several tissues of turkeys and broilers (Jestoi, 2008).

In vitro bioassays may be used to investigate the toxicity and endocrine disrupting potential of compounds (Connolly et al., 2011). The emerging technology, High Content Analysis (HCA) is a highly powerful multi-parameter bio-analytical based tool incorporating fluorescent microscopy with automated *in vitro* cell analysis software. HCA provides assays with high sensitivity and specificity for pre-lethal cytotoxicity and multiple biological endpoints for use as a high throughput-screening tool to monitor the cytotoxicity, endocrine disruption and biological effects of compounds on exposed cells (Clarke et al., 2015).

In this study, we have investigated the endocrine disrupting and cytotoxic potential of FB1 and BEA using various *in vitro* bioassays. Reporter gene assays (RGAs) utilising human mammary gland cells with natural steroid hormone receptors for oestrogens, androgens, progestagens and glucocorticoids (Willemsen et al., 2004) are employed for the identification of endocrine disruption at the level of nuclear receptor transcriptional activity. HCA is used to detect early cytotoxicity, via multiple markers in the progestagen responsive (TM-Luc) cell line exposed to 0.001-10 µM BEA, to ensure that a reduction in transcriptional activation of endocrine receptors is not correlated with pre-lethal toxicity. HCA is also used to assess cytotoxicity in colon adenocarcinoma (Caco-2) cells because the ingestion of food contaminated with FB1 and BEA is the main exposure route for animals and humans. Moreover, the Caco-2 cell line has been extensively used as a model of the intestinal barrier. The parental cell line undergoes spontaneous differentiation that leads to cells expressing morphological and functional characteristics of the mature enterocyte.

2. MATERIALS AND METHODS

2.1 Reagents

Methanol, thiazolyl blue tetrazolium bromide (MTT), FB1, BEA and the steroid hormones 17β-estradiol, testosterone, progesterone and hydrocortisone were obtained from Sigma–Aldrich (Poole, Dorset, UK). Cell culture reagents were obtained from Life Technologies (Paisley, UK). Multiparameter cytotoxicity 2 multiplex kit (8400202) containing mitochondrial probe and cell membrane permeability dye was supplied by Thermo Scientific, UK. Stock solutions of FB1 and BEA were prepared in methanol and stored at -20ºC. FB1 and BEA were dissolved in methanol at a final concentration of 0.5% (v/v) in media for the RGAs, MTT assays and HCA.

2.2 Cell culture

All cells were routinely cultured in 75 cm2 tissue culture flasks (Nunc, Roskilde, Denmark) at 37 ° with 5% CO_2 and 95% humidity. Four RGA cell lines were previously developed by the transformation of human mammary gland cells with the luciferase gene under the control of a steroid hormone inducible promoter (Willemsen et al., 2004). The MMV-Luc cell is specific for the detection of oestrogens, TARM-Luc for androgens and 313rogestogens, TM-Luc for 313rogestogens and TGRM-Luc for glucocorticoids and 313rogestogens. The RGA cells were routinely grown in cell culture medium containing Dulbecco's Modified Eagle Medium (DMEM), 10% foetal bovine serum (FBS) and 1% penicillin streptomycin. As phenol red is a weak oestrogen, DMEM without phenol red was used when culturing the MMV-Luc cells. Cells were transferred prior to RGA analysis into assay media, which was composed of DMEM and 10% hormone depleted serum. The Caco-2 cell line (ATCC HTB-37) was routinely grown in DMEM medium, 10% FBS and 1% penicillin streptomycin.

2.3 Reporter gene assay (RGA)

RGAs were carried out as previously described by Frizzell et al. (2011). Briefly, cells were seeded at a concentration of 4×10^5 cells/ml, 100 µl/well, into white walled 96 well plates with clear flat bottoms (Greiner Bio-One, Germany). The cells were incubated for 24 h and then exposed to BEA and FB1 (0.001, 0.01, 0.1, 1, 10 µM) for the agonist test. The positive control used with each cell line (in the agonist and antagonist test) was as follows: 1.35 ng/ml 17 β-estradiol (0.005µM) (MMV-Luc cells), 14.5 ng/ml testosterone (0.005µM) (TARM-Luc cells), 157 ng/ml progesterone (0.5µM) (TM-Luc cells) and 181 ng/ml hydrocortisone (0.5µM) (TGRM-Luc cells). A solvent control 0.5% (v/v) methanol in media was also added to each plate. Antagonist tests were carried out by incubating BEA and FB1 (0.001, 0.01, 0.1, 1, 10 µM) with the relevant positive control for each cell

line. The cells were incubated for 48 h, after which, the media was discarded and the cells washed once with phosphate buffered saline (PBS). The cells were lysed with 30 µl cell culture lysis buffer (Promega, Southampton, UK) and then 100 µl luciferase (Promega, Southampton, UK) injected into each well and the response measured using the Mithras Multimode Reader (Berthold, Other, Germany). The response of the cells to the various compounds was measured and compared with the solvent control.

2.4 Cell viability assay

The MTT assay, based on the ability of viable cells to metabolize the yellow tetrazolium salt to a blue formazan product by the mitochondria, was performed in parallel to the RGA assays to monitor for cytotoxic effects of the mycotoxins and their concentrations tested.

Briefly, the cells were exposed exactly as for the RGAs but in clear flat bottomed 96 well plates (Nunc, Roskilde, Denmark). Following removal of the media, 50 µL of MTT solution (2 mg/ml stock in PBS diluted 1:2.5 in assay media) was added to each well and incubated for 4 h. The supernatant was removed and 200 µL/well of DMSO added to dissolve the formazan crystals. The absorbance was measured at 570nm and a reference absorbance of 630nm using an automatic plate reader (Tecan, Safire, USA). Cell viability was calculated as a percentage absorbance of the sample when compared to the absorbance of the solvent control (0.5% (v/v) methanol in media).

2.5 HCA multi-parameter assay

HCA is a rapid and robust technology which can determine multiple cytotoxic effects, including early (pre-lethal) as well as late-stage occurrences of cytotoxicity simultaneously. The cytotoxicity of BEA and FB1 was assessed on Caco-2 cells as an effective indicator of toxicity to the human gut. The TM-Luc cell

line was also investigated by HCA to confirm whether pre-lethal toxicity was inducing the antagonist response observed at 1 µM.

Briefly, cells were seeded at a concentration of 2 × 10^4 cells/ml, 100 µl/well, into 96 well plates (Nunc, Roskilde, Denmark). The cells were incubated for 24 h and then exposed to (0.001, 0.01, 0.1, 1, 10 µM) of BEA (TM-Luc cells for 48 h) and BEA or FB1 (Caco-2 cells for 24 and 48 h).

Cellomics® HCA reagent series multi-parameter cytotoxicity dyes were utilised. Mitochondrial membrane potential dye was prepared by adding 117 µl of anhydrous DMSO to make a 1 mM stock. Permeability dye was used as provided in the-multiparameter cytotoxicity 2 multiplex kit (8400202). The live cell staining solution was prepared by adding 2.1 µl permeability dye to 6 ml of complete media that had been preheated to 37ºC, and then 21 µl of mitochondrial membrane potential dye (final concentration 3.5 mM). Nuclear stain solution was prepared by adding 5.5 µl Hoechst 33342 dye to 11 ml 1X Wash Buffer.

After incubation, 50 µl of live cell staining solution was added to each well. Cells were incubated in the dark at 37ºC and 5% CO_2 for 30 min. The staining solution was aspirated and 100 µl of 10% formalin solution (fixation solution) added. The cells were incubated for 20 min at room temperature before discarding the fixation solution and washing the cells with 100 µl of PBS. Nuclear staining solution (100 µl) was then added, and the cells incubated for 10 min at room temperature protected from light. The cells were then washed twice and the wells filled with 100 µl of PBS. Cell number (CN), nuclear area (NA), nuclear intensity (NI), plasma membrane permeability (PMP), mitochondrial membrane potential (MMP) and mitochondrial mass (MM) were measured using the CellInsight™ NXT High Content Screening platform (Thermo Fisher Scientific, UK).

2.6 Statistical analysis

Assay exposures were carried out in triplicate wells and in three independent experiments. Results were expressed as the mean ± standard error of the mean (SEM) of the triplicate exposures. For the RGAs, data was analysed using Microsoft Excel and Graphpad PRISM software (San Diego, CA). A one way analysis of variance (ANOVA) and Dunnett's multiple comparison test was used to determine significant differences between the treatments and the corresponding controls in the RGAs, MTT assays and HCA. The mean concentrations were tested for significant difference at the 95% confidence level. A p value of < 0.05 was considered statistically significant, $p = \leq 0.05$ (*), ≤ 0.01 (**) and ≤ 0.001 (***).

3. RESULTS

3.1. Cell viability

The MTT assay was used to determine the viability of the RGA cells following exposure to FB1 or BEA (0.001-10 µM). No cytotoxicity was observed in any of the RGA cell lines exposed to 0.001-10 µM FB1 (Fig.1) or 0.001-1 µM BEA. However, at 10 µM BEA, a decrease in cell viability for all RGA cell lines was observed ($p \leq 0.001$) (Fig. 1).

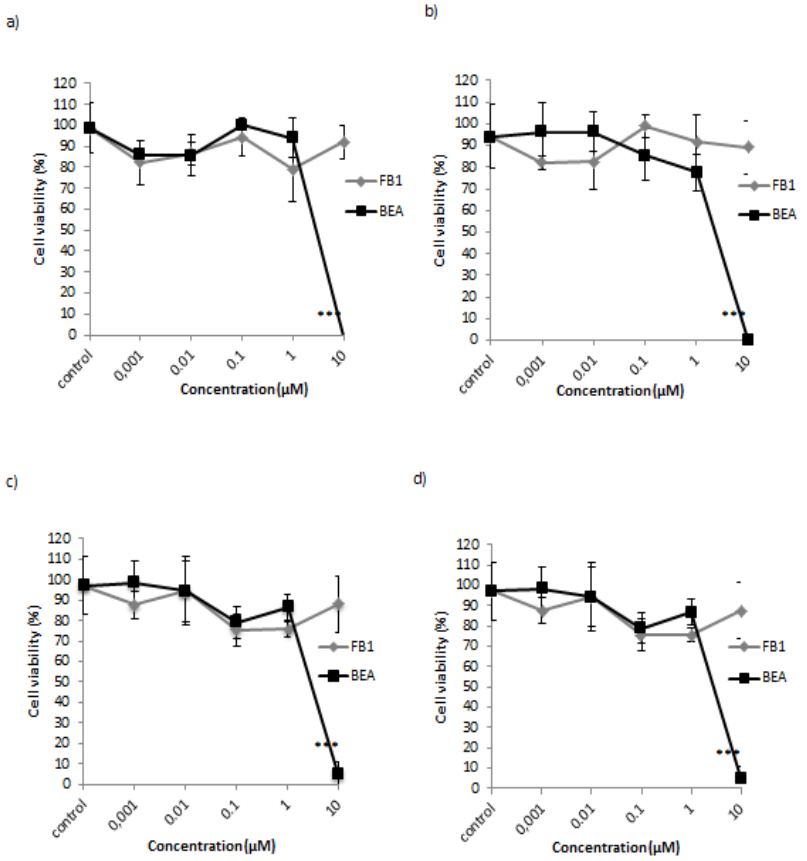

Fig.1 Viability of the RGA cell lines a) MMV-Luc b) TARM-Luc c) TM-Luc and d) TGRM-Luc following exposure to 0.001-10 µM of FB1 and BEA for 48 h and compared to the solvent control, as determined in the MTT assay. Values are means ± SEM for the three separate experiments (n=3), $p \leq 0.001$ (***).

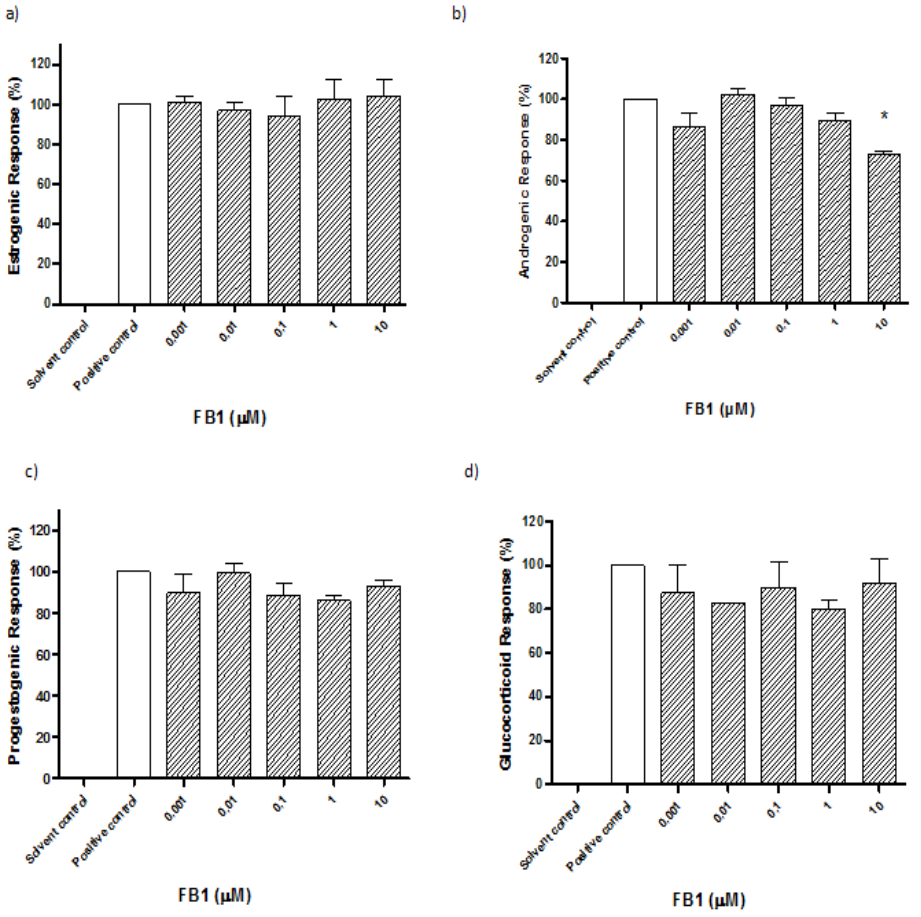

Fig.2 Results of RGA antagonistic test following co-exposure of the positive control with FB1 (0.001-10 µM) in the a) MMV-Luc (oestrogen responsive), b) TARM-Luc (androgen responsive), c) TM-Luc (progestogen responsive) and d) TGRM-Luc (glucocorticoid responsive) RGA cells. Responses measured are compared to the solvent and the positive control (1.36 ng/ml 17 β-estradiol, 14.5 ng/ml testosterone, 157 ng/ml progesterone and 181 ng/ml cortisol, respectively). Results are expressed as the mean percentage response ± SEM for the three separate experiments (n=3), $p \leq 0.05$ (*).

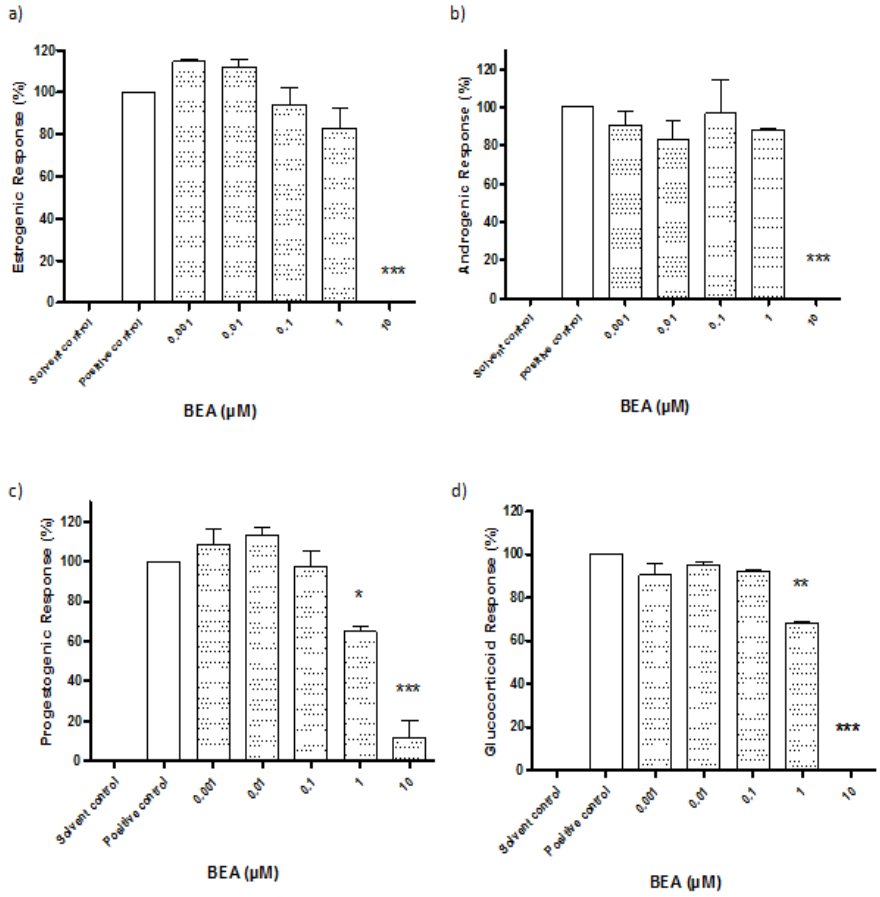

Fig.3 Results of RGA antagonistic test following co-exposure of the positive control with BEA (0.001-10 µM) in the a) MMV-Luc (estrogen responsive), b) TARM-Luc (androgen responsive), c) TM-Luc (progestogen responsive) and d) TGRM-Luc (glucocorticoid responsive) RGA cells. Responses measured are compared to the solvent and relevant positive controls (1.36 ng/ml 17 β-estradiol, 14.5 ng/ml testosterone, 157 ng/ml progesterone and 181 ng/ml cortisol, respectively). Responses are expressed as the mean percentage response ± SEM for the three separate experiments (n=3), $p \leq 0.05$ (*), ≤ 0.01 (**), ≤ 0.001 (***).

3.3 High Content Analysis (HCA)

In the TM-Luc (progestagen responsive) cell line, BEA (10 µM) was not possible to analyse due to lethal cytotoxic effects. BEA (1 µM) did not show any significant differences when compared to the control. Therefore, no pre-lethal toxicity was observed at 1µM BEA, confirming that the antagonism observed in the progestagen RGA was a true response (Fig. 4).

Exposure of Caco-2 cells to 0.001-10 µM FB1 or BEA revealed that 1 µM BEA caused a significant ($p \leq 0.01$) decrease in the CN (Fig. 5). Nevertheless, 10 µM BEA was not possible to analyse due to lethal cytotoxic effects on the Caco-2 cells.

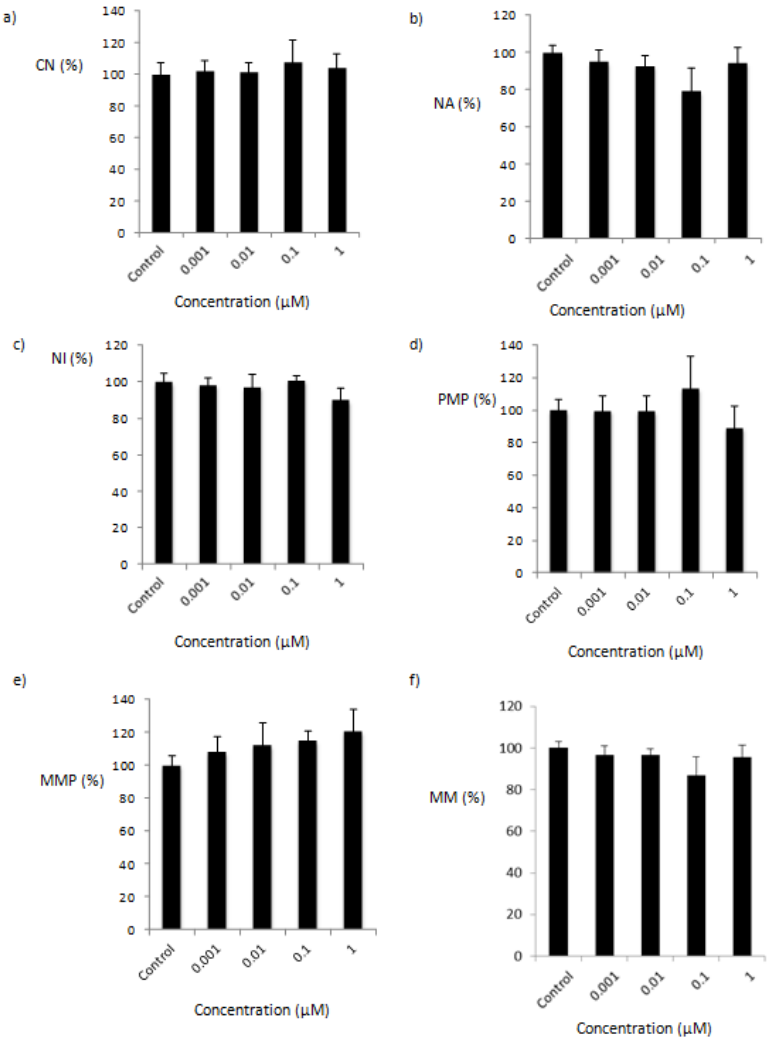

Fig.4 Quantification of the cytotoxic effects of 0.001-1 µM BEA in the progestagen responsive TM-Luc cells as measured by HCA. a) cell number (CN) b) nuclear area (NA), c) nuclear intensity (NI), d) plasma membrane permeability (PMP), e) mitochondrial membrane potential (MMP) and f) mitochondrial mass (MM). Data are expressed as mean values ± SEM for the three separate experiments (n=3).

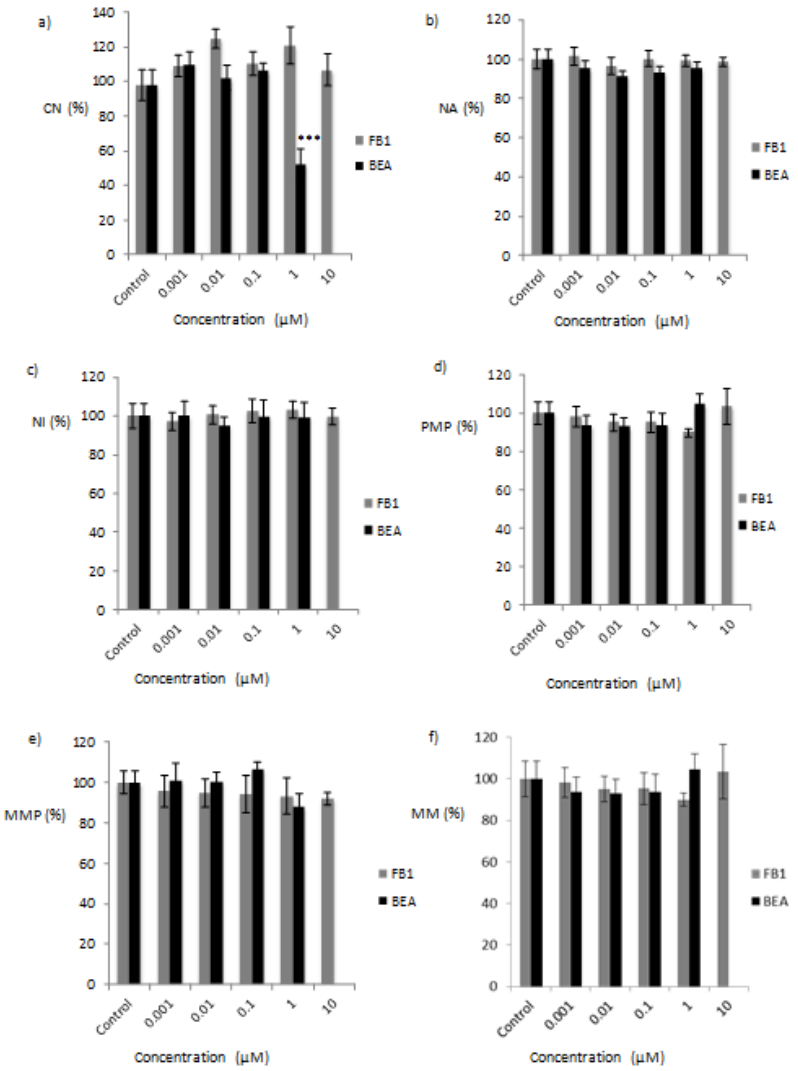

Fig.5 Quantification of the cytotoxic effects of 0.001-10 µM FB1 and BEA in the gut derived Caco-2 cells after 48 h exposure as measured by HCA. a) cell number (CN) b) nuclear area (NA), c) nuclear intensity (NI), d) plasma membrane permeability (PMP), e) mitochondrial membrane potential (MMP) and f) mitochondrial mass (MM). Data are expressed as mean values ± SEM for the three separate experiments (n=3). P ≤ 0.001 (***) indicate significant differences from the solvent control.

4. DISCUSSION

The MTT assay confirmed that FB1 (0.1 -10 µM) was not cytotoxic to any of the four RGA cell lines. This value is consistent with other publications, Meca et al., (2010) showed that exposure of Vero cells (monkey kidney) to 0-100 µM FB1 for 24 h decreased cellular viability to 60 % at 100 µM when compared to the control. In addition, Wan et al., (2013) did not observed a reduction of viability from 0 to 20 µM FB1 in IPEC-J2 (porcine jejunal epithelial) cell line after 48 h of exposure.

BEA reduced cell viability at a concentration of 10 µM in all of the RGA and Caco-2 cell lines. BEA (1 µM) also decreased viability in the Caco-2 cell line upon 48 h exposure. This data is consistent with previous studies whereby 24 and 48 h 0-30 µM BEA exposure of Caco-2 cells decreased viability to 80% and 87% respectively and HT-29 (human colon adenocarcinoma) cells presented a decrease of 85% at 24 h and 90% at 48 h (Prosperini et al., 2012). Similar results were obtained by Calo et al. (2004) with two human cell lines of myeloid origin (U-937 and HL-60 cells) and Ferrer et al. (2009) who investigated 0-100 µM BEA exposure on Chinese hamster ovary cells (CHO-K1). They observed a decline in viability at a concentration of 10 µM or higher after 24 h.

The application of HCA in toxicity studies is based on the parallel analysis of multiple markers for cytotoxicity, which allows early reversible and late irreversible effects to be distinguished, and thus provides a more detailed analysis of compound-induced toxicity (Ramirez et al. 2010; Tolosa et al., 2015). In this context, HCA can identify gross toxicity and pre-lethal toxicity, whereby exposed cells are not dead but are becoming unhealthy. While traditional end-point toxicity assays such as MTT can identify gross toxicity, they cannot do so for pre-lethal toxicity.

In the current study, an antagonist response was observed in the progesterone responsive TM-Luc cell line after exposure to 1 □M BEA. While the MTT assay was able to confirm cytotoxicity via BEA exposure at 10 µM but not at 1 □M, the potential for pre-lethal toxicity being responsible for the perceived antagonist response was considered. Consequently, HCA analysis was utilised to confirm the absence of pre-lethal toxicity and thus confirm the validity of the progesterone receptor antagonist response.

The Caco-2 cell line is a well-recognised human gut cell model (Sambuy et al., 2004) and as such is suited to investigating the toxic effects of food contaminants. HCA analysis confirmed that FB1 was not cytotoxic at any of the concentrations tested on the Caco-2 cell line. However, BEA exhibited cytotoxicity at 1 □M on the Caco-2 cell line. Furthermore, in this study was observed a slight decrease in MMP at 1 □M BEA. According to Jow et al. (2004), Ca2+-dependent pathway by BEA involves cell death, in which it induced an increase in intracellular [Ca2+] that leads to a combination of cellular apoptosis and necrosis responses. Moreover, Tonshin et al., (2010) in isolated mitochondria BEA induced a loss of MMP where K+ inflow into the mitochondrial matrix and uncoupling of oxidative phosphorylation, followed by induction of apoptosis. In addition, Prosperini et al., (2013) investigated that Caco-2 cells exhibit mitochondrial dysfunction leading a stable depolarized state of MMP and cell death after exposure of 1.5 and 3 □M BEA. Low BEA concentrations might be reached due to food consumption and based on tissue accumulation (Jestoi et al., 2007). Moreover, with regard to food intake, BEA might increase the absorption of commonly co-occurring mycotoxins probably leading to higher toxicity. Thus, exposure to low BEA concentrations activates diverse cellular stress response and protection systems (Mallebrera et al., 2014). This indicates that continuous exposure to BEA might lead to alter the intestinal epithelial barrier (Dornetshuber et al., 2009).

Antagonism of the androgen receptor in the TARM-Luc cell line was observed following exposure to 10 µM FB1. A reduction in the transcriptional activity of the androgen, glucocorticoid, oestrogen and progestogen receptor was correlated to the cytotoxic effects of BEA at 10 µM rather than true antagonism. An antagonistic response was also observed in the TGRM-Luc (glucocorticoid) and TM-Luc (progesterone) cell lines following exposure to 1 µM BEA. HCA established that no pre-lethal toxicity was evident in the TM-Luc cell line at 1 µM BEA and thus the reduction in progesterone receptor transcriptional activity was confirmed as a true antagonist response. To the authors' knowledge, this is the first study investigating the endocrine disrupting effects of FB1 and BEA at the level of nuclear receptor activity.

The actions of progesterone, glucocorticoid and androgen are mediated by its receptor. In the target cell, progesterone, glucocorticoid and androgen produce a change in conformation of its receptors that is associated with transforming receptors from a non-DNA binding form to one that will bind to DNA (Spitz et al., 2003). This transformation is go with a loss of associated heat shock proteins and dimerization. The activated receptors dimers then binds to specific DNA sequences within the promotor region of progesterone, glucocorticoid and androgen responsive genes. Antagonist impair the ability of receptors to interact with coactivators allowing the recruitment of corepressors (Liu et al., 2002). The antagonist activity of an antihormone may depend on the cell or tissue type. In addition, these transformations in the structure and function of the receptor results in numerous endocrine disorders. Many antagonists of progesterone receptor display antiproliferative effects in the endometrium by suppressing follicular development and blocking the LH flood. Moreover, progesterone antagonists are potent antiglucocorticoid agents (Neulen et al., 1996). GR signalling is requiered for homeostatic control of pyramidal neurons. Thus, GR

hormone influence memory, mood, and neuronal survival (Savory et al., 2001) Therefore, inhibition of the GR may affect the peripheral glucose metabolism, the stress response, and the regulation of the hypothalamic pituitary axis (Honer et al., 2003; Deroche-Gamonet et al., 2003). The regulatory steroidal sex hormones role in developmental processes such as sex determination and differentiation is of particular interest with regard to endocrine disruption (Kelce et al., 1995; 1997). Androgens, through interaction with the androgen receptor, play decisive roles in sexual differentiation of the male reproductive tract, accessory reproductive organs, and other tissues during fetal development. They also influence male pubertal maturation and the maintenance of secondary sex characteristics in adults (Wilson et al., 2001).

This *in vitro* investigation has demonstrated the potential for FB1 and BEA to modulate the endocrine system by antagonism of nuclear transcriptional activity as observed for BEA (1 μM) on the glucocorticoid and progesterone receptor and FB1 (10 μM) on the androgen receptor. HCA has also proven to be an added value cytotoxic assessment tool in establishing pre-lethal toxicity in exposed cells and confirming antagonistic responses. In addition, while FB1 did not show any significant cytotoxic effects on mammalian gut cells, BEA did at a concentration of 1 □M. Further investigation is needed to investigate the risk of BEA and FB1 exposure in humans and animals.

Conflict of interest

The authors declare that there are no conflicts of interest.

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3.11 Reaction of zearalenone and α-zearalenol with allyl isothiocyanate, characterization of reaction products, their bioaccessibility and bioavailability *in vitro.*

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Abstract

This study investigates the reduction of zearalenone (ZEA) and α-zearalenol (α-ZOL) on a solution model using allyl isothiocyanate (AITC) and also determinates the bioaccessibility and bioavailability of the reaction products isolated and identified by MS-LIT. Mycotoxin reductions were dose-dependent, and ZEA levels decreased more than α-ZOL, ranging from 0.2 to 96.9% and 0 to 89.5% respectively, with no difference ($p \leq 0.05$) between pH 4 and 7. Overall, simulated gastric bioaccessibility was higher than duodenal bioaccessibility for both mycotoxins and mycotoxin-AITC conjugates, with duodenal fractions representing ≥63.5% of the original concentration. Simulated bioavailability of reaction products (α-ZOL/ZEA-AITC) were lower than 42.13%, but significantly higher than the original mycotoxins. The cytotoxicity of α-ZOL and ZEA in Caco-2/TC7 cells was also evaluated, with toxic effects observed at higher levels than 75 μM. Further studies should be performed to evaluate the toxicity and estrogenic effect of α-ZOL/ZEA-AITC.

Keywords: zearalenone, α-zearalenol, allyl isothiocyanate, reaction products, chemical reduction.

1. INTRODUCTION

Zearalenone (ZEA) and its derivative α-zearalenol (α-ZOL) are non-steroidal estrogenic mycotoxins produced by fungi belonging to the genus *Fusarium and Gibberella* (Yang, Wang, Liu, Fan, & Cui, 2007). These fungal species contaminate pre and postharvest cereal crops such as corn, barley, wheat, rice and oats mainly from temperate and warm regions (Zinedine, Soriano, Molto, & Mañes, 2007). Several studies have demonstrated hepatotoxic, haematotoxic, immunotoxic,

genotoxic and teratogenic effects of these mycotoxins to a number of mammalian species (Zinedine et al., 2007). ZEA elicit estrogenic response upon binding to the estrogen receptor (Drzymala et al., 2015). Moreover, it can be metabolized in the human body to α-ZOL, which possesses three to four times higher estrogenic activity (Wang, et al. 2014).

There are some strategies proposed for the detoxification and biodegradation of ZEA in foods through chemical/enzymatic methods using ozone (McKenzie et al., 1997), H_2O_2 (Abd Alla, 1997) and lactonohydrolase (Takahashi-Ando, Kimura, Kakeya, Osada, & Yamaguchi, 2002); biological methods using lactic acid bacteria (Mokoena, Chelule, & Gqaleni, 2005), *Aspergillus niger* strain FS10 (Sun et al., 2014) and *Lactobacillus plantarum* Lp22, Lp39 and Lp4 (Zhao et al., 2015); and physical methods such as the use of adsorbent materials (Avantaggiato, Havenaar, & Visconti, 2003; Ramos, Hernández, Plá-Delfina, & Merino, 1996) or extrusion (Cetin, & Bullerman, 2005). However, there is little information concerning the metabolites produced through these processes and their potential toxicity.

Glucosinolates (GLs) are a group of phytochemicals found in vegetables of the *Brassicaceae (Syn. Cruciferae)* family, which includes broccoli, cauliflower, mustard and horseradish (Meca, Luciano, Zhou, Tsao, & Mañes, 2012). Damage to the plant tissue leads to the hydrolysis of GLs by endogenous myrosinase producing numerous biologically active compounds, including isothiocyanates (ITCs), thiocyanates and nitriles (Borges, Simões, Saavedra, & Simões, 2014). ITCs have several biological activities including plant defense (against insects and microbial infections) (Luciano, & Holley, 2009; Mansour et al., 2012; Santos, Faroni, Sousa, & Guedes, 2011), benefits to human health (chemopreventive and anti-angiogenic properties) (Cavell, Sharifah, Donlevy, & Packham, 2011; Fimognari, Turrini, Feruzzi, Lenzi, & Hrelia, 2012; Zhang, 2004) and might be used

as natural food preservatives (Borges et al., 2014; Saavedra et al., 2010). Allyl isothiocyanate (AITC) is one of the most common ITC, which has been reported as potent antimicrobial (Luciano, & Holley, 2009). Previous studies have also demonstrated its capacity to react with mycotoxins such as beauvericin (BEA) (Meca, Luciano, et al., 2012) and fumonisins (FBs) (Azaiez, Meca, Manyes, Luciano, Fernández-Frazón, 2013) in buffered solutions and in food matrices. AITC was able to react with both mycotoxins forming adducts, which may reduce their toxicity.

Toxins ingested through food products can be degraded or modified by metabolic processes of the human body, and only a fraction of the initial content may be accessible for absorption (Angelis, Monaci, Mackie, Salt, & Visconti, 2014). In this sense, bioavailability is defined as the portion of ingested contaminant that reaches the bloodstream (Kabak, & Ozbey, 2012). These studies in combination with cell models can provide important information concerning the impact of these compounds on the human health (Meca, Mañes, Font, & Ruiz, 2012). Bioavailability and toxicity evaluated through cellular systems has been widely used by rapid and cost-effective assays of easy standardization, which reduce the use of experimental animals and enables the investigation of specific mechanisms using different cultured cells (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009).

The objective of the present study was to assess the potential of AITC to react with α-ZOL and ZEA in buffered solutions and to determine the bioaccessibility and bioavailability *in vitro* of the reaction products.

2. MATERIALS AND METHODS

2.1. Materials and apparatus

ZEA (MW = 318.36 g/mol; ≥ 98% purity) and α-ZOL (MW = 320.38 g/mol; 97 % purity) standards, AITC (MW = 99.15 g/mol; 95% purity), formic acid (HCOOH), potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulfate ($NaSO_4$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amylase, hydrochloric acid (HCl), pepsin, pancreatin and bile salts were obtained from Sigma–Aldrich (St. Louis, MO, USA). The stock solutions were prepared in methanol and kept at -20°C. Acetonitrile, methanol and ethyl acetate of LC-MS grade were purchased from Fisher Scientific (New Hempshire, USA). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Cell culture materials including Dulbecco's modified Eagle's medium (DMEM), penicillin, amphotericin B, HEPES, no essential aminoacids (NEAA), streptomycin, phosphate buffer saline (PBS), Hank's balanced salt solution (HBSS) and dimethyl sulfoxide (DMSO) were also provided by Sigma-Aldrich. Fetal calf serum (FCS) was purchased from Cambrex Co. (Belgium).

2.2. Reduction of α-ZOL and ZEA with AITC *in vitro*

ZEA and α-ZOL standards were diluted to 78 µM phosphate buffer at pH 4 or 7. AITC at 2, 20, 100 or 200 mM was added to the reaction vials (final volume of 1 ml), which were tightly closed, shaken with the use of a vortex for 1 min and kept at room temperature. Aliquots were draw after 0, 4, 8, 24 and 48 h of reaction for further analyses. Assays were carried out in triplicate and compared with a standard curve ranging from 0.3 to 300 µM. The results were expressed in percentage (%) of reduction of mycotoxins based on a control sample prepared with the mycotoxin standard.

2.2.1. HPLC analysis

ZEA and α-ZOL were determined using Merk HPLC with a diode array detector (LC-DAD) L-7455 (Merk, Darmstadt, Germany) at 236 nm and Hitachi Software Model D-7000 version 4.0 was used for data analysis. A Gemini C_{18} column (Phenomenex, Torrance, USA) 4.6 × 150 mm, 3 µm particle size was used as the stationary phase. The isocratic mobile phase was consisted of water/acetonitrile (55:45, v/v) with a flow rate of 0.7 ml/min. The samples were filtered through 0.22 µm nylon membrane and 20 µL was injected into HPLC system. There was a new peak identified on LC-DAD chromatograms corresponding to the reaction product of AITC and either α-ZOL or ZEA. The structures of these compounds were confirmed by linear ion trap spectrometer (MS-LIT). Assuming that 1 mole of AITC and 1 mole of α-ZOL or ZEA produces 1 mole of adducts, the molecular weight (MW) was considered 418.7 and 418.4 g/mol of ZEA-AITC and α-ZOL-AITC respectively. This ratio was used to calculate its theoretical concentration.

2.2.2. MS-LIT characterization of α-ZOL and ZEA-AITC adducts

A 3200 QTRAP™ linear ion trap mass spectrometer (AB SCIEX Concord, Ontario, Canada) coupled to a Turbo Ion Spray source was used. This instrument is based on a triple-quadrupole path (QqQ) in which the third quadrupole also operates as a linear ion trap (QqLIT) with improved performance. In the QqLIT configuration, Q TRAP™ operates in enhanced resolution (ER) and enhanced product ion (EPI) scan modes. Applied Biosystem/MDS SCIEX Analyst software version 1.3.2 was used for data acquisition and processing.

The electrospray ionization mass spectrometry (ESI-MS) analyses were performed in positive ion mode. The equipment was set as follows: ESI needle voltage at 5500 V, curtain gas at 35 (arbitrary units), GS1 and GS2 were set to 35 and 40 ºC, respectively, and probe temperature at 350 ºC. Nitrogen served both as turbo-gas and collision gas. The method was optimized based on mycotoxin

reduction, and α-ZOL/ ZEA-AITC reaction products were obtained from the combination of 200 mM of AITC and 78 µMof of each mycotoxin (α-ZOL or ZEA). Products were extracted from the buffer solution with 3 ml of ethyl acetate (99.9%). The extraction was repeated with another 3 ml of ethyl acetate and the solvent was evaporated using nitrogen flow. The isolate was diluted in 1 ml of methanol and infused into the ion source at a flow rate of 20 µL/min introduced via a model 11 Harvard infusion pump. Full-scan spectra were analyzed for the identification of products formed through the reaction between ZEA or α-ZOL and AITC. Spectra were preliminarily recorded by connecting the Harvard infusion pump to the interface. The characterization of isolated compounds were performed using the modality of ER scan, the mass range from 200 to 500 Da to obtain the general spectra of the molecule. The utilization of the mass spectrometry associated at the detection with the linear ion trap allowed the total characterization of the isolated compounds.

2.3. *In vitro* digestion model

Then, digestion started by adding 0.6 mL of artificial saliva [10 ml KCl (89.6 g/l), 10 ml KSCN (20 g/l), 10 ml NaH_2PO_4(88.8 g/l), 10 ml Na_2SO_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) and 290 mg of α-amylase completed to 0.5 L and adjusted the pH to 6.8 to the tubes]. The solution was homogenized, added with 10 ml of water and pH was adjusted to 2 with HCl 1 N. Immediately after, 0.05 ml of pepsin solution (0.04 g/ml in HCl 0.1 N) was added and the samples were incubated at 37 °C for 2 h in a shaker water bath (100 rpm) (Stuart, SBS30, Staffordshire, UK). To simulate the duodenal compartment, 20 ml of water was added and the pH was increased to 6.5 with $NaHCO_3$ 1 N, followed by addition of 0.125 ml of a solution of pancreatin (4 g/l) and bile salts (25 g/l) (1:1; v/v). The mixture was homogenized and incubated at

37 °C for 2 h in a water bath with orbital shaker at 100 rpm. Samples of 5 ml were drawn to evaluate the concentrations of the compounds after the gastric and duodenal digestion (bioaccessibility). These aliquots were centrifuged at 4000 rpm and 4 °C during 5 min. The supernatant obtained was filtered and injected into LC to quantify the mycotoxins and reaction products.

2.4. *Cell culture*

Caco-2/TC7 cell were routinely maintained and grown with DMEM supplemented with 25 mM HEPES, 1% NEAA, 100 U/ml penicillin, 100 mg/ml streptomycin, 2.5 µg/ml amphotericin B, and 10% heat inactivated FBS. Incubation conditions were pH 7.4, 37 ^{o}C and 5% CO_2 in a 95% relative humidity atmosphere. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St. Louis-MO, USA).

2.5. *In vitro* cytotoxicity assays

The cytotoxicity of ZEA and α-ZOL towards Caco-2/TC7 cells was determined by MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylte trazolium bromide and protein content (PC) assays. MTT assay measures the ability of live cells, but not dead cells, to reduce a colourless tetrazolium salt to purple formazan salt by the mitochondria; whereas PC assay indicates the relative determination of cell number by measuring the collective protein content on a cell culture dish. The MTT assay was performed as described by Ruiz, Festila, & Fernández (2006) with some modifications and PC assay was analyzed according to the procedure described by Pichardo et al. (2007). Caco-2/TC7 cells were cultured in 96-well tissue culture plates at a density of $3x10^4$ cells per well and grown to 90% confluence. The growth medium was removed and ZEA and α-ZOL, at concentrations from 6.25 to 100 µM, were added to the medium, allowing uptake to proceed for 24 h. Then, the medium was removed and each well received 200µL of fresh medium containing 50 µL of MTT for 4 h (37 °C in

darkness). The resulting formazan was solubilized in DMSO. The cell viability or protein content was determined using an automatic ELISA plate reader MultiscanEx (Thermo Scientific, MA, USA) wit wavelength of 570 nm.

PC assay was also conducted spectrophotometrically at 24 and 48 h in the same 96-well culture plates where the MTT assay was performed. The medium with formazan was then removed and the cells were washed with PBS and homogenized in NaOH for 2 h at 37 °C. Then, the NaOH was removed and an acidic solution of Coomassie Brilliant Blue was added at room temperature. Protein content was measured after 30 min at 620 nm using an automatic ELISA plate reader MultiscanEx (Thermo Scientific, MA, USA). Results were expressed as percentage relative to control cells (1% DMSO). Mean inhibition concentration (IC50) values were calculated from full dose-response curve.

2.6. *In vitro* bioavailability

Differentiated Caco-2/TC7 were exposed to α-ZOL/ZEA-AITC adducts produced through α-ZOL/ZEA and AITC reaction to analyze the *in vitro* bioavailability of these compounds according to Meca, Mañes, et al. (2012). Briefly, Caco-2/TC7 cells were seeded at 1.35 x 10^6 cells/cm^2 on a 6-well Transwell Permeable Supports, 12 mm diameter (Corning, NY, USA) and 0.4 mm of pore size, and grown for 21 days until morphological differentiation. The medium was renewed every 2–3 days. After this period, apical (upper compartment) and basolateral (lower compartment) medium were removed, and cells were washed twice with PBS, followed by a final wash with HBSS (transport medium). The apical solution composed by 1.5 ml of HBSS and a α-ZOL/ZEA-AITC adducts (at initial levels of 15, 30 and 60 µM) was subjected to simulated duodenal digestion (Fig. 1) and digested sample obtained was diluted in HBSS (1:1, v/v). Bioavailability was assessed by transepithelial passage of this solution to the basolateral side, which contained 0.5 ml of HBSS. Control samples composed by transport medium with

methanol 1%, were also evaluated. Aliquots (150 µL) were drawn from the basolateral compartment after 0, 1, 2, 3 and 4 h of incubation and analyzed by LC-DAD.

2.7. Statistical analysis

Graphpad Prism version 6.0 (Graphpad Software Inc., La Jolla, CA, USA) was used for the statistical analysis of data. All experiments were performed in triplicate and differences between groups analyzed with one-way ANOVA followed by the Tukey HSD post-hoc test for multiple comparisons. The level of significance considered was $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. ZEA and α-ZOL *in vitro* reduction

The reaction between α-ZOL/ZEA (78 µM) and AITC (2, 20, 100 or 200 mM) were monitored by LC-DAD in PBS at different pH levels (4 and 7) and incubation times (0, 4, 8, 24 and 48 h). As shown on Table 1, the reduction ranged from 0 to 89.5 ± 1.2% for α-ZOL and 0.2 ± 0.3% to 96.9 ± 2.4% for ZEA. There was no difference ($p \leq 0.05$) between the pH levels investigated. The effect of AITC was dose-dependent towards both mycotoxins. However, the reaction occurred rapidly with most doses reaching a plateau already at 0 h, with a few exceptions that presented this same reaction plateau after 4 h.

AITC seems to be more reactive with ZEA than α-ZOL. At 20 mM (pH 4), AITC was able to reduce more than half of ZEA's initial concentration, whereas α-ZOL was reduced by a maximum of 28.9 ± 8.9% with this same dose. ZEA was also reduced by 74.5 ± 10.3% (pH 4) and 77.2 ± 3.6% (pH 7) promptly after addition of 100 mM of AITC, while α-ZOL was reduced by 54.1 ± 3.8% and 67.7 ± 6.4% at pH 4 and 7, respectively. ZEA and α-ZOL are important mycotoxins in animal production, and the use of allyl isothiocyante in animal feed could be an

alternative to mitigate this problem, since 20 mM AITC can reduce levels up to 68.0% *in vitro*.

Previous studies have reported the use of gaseous allyl, benzyl and phenyl isothiocyanates (ITCs) to reduce mycotoxins levels. Meca, Luciano, et al. (2012), evaluated the reduction of beauvericin (BEA) by AITC in buffered solutions and in wheat flour. AITC at 1 mM was able to reduce this mycotoxin (25 mg/l) in buffered solutions by 100% after 48 h. Similar to the results obtained in this study, no significant difference was found for the extent of reaction between AITC and BEA at pH 4 and 7. In addition, wheat flour treated with gaseous AITC (50, 100 and 500 µL/l) was able to reduce the initial BEA concentration (25 mg/Kg) by 10-65% in a dosedependent fashion.

Reaction of isothiocyanates (allyl, benzyl and phenyl – 1 mg/l) and fumonisins (FB_1, FB_2 and FB_3 – 1 mg/l) at pH 4, 7 and 9 was also surveyed (Azaiez, et al., 2013). The reduction of FB_1 and FB_2 in solution ranged from 42% to 100% in a time-dependent manner and was greatly influenced by pH. In general, lower pH levels facilitated the reaction between ITCs and FBs, where four reaction products were identified. Moreover, gaseous ITCs were used to fumigate corn kernels and corn flour contaminated with FBs. ITC fumigation (50, 100 and 500 µL/l) was able to reduce 53%-96% of FB_1, 29%-91% of FB_2 and 29%-96% of FB_3. Reduction of these FBs could be due to the free amino group contained in these mycotoxin structures, which act as an electron donor and react with the electrophile carbon present within the isothiocyanate (ITC) group.

Table 1. Reduction of α-ZOL and ZEA through *in vitro* reaction with AITC at different pH and incubation time.

Time (h)	pH 4				pH 7			
	AITC concentration (mM)[a]							
	2	20	100	200	2	20	100	200
α-ZOL reduction ± SD (%)[b]								
0	NE	$27.0^{aA} \pm 7.1$	$54.1^{aB} \pm 3.8$	$83.3^{aC} \pm 4.7$	NE	$17.3^{aA} \pm 3.2$	$67.7^{aBC} \pm 6.4$	$80.3^{aC} \pm 2.0$
4	NE	$28.9^{aA} \pm 8.9$	$74.2^{bBC} \pm 3.5$	$89.5^{bBC} \pm 1.2$	NE	$17.8^{aA} \pm 4.2$	$67.9^{aB} \pm 5.1$	$81.5^{aC} \pm 2.8$
8	NE	$24.4^{aA} \pm 6.6$	$73.3^{bC} \pm 2.8$	$89.5^{bC} \pm 0.5$	NE	$16.4^{aA} \pm 6.0$	$65.4^{aB} \pm 2.7$	$81.6^{aC} \pm 3.4$
24	NE	$26.4^{aA} \pm 5.2$	$74.5^{bB} \pm 3.1$	$88.5^{bB} \pm 1.1$	NE	$19.6^{aA} \pm 9.8$	$70.6^{aB} \pm 2.3$	$78.9^{aBC} \pm 7.7$
48	NE	$27.7^{aA} \pm 6.5$	$73.0^{bB} \pm 2.7$	$88.6^{bB} \pm 2.1$	NE	$23.2^{aA} \pm 6.9$	$69.3^{aB} \pm 4.9$	$77.6^{aBC} \pm 8.2$
ZEA reduction ± SD (%)[b]								
0	$0.4^{aA} \pm 0.5$	$51.6^{aBC} \pm 6.5$	$74.5^{aCD} \pm 10.3$	$83.2^{aD} \pm 11.9$	$1.5^{aA} \pm 1.5$	$44.5^{aB} \pm 2.2$	$77.2^{aD} \pm 3.6$	$82.1^{aD} \pm 5.3$
4	$0.2^{aA} \pm 0.3$	$54.9^{aB} \pm 10.5$	$92.2^{aC} \pm 3.4$	$96.9^{aC} \pm 2.4$	$1.6^{aA} \pm 1.4$	$41.5^{aB} \pm 3.4$	$88.4^{abC} \pm 7.6$	$95.6^{bC} \pm 2.6$
8	$0.4^{aA} \pm 0.6$	$52.2^{aB} \pm 9.2$	$90.6^{aC} \pm 3.7$	$96.8^{aC} \pm 2.7$	$1.1^{aA} \pm 0.5$	$31.9^{aB} \pm 11.0$	$90.4^{bC} \pm 3.3$	$95.2^{bC} \pm 2.1$
24	$0.9^{aA} \pm 0.6$	$60.2^{aB} \pm 9.7$	$90.8^{aC} \pm 8.0$	$92.3^{aC} \pm 3.0$	$0.7^{aA} \pm 1.0$	$47.0^{aB} \pm 15.9$	$92.2^{abC} \pm 6.9$	$95.4^{bC} \pm 2.3$
48	$0.4^{aA} \pm 0.6$	$68.0^{aBC} \pm 5.5$	$87.1^{aCDE} \pm 10.5$	$95.4^{aE} \pm 1.4$	$0.5^{aA} \pm 0.5$	$47.8^{aB} \pm 15.5$	$85.4^{abD} \pm 3.3$	$94.1^{abDE} \pm 3.5$

NE: no effect observed in mycotoxins levels.

Different lower case letters show significant difference ($p \leqslant 0.05$) in the same column, whereas different capital letters denote significant difference ($p < 0.05$) within the same row.

[a] Concentrations of AITC (allyl isothiocyanate) evaluated (2, 20, 100, 200 mM) to reduce 78 μM of α-ZOL (α-Zearalenol)/ZEA (zearalenone) in different incubation time.

[b] Percentage of reduction was calculated based on a blank prepared for each assay.

3.2. *Identification of ZEA/α-ZOL-AITC reaction products*

MS-LIT total ion chromatograms of isolated α-ZOL-AITC and ZEA-AITC reaction products are shown in Fig. 1a and b. The results obtained in the present study show that ZEA and α- ZOL can react with AITC and form adducts. Despite the structural similarity, the reduction of ZEA was higher than α-ZOL (Table 1). ZEA ($C_{18}H_{22}O_5$) and its metabolite α-ZOL ($C_{18}H_{24}O_5$) contain some nucleophile groups that may react with the central carbon.

Fig. 1 shows the mass spectra obtained of ZEA-AITC and α-ZOL-AITC adduct in enhanced resolution (ER). Several diagnostic fragments were found in the spectra, confirming the structure of the compound. The molecular weight of the reaction compound ZEA-AITC is the fragment with *m/z* of 419.2 $[M + H]^+$. The fragment with *m/z* of 437.0 corresponds to $[M + 2H + H_2O]^+$, whereas the fragment with *m/z* of 461.0 represents the sodium adduct $[M +2H + H_2O + Na]^+$. Another important fragment is the ion with *m/z* of 478.9 that represents the ion corresponding to the *m/z* of 461.0 with the addition of water $[M + 2H + 2H_2O + Na]^+$.

The characterization of the reaction product between α-ZOL and AITC is shown on Fig. 1b, with the ER mass spectra of the isolated compounds. The ion with *m/z* of 461.3, corresponding to the sodium adduct of α-ZOL-AITC plus a molecule of water $[M + H_2O + Na]^+$ confirm the correct identification of the α-ZOL adduct.

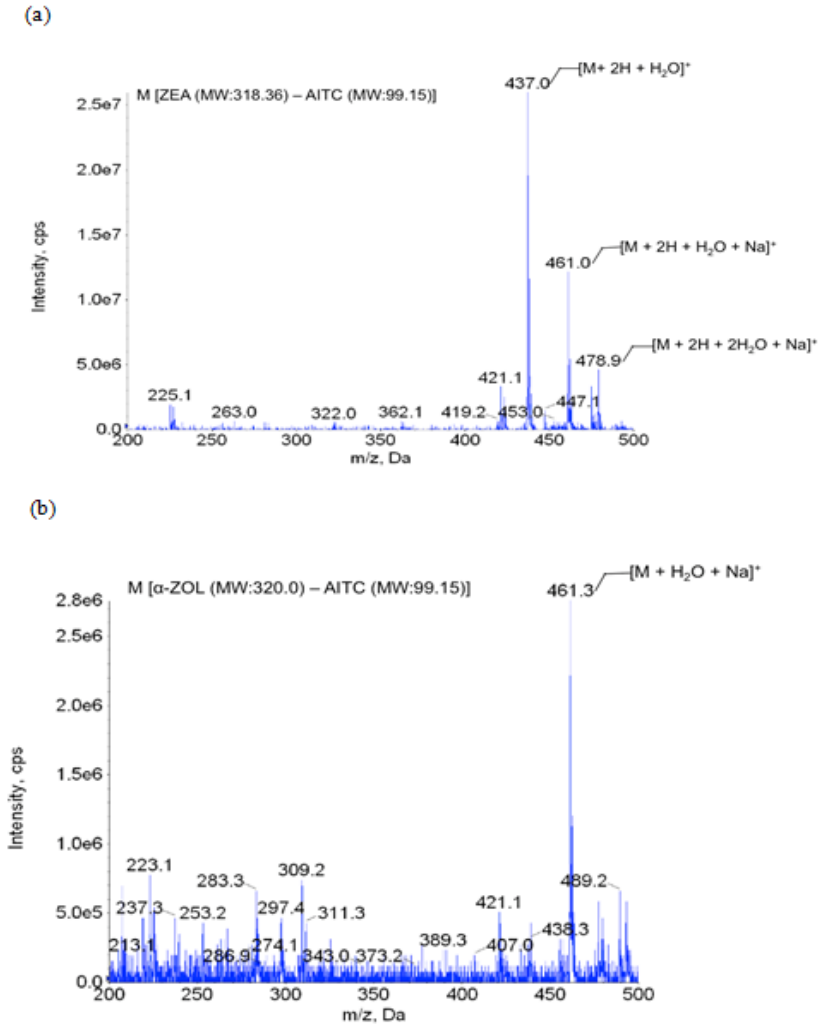

Figure 1. ER mass spectra of reaction products: (a) ZEA with AITC and (b) α-ZOL with AITC.

3.3 *Bioaccessibility of α-ZOL/ZEA-AITC adducts*

The bioaccessible fraction is the amount of an ingested compound that is available for absorption in the body after digestion (Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005). In this sense, the amount of α-ZOL, ZEA and α-ZOL/ZEA-AITC adducts under simulated human digestive fluids were determined and are presented on Table 2. α-ZOL showed higher gastric bioaccessibility than α-ZOL-AITC at 30 μM. Duodenal bioaccessibility was also higher for α-ZOL (80.9 ± 1.0% and 84.8 ± 5.9%) than α-ZOL-AITC (63.7 ± 7.3% and 71.4 ± 2.8%) at 30 and 60 μM, respectively. At 15 μM, α -ZOL had no difference with α -ZOL-AITC for the gastric fraction and duodenal fraction.

Gastric bioaccessibility of ZEA and ZEA-AITC were also similar to all levels investigated. The results of gastric and duodenal bioaccessibility of ZEA obtained in the present study are higher than Ferrer, Manyes, Manes, and Meca (2015), which recovered 54.6 ± 3.2% and 44.3 ± 2.5% of ZEA content administered *in vitro* under gastric and duodenal model. These compounds can be structurally transformed or degraded during the digestion, but this mechanism is difficult to assess by the complex processes that comprise the digestion system (Versantovoort et al., 2005).

Table 2. In vitro bioaccessibility assessment of α-ZOL, ZEA and α-ZOL-AITC and ZEA-AITC adducts

Fraction	Bioaccessibility *in vitro* (%) (Mean ± SD)					
	α-ZOL (μM)*			α-ZOL-AITC (μM)*		
	15	30	60	15	30	60
Gastric	85.0 ± 12.4^{abA}	92.4 ± 3.1^{bA}	91.2 ± 5.2^{bA}	70.5 ± 5.4^{aA}	72.8 ± 3.8^{aA}	85.4 ± 2.1^{bA}
Duodenal	65.0 ± 10.4^{abA}	80.9 ± 1.0^{cB}	84.8 ± 5.9^{cA}	57.8 ± 3.4^{aB}	63.7 ± 7.3^{abA}	71.4 ± 2.8^{bB}
	ZEA (μM)*			ZEA-AITC (μM)*		
	15	30	60	15	30	60
Gastric	89.1 ± 11.7^{abcA}	88.1 ± 2.3^{bA}	94.2 ± 1.0^{cA}	71.0 ± 3.9^{aA}	77.4 ± 1.7^{aA}	78.5 ± 3.6^{aA}
Duodenal	81.5 ± 7.3^{abA}	77.8 ± 4.4^{abA}	81.8 ± 1.7^{bB}	63.5 ± 11.3^{aA}	67.3 ± 1.3^{aB}	69.6 ± 4.0^{abB}

ND: no detected levels.

Different capital letters show significant difference ($p \leqslant 0.05$) in the same column, whereas different lower case letters denote significant difference ($p < 0.05$) within the same row.

* Initial concentration of α-ZOL, ZEA, α-ZOL-AITC (allyl isothiocyanate), ZEA-AITC (15, 30 and 60 μM) evaluated bioaccessibility through *in vitro* digestion model (gastric 2 h and duodenal 2 h).

3.4. *In vitro cytotoxicity and duodenal bioavailability*

Caco-2/TC7 cell monolayers were used in the design of a simulated *in vitro* intestinal model in order to assess the absorption of α-ZOL/ZEA-AITC adducts. However, ZEA and α-ZOL are cytotoxic and could reduce the viability of Caco-2/TC7. This could interfere with the interpretation of the results, since the absorption should occur through viable cells. Therefore, cytotoxicity of ZEA and α-ZOL towards Caco-2/TC7 was analyzed. MTT assay shows that ZEA did not decrease cell viability at doses up to 100 μM ($p \geq 0.05$) at 24 h and ≥ 50 μM were necessary to affect ($p \leq 0.05$) Caco-2/TC7 viability at 48h. However, α-ZOL at ≥ 37.5 μM and ≥ 18.75 μM were able to reduce cell viability ($p \leq 0.05$) after 24 and 48 h, respectively (Fig. 2). The IC_{50} value of 95 μM was obtained for α-ZOL after 48 h of exposure. Results from PC assays show that ZEA did not damage the cells at doses up to 100 μM after 24 and 48 h (Fig. 3a). Moreover, α-ZOL only damaged the cells at 100 μM and ≥ 18.75 μM after 24 and 48 h of exposure, respectively (Fig. 3b).

The evaluation of the duodenal bioavailability of α-ZOL/ZEAAITC conjugates was carried out by determining the concentrations in the basolateral compartment after 1, 2, 3 and 4 h considering the initial concentrations of each compound.

The initial concentrations applied to the *in vitro* digestion model were 15, 30 and 60 μM of each compound. However, the gastric and pancreatic juices diluted the toxins. If these compounds were totally bioaccessible, their concentration in the simulated intestine would be 0.27, 0.54 and 1.08 μM in respect to their initial concentration of 15, 30 and 60 μM, respectively. As presented in Figs. 2 and 3, much higher concentrations were necessary to cause cell damage and to reduce cell viability after 24 h.

As it can be observed in Table 3, ZEA-AITC presented a higher bioavailability than α-ZOL-AITC. The former compound presented a similar profile among all

concentrations studied. The bioavailability obtained for the initial concentration of 15 μM ranged from 3.92% after 2 h to 15.84% after 4 h for α-ZOL-AITC and from 8.61 to 37.61% for ZEA-AITC. α-ZOL-AITC showed a bioavailability ranging from 0.98 (1 h) to 15.67% (4 h) for the initial concentration of 30 μM and from 0.74 to 12.72% using an initial concentration of 60 μM. Bioavailability ranging from 9.99 to 36.15% (0–4 h; 30 μM) and 10.14 to 42.13% (0–4 h; 60 μM) were obtained for ZEA-AITC. In addition, the mycotoxins themselves presented significantly lower bioavailability than the reaction products or the original mycotoxins were metabolized to a higher extent.

Videman, Mazallon, Tep and Lecouer, (2008) evaluated the metabolism and transfer of ZEA using Caco-2 cell line as a model of intestinal epithelial barrier demonstrating that ZEA easily crosses the cell barrier. After 3 h of ZEA exposure at 10 μM, about 30% crossed the cell monolayer. The metabolites produced were evaluated and the composition was as follows: 40.7 ± 3.1% α-ZOL, 31.9 ± 4.9% β-ZOL, 8.2 ± 0.9% ZEA-glucuronide and 19.1 ± 1.3% α-ZOL-glucuronide.

Another study Pfeiffer, Kommer, Dempe, Hilebrand, & Metzier, (2011) determined theabsorption of ZEA and α-zearalanol (α-ZAL) *in vitro* using monolayers of differentiated Caco-2 cells. Cells were exposed to ZEA or α-ZAL (10, 20, 30 and 40 μM) for 6 h. Unconjugated ZEA appeared to decrease with first-order kinetics at the apical side, while basolateral reached a plateau after 2 h. After 3-h incubation of 40 μM of ZEA, 57.5% were recovered unconjugated, 11.1% were metabolized to glucoronides and 26.2% were found as α- or β-ZOL. *In vivo* and *in vitro* studies of ZEA kinetics showed early appearance of dietary ZEA in the plasma demonstrating that ZEA can be efficiently absorbed in the proximal part of the small intestine (Avantaggiato et al., 2003; Kuiper-Goodman, Scott, & Watanabe, 1987; Ramos et al., 1996). Furthermore, α-ZOL, β-ZOL, ZEA-glucuronide and α-ZOL-glucuronide are rapidly produced and easily cross the cell

membranes, being detectable at both apical and basolateral sides since the first hour of exposure to ZEA. α-ZOL is the main metabolite produced by the Caco-2 cells and it shows the strongest estrogenic activity (Videmann, Mazallon, Tep, & Lecoeur, 2008). The present study suggests that the reaction products of the mycotoxins with AITC were more stable during the bioavailability study, and perhaps it could avoid the formation of more estrogenic metabolites. However, the toxic effects of these compounds still need to be assessed.

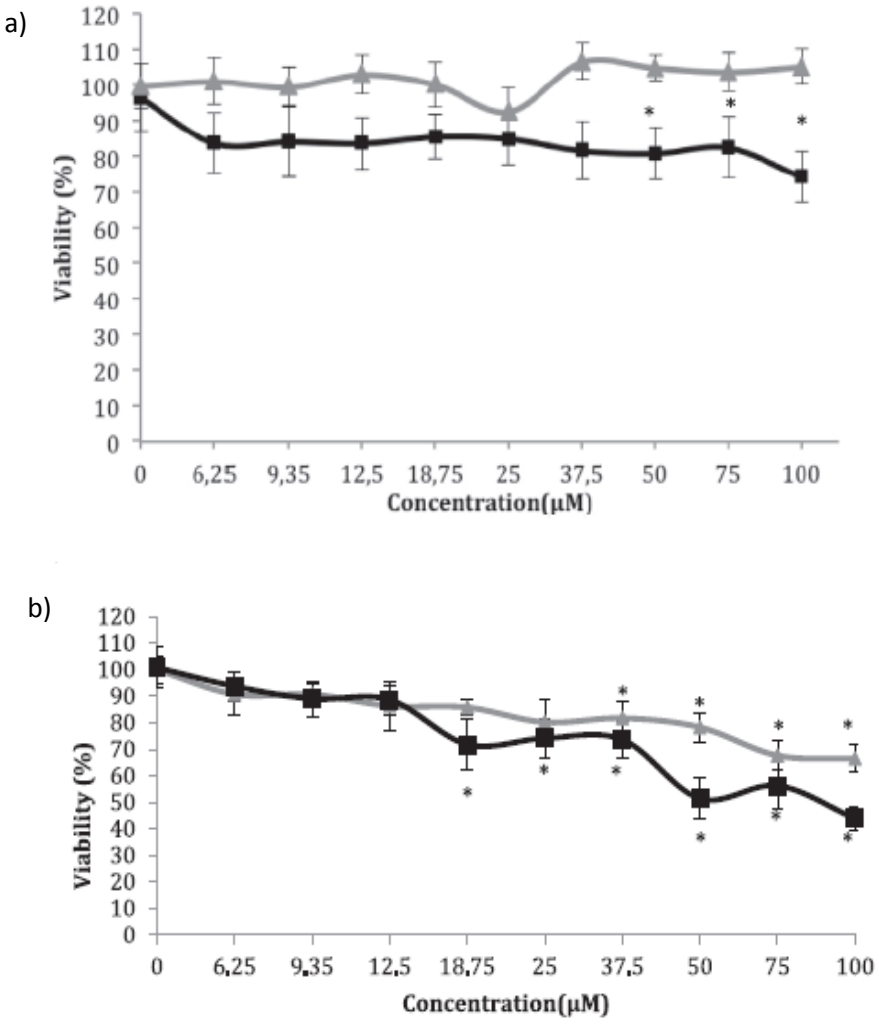

Figure 2. Dose response curve of Caco-2/TC7 cells viability in the presence of a) ZEA and b) α-ZOL measured by MTT. Cells were incubated for 24 (–▲–) and 48 h (–■–). All values are expressed as mean ± SD of 3 replicates. *Represent significant difference ($p \leq 0.05$) between the treatment and the control (100% viability).

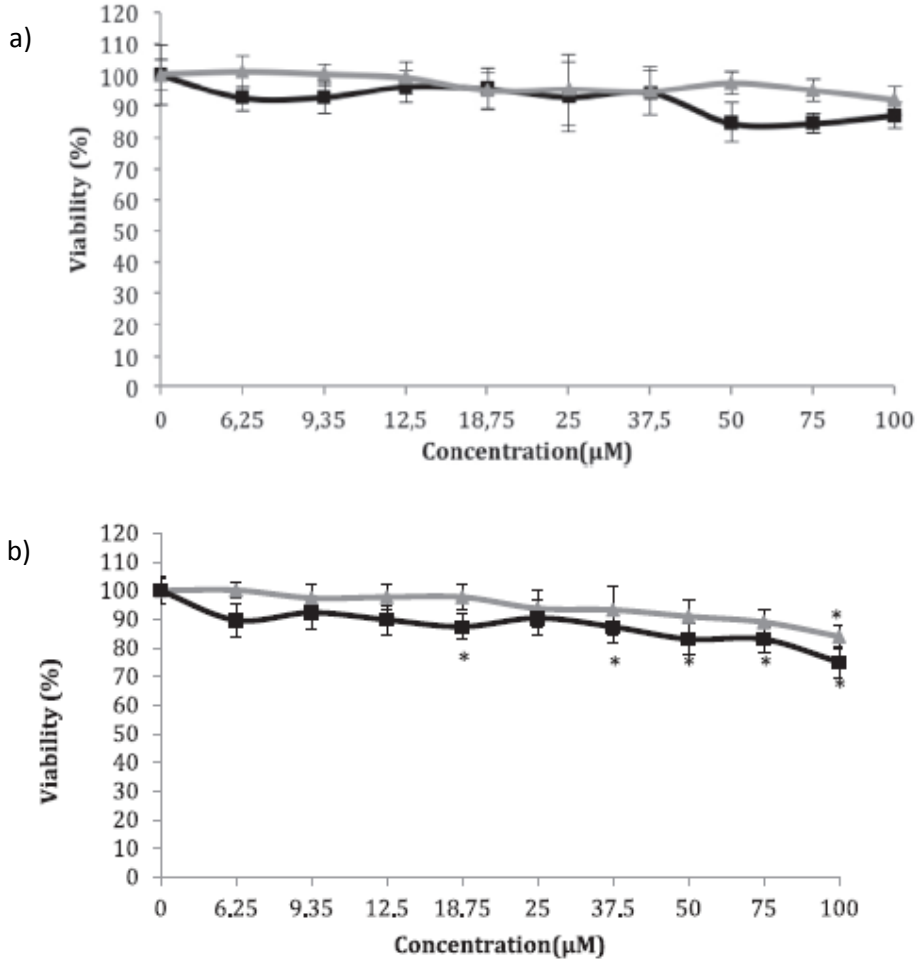

Figure 3. Dose response curve of Caco-2/TC7 cells viability in the presence of a) ZEA and b) α-ZOL measured by PC. Cells were incubated for 24 (–▲–) and 48 h (–■–). All values are expressed as mean ± SD of 3 replicates. *Represent significant difference ($p \leq 0.05$)between the treatment and the control (100% viability).

Table 3. Bioavailability of the products obtained by α-ZOL/ZEA and AITC reaction products after digestion treatment through Caco-2/TC7 cell monolayers

Hours	Bioavailability %			
	α-ZOL (μM)	α-ZOL – AITC (μM)		
	60 (Control)	15	30	60
1	0.06 ± 0.04^{aA}	ND	0.98 ± 0.2^{bA}	0.74 ± 0.47^{abA}
2	0.28 ± 0.15^{aAB}	3.92 ± 2.69^{aA}	3.79 ± 2.8^{aB}	3.31 ± 0.40^{aA}
3	0.29 ± 0.11^{aAB}	9.52 ± 4.00^{bAB}	8.80 ± 2.8^{bBC}	4.41 ± 1.43^{abA}
4	0.48 ± 0.21^{aB}	15.84 ± 5.9^{bC}	15.67 ± 3.7^{bC}	12.72 ± 2.65^{bB}
Hours	ZEA (μM)	ZEA – AITC (μM)		
	60 (Control)	15	30	60
1	0.78 ± 0.15^{aA}	ND	9.99 ± 1.07^{bA}	10.14 ± 0.52^{bA}
2	1.06 ± 0.24^{aA}	8.61 ± 3.8^{bA}	13.82 ± 3.61^{bA}	19.34 ± 9.95^{bAB}
3	1.16 ± 0.27^{aA}	13.05 ± 4.96^{bA}	17.54 ± 6.55^{bA}	25.02 ± 4.73^{bBC}
4	1.23 ± 0.39^{aA}	37.61 ± 4.30^{bB}	36.15 ± 4.90^{bB}	42.13 ± 2.51^{bC}

ND: no detected levels.

Caco-2/TC7 cells were apically exposed to the duodenal fraction (initial concentrations of 60 μM of mycotoxin – control – and 15, 30, 60 μM of mycotoxin-AITC). The compounds evaluated were measured in the basolateral (BL) compartment after 1, 2, 3, 4 h.

Different capital letters show significant difference ($p \leqslant 0.05$) in the same column, whereas different lower case letters denote significant difference ($p < 0.05$) within the same row.

4. CONCLUSION

The results of this trial indicate that allyl isothiocyanate can react and reduce α-ZOL and ZEA *in vitro* at levels up to 96.9%, and form ZEA/α-ZOL-AITC conjugates. Reduction of mycotoxins and their reaction products were identified after gastric and duodenal treatments, probably due structural transformation during digestion. Moreover, the data obtained in this study suggested a lower bioavailability *in vitro* of ZEA, α-ZOL in comparison to their AITC conjugates. Further investigation may focus on the evaluation of the possible utilization of AITC to control ZEA and α-ZOL levels in food and animal feed. In addition, the toxicological assessment of the ZEA-AITC and α-ZOL-AITC must be performed.

Conflict of interest

The authors declare that there are no conflicts of interest.

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4.DISCUSIÓN GENERAL

GENERAL DISCUSSION

4. DISCUSIÓN GENERAL

Con el objetivo de conocer los mecanismos de toxicidad del AOH, se estudió la citotoxicidad del alternariol en las células Caco-2 y cómo altera su efecto tóxico en dichas celulas la combinación con otras micotoxinas de *Fusarium*. A continuación, se determinó el estrés oxidativo mediante la generación de ROS y LPO. Debido al incremento de la toxicidad y del estrés oxidativo, el siguiente objetivo fue evaluar los efectos toxicológicos del AOH a través de posibles alteraciones del ciclo celular, daño al ADN, inducción de apoptosis/necrosis y la alteración de la permeabilidad de la membrana mitocondrial (MMP) en las células Caco-2. Teniendo en cuenta que el sistema antioxidante celular tiene por objetivo mantener los niveles normales de ROS, se evaluó el sistema de defensa intracelular enzimático (SOD, CAT, GPx, GR y GST) y no enzimático (GSH), así como el papel que ejercen algunos antioxidantes de la dieta (Quer, SS-I, tirosol, aceite de oliva extra virgen y oleuropeina) para disminuir el exceso de estrés oxidativo.

Se evaluó la citotoxicidad individual de las micotoxinas de *Alternaria* AOH y AME y de *Fusarium* ENN B, DON, ZEA, α-ZOL, FB1, BEA en las células humanas Caco-2, Caco-2/TC7 y RGA durante un periodo de exposición de 24, 48 y 72 h. Los parámetros que se utilizaron para determinar la citotoxicidad fueron el MTT, el NR, el PC y el High Content Analysis.

El AOH, AME, DON y ENN B disminuyen la viabilidad en las células Caco-2 dependiendo del tiempo y de la concentración tras la exposición a 24, 48 y 72 h. El funcionamiento mitocondrial (MTT) se ve más afectado que la alteración lisosomal (NR) y las proteínas totales (PC) tras la exposición del AOH en las células Caco-2. La concentración ensayada más alta (100 µM) redujo un 15% la viabilidad tras 72 h.

El AME redujo la viabilidad en un 17% tras 48 h de exposición de forma similar al AOH. Sin embargo, no se obtuvieron valores de IC_{50} para el AOH ni para el AME. Los resultados obtenidos están en concordancia con los observados por Juan-Garcia et al., (2015), Tiessen et al., (2013) y Tieman et al., (2009), quienes estudiaron la citotoxicidad del AOH y AME y no obtuvieron valores de IC_{50} en un rango de concentraciones entre 0 y100 µM en las células HepG2, HT-29 Y P4. Por el contrario, Bensassi et al., (2011 y 2012), obtuvo valores de IC_{50} de 65 y 120 µM en las células HCT116 expuestas a AOH y AME, respectivamente, mediante el ensayo del FDA.

Con la ENN B y el DON se observó, a 24 h de exposición, una disminución aproximada del 20% y 35% de la viabilidad a partir de una concentración de 2.5 y 1.25 µM, respectivamente. Tras 48 h de exposición, la viabilidad disminuyó aproximadamente un 38% y 30% a partir de 1.25 y 0.312 µM y después de 72 h de exposición, la disminución de la viabilidad fue del 90% y 55% respectivamente. Los valores de IC_{50} obtenidos para la ENN B y el DON fueron de 3.9 µM y 5.5 µM. Valores similares de IC_{50} fueron obtenidos por otros autores para la ENN B; Wätjen et al. (2014) observaron valores de IC_{50} de 2.5 µM en las células H4IIE e Ivanova et al. (2006) obtuvieron valores de 3.6 y 9.8 µM en las células MRC-5 mediante el ensayo de BrdU y AB, respectivamente. Los resultados obtenidos para el DON son comparables a los resultados obtenidos en la literatura. Nielsen et al. (2009), mediante el ensayo de WST-1, obtuvieron valores de IC_{50} de 1.0 y 4.5 µM en las células Caco-2 y HUEVEC. Ruiz et al. (2011a, 2011b) obtuvieron valores de IC_{50} de 1.83 y 5.05 µM en las células CHO-K1 y Vero, respectivamente.

La ZEA y el α-ZOL se estudiaron tras 24 y 48 h de exposición en las células Caco-2/TC7 con el ensayo del MTT y el de las PC. Se observó una disminución de la

viabilidad celular tras 48 h a concentraciones superiores de 50 µM de ZEA mediante el ensayo del MTT. No obstante, la ZEA no produce valores de $IC_{50.}$ Por otra parte, 37.5 µM de α-ZOL disminuyó la viabilidad a partir de 24 h de exposición, presentando un IC_{50} de 95 µM tras 48 h de exposición mediante el ensayo del MTT. No se observó disminución de la viabilidad en las células Caco-2/TC7 expuestas a ZEA ni a 24 ni a 48 h mediante el ensayo de las PC. En las células expuestas a α-ZOL, la viabilidad celular disminuyó con el ensayo de las PC a las concentraciones de 100 y 18.75 µM tras 24 y 48 h de exposición. De acuerdo con los resultado de este estudio, Marin et al. (2015) y Tatay et al. (2014) obtuvieron que la ZEA es menos citotóxica que el α-ZOL cuando se exponen a células IPEC-1 y CHO-K1, respectivamente. De igual modo, Sun et al. (2015) y Taranu et al. (2015) obtuvieron valores de IC_{50} de 100 µM y 163.8 µM para la ZEA en las células BRL3A y IPEC-1. Sin embargo, Tatay et al. (2014) obtuvieron, tras 48 h de exposición, valores de IC_{50} de 60.30 µM y 32.00 µM para la ZEA y el α-ZOL, respectivamente.

Se evaluó la citotoxicidad de la FB1 y la BEA en las células RGA mediante el MTT tras 48 h de exposición. La FB1 no disminuyó la viabilidad en ninguna de las concentraciones ensayadas. Sin embargo, la BEA a 10 µM disminuyó la viabilidad en un 100% en las células RGA.

La FB1 y la BEA se estudiaron tras 48 h de exposición mediante el ensayo del HCA en las células Caco-2. La FB1 no disminuyó la viabilidad a ninguna de las concentraciones expuestas (0-10 µM). Sin embargo, la BEA a 1 µM disminuye la viabilidad en un 45.8 %. Meca et al. (2010c) y Wan et al. (2013) estudiaron la citotoxicidad de la FB1 en las células Vero y IPEC-J2 sin observar disminución de la viabilidad a las mismas concentraciones ensayadas. Estudios realizados con DON

evidencian resultados similares. Calo et al (2004) y Ferrer et al. (2009) expusieron células de origen mieloide (U-937 y HL-60) y CHO-K1 a concentraciones entre 0-100 µM de BEA y tras 24 h de exposición disminuyó la viabilidad a partir de 10 µM.

Después de los estudios de citotoxicidad realizados, se puede concluir que el AOH, el AME, la BEA, el DON, la ENN B, la ZEA y el α-ZOL ocasionaron un efecto citotóxico dependiendo del tiempo y concentración ensayadas en las células Caco-2, Caco-2/TC7 y RGA. De los datos obtenidos se puede concluir que el efecto citotóxico se produjo en el siguiente orden: ENNB>DON>BEA>α-ZOL>ZEA>AME>AOH. La FB1 no presentó efectos citotóxicos a las concentraciones ensayadas.

La co-presencia de micotoxinas es muy importante porque los efectos tóxicos que se pueden producir con la combinación de estas pueden ser distintos a los efectos tóxicos producidos por las micotoxinas de manera individual, ya que pueden dar lugar a efectos de sinergismo, adición y/o antagonismo.

Las micotoxinas están presentes de manera ubicua en los alimentos y piensos alrededor de toda Europa, superando en ocasiones los niveles máximos permitidos por la UE. Teniendo en cuenta el hecho de que los hongos micotoxigénicos son capaces de producir más de una micotoxina y que los alimentos y piensos pueden estar infectados por diversas especies de hongo, se puede sospechar la presencia de varias micotoxinas en un mismo alimento. Por ello, es importante el estudio de las combinaciones (binarias y terciarias) para poder analizar posibles interacciones entre ellas y el aumento de los efectos tóxicos. Para llevar a cabo este objetivo, se seleccionó el método MTT para los estudios de las mezclas de micotoxinas, ya que previamente se observó el efecto

de las micotoxinas sobre la función mitocondrial. Para este propósito se utilizaron las células Caco-2 a las que se expusieron las combinaciones binarias y terciarias de AOH+ ENN B + DON durante 24, 28 y 72 h y la mezcla AME + AOH durante 24 y 48 h de exposición. Tras los resultados obtenidos, se aplica el método matemático de las isobolas basado en el índice de combinación (IC) introducido por Chou (2006) y Chou y Talalay (1984) a las combinaciones seleccionadas.

Las interacciones binarias y terciarias producidas por AOH, ENN B, DON y AME no siguieron un patrón uniforme a lo largo de la fracción afectada y el tiempo de exposición. En las interacciones DON+AOH, ENN B+AOH y AOH+AME se observó un efecto sinérgico a las concentraciones más bajas ensayadas, que ligeramente se transformó en un efecto adición a las concentraciones más elevadas. Diferentes autores observaron este mismo efecto en mezclas de DON con otras micotoxinas como la FB1, la BEA y la ZEA (Kouadio et al., 2007; Ficheux, Sibiril y Parent-Massin, 2012). Teniendo en cuenta los resultados obtenidos, se puede indicar que el AOH aumenta su toxicidad cuando está en combinación con otra micotoxina. Estos resultados guardan relación con el estudio de Da Motta y Valente Soares (2000), quienes sugieren un efecto sinérgico en la combinación de AOH+AME. Sin embargo, Bensassi et al. (2015) observaron un efecto aditivo en la combinación AOH+AME. El efecto sinérgico observado se produce en las combinaciones binarias con AOH y en la combinación terciaria tras 72 h de exposición a bajas concentraciones. Esto podría deberse a que una micotoxina aumenta la actividad de otra en la mezcla o a que las micotoxinas actúan sobre sitios de receptores diferentes dentro de la misma ruta. Por el contrario, la combinación binaria DON+ENN B y la terciaria DON+ENN B+AOH presentaron un efecto antagonista. El DON+ENN B a 24 h produce un efecto de adición cuyo resultado fue muy similar al obtenido por Ruiz et al. (2011b) al estudiar la mezcla

DON+BEA tras 24 h. Por otra parte, Wan et al. (2013) evaluaron la citotoxicidad de una mezcla de micotoxinas compuesta por DON, NIV, ZEA y FB1 en las células intestinales IECs y observaron que en las mezclas donde estaba presente el DON no había aumento de la citotoxicidad en comparación con el DON de manera individual, produciendo un efecto antagonista a mayor tiempo de exposición en combinación con las micotoxinas más citotóxicas. Este efecto puede ser debido a una competición por el sitio activo en el lugar de unión de la micotoxina. La disminución de la viabilidad celular en las mezclas en orden creciente fue el siguiente: (DON+ENN B)> (ENN B+AOH)> (DON+AOH)> (DON+AOH+ENN B).

Es difícil dar una explicación de las interacciones que no siguen un mismo patrón, ya que el método de las isobolas sólo permite una determinación cuantitativa y no aporta información sobre el mecanismo por el cual se produce la interacción entre las micotoxinas de la mezcla (Ince et al., 1999; Cheng y Lu, 2002; Chen et al., 2014). El comportamiento sinérgico para algunas mezclas puede suponer un riesgo asociado a la concurrencia de micotoxinas en alimentos, lo que indica que la evaluación del comportamiento toxicológico de una única micotoxina puede dar lugar a la subestimación de su efecto tóxico potencial.

Los estudios de viabilidad celular con las diferentes micotoxinas ponen de manifiesto los efectos tóxicos en los cultivos celulares, ya sea de forma individual o en combinación. Debido a que la disminución de la viabilidad celular se debe a procesos moleculares y considerando que la identificación de dichos eventos subyacentes conducen desde la exposición inicial de las micotoxinas a la manifestación final de la lesión tóxica a nivel celular, se llevan a cabo los siguientes objetivos planteados con el AOH; estudio de la posible interacción con los componentes y actividades celulares, tales como interacción con el ciclo

celular y, por lo tanto, sobre la proliferación celular, alteración del potencial de membrana celular y mitocondrial con consecuencias en los procesos de apoptosis y necrosis; daño al ADN y estudio de la posible alteración sobre el sistema endocrino. Además, se estudia la interacción a nivel molecular pueden generar las especies radicalárias de oxígeno y las consecuencias que tienen sobre todos los componentes celulares.

Los resultados obtenidos sobre el ciclo celular señalaron que tras 24 h de exposición al AOH, se produjo una mayor proporción de células Caco-2 en la fase G2/M (33%) comparado con el control (24%), mientras que las células en fase G1 (27%) disminuyeron comparando con el control (51%). Tras 48 h de exposición, el AOH ocasionó una detención del ciclo celular en G2/M y un aumento significativo en la fase S (35%) comparado con el control (24%) y en la fase Sub G1 (4%) frente al control (1%). La reducción de células en la fase G1 se mantuvo a través de todos los tiempos de exposición. La detención del ciclo celular en la fase G2/M y/o en la fase S es una respuesta común de la mayoría de células eucarióticas frente a un riesgo genotóxico (Shackelford et al. 1999). En la fase G1 se lleva a cabo una intensa actividad biosintética donde las células que no entran en mitosis para completar el ciclo celular permanecen en la fase Sub G1 (estado de reposo o quiescencia). Por tanto, dependiendo del daño al ADN, el ciclo celular puede bloquearse en la fase G1 (Bartek et al. 2001). Asimismo, Solhaug et al. (2013) observaron que el AOH produce la inducción en cascadas de la señalización incluyendo las quinasas de mamíferos Chk1 y Chk2 con la subsiguiente activación de la proteína p53. La activación de esta proteína está implicada en la detención del ciclo celular y es un mediador importante en la fase G2/M y G1/S que controla los daños en el ADN y evita la replicación de las células dañadas. Si el daño en el

ADN no se repara, las células pueden verse sometidas a un proceso de necrosis o apoptosis.

Los resultados de este estudio muestran un bloqueo en la fase G2/M del ciclo celular. Estos resultados están de acuerdo con la mayoría de los estudios previos realizados con AOH en células de linfoma de ratón (L5178Y TK +/-), células de hámster chino (V79), Ishikawa y macrófagos (RAW 264.7) (Brugger et al, 2006; Lehmann et al, 2006; Fleck et al, 2012; Solhaug et al, 2012). Wollenhaupt et al. (2008) también demostraron una acumulación de células en la fase G1 de células del endometrio.

La parada del ciclo celular en la fase G2/M puede ser consecuencia de un retraso en el ciclo celular debido a un posible proceso de adaptación al cambio celular producido por el AOH con un daño en el ADN (Abid-Essefi et al., 2003). Por lo tanto, el siguiente objetivo fue el estudio de la apoptosis/necrosis en las células Caco-2 tras la exposición al AOH. Después de 24 y 48 h de exposición, el AOH no indujo la apoptosis temprana en las células Caco-2 a ninguna de las concentraciones ensayadas. Sin embargo, en todos los tiempos de exposición y concentraciones ensayadas aumentaron las células en estadio apoptótico/necrótico (7% - 28%) respecto al control (aprox. 2%). El mayor incremento de células apoptóticas/necróticas se obtuvo a 60 µM de exposición. Las células necróticas aumentaron después de 24 h de exposición entre un 28% y 74% y tras 48 h entre un 12% y 34% respecto al control (1% y 2%), respectivamente. Estos resultados afirman que el AOH induce apoptosis tardía. En las etapas celulares de apoptosis/necrosis o después de un daño celular traumático, se conduce a la necrosis donde las células ya no son capaces de mantener la integridad de la membrana celular. Por lo tanto, la pérdida de

equilibrio entre el medio intracelular y extracelular caracteriza la última etapa del proceso de muerte celular por necrosis (Berghe et al., 2010). Igualmente, Soulhaug et al., 2012 observaron un aumento de células RAW 264.7 en estadio necrótico después de 48 h de exposición a 60 µM de AOH. Esta investigación podría apoyar la inducción de apoptosis/necrosis y necrosis a través de la vía mitocondrial.

La mitocondria tiene un papel importante en el proceso de muerte celular al liberar factores implicados en la inducción de apoptosis en las células. Por ello, en este estudio se planteó el objetivo de evaluar el ΔΨm tras la exposición de las células Caco-2 al AOH. A las 24 h de exposición, no se produjo la pérdida del ΔΨm con ninguna de las concentraciones ensayadas. Sin embargo, a las 48 h de exposición, las células tratadas con las concentraciones más elevadas de AOH (30 y 60 µM), mostraron una significativa pérdida del ΔΨm. Estos resultados sugieren que la mitocondria desempeña un papel importante en la inducción de la apoptosis viéndose afectada por el AOH. De acuerdo con Bensassi et al. (2012), la exposición celular a 50 µM de AOH induce la apertura de PTPC en las células HCT 116. Como resultado de la apertura de PTPC, se produce la disipación del ΔΨm. Asimismo, el ΔΨm disminuye con la liberación de proteínas, como por ejemplo el citocromo c. Por otra parte, las ROS modulan la apertura de PTPC oxidando grupos tioles (-SH) (Vianello et al., 2012), lo que podría sugerir que las ROS también están relacionados con el estrés oxidativo y la actividad mitocondrial.

La parada de las fases G2/M y S del ciclo celular se correlaciona con daños a nivel del ADN, los cuales, si no se reparan por mecanismos celulares, pueden inducir a apoptosis. En este estudio se evaluó un posible daño producido por el AOH a nivel del ADN mediante el ensayo del cometa y se observó un incremento

del daño al ADN dependiente de la concentración del AOH durante 24 h de exposición. El porcentaje de ADN en la cola aumento significativamente en un 70%, 78% y 85% después de 15, 30 y 60 µM de AOH respecto al control. Este resultado indica que el AOH es un compuesto clastogénico capaz de romper la cadena de ADN de las células Caco-2. Resultados similares obtuvieron Fleck et al. (2012) y Solhaug et al. (2012), quienes demostraron que el AOH en las células V79 y RAW 264.7 induce roturas de la cadena de ADN después de 24 h de incubación. Además, de modo similar a nuestros resultados, demostraron que el daño al ADN producido por el AOH está estrechamente relacionado con niveles altos de ROS y con el bloqueo de puntos de control en el ciclo celular (G2/M y G1/S), inducción de apoptosis/necrosis y pérdida de la integridad de la membrana mitocondrial.

En general, las micotoxinas son biodisponibles, aunque algunas se absorben con más rapidez que otras ejerceciendo efectos tóxicos. Por ello, se determina el efecto de algunas micotoxinas sobre la función endocrina. Los ED pueden interferir en el sistema y en la función endocrina de distintas maneras. La perturbación de la homeostasis endocrina produce consecuencias como trastornos metabólicos, anomalías o disfunción hormonal. Hasta la fecha, diferentes autores han evaluado la ENN B, el DON, el AOH, la ZEA y sus metabolitos como posibles ED (Frizzell et al., 2011; Ndossi et al., 2012; Frizzell et al., 2013ab; Frizzell et al., 2014; Kalayou et al., 2015). Teniendo en cuenta que la FB1, la BEA, el AOH y el AME son unas de las micotoxinas más prevalentes en los alimentos, se realizaron varios bioensayos para evaluar sus efectos como posibles ED (OECD, 2012b). Los resultados obtenidos con el AOH y el AME no fueron concluyentes, por lo que no son mostrados a lo largo de esta tesis. En los resultados obtenidos para la FB1 y BEA no se observó ninguna respuesta agonista a ninguna de las concentraciones ensayadas desde 0.001 hasta 10 µM durante 48

h de exposición. Sin embargo, 10 µM de FB1 exhibió un efecto antagonista sobre el receptor de andrógenos, 1 µM de BEA presentó un efecto antagonista sobre los receptores progestogénicos y glucocorticoides y 10 µM de BEA presentó un fuerte antagonismo en todos los receptores (estrogénico, andrógenos, progestogénico y glucocorticoide). Se comprobó la toxicidad de dichas micotoxinas por los métodos MTT y HCA, observandose que la respuesta antagonista a 10 µM de BEA en todos los receptores se debía a la citotoxicidad ocasionada por ésta, y no a su efecto como ED.

En relación con las concentraciones ensayadas, la BEA tiene un efecto mayor sobre la función endocrina que la FB1. En cuanto a los receptores afectados, la BEA actúa sobre receptores específicos de progesterona y glucocorticoides, mientras que la FB1 interviene sobre receptores específicos de andrógenos (OECD 455).

Otros autores han investigado diferentes micotoxinas como posibles ED a través del test RGA (OECD 455). Frizzell et al., (2011) estudiaron la ZEA, el β-ZOL, y el α-ZOL, observando una respuesta agonista en los receptores estrogénicos y antagonista en los receptores androgénicos, siendo mayor el efecto del β-ZOL que de la ZEA y que del α-ZOL. Estos mismos autores estudiaron otras micotoxinas de *Fusarium*, la OTA, el DON, la T-2 y la HT-2, las cuales no presentaron respuesta agonista. Por el contrario, sí se observó una respuesta antagonista en todos los receptores (estrógenos, andrógenos, corticoides y progesterona) (Ndossi et al., 2012; Frizzell et al., 2013). La ENN B y la PAT siguieron un patrón muy parecido a los anteriores porque no presentaron respuesta agonista y se manifestó la respuesta antagonista en los receptores de estrógenos, andrógenos y progesterona (Frizzell et al., 2014, Kalayou et al., 2015). Por el contrario, en los

ensayos para evaluar la respuesta antagonista con el AOH. se observó una respuesta estrogénica débil en los receptores de andrógenos, glucocorticoides y progesterona (Frizzell et al., 2013).

Teniendo en cuenta que el AOH es citotóxico, bloquea puntos de control del ciclo celular, induce apoptosis tardía y necrosis, altera el potencial de membrana y causa daño al ADN, se planteó estudiar si esta toxicidad podía ser debida a la capacidad del AOH de generar ROS y causar LPO. Los resultados obtenidos mediante la sonda fluorescente H_2-DCFDA demostraron que todas las concentraciones del AOH (15, 30 y 60 µM) produce ROS tras la exposición en las células Caco-2, siendo la máxima producción de 1.4 veces superior al control, determinada tras la inmediata exposición del AOH. Desde 5 a 120 min. la producción de ROS aumentó ligeramente (1.2 veces al control).

Una de las consecuencias más estudiada producida por las ROS es la oxidación de los lípidos de las membranas celulares, es decir, la generación de LPO. Los resultados obtenidos demostraron que el AOH aumentó significativamente la producción de LPO dependiendo de la concentración. El aumento de la LPO varió desde 130% (30 µM) a 250% (60 µM), respecto al control tras 24 h de exposición.

Estos resultados son consistentes con los de Tiessen et al., (2013) y Schwarz et al., (2012), que mostraron que la exposición de las células HT-29 a concentraciones desde 10 a 50 µM de AOH induce un aumento en la generación de ROS 2.5 veces superior al control. Bensassi et al. (2012) llevaron a cabo diferentes ensayos para determinar el producto predominante de las ROS generado después de la exposición de las células HCT116 a 50 µM de AOH, y

descubrieron que el AOH no indujo una producción significativa H_2O_2, pero si un aumento de $O2^{\bullet -}$ mitocondriales 4 veces superior al control.

La producción de ROS y LPO demostraron implicación del AOH en la toxicidad celular. La hidroxilación del AOH conduce a la formación de catecoles o hidroquinonas, que podrían producir estrés oxidativo (Schreck et al. 2012). De la misma manera, Fehr et al. (2009) y Solhaug et al. (2012) sugirieron que el aumento de las ROS en células tratadas con AOH podrían deberse a los productos del metabolismo del AOH.

A continuación, teniendo en cuenta la citotoxicidad de las micotoxinas estudiadas, se plantea cuál es la bioaccesibilidad de las micotoxinas a nivel gastro entérico y su absorción (biodisponibilidad).

En este estudio se evaluó la bioaccesibilidad y biodisponibilidad del AOH, la ZEA y α-ZOL, ya que la biodiponibilidad de la ENN B, BEA, FB1 y DON ya se han determinado en las células Caco-2 previamente por nuestro grupo de investigación.

Para evaluar la bioaccesibilidad de la ZEA y el α-ZOL, se aplico el método de digestión estático *in vitro*, el cual fue optimizado previamente por nuestro laboratorio. La optimización del método de digestión gástrica *in vitro* muestra que la ZEA y el α-ZOL son bioaccesibles en un rango que varía entre 1.9±0.1% y 3.2±1.4% y entre 3.6±0.2 y 5.8±0.6%, respectivamente, siendo el α-ZOL más bioaccesible a nivel gástrico que la ZEA considerando las tres concentraciones ensayadas (15, 30 y 60 µM). Los valores medios obtenidos en la bioaccesibilidad duodenal demuestran un aumento de la bioaccesibilidad de la ZEA y el α-ZOL que varía, respectivamente, entre 4% y 19.8%.

Los datos muestran que el contenido de la ZEA y α-ZOL, tras el proceso de digestión completo, se reduce. Esto tiene gran interés en la evaluación de la fracción biodisponible en la absorción por parte del hombre y/o animal. Sin embargo, hay que considerar las diferencias que pueden obtenerse debido a los tipos de métodos de digestión *in vitro* aplicados, los procedimientos, pH utilizados, estructura de las micotoxinas y la composición de los alimentos, pues todo ello podría contribuir a variar los valores de bioaccesibilidad obtenidos.

Teniendo en cuenta la bioaccesibilidad y el efecto citotóxico obtenido tras la exposición de las micotoxinas de forma individual y combinada, se plantea el estudio de la biodisponibilidad para el AOH, la ZEA y el α-ZOL. Atendiendo, a que el epitelio intestinal es la primera barrera para la absorción y donde posiblemente puede tener lugar el metabolismo de alguna micotoxina, se utilizaron en este estudio las células Caco-2/ TC7, ya que son un clon celular de las Caco-2, poseen una población más homogénea y tienen más desarrolladas las funciones de los enterocitos, siendo la línea celular de elección para este tipo de ensayos.

Los porcentajes de transporte desde el compartimento apical (AP) al basolateral (BL) en el modelo *in vitro* fueron muy bajos para todas las micotoxinas. La ZEA y el α-ZOL son transportadas desde el compartimento AP al BL en un 1.2% y 0.5% a las 4 h, respectivamente. El contenido de ZEA y α-ZOL en el compartimento AP fue del 2.18% y 0.5%, respectivamente. Estos resultados sugieren que ambas micototoxinas han sufrido un proceso de degradación en el compartimento AP o en las células Caco-2 TC7. Videman et al. (2008) evaluaron el metabolismo y transporte de la ZEA en las células Caco-2 y, tras 3 h de incubación con 10 μM de ZEA, obtuvieron un trasporte del 30% (concentración en el compartimento BL). Estos autores también evaluaron procesos de metabolización,

obteniendo diferentes concentraciones de metabolitos en el medio celular. El análisis de metabolitos correspondía a α-ZOL en un 41%, un 32% al β-ZOL, un 8% a ZEA-glucurónido y un 19% a α-ZOL-glucurónido.

Respecto al AOH, los porcentajes de transporte desde el compartimento AP al BL variaron entre un 13% y un 21% durante 3 h de exposición a las concentraciones de 15, 30 y 60 µM. Por otra parte, el contenido del AOH en la parte AP varió entre 15% y 18.2%, dependiendo del tiempo de exposición y la concentración ensayada. La biodisponibilidad a nivel del colon fue mayor que la duodenal, variando el transporte de 10% a 31% y el contenido en la parte AP entre 11% y 25% durante 3 h de exposición a las concentraciones de 15, 30 y 60 µM. Las diferencias obtenidas entre el AOH, la ZEA y el α-ZOL pueden deberse a la diferencia en la estructura química de estas micotoxinas y al mayor peso molecular de estas últimas.

Tras los resultados obtenidos de la citotoxicidad del AOH, debido al incremento de la producción de LPO y ROS, y considerando que las micotoxinas son bioaccesibles, se procedió a determinar la eficacia del sistema de defensa intracelular frente al estrés oxidativo. Se evaluó tanto el mecanismo enzimático como el no enzimático. El sistema enzimático de primera línea de defensa frente a los radicales libres está compuesto principalmente por la SOD, la CAT y la GP_X. Los resultados obtenidos indicaron un incremento de la actividad de la SOD a todas las concentraciones (15, 30 y 60 µM) de AOH expuestas en las células Caco-2. El aumento de actividad fue concentración dependiente, desde 67% a 93% respecto al control. La eliminación de H_2O_2 tras la exposición al AOH fue demostrada por la alteración de la actividad de la CAT y GPx. La actividad de la CAT disminuyó a 30 y 60 µM de AOH entre un 53% y 56% respecto al control. Sin embargo, no se

obtuvieron diferencias con 15 µM. Medir el estrés oxidativo puede tener gran complejidad debido a que el sistema de defensa antioxidante reduce rápidamente los productos generados en las células. Por otra parte, tal y como sugiere Matés et al. (2000), grandes cantidades de H_2O_2 pueden producir un bloqueo de la actividad de la CAT. Teniendo en cuenta que la GPx trabaja simultáneamente con la CAT para eliminar H_2O_2, se estudió la actividad de la GP_X, la cual no produjo diferencias respecto al control ni a 15 ni a 30 µM de AOH. Sin embargo, a 60 µM se observó una disminución en la actividad enzimática del 30% respecto al control. Estos resultados sugieren que la actividad enzimática de la CAT es más eficaz que la de la GPx, ya que se activa tras la exposición de 30 µM de AOH, mientras que la GPx actúa tras la exposición de 60 µM. Debido al consumo de dichas enzimas, se produce una saturación de las mismas a 60 µM de AOH. Los resultados globales del sistema de defensa enzimático demuestran que la SOD es más eficiente eliminando las $O2^{\bullet -}$ que la CAT y la GPx eliminando H_2O_2. Los resultados observados son comparables a aquellos obtenidos en estudios con otras micotoxinas; Ramyaa et al. (2014) observaron un aumento de LPO y una disminución de la actividad enzimática de la CAT a 10 µM de OTA en las células HepG2. Kono y Fridovich, (1982) afirman que la disminución de la actividad enzimática de la CAT puede ser debida a una acumulación de $O2^{\bullet -}$.He et al., (2011) y Theumer et al., (2010) observaron un incremento en la actividad de la SOD causada por la T-2, FB1 y AFB1 en las células GPCs Y SMC durante 48 h de exposición. Por lo tanto, podemos concluir que el sistema de defensa enzimático tiene un papel muy importante en la reducción del estrés oxidativo en las células Caco-2 expuestas a AOH.

Considerando que el GSH es una de las líneas de defensa antioxidante no enzimática más importante a nivel celular, se procedió a estudiar los niveles de

GSH tras la exposición del AOH en células Caco-2. El GSH es el principal antioxidante endógeno producido por las células (Yang et al., 2014) y participa directamente en la neutralización de los radicales libres y ROS. Igualmente, está implicado en decisiones determinantes a nivel celular, tales como la proliferación del ciclo celular o la apoptosis y necrosis. A 60 µM de AOH se produjo una disminución de los niveles de GSH (43%) respecto al control. Este resultado sugiere que el GSH participa de manera activa en la defensa celular frente al AOH. En condiciones fisiológicas normales, el GSSG se reduce a GSH por la GR a expensas de NADPH, formándose, de este modo, un ciclo redox. Por lo tanto, se determina la actividad de la GR para conocer si se regenera GSH ante la presencia de AOH en las células Caco-2. Los resultados obtenidos mostraron una diminución (23%) de la actividad de la GR tras 60 µM de AOH respecto al control, indicando que la recuperación del GSH no pudiera ser total. Por tanto, estos efectos pueden conducir a un desequilibrio oxidativo en el ciclo del GSH y, como consecuencia, contribuir al proceso oxidativo.

Además, se estudió la actividad de la enzima GST. La GST tiene la capacidad de detoxificar productos que producen toxicidad a nivel celular con participación del GSH. Una reducción de la GST puede producirse por una inhibición catalítica debido a los altos niveles de ROS o al agotamiento de la disponibilidad de GSH. En este estudio se observó que a las concentraciones bajas ensayadas (15 µM) se produce una estimulación de la enzima (22%) respecto al control, sugiriendo que la GST detoxifica el AOH en las células Caco-2. Sin embargo, a concentraciones altas (60 µM), la GST disminuyó (30%) su actividad respecto al control, ya que como se ha demostrado previamente, los niveles de GSH a esta concentración de AOH disminuyen también. Teniendo en cuenta todos los resultados obtenidos, el GSH, la GST, la CAT y la SOD contribuyen en la

protección de las células Caco-2 frente al estrés oxidativo producido por el AOH. Resultados similares fueron obtenidos por Tiessen et al., (2013) al exponer las células HT-29 a concentraciones entre 10 y 50 µM de AOH, donde los niveles de GSH disminuyeron hasta un 26% tras 1 h de exposición. Diversos autores sugieren los mismos resultados con micotoxinas de otros géneros (*Penicillium, Aspergillus y Fusarium*), como el DON en los linfocitos de sangre periférica humana (PBL) (Yang et al., 2014) y células HEK-293 (Dinu et al., 2011); la BEA en las células Caco-2 (Prosperini et al, 2013) y en las células CHO-K1 (Mallebrera et al, 2014); la ZEA en células HepG2 (Hassen et al, 2007); y la OTA en células LLC-PK1 (Schaaf et al., 2002). Además, Tiessen et al., (2013) demostraron que el AOH únicamente produce niveles significativos de GST después de la exposición a 50 µM. Por ello, sugieren que este aumento de actividad podría ser debido a la formación de metabolitos, los cuales podrían generar catecoles o hidroquinonas y, como consecuencia, la formación de las ROS.

Por otra parte, la dieta mediterránea se caracteriza por el uso de alimentos locales, frescos y de temporada. Las verduras, legumbres, frutas, el aceite de oliva, tubérculos, cereales y frutos secos conforman la base de esta dieta. La abundancia de productos vegetales propicia la presencia de fibra, vitaminas y antioxidantes. En este estudio se ha querido determinar el efecto antioxidante de los componentes de dicha dieta sobre los efectos citotóxicos del AOH.

El aceite de oliva virgen (AOVE) es uno de los componentes de la dieta mediterránea y se considera una importante fuente de compuestos fenólicos como el hidroxitirosol, tirosol y la oleuropeína (Driss y El-Benna. 2010). En este estudio se evaluó el efecto citprotector del tirosol y oleuropeína a

concentraciones de 25, 50 y 100 µM y de un extracto de AOVE frente a 12.5, 25, 50 y 100 µM de AOH en células Caco-2. Los resultados demuestran que los antioxidantes del AOVE previenen del daño celular producido por el AOH. El extracto del AOVE aumentó la viabilidad celular en todas las diluciones ensayadas. Sin embargo, 100 µM de AOH bloquea la función citoprotectora del AOVE, ya que no muestra diferencias significativas respecto al AOH de manera individual. Asimismo, el extracto del AOVE puro mostró ser un potente reductor de las ROS originado por el AOH en las células Caco-2. Por lo tanto, estos resultados sugieren que el extracto del AOVE posee propiedades antioxidantes debido al elevado contenido de compuestos fenólicos. Estos resultados están de acuerdo con otros autores que sugieren que el AOVE y sus compuestos fenólicos son muy eficaces eliminando radicales de hidroxilo, superóxido y radícales de peróxido (Gioffi et al, 2010; Driss y El-Benna, 2010; Sarria et al, 2012).

Teniendo en cuenta el efecto citoprotector del AOVE, se estudió el efecto antioxidante de los principales polifenoles, la oleuropeína y el tirosol. 50 µM de oleuropeína en combinación con 100 µM de AOH protege respecto a 100 µM de AOH ensayado directamente sobre las células Caco-2. Sin embargo, cuando se aumenta la concentración de oleuropeína (100 µM) disminuye la proliferación celular entre un 10% y 20 % en todas las concentraciones de AOH ensayadas. Por otra parte, respecto al tirosol, no se observaron diferencias significativas cuando fue expuesto simultáneamente con el AOH. Además, tras la exposición de 25 µM de tirosol con 100 µM de AOH, aumentó la toxicidad de forma considerable, ya que disminuye la viabilidad celular obteniéndose una IC_{50} de 70 µM comparado con la exposición individual de AOH que no produce IC_{50} a las mismas concentraciones ensayadas (100 µM). Teniendo en cuenta que el AOH produce estrés oxidativo, se estudió la eficacia de ambos antioxidantes frente a las ROS.

Los resultados obtenidos demostraron que tras la combinación de AOH (15, 30 y 60 µM) y oleuropeína (1, 2.5 y 25 µM) o tirosol (1, 2.5 µM) no se produjeron ROS. Por contra, 25 µM de tirosol provocó un aumento de ROS del 20% desde los 5 min de exposición respecto al control. Otros autores han analizado el efecto de la oleuropeína y tirosol en diferentes líneas celulares. Bulotta et al., (2011) estudiaron el efecto de la oleuropeína en las células de mama humanas encontrando resultados contradictorios, ya que en algunos casos disminuye la viabilidad celular (células T-47D) y en otras no produce alteración en las células (MCF-7). Manná et al., (2000) demostraron que las diferencias entre ambas sustancias pueden deberse a su proceso de absorción celular. El transporte de tirosol se produce a través de difusión pasiva mientras que, por el contrario, la oleuropeína tiene menor absorción en el intestino delgado, teniendo además que ser metabolizada en hidroxitirosol, su principal producto final (Covas et al., 2010; Corona et al., 2014). Bulotta et al., 2011 y Di Benedetto et al., (2007) observaron disminución de las ROS en presencia de oleuropeína y tirosol en las células T-47D y MCF.

La Quer es un polifenol presente en los frutos rojos y otras bayas con efectos antioxidantes. Con el objetivo de estudiar el efecto citoprotector de la Quer, se evaluaron concentraciones entre 1.56 y 100 µM frente a concentraciones entre 1.56 y 100 µM de AOH y AME tras 24 y 48 h de exposición. Respecto a las combinaciones binarias, la Quer no protegió a las células Caco-2 frente al AOH y el AME a ningún tiempo de exposición ensayado. La combinación AOH+Quer disminuyó la viabilidad respecto al AOH de manera individual después de 24 h exposición. La combinación terciaria (AOH+AME+Quer) también disminuyó la viabilidad, siendo la mayor reducción de un 47% tras 24 h y de un 26% tras 48 h de exposición. Estos resultados corresponden con los observados por

Robaszkiewicz et al. (2007) y Lombardi et al. (2012), quienes obtuvieron una reducción de la viabilidad celular a 50 y 100 µM de Quer en las células A549 y CHO-K1, respectivamente. Además, Barcelos et al. (2011) estudiaron la Quer en combinación con AFB1 en las células HepG2 y demostró que la Quer a altas concentraciones actúa como prooxidante. No obstante, concentraciones de Quer menores de 100 µM no alteran la viabilidad celular cuando se exponen al AOH y AME de manera combinada. Hundhausen et al. (2005), y Sergent et al. (2005) estudiaron el efecto de la Quer en células HepG2 y Caco-2 expuestas a OTA y concluyeron que la Quer no muestra ningún efecto citoprotector cuando se exponen de manera simultánea.

Las legumbres son otro de los componentes habituales de la dieta mediterránea. La SS-I es la saponina del grupo B más abundante y se encuentra en gran número de plantas y leguminosas. La SS-I posee actividad antioxidante e inhibe el crecimiento de los hongos entre otras propiedades. Se estudió el efecto antioxidante de la SS-I mediante pre-tratamiento y en combinación simultánea con el AOH en las células Caco-2. Los resultados obtenidos del pre-tratamiento con SS-I demostraron un incremento en la viabilidad celular (30%) respecto al control después de la exposición de 50 µM de AOH. Cuando la SS-I se expuso de manera simultánea con el AOH (1:1) en las células Caco-2, se produjo un aumento de la proliferación celular (entre 6.25 y 25 µM), siendo dicho incremento del 20% al 35% respecto al AOH ensayado de manera individual. Hasta la fecha, no hay estudios que relacionen el poder antioxidante de SS-I frente a las micotoxinas. Sin embargo, existen varios estudios *in vivo* que demuestran que las soyasaponinas disminuyen el colesterol e inhiben la reabsorción intestinal de los ácidos biliares (Oakenfull et al. 1984, Lee et al. 2005). La forma aglicona de SS-I es la soyasapogenol B, la cual es más bioactiva que su glucósido y es una sustancia

insoluble en agua (Hu et al., 2004). Cuando los compuestos no son solubles en agua, un paso crucial en su absorción luminal es la solubilidad en los fluidos intestinales y el transporte de compuestos solubilizados hacia la superficie de la membrana. Por lo tanto, una hipótesis alternativa asume que soyasapogenol-B y AOH, ambos con propiedades lipofílicas, compiten por su incorporación en micelas mixtas de la dieta, como puede ocurrir con el colesterol. En consecuencia, se forma un complejo SS-I-AOH que podría inhibir la absorción de AOH y contribuir a disminuir su potencial tóxico a nivel celular.

En conclusión, el efecto citoprotector de los componentes de la dieta mediterránea (antioxidantes, leguminosas, etc.) depende de la concentración de los mismos y de las micotoxinas en el alimento, de la proporción de la mezcla de ambos y de la posible interacción entre ellos, pudiendo contribuir su presencia a disminuir el efecto tóxico potencial de las micotoxinas.

Para concluir, se han estudiado diferentes estrategias para prevenir el crecimiento de los hongos en los alimentos y evitar la presencia de micotoxinas en la dieta humana y/o animal. En esta tesis se investiga la reducción de la ZEA y α-ZOL (78 µM) en un modelo de digetión estático *in vitro* usando AITC (2, 20, 100 y 200 mM). El efecto de la AITC es concentración dependiente. Tras la administración del AITC, la reducción de la ZEA y el α-ZOL varían del 0.2% al 96.9% y del 0% al 89.5%, respectivamente, sin diferencias significativas entre los diferentes pH ensayados (4 y 7). El AITC reacciona mejor con la ZEA que con el α-ZOL, ya que 20 mM de AITC (pH 4) es capaz de reducir más la mitad de la concentración inicial de ZEA, mientras que α-ZOL se redujo como máximo hasta el 29% con esta misma dosis.

Estudios previos han indicado que el uso de alil, bencil y fenil isotiocianatos gaseosos (ITC) reduce los niveles de micotoxinas. Meca et al., (2012) evaluaron la reducción de BEA con soluciones tamponadas de AITC (1mM) y en harina. El AITC fue capaz de reducir la BEA en la solución tamponada en un 100% tras 48 h de exposición, sin observarse diferencias significativas entre pH 4 y 7. Además, la harina de trigo tratada con AITC gaseoso redujo entre un 10% y un 65% la concentración de BEA dependiendo de la concentración. La reacción de ITC con la FB1, FB2 y FB3 fue estudiada por Azaiez et al., (2013), observándose reducción de FB1 y FB2 entre el 42% y el 100%, dependiendo del tiempo de exposición y de la influenciada del pH. En general, los niveles de pH más bajos facilitaron la reacción entre ITC y FBs.

Asimismo, los ITC se utilizaron para fumigar granos y harina de maíz contaminado con las FBs, obteniéndose una reducción entre un 53% y un 96% de la FB1, entre un 29% y un 1% la FB2 y entre un 29% y un 96% para la FB3. Teniendo en cuenta los resultados obtenidos, el uso de alil, bencil y fenil isotiocianatos podría ser una alternativa para mitigar el problema de la contaminación por micotoxinas en los alimentos.

5.CONCLUSIONES

CONCLUSIONS

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1. El alternariol, alternariol monometil éter, beuavericina, deoxinivalenol, eniatina B, zearalenona y α-zearalenol ocasionaron un efecto citotóxico de manera tiempo y concentración dependiente en las células Caco-2. El efecto citotóxico se produjo en el siguiente orden: eniatina B>deoxinivalenol>beauvericina>α-zearalenol>zearalenona>alternariol monometil éter>alternariol. De esta forma, queda demostrado que estos componentes son potencialmente tóxicos.

2. Las combinaciones alternariol+alternariol monometil éter, deoxinivalenol+alternariol y eniatina B+alternariol causaron un efecto sinérgico. La combinación deoxinivalenol+eniatina B causó un efecto de adición y la combinación terciaria deoxinivalenol+alternariol+eniatina B causó un efecto antagonista. Estos resultados ponen de manifiesto la complejidad de las interacciones por la presencia simultánea de estos compuestos.

3. Se observó estrés oxidativo tras la exposición del alternariol en células Caco-2 consecuencia del aumento de las especies reactivas de oxígeno, peroxidación lipídica, pérdida de la integridad de la membrana celular, daño del ADN celular, células proapoptoticas y necróticas.

4. La beauvericina muestra mayor alteración endocrina que la fumonisina B1.

5. La zearalenona y el α-zearalenol presentan baja bioaccesibilidad. Se demuestra baja biodisponibilidad del alternariol, la zearalenona y el α-zearalenol, siendo el alternariol el más biodisponible.

6. El sistema de defensa enzimático protege frente al estrés oxidativo producido por el alternariol. El glutatión, el glutatión-S-transferasa y la superóxido dismutasa son las enzimas más implicadas en la protección celular.

7. Entre los sistemas de protección aportados por la dieta, se observa que el aceite de oliva virgen extra y la soyasaponina-I previenen el daño celular producido por el alternariol en las células Caco-2. Sin embargo, la quercetina no protege a las células frente a las micotoxinas de Alternaria.

8. Se observa una elevada reducción de la zearalenona y el α-zearalenol con isotiocianatos, por lo que estos podrían ser una buena alternativa para mitigar la contaminación por micotoxinas en los alimentos.

9. La presencia de micotoxinas en los alimentos supone un riesgo potencial de salud pública. Debido a que no se conocen los mecanismos de acción de algunas de ellas y no se dispone de legislación, se deberían realizar más estudios *in vitro* e *in vivo* para conocer las implicaciones en la salud de la población y prevenir los efectos.

5. CONCLUSIONS

1. The alternariol, alternariol monomethyl ether, beuavericin, deoxynivalenol, eniatine B, zearalenone and α-zearalenol caused a cytotoxic effect in time and concentration dependent on Caco-2 cells. The cytotoxic effect occurred in the following order: eniatine B> deoxynivalenol> beauvericin> α-zearalenol> zearalenone> alternariol monomethyl ether> alternariol. It is demonstrated that these compounds are potentially toxic.

2. The combinations alternariol + alternariol monomethyl ether, deoxynivalenol + alternariol and eniatine B + alternariol caused a synergistic effect. The combination of deoxynivalenol + eniatin B caused an addition effect and the tertiary combination deoxynivalenol + alternariol + eniatin B caused an antagonistic effect. These results demonstrate the complexity of the interactions by the simultaneous presence of these compounds.

3. Oxidative stress was observed following the exposure of alternariol on Caco-2 cells related to high levels of reactive oxygen species, lipid peroxidation, loss of membrane integrity, cellular DNA damage, proapoptotics and necrotics cells.

4. Beauvericin shows greater endocrine disturbance than fumonisin B1.

5. Zearalenone and α-zearalenol have low bioaccessibility. Low availability of alternariol, zearalenone and α-zearalenol is demonstrated, with alternariol being the most bioavailable

6. The enzymatic defense system protects against the oxidative stress produced by alternariol, being glutathione, glutathione-S-transferase and superoxide dismutase, the enzymes most involved in cell protection.

7. Among the protection systems provided by diet, It is observed that extra virgin olive oil and soyasaponin-I prevent cell damage caused by alternariol in Caco-2 cells. However, quercetin does not protect cells against Alternaria mycotoxins.

8. A high reduction of zearalenone and α-zearalenol with isothiocyanates is observed, so it may be a good alternative to mitigate the problem of mycotoxin contamination in food.

9. The presence of mycotoxins in food represents a potential public health risk because some mechanisms of action are not known and no legislation is available. Therefore, more *in vitro* and *in vivo* studies would be necessary to know the implications in food safety.

6.REFERENCIAS

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